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Introduction

Early life encompasses critical windows of development that can have long lasting effects on the health of the offspring. Stress during early life can critically impact development. Specifically, maternal psychological and nutritional stressors lead to suboptimal offspring health (Reynolds, 2013; Moisiadis & Matthews, 2014*a*; Kim *et al.*, 2016). Psychological stress during pregnancy can impact fetal growth. Women with higher corticotropin-releasing hormone at midgestation, were 7.5 folds more likely to deliver preterm (Inder *et al.*, 2001). Additionally, maternal nutritional stress increases the relative risk of neonatal death by two folds for women with severe obesity (Aune *et al.*, 2014).
The placenta and mammary glands are primary sites of nutrient delivery to the developing offspring. Given their crucial role in development, the placenta and mammary glands are highly affected by maternal psychological and nutritional stressors. Studies assessing the mechanisms by which maternal stressors alter placental and lactational development and function remain limited.

My objective is to determine the role of maternal stressors on offspring health mediated through placental and mammary function. I will test the hypothesis that ***maternal psychological and nutritional stressors alter placental and mammary gland functions, ultimately affecting offspring health.*** This hypothesis is supported by data showing that maternal psychological stress can lead to reduced birthweight, altered offspring hypothalamic-pituitary-adrenal axis activity, reduced milk immune components, and earlier termination of lactation (Levine, 1967; Edwards *et al.*, 1993; Matthews, 2000; Li *et al.*, 2008; Thibeau *et al.*, 2016). Furthermore, maternal nutritional stress is associated with increased risk of fetal macrosomia, increased likelihood of earlier weaning, and altered milk lipid composition (Owens *et al.*, 2010; Panagos *et al.*, 2016; Castillo *et al.*, 2016). Our preliminary data demonstrate that stressed dams exposed to glucocorticoids starting a week prior to conception give birth to small, non-viable pups. Also, hyperactivation of mammary adipocyte mechanistic target of rapamycin 1 (mTORC1) showed increased milk macronutrients. I will test my central hypothesis via the following four aims:

Aim 1: Determine the effects of maternal glucocorticoid-induced stress on placental function. I will expose pregnant dams to the synthetic glucocorticoid, dexamethasone, and then collect pre-term placentas in order to evaluate nutrient transport and endocrine function. In separate cohorts, I will monitor how gestational dexamethasone exposure affects offspring metabolic health.
Aim 2: Identify the relationship between glucocorticoid exposure and mammary function. I will expose dams to dexamethasone during lactation then assess mammary gland development, milk volume and composition. I will then monitor the growth and health of the offspring of the exposed dams.
Aim 3: Elucidate the consequences of placental mTORC1-hyperactivation nutritional stress on placental role. To model 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 assess placental hormone production and evaluate nutrient transport, along with offspring metabolic health.
Aim 4: Detect the effects of nutritional stress via mTORC1 hyperactivation on lactation. Milk volume and composition along with offspring health from dams with adipocyte mTORC1 hyperactivation will be measured.

As a result of these studies I will learn how our models of maternal psychological and nutritional stressors influence placental and mammary gland functions. I will also determine how early life exposures, demonstrated by gestation and lactation periods, affect offspring health.
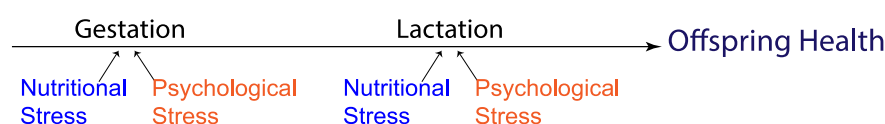
Strengths and Limitations of Proposed Models

Dexamethasone as a Model of Stress

There are two types of stress, acute and chronic. Acute stress, in response to an immediate threat, is processed through the sympathetic nervous system to trigger a fight-or-flight response. The acute stressor triggers the hypothalamic-pituitary-adrenal axis which releases the glucocorticoids cortisol/corticosterone in the cascade shown in *Figure 1*. If acute stress persists, this leads to chronic stress whereby cortisol/corticosterone levels remain elevated (Stephens & Wand, 2012).

The majority of research has focused on glucocorticoid exposure during midgestation in animal models, long after placental development is complete. Lifestyle stressors that pregnant women may encounter like low socioeconomic status, institutional racism, shootings, and other traumatizing events that can affect health of the mother and child are common but not well studied. For example, institutional racism and the events of 9/11 in New York City caused reductions in birthweight (Collins *et al.*, 2004; Eskenazi *et al.*, 2007). These chronic stressful events are not yet well explored in animal models throughout pregnancy, and the mechanisms of action by which stress influences placental development are not understood. Furthermore, stress during lactation is scarcely studied.

Our model of chronic stress is providing a constant supply of the glucocorticoid dexamethasone. Dexamethasone is a potent synthetic glucocorticoid that mimics endogenous cortisol/corticosterone, and is a common therapeutic. When given at our dose of 1mg/kg/day, dexamethasone causes a state of elevated glucocorticoids and mimics a state of stress (De Souza *et al.*, 1973). My aim is to determine the exact mechanisms by which dexamethasone, a potent synthetic glucocorticoid substrate used to mimic chronic stress, namely affects placentation (Aim 1) prior to the full development of the placenta and its effect on mammary gland function (Aim 2). Based on our results that will examine the mechanisms, our data can further be used to better understand the effect of stressors during different time points in early life.

Figure 1: the HPA axis from "Know your brain: HPA axis", 2014 [1]

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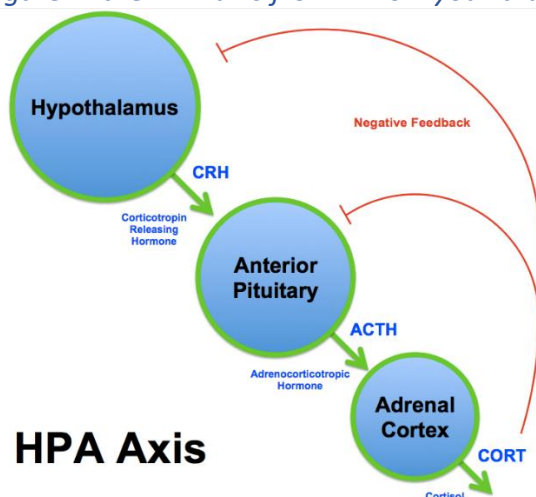
[1] Know your brain: HPA axis, NEUROSCIENTIFICALLY CHALLENGED, Published June 04,2014. Retrieved from: https://www.neuroscientificallychallenged.com/blog/2014/5/31/what-is-the-hpa-axis

mTORC1 Hyperactivation as a Model of Obesity

Obesity is a major public health crisis affecting 37.7% of Americans (Flegal *et al.*, 2016). There are many molecular and physiological changes associated with obesity, but I will focus on the nutrient sensing kinase mTORC1. In most cells mTORC1 is activated by anabolic signals and an excess of nutrients.

Placental mTORC1

Mechanistic target of rapamycin 1 (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). Placental mTORC1 has been identified as being upregulated in maternal obesity and was implicated as the main driver of offspring phenotype in maternal obesity (Jansson *et al.*, 2013). My model of mTORC1 hyperactivation in the placenta allows us to better test the mechanisms by which placental mTORC1 affects placental function and offspring outcome (Aim 3). This model further elucidates exact mechanisms of mTORC1 without the confounding variables stemming from maternal obesity such as altered insulin sensitivity, glucose homeostasis, adipokine and hormone levels, and inflammatory profile.

Adipocyte mTORC1

mTORC1 is a nutrient sensor and a main regulator of protein and lipid synthesis (Wang & Proud, 2006; Cai *et al.*, 2016). Obesity, identified as having excess fat mass, promotes mTORC1 activity (Catania *et al.*, 2011). In obese subjects, gene expression of mTORC1 was upregulated in the visceral fat compartments (Catalán *et al.*, 2015). My model of adipocyte mTORC1 hyperactivation mimics the obesogenic environment and better allows us to understand the mechanisms by which milk composition and volume are altered in a nutrient-excess medium with mTORC1 hyperactivation (Aim 4). It is worth noting that our model has mTORC1 hyperactivation in all adipocytes and is not specific to the mammary adipocytes, as no mammary-gland-specific adipocyte driver has been identified yet.

Strengths and Limitations of Using Mouse Model

Mice vs Humans

Human and mice are both mammals that develop very similarly[2]. The mouse and human genome is also 85% similar[3], and both mammals share very similar organ and system functions (Rangarajan & Weinberg, 2003). Genome editing is possible in a mouse model but not in humans, allowing us to better understand mechanisms. Furthermore, tissue collection in mice is feasible at any point in life. The proposed experiments in my proposal would be impossible and unethical to conduct with human subjects. Additionally, human samples (i.e. placentas) cannot be easily collected anytime during pregnancy due to ethical considerations. Even with human samples available, it is very hard to discern the exact mechanisms at play since humans are complex beings that are influenced by multiple exposures and lifestyle habits. Mice, on the other hand, are easier to control, manipulate, and assess

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[2] Why are mice considered excellent models for humans?, The Jackson Laboratory. Retrieved from https://www.jax.org/why-the-mouse/excellent-models

[3] The Mouse Model: Less than Perfect, Still Invaluable, Institute for Basic Biomedical Sciences, JOHNS HOPKINS MEDICINE. Retrieved from https://www.hopkinsmedicine.org/institute_basic_biomedical_sciences/news_events/articles_and_stories/model_organisms/201010_mouse_model.html

environmental or dietary exposures for, which makes our analysis less confounded by multiple variables. Despite their great use, mice are not a perfect model to replicate human development, but they remain an invaluable resource to elucidate potential mechanisms at play.

Mice vs Cells

Studying cells in media has advanced science whether it is using human/animal cell lines or tissue explants. Cell studies are cheaper, easier to purchase, and easier to manipulate by changing the media constituents[4]. Nonetheless, cell studies remain very limited and far removed from the effects of the bodily systems, making extrapolating data to humans very challenging. This is especially because cells are studied outside their natural environment, which otherwise is inside the body, where they would normally be in contact with other cell types and systems (Kaur & Dufour, 2012). Mouse models still offer a better option as opposed to cell studies as the mouse allows for broader understanding of organ functionality and role when the full body is otherwise intact and functioning.

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[4] Mouse Cell Line, ingenious targeting laboratory. Published January 2, 2018. Retrieved from
https://www.genetargeting.com/stem-cell/mouse-cell-line/

Specific Aim 1

Determining the effects of glucocorticoid-induced stress on placental transport of nutrients and endocrine function.

The placenta is the direct and only site of communication between mother and fetus during *in utero* development (Brett *et al.*, 2014). It 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), and it is highly regulated to ensure adequate growth of the fetus in normal pregnancies (Napso *et al.*, 2018*a*). In cases of maternal psychological or glucocorticoid-induced stress, placental nutrient transport and endocrine function are compromised leading to potentially suboptimal fetal growth (Eskenazi *et al.*, 2007; Kipmen-Korgun *et al.*, 2012; Waffarn & Davis, 2012). In Denmark, 20% of women reported use of corticosteroids from 4 weeks prior to delivery until delivery between 1996-2008 (Hviid & Mølgaard-Nielsen, 2011). The mechanisms by which maternal corticosteroids influence fetal health and placental function are understudied (Kemp *et al.*, 2015). Some side effects like reduced birthweight, offspring hypertension, mental illness and higher childhood HPA axis activity remain controversial (Alexander *et al.*, 2012; Waffarn & Davis, 2012; Duthie & Reynolds, 2013; Reynolds, 2013; Braun *et al.*, 2013*a*; Moisiadis & Matthews, 2014*b*). My hypothesis is that maternal glucocorticoid treatments prior to conception and/or during conception cause altered placental transport and hormonal function in a time-dependent manner by which an early and prolonged exposure during pregnancy has more prominent side effects on the placenta, fetus, and offspring. To test this hypothesis, I will examine a) how maternal dexamethasone exposure affects placental development and function, b) how maternal dexamethasone exposure affects fetal and offspring development and health, and c) the role of placental glucocorticoid receptor (GR) in mitigating the effects of maternal dexamethasone exposure.

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)
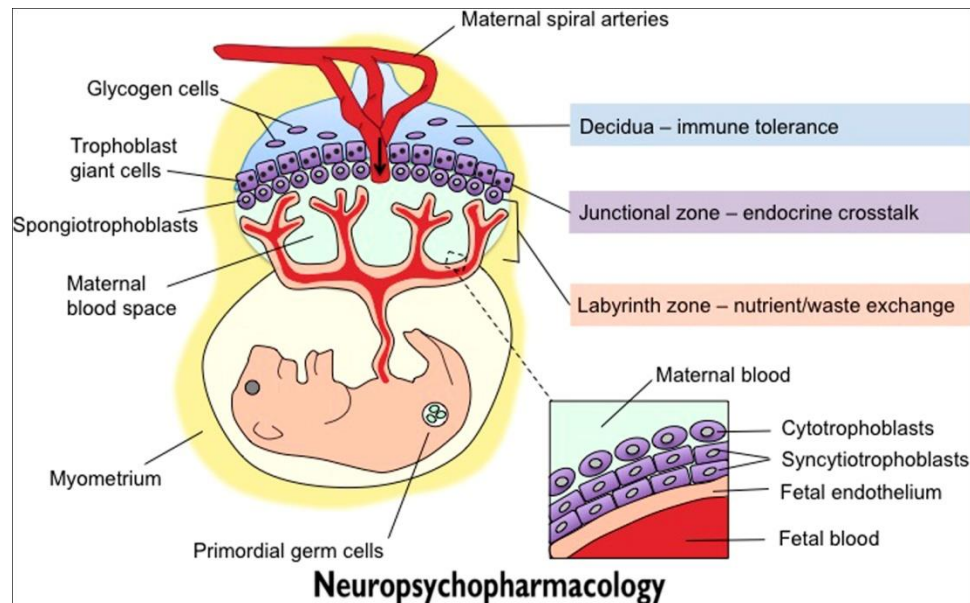
Cortisol/Corticosterone Levels in Pregnancy

During human pregnancy, mean cortisol rises gradually as pregnancy progresses (Carr *et al.*, 1981). Mean cortisol levels increase in humans during the first, second and third trimester by 1.6, 2.4 and 2.9 folds, respectively (Jung *et al.*, 2011). The increased cortisol levels may be explained by placental secretions of estrogen stimulating maternal cortisol production and mitigating maternal negative feedback (Lindsay & Nieman, 2005; Duthie & Reynolds, 2013) and/or by placental production of corticotropin-releasing hormone (CRH) into the maternal circulation in mid- and late gestation (Duthie & Reynolds, 2013). Maternal cortisol promotes placental CRH production, which in turn promotes maternal HPA axis activity thus acting as a feed-forward positive mechanism.

However, in mouse pregnancy, corticosterone levels do not increase as much as humans near term although there are still increases. In pregnant control mice, corticosterone levels were not significantly different at E11 and E17 despite slightly higher levels at E17 (Jafari *et al.*, 2017). Other studies showed an increase in corticosterone levels at E19 compared to E16 in control unstressed mice (Vaughan *et al.*, 2012). Unstressed pregnant mice had higher corticosterone levels with peak levels at E16 being 60 times higher than non-pregnant mice (Barlow *et al.*, 1973). The levels then dropped after E16 until delivery at E19 (Barlow *et al.*, 1973).

Fetal HPA Axis Development

The human fetal hypothalamic-pituitary axis activity is detected as early as 8-12 weeks of gestation (Ng, 2000) and is fully developed in the second trimester of pregnancy (Moisiadis & Matthews, 2014*b*). In early pregnancy, fetal cortisol is thought to primarily be attained from maternal cortisol, as the fetus is believed to sufficiently produce cortisol at 22 weeks of gestation (Buss *et al.*, 2012). Given the critical developmental window by which fetal organs and HPA axis are developing, it is possible that increased

maternal cortisol levels in early pregnancy compared to late pregnancy may have more deleterious effects on fetal development (Barker, 2007; Braun *et al.*, 2013*a*). In mice, the offspring HPA develops postnatal in two phases. On postnatal day (PND) 1 through 12, the mouse HPA is considered hypo-responsive, and after PND 12 the HPA system matures (Schmidt *et al.*, 2003).

Glucocorticoid Treatments in Pregnancy

In addition to the naturally increasing cortisol levels in pregnancy, glucocorticoid (GC) treatments are further prescribed during pregnancy for multiple reasons. A single course of synthetic corticosteroid treatment is prescribed to women who are at risk of delivering premature babies. The treatment is proven to increase offspring chances of survival post-delivery (Doyle *et al.*, 2000; Baisden *et al.*, 2007*a*). Glucocorticoid treatments are prescribed as they enhance fetal growth, specifically fetal lung maturation to prevent respiratory distress syndrome (RDS), and aid in overall embryogenesis to prevent perinatal death due to hemorrhages, heart failure and other underlying causes associated with preterm birth (Lunghi *et al.*, 2010; Singh *et al.*, 2012). Specifically, betamethasone, dexamethasone, prednisolone, corticosteroids, or cortisol are prescribed to women who have acute asthma or asthma, hyperemesis gravidarum, depression, stress, or are at risk of delivering preterm babies (Singh *et al.*, 2012). The use of corticosteroids is widespread. In a Danish cohort study encompassing all births in Denmark from 1996-2008, about 20% of women reported use of corticosteroids from 4 weeks prior to delivery until delivery (Hviid & Mølgaard-Nielsen, 2011). In an American cohort study including 152,531 pregnancies between 1996-2000, 3.5% of pregnant women who had a documented diagnosis associated with preterm birth used corticosteroids, while 1.7% of pregnant women who did not have a documented diagnosis used corticosteroid (Andrade *et al.*, 2004). Despite the placenta's function to protect the fetus from excess maternal corticosteroid, synthetic corticosteroids used in preterm treatments can readily cross the placenta bypassing inactivation by HSD11B2(Cuffe *et al.*, 2011*a*; Singh *et al.*, 2012).

Effects of Glucocorticoid Exposure on Placental and Fetal Development

Pregnant rats treated with dexamethasone at E13 until E20 showed reduced placental and fetal weights (Ain *et al.*, 2005*a*). Despite the evident placental and fetal growth restriction, dexamethasone did not affect litter size or fetal viability (Ain *et al.*, 2005*a*). Rats exposed to triamcinolone once at E16 had 53% reduction in placental weight and 73% reduction in fetal weights (Hahn *et al.*, 1999*a*). Mice exposed to a sound stressor on E10.5, E12.5, and E14.5 showed reduced fetal body weight and had growth restriction that was more evident in female fetuses (Wieczorek *et al.*, 2019). Pregnant mice exposed to dexamethasone on E15, E16, and E17 had reduced placental and fetal weights and trophoblast swelling in the junctional and labyrinth zones (Baisden *et al.*, 2007*a*). Furthermore, mice given dexamethasone at E11-E16 had reduced fetal and placental weights at E16, but the volume of the placental junctional and labyrinth zone was unchanged despite less fetal capillaries in the labyrinth zone (Vaughan *et al.*, 2012). On the contrary to the evident reduction in placental and fetal weights seen in some papers, mice treated with dexamethasone on E13.5 and E14.5 showed no effect on fetal weight at E15.5, E18.5, or at birth (Audette *et al.*, 2011*a*). There was also no effect on placental weights at E15.5, E17.5 in male or female placentas, but at E18.5 female placentas had reduced weight but male placentas were not different (Audette *et al.*, 2011*a*). However, placental junctional and labyrinth zone proportions with respect to the total placental area was unchanged (Audette *et al.*, 2011*a*).

Effect of Glucocorticoid Exposure on Placental Nutrient Transporters

Since the placenta is the only source of fetal nutrient acquisition, transporter expression may reflect the efficiency at which maternal nutrients pass through the placenta to the fetus. To pass to the fetus, the

nutrients need to bypass the three placental exchange barriers including two syncytiotrophoblast layers and the fetal endothelial cell layer (Georgiades *et al.*, 2002). The findings below highlight the conflicting evidence within species and between models regarding placental transporter expression, which emphasizes the need for further studies.

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*a*). GLUT1 and GLUT3 are the most extensively studied transporters in the placenta. Rats exposed to triamcinolone (TA) at E16 had reduced mRNA and protein expression of GLUT1 and GLUT3 at E21 (Hahn *et al.*, 1999*a*). Inversely, pregnant rats exposed to 100 or 200 ug/kg body weight/ day of dexamethasone starting at E15 showed increased placental GLUT1 protein expression by 1.6 and 1.9 fold, respectively at E21 indicating a dose-dependent effect (Langdown & Sugden, 2001).

Mice exposed to corticosterone in their drinking water at E11-E16 had increased placental GLUT1 and GLUT3 gene expression at E16, while mice exposed at E14-E19 showed unchanged expression (Vaughan *et al.*, 2015). Unlike the transporter expression at both timepoints, transplacental clearance of radiolabeled glucose was unchanged at E16 after the exposure from E11-E16, but clearance was reduced by 33% at E19 after exposure from E14-E19 (Vaughan *et al.*, 2015). This suggests that other glucose transporters may be involved in placental glucose uptake (Vaughan *et al.*, 2015). Opposite results show that mice treated with dexamethasone for 60 hours via a minipump starting at E12.5 showed unaltered gene expression of GLUT1 and GLUT3 at E14.5 and at E17.5 (Cuffe *et al.*, 2011*a*).

Human placental extracts from term deliveries further showed reductions in GLUT1 mRNA and protein expression after TA treatment along with reduced protein expression of GLUT3 but unaltered GLUT3 mRNA expression (Hahn *et al.*, 1999*a*). Another study showed that human placental extracts from term deliveries had unaltered GLUT1 mRNA expression but increased GLUT1 protein expression when treated with 1, 2, and 20 mg/ml of hydrocortisone (Mateos *et al.*, 2018). GLUT3 mRNA expression was increased when placental explants were treated with 2mg/ml hydrocortisone only (Mateos *et al.*, 2018). Despite the increased GLUT3 mRNA expression at 2mg/ml and the increased GLUT1 protein expression at all doses of hydrocortisone, placental uptake of radiolabeled glucose was decreased by 30-40% when explants were treated with 2 and 20 mg/ml hydrocortisone (Mateos *et al.*, 2018).
Refer to *Appendix A* for a table summary of results.

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.

Midgestation administration of dexamethasone in mice at E13.5 and E14.5 caused unaltered placental System A transfer of radiolabeled amino acid at E15.5 and E17.5 along with unchanged mRNA expression of SNAT1, SNAT2 and SNAT4 in male and female placentas (Audette *et al.*, 2011*a*). However, at E18.5, system A mediated amino acid transfer of radiolabeled amino acid was reduced in male and female placentas despite no significant changes in SNAT1, SNAT2, and SNAT4 transporter expression at E18.5 (Audette *et al.*, 2011*a*). This indicates a potential long-term effect of midgestational dexamethasone exposure on placental system A amino acid transfer (Audette *et al.*, 2011*a*). Furthermore, at E16, mice treated with dexamethasone in their drinking water at E11-E16 showed unchanged fetal accumulation of radiolabeled amino acid despite a 35% increase in placental radiolabeled amino acid accumulation and increased SNAT1 and SNAT2 gene expression but unaltered SNAT4 expression (Vaughan *et al.*, 2012). At E19, mice given dexamethasone at E14-E19 had reduced fetal and placental accumulation of radiolabeled amino acid by 40-50% despite showing increased SNAT1 expression with unchanged SNAT 2 and SNAT4 expression (Vaughan *et al.*, 2012). Mouse placentas at E19 from those given dexamethasone at E11-E16 had a 38% increase in fetal accumulation of amino acid despite no change in placental accumulation and no change in SNAT1, SNAT2, and SNAT4 expression (Vaughan *et al.*, 2012). This further indicates a time-dependent effect on amino acid transporter expression, placental transport, and fetal accumulation. No change in gene expression of SNAT1, SNAT2, and SNAT4 at E14.5 and at E17.5 was detected in mice given dexamethasone for 60 hours starting at E12.5 (Cuffe *et al.*, 2011*a*).

Human placental explants from term pregnancies were incubated with dexamethasone for 48 hours showed 30% increase in placental uptake of radiolabeled amino acid at 10^{-6} M but not at 10^{-8} M despite no changes in mRNA expression of SNAT1, SNAT2 or SNAT4 at both concentrations (Audette *et al.*, 2010). Human term placental extracts from women who were treated with glucocorticoids during gestation showed varied effects depending on the intermittent time between the treatment and delivery (Audette *et al.*, 2014). Uptake of radiolabeled amino acid by placentas of mothers who delivered 14 days after the GC treatment but prior to term was lower than uptake from delivered placentas within less than 14 days of treatment (Audette *et al.*, 2014). Compared to term controls of untreated mothers, term placentas from GC treated mothers had significantly lower system A transport (Audette *et al.*, 2014). Gene expression revealed no effect on SNAT1 and SNAT2 across all treatment groups and the control, but GC-treated term placentas had reduced SNAT4 expression compared to GC-treated placentas delivered after 14 days of treatment but prior to term (Audette *et al.*, 2014). This further suggests that long-term effects of dexamethasone may be more critical given that the reduced transport was amplified in GC-treated placentas delivered after 14 days of treatment but prior to term and in GC-treated term placentas compared to placentas delivered within 14 days of treatment (Audette *et al.*, 2014).

In humans, system A transport of radiolabeled amino acids was significantly lower in placental explants of IUGR deliveries (Shibata *et al.*, 2008). Additionally, placental uptake of lysine was reduced at the basolateral membrane, while system-L-mediated uptake of leucine was reduced in the microvillous and basal membranes of placental explants from IUGR pregnancies (Jansson *et al.*, 1998). IUGR placentas had 34% lower sodium-dependent taurine transport in the microvillous membrane compared to healthy controls (Norberg *et al.*, 1998).

BeWo choriocarcinoma human placental cell lines showed higher radiolabeled sodium-dependent amino acid transfer between membranes when incubated with 1000nM cortisol (Jones *et al.*, 2006). SNAT1 mRNA expression was unchanged when BeWo cells were incubated with cortisol at 20, 50, 1000, and 2500 nM, but SNAT2 mRNA expression increased by 21% and 30% when incubated with cortisol at

1000nM and 2500nM (Jones *et al.*, 2006). SNAT2 protein expression further showed an 11% increase with 1000nM cortisol (Jones *et al.*, 2006).
Refer to *Appendix A* for a table summary of results.

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).

To my knowledge, lipid transporter expression and transport activity have not been assessed after antenatal GC exposure, but one study did assess lipoprotein lipase activity along with fatty acid esterification and oxidation (Mateos *et al.*, 2018).Using placental explants from term deliveries, fatty acid utilization and storage was altered when cells were treated with hydrocortisone. Fatty acid oxidation was reduced by 25, 50 and 75% in explants treated with 1, 2 and 20 mg/ml hydrocortisone (Mateos *et al.*, 2018). Fatty acid esterification was also reduced at all doses used. Lipoprotein lipase (LPL) activity showed reductions by 40 and 80% when cells were incubated with 2 and 20 mg/ml hydrocortisone (Mateos *et al.*, 2018). Hence, fatty acid uptake, storage and oxidation were all impaired. This same study showed reductions in radiolabeled glucose uptake at 2 and 20 mg/ml doses, and the reduced fatty acid metabolism capacity further suggests failed placental compensatory mechanism to utilize fatty acids when glucose uptake is compromised, despite the availability of nutrients (Mateos *et al.*, 2018).

In humans, microvillous membrane LPL activity was reduced by 47% in placentas of IUGR preterm pregnancies (Magnusson *et al.*, 2004). Additionally, LDL receptor protein levels were reduced in placentas from pregnancies with IUGR (Wadsack et al., 2007).
Finally, ultrasound images of human growth-restricted fetuses showed reduced fat and lean mass, suggesting fetal nutrient deprivation (Padoan *et al.*, 2004).
Refer to *Appendix A* for a table summary of results.

Effect of Glucocorticoid Exposure on Placental mTORC1 Function

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). Fewer studies have assessed the relationship between maternal GC exposure and placental mTORC1 activity in rodents or humans, but its activity is reduced in intrauterine growth restriction (Roos *et al.*, 2007). Mice exposed to corticosterone at E14-E19 had reduced mTORC1 activity at E19 evident by the reduced p4E-BP1 and pS6K expression, downstream targets of mTORC1, and increased REDD1 expression which is an inhibitor of mTORC1 signaling (Vaughan *et al.*, 2015). Mice exposed to corticosterone at E11-E16 had reduced pAKT levels but unchanged total AKT levels and unchanged REDD1 expression, suggesting a minimal effect on mTORC1 function at E16 (Vaughan *et al.*, 2015).

Effect of Glucocorticoid Exposure on Placental Endocrine Function

Pregnant rats treated with dexamethasone at E13 had reduced *Igf2* mRNA expression in the junctional zone but unaltered expression in the labyrinth zone (Ain *et al.*, 2005*a*). Conversely, pregnant mice

exposed to glucocorticoids at midgestation showed no change in placental *Igf2* gene expression (Baisden *et al.*, 2007*a*; Cuffe *et al.*, 2011*a*; Vaughan *et al.*, 2015).

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 GC or psychological stress exposures. 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). Based on our results, other placental hormones may be assessed in the future.

Effect of In Utero Glucocorticoid Exposure on Offspring

Women with higher corticotropin-releasing hormone (CRH) at midgestation, were 7.5 fold more likely to deliver preterm (Inder *et al.*, 2001). However, offspring outcome remains conflicting. In humans, antenatal corticosteroid exposure caused higher systolic and diastolic blood pressure in children ages 14 years (Doyle *et al.*, 2000). At 30 years of age, offspring of mothers who received antenatal betamethasone had higher insulin levels 30 minutes after a glucose tolerance test with lower glucose concentrations at 120 minutes, but offspring did not have altered cortisol levels, lipid profile or blood pressure (Dalziel *et al.*, 2005). This suggests an impaired insulin sensitivity (Dalziel *et al.*, 2005). Additionally, body composition of children of mothers who had higher cortisol levels during pregnancy showed increased fat mass index in girls but a lower fat mass index in boys indicating a sex-difference (Van Dijk *et al.*, 2012). Maternal third trimester cortisol levels were positively associated with infant body fat percent increase from age 1-6 month suggesting programmed adiposity that can contribute to childhood obesity (Entringer *et al.*, 2016). Antenatal GC treatment showed a blunted HPA axis activity in infants ages 3-6 days after a stressful exposure (Davis *et al.*, 2004). Despite showing reduced cortisol levels in newborns exposed to antenatal glucocorticoids, long-term effects vary. Children ages 6-11 years who were exposed to antenatal glucocorticoids had higher cortisol levels in response after a standardized stressful test, and this difference was mainly influenced by higher salivary cortisol in females, indicating a potential sex-dependent elevation in HPA axis activity effect (Alexander *et al.*, 2012). Studies have shown multiple offspring outcomes including increased blood pressure in children, increased risk of preeclampsia, impaired mental development in infants, increased infant cortisol, reduced fetal weight, and other symptoms associated with timing, dosage and type of corticosteroid treatment during pregnancy (Singh *et al.*, 2012).

In mice, male offspring exposed to dexamethasone for 60 hours starting at E12.5 had lower fetal weights at E14.5 but not at E17.5 (O'Sullivan *et al.*, 2013). The male offspring from similarly treated dams had similar weights at 2 weeks, 4 weeks, and 3 and 6 months of age (O'Sullivan *et al.*, 2013). Twenty-one-day-old rats exposed to antenatal dexamethasone at E15 till delivery had 66% lower body weights than controls, lower corticotropin releasing hormone content and concentrations, lower adrenal and plasma corticosterone levels and severe adrenal atrophy (Dupouy *et al.*, 1987). Conversely, rats exposed to antenatal glucocorticoids showed unaltered ACTH and corticosterone plasma levels at PND1,7,9, and 20 but had a suggested increased HPA axis activity when stress was induced during adulthood (Bakker *et al.*, 1995). Offspring of physically stressed rats during gestation showed higher plasma glucose levels at 24 months of age despite similar insulin secretion when challenged with oral glucose tolerance test (Lesage *et al.*, 2004).

Experimental Design

To determine how glucocorticoid exposure affects placental function, we will obtain a total of 80 female and 80 male mice (n=10 females and 10 males per group- 8 groups total)[5] that are 8 week-old C57BL/6 virgin mice from Jackson laboratory. Mice will be given two weeks to acclimatize with *ad libitum* access to normal chow diet and water. After acclimatization, mice will then be single-housed and randomized into one of the following groups, to assess placental morphology (at E14.5) and effects on offspring (from delivery to 6 weeks of age). The experimental design is represented in Figure 2. Pending these results other groups may be evaluated at different gestation time points.

Cohort A of groups treated one week prior to gestation:
1. *Water Pre-gestation till E14.5:* control group on water one week prior to conception and until midgestation at embryonic day 14.5
2. *Dexamethasone Pre-gestation till E14.5*: experimental group exposed to dexamethasone in drinking water a week prior to conception and until midgestation at embryonic day 14.5
3. *Water Pre-gestation till Delivery*: control group on water one week prior to conception and until delivery
4. *Dexamethasone Pre-gestation till Delivery*: experimental group exposed to dexamethasone in drinking water a week prior to conception and until delivery

Cohort B of groups treated at conception:
1. *Water Conception till E14.5*: control group on water starting at conception and until midgestation at embryonic day 14.5
2. *Dexamethasone Conception till E14.5*: experimental group exposed to dexamethasone in drinking water starting at conception and until midgestation at embryonic day 14.5
3. *Water Conception till Delivery*: control group on water starting at conception and until delivery
4. *Dexamethasone Conception till Delivery*: experimental group exposed to dexamethasone in drinking water starting at conception and until delivery

All groups will have *ad libitum* access to normal chow diet and water or dexamethasone depending on treatment arm. Experimental groups will receive 1mg/kg/day dexamethasone in their drinking water with *ad libitum* access. For groups of Cohort A (receiving dex or water a week prior to conception), female mice will be mated with age-matched male mice after one week of treatment. A copulatory plug will be checked daily to identify E0.5 day. For groups of Cohort B (receiving dex or Water at conception), 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 to determine treatment initiation. Once a copulatory plug is identified, mice will be placed on dexamethasone or water based on their assigned group.

Males will be removed from the cage after a copulatory plug is detected to minimize male exposure to treatment and to better detect potential miscarriages. 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 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.

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[5] Powered to see 1.2SD effect size in placental and offspring weights, we need 10 female mice and 10 male mice per group (8 groups total) to get a power of 0.825.

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 that will be allowed to complete their pregnancy and deliver their pups at around E21.5, survival and birth rates will be noted. Water and dex groups that will complete their pregnancy and deliver their pups will have *ad libitum* access to normal chow diet and will be placed on regular water immediately after parturition and during lactation (no dex exposure during lactation). Pups will be sexed and culled to 4 (ideally 2 males and 2 females) 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 treatment group. 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 (gonadal white adipose tissue-gWAT and inguinal white adipose tissue-iWAT) will be collected and weighed to determine adiposity.
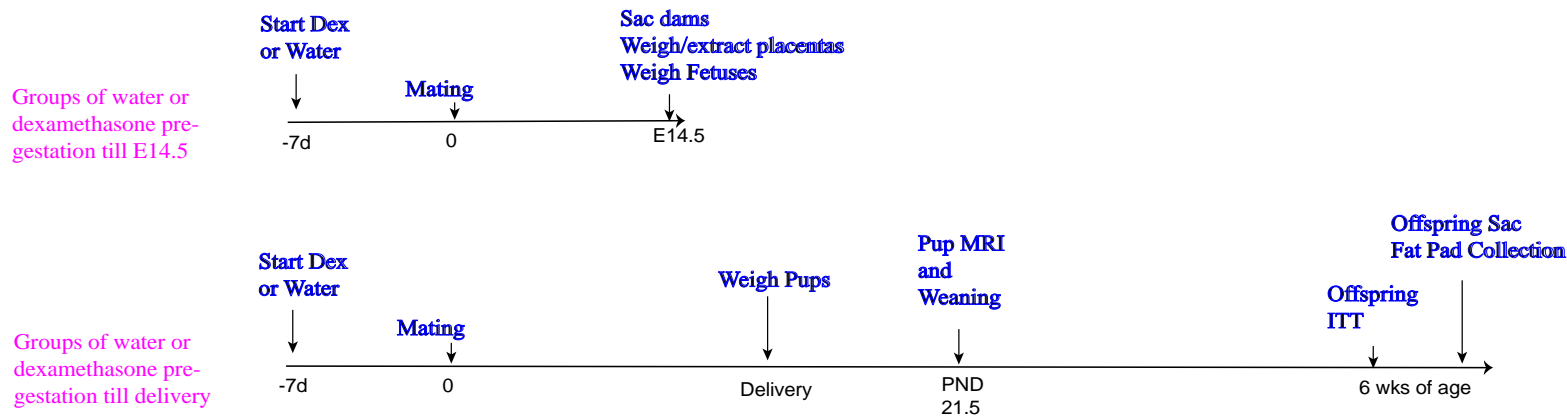
To determine if the effects of dexamethasone exposure on the placenta and the fetus can be rescued, we will develop a placenta-specific glucocorticoid receptor knockout (KO) model. To isolate placental from fetal and maternal glucocorticoid signaling, our knockout model will ablate GR conditionally in the placenta. To my knowledge, this is the first time such a model has been generated. To generate the GR-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 exon 2 of *Nr3c1* will be crossed with a male having placental driver *Cyp19a1-Cre*$^{Tg/+}$ (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 (KO) *Nr3c1* $^{fl/fl}$; *Cyp19a1-Cre*$^{Tg/+}$, conditionally heterozygous *Nr3c1* $^{fl/+}$; *Cyp19a1-Cre*$^{Tg/+}$, and wild-type *Nr3c1* $^{fl/fl}$; *Cyp19a1-Cre* $^{+/+}$, *Nr3c1* $^{fl/+}$; *Cyp19a1-Cre* $^{+/+}$, *Nr3c1* $^{+/+}$; *Cyp19a1-Cre* $^{+/+}$, or *Nr3c1* $^{fl/fl}$; *Cyp19a1-Cre* $^{Tg/+}$ (no Cre transgene) at an expected ratio of 1:2:5 with the knockout (*Nr3c1* $^{fl/fl}$; *Cyp19a1-Cre*$^{Tg/+}$) and wild-type (*Nr3c1* $^{fl/fl}$; *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 but a phenotypically WT embryo.

The dams with GR-KO will be treated with dexamethasone similar to the previous groups in cohorts A and B to determine placental, embryonic, and offspring function and growth.

Figure 2: Diagram representing the experimental design and respective timeline

Cohort A

Groups of water or dexamethasone pre-gestation till E14.5

Start Dex or Water
Mating
Sac dams
Weigh/extract placentas
Weigh Fetuses

-7d   0   E14.5

Groups of water or dexamethasone pre-gestation till delivery

Start Dex or Water
Mating
Weigh Pups
Pup MRI and Weaning
Offspring ITT
Offspring Sac
Fat Pad Collection

-7d   0   Delivery   PND 21.5   6 wks of age

Cohort B

Groups of water or dexamethasone at conception till E14.5

Start Dex or Water
Mating
Sac dams
Weigh/extract placentas
Weigh Fetuses

0   E0.5   E14.5

Groups of water or dexamethasone at conception till delivery

Start Dex or Water
Mating
Weigh Pups
Pup MRI and Weaning
Offspring ITT
Offspring Sac
Fat Pad Collection

0   E0.5   Delivery   PND 21.5   6 wks of age

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

♀ $Nr3c1^{fl/fl}$;$Cyp19a1$-$Cre^{+/+}$ x   ♂ $Nr3c1^{+/+}$;$Cyp19a1$-$Cre^{Tg/+}$

9-12 weeks expected timeline

$Nr3c1^{fl/+}$;$Cyp19a1$-$Cre^{+/+}$ & $Nr3c1^{fl/+}$;$Cyp19a1$-$Cre^{Tg/+}$
1WT : 1Het

♀ $Nr3c1^{fl/+}$;$Cyp19a1$-$Cre^{+/+}$ x   ♂ $Nr3c1^{fl/+}$;$Cyp19a1$-$Cre^{Tg/+}$

9-12 weeks expected timeline

$Nr3c1^{fl/fl}$; $Cyp19a1$- $Cre^{Tg/+}$ — 1

$Nr3c1^{fl/fl}$; $Cyp19a1$- $Cre^{+/+}$ — 1

$Nr3c1^{fl/+}$; $Cyp19a1$- $Cre^{Tg/+}$ — 2

$Nr3c1^{fl/+}$; $Cyp19a1$- $Cre^{+/+}$ — 2

$Nr3c1^{+/+}$; $Cyp19a1$- $Cre^{Tg/+}$ — 1

$Nr3c1^{+/+}$; $Cyp19a1$- $Cre^{+/+}$ — 1

1KO : 2Het : 5WT

♀ $Nr3c1^{fl/fl}$;$Cyp19a1$-$Cre^{+/+}$ x   ♂ $Nr3c1^{fl/fl}$;$Cyp19a1$-$Cre^{Tg/+}$

2-12 weeks expected timeline

$Nr3c1^{fl/fl}$;$Cyp19a1$-$Cre^{+/+}$ & $Nr3c1^{fl/fl}$;$Cyp19a1$-$Cre^{Tg/+}$
1WT : 1KO

Methods

Dexamethasone Exposure

Water-soluble dexamethasone (Sigma) will be prepared at a concentration of 53 mg/L, which our previous work shown results in a dose of approximately 1 mg/kg/day in non-pregnant mice.

If the dam is single housed or nursing pups:

> (the new added total water/dexamethasone- the last measurement's water/dexamethasone) / # of days between measurements

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

> (the new added total water/dexamethasone - the last measurement's water/dexamethasone) * #of days between measurements / sum of days spent by each mouse in that cage between measurements

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, PCR will be performed for *Sry* to determine the sex of the placentas/fetuses using fetal tails. We will use sequence-specific primers to amplify *Sry* gene. Briefly, the tail will be homogenized and treated to collect the DNA. Sample, forward and reverse *Sry* primers, dNTP, polymerase, sterile water, and manufacturer buffer will be mixed. Thermal cycler will be run at various temperatures for ~4 hours.

RNAseq

To determine gene expression of placentas exposed to antenatal GC, we will perform RNAseq studies. We will use 5 male and 5 female placentas from each group exposed to water or dexamethasone. RNAseq will be done by the University of Michigan DNA Core. We will align reads to the mouse genome using a TopHat/DESeq/GSEA pipeline to identify differentially expressed placental genes. To determine the enriched core, we will use the genes of interest involved in nutrient transport and endocrine function of the placenta. We will determine if dexamethasone effects on the placenta are sex-dependent. We will use network analysis leveraging TRANSFAC and the Signaling Pathway Project to identify potential transcription factors and pathways at play. Our lab has extensive experience in performing RNAseq studies (Lu *et al.*, 2014; Hochberg *et al.*, 2015; Urraca *et al.*, 2015; Ponnusamy *et al.*, 2017; Ochsner *et al.*, 2019).

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 *Nr3c1* gene will be conducted to determine gene expression. For fetal/placental genotyping, fetal tail will be entirely clipped for DNA analysis along with a section of the placenta to confirm expression of *Nr3c1.*

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 1.1: How does maternal GC exposure affect placental development, fetal growth, and fetal survival?

I hypothesize that our prolonged dexamethasone exposure will reduce placental and fetal weights along with reduced placental labyrinth zone area causing intrauterine growth restriction (IUGR) in group of dexamethasone pre-gestation till E14.5 and in the group of dexamethasone conception till E14.5 with more pronounced effect in the groups treated with dexamethasone pre-gestation over groups treated at conception. Pregnant mice exposed to dexamethasone on E15, E16, and E17 had reduced placental and fetal weights and trophoblast swelling in the junctional and labyrinth zones (Baisden *et al.*, 2007*a*). Furthermore, mice given dexamethasone at E11-E16 had reduced fetal and placental weights at E16, but the volume of the placental junctional and labyrinth zone was unchanged despite less fetal capillaries in the labyrinth zone (Vaughan *et al.*, 2012), which suggests that our prolonged exposure will have more drastic effects on placental labyrinth area. To my knowledge, studies assessing effects of glucocorticoid treatment pre-gestation or very early in gestation are lacking. My preliminary data shows that mice treated with 1 mg/kg of the synthetic glucocorticoid dexamethasone in the drinking water from 1 week prior to mating through pregnancy had 1) dramatically reduced fertility whereby litter size from treated dams was 34% lower and had 2) 37% reduction in offspring birth weight. I thus expect fetal survival to be reduced as evident by resorption more so in the dexamethasone pre-gestation group than in the conception group.

Aim 1.2: How does maternal GC exposure affect the expression of placental nutrient transporters?

The experiments conducted in this aim will examine the effect of dexamethasone treatment on placental transport and transporters. I predict that placental glucose transporters, GLUT1 and GLUT3, will have increased gene expression. This is supported by increased placental GLUT1 protein expression at E21 in rats exposed to 100 and 200 ug/kg body weight/ day of dexamethasone starting at E15 (Langdown & Sugden, 2001). Furthermore, mice exposed to corticosterone in their drinking water at E11-E16 had increased placental GLUT1 and GLUT3 gene expression at E16 (Vaughan *et al.*, 2015). This exposure timing (E11-E16) covers our window of interest at E14.5, but it is worthy to note that not all

studies detected changes in glucose transporter expression during pregnancy using various exposure windows and doses (Cuffe *et al.*, 2011*a*; Vaughan *et al.*, 2015; Mateos *et al.*, 2018).

Since system A amino acid transport is the most associated with growth restriction (Glazier *et al.*, 1997), which is expected to occur after our exposure (from *Aim* 1), my hypothesis will focus on SNAT1, SNAT2, and SNAT4 expression. I predict increased placental system A amino acid transporter expression of SNAT1, SNAT2 but unchanged SNAT4 with our GC exposures. This hypothesis is supported by increased SNAT1 and SNAT2 gene expression but unaltered SNAT4 expression at E16 in mice treated with dexamethasone in their drinking water at E11-E16, which covers our window of interest at E14.5 (Vaughan *et al.*, 2012). At E19, mice given dexamethasone at E14-E19 also showed increased SNAT1 expression but unchanged SNAT2 and SNAT4 expression (Vaughan *et al.*, 2012). This further indicates a time-dependent effect on amino acid transporter expression, by which our longer exposure starting at pre-gestation or at conception will yield more similar results to the exposure given at E11-E16. It is worth noting that different exposure periods and pregnancy timepoints did not show a change in expression of all the SNAT genes (Jones *et al.*, 2006; Audette *et al.*, 2010, 2011*a*, 2014; Cuffe *et al.*, 2011*a*).

I hypothesize that LPL and LDL receptor will be downregulated in mouse placentas. This hypothesis is based on findings of reduced placental LPL activity in hydrocortisone treated term placental explants (Mateos *et al.*, 2018) and reduced LDL receptor expression in placentas from IUGR pregnancies (Wadsack *et al.*, 2007).

Aim 1.3: Is placental mTORC1 signaling altered after maternal GC exposure?
I hypothesize that placental mTORC1 activity will be reduced in placentas exposed to dexamethasone. This is because mice exposed to corticosterone at E14-E19 had reduced expression of downstream targets of mTORC1 and increased expression of REDD1, an inhibitor of mTORC1 signaling (Vaughan *et al.*, 2015). Furthermore, the expected IUGR (from *Aim 1*) may be supportive of reduced mTORC1 signaling.

Aim 1.4: How does maternal GC exposure affect placental endocrine function?
I hypothesize GDF15, an anorexic hormone, to be downregulated in the placentas of dexamethasone-treated animals. 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 activity is predicted to be reduced (from *Aim 3*), then GDF15 levels should decrease accordingly.
I expect IGF2 expression to be reduced since pregnant rats treated with dexamethasone at E13 had reduced *Igf2* mRNA expression in the junctional zone despite unaltered expression in the labyrinth zone (Ain *et al.*, 2005*a*). Since our exposure is more chronic and placental weight and development is expected to be reduced (from *Aim 1*), then placental *Igf2* should be reduced.

Aim 1.5: Is offspring survival, weight, body composition and insulin sensitivity altered with maternal GC exposure?
Given the severe expected IUGR, reduced fetal weights, and increased resorption (from *Aim 1*), I predict that offspring survival will be reduced. Our preliminary data shows that pre-gestational exposure will dramatically reduce offspring survival at PND0.5-1 with 100% lethality of all pups from dexamethasone-treated dams. Dexamethasone exposure at conception is similarly predicted to reduce offspring survival within PND0.5-1 but to a lesser degree than the pre-gestational exposure. I predict offspring body

weight to be relatively similar at 6 weeks as seen in adult rats (Lesage *et al.*, 2004; O'Sullivan *et al.*, 2013), but the offspring will have higher fat mass as seen in human studies (Van Dijk *et al.*, 2012). Furthermore, since our exposure is longer than that of most studies, offspring may show increased insulin levels during the ITT at 6 weeks of age. In support of this hypothesis, 30-year-old human offspring of mothers who had received antenatal betamethasone had higher insulin levels 30 minutes after a glucose tolerance test (Dalziel *et al.*, 2005). On the other hand, adult rat offspring of antenatally stressed dams had unaffected insulin secretion in response to an OGTT (Lesage *et al.*, 2004), but since our exposure is more chronic prior to or at gestation, then I expect insulin levels to be higher indicating less insulin sensitivity.

Aim 1.6: Does a placental GR-KO model rescue the placental and fetal effects of GC exposure?
Based on the results of Aims 1.1-1.5 we will have identified critical glucocorticoid-induced changes in placental gene expression, signaling, placental and fetal size and offspring health. Those models however do not separate effects of dexamethasone on the mother from those on the placenta. To separate these we will use placental GR knockouts and repeat these studies. We expect that placental-derived glucocorticoid actions will be blocked by GR knockout in the placenta, but maternal glucocorticoid actions will be retained.

Potential Pitfalls and Alternate Approaches (Aims 1.1-1.6)
It is possible that mice in the pre-gestation dexamethasone will not conceive immediately upon mating thus causing their 1 week dexamethasone exposure prior to mating to be prolonged. We will have to eliminate all dams that will be exposed to pre-gestational dexamethasone for more than a week and the half prior to conception, thus we may need more mice. It is also possible for both groups that the mice may have spontaneous abortions and resorptions due to induced dexamethasone-induced stress even prior to the E14.5 time point. We may thus need to alter our exposure time and try different exposure windows for shorter periods of time during gestation. It is also possible that our placental GR KO model may prove lethal. In that case, we will use a different parental strain of Hets (Het x Het) to generate a partial knockout that may prove viable.

Paper	Methods/Exposure	Results
(Hahn *et al.*, 1999*b*)	Human placental extracts from term pregnancy treated with triamcinolone (TA) E21 Rat placentas from rats injected with 0.38mg/kg TA once at E16 Mouse E17 placentas from GR transgenic mice using antisense RNA – this antisense is in the mother, but in placenta GR protein expression was reduced by 28%	Human TB cells had GLUT1 on MVM, GLUT3 on endothelial cells GLUT1 mRNA and protein reduced after TA GLUT3 mRNA unaffected, but protein decreased In rat and mouse, GLUT1 and GLUT3 localized in STB, CTB and endothelial cells(weakest in CTB) In rats, fetal and placental weights reduced by 73% and 53%, respectively at E21. Implantation number unaffected GLUT1 and GLUT3 mRNA and protein reduced after TA Placental wt of transgenic mice reduced by 28%, offspring of transgenic mice were 20% lighter GLUT1 mRNA and protein was reduced GLUT3 mRNA and protein increased
(Vaughan *et al.*, 2015)	2 Mice cohorts given corticosterone in drinking water at two intervals: 1. E11-E16 2. E14-E19 The dose was designed to produce plasma cort levels that are high and similar to concentrations reported in heat/light stressed dams Unidirectional materno-fetal clearance of non-metabolizable glucose was assessed	On D19, transplacental 3Hmethyl-D-glucose clearance decreased by 33% Cort reduced fetal weight by 8% and 19% at D16 and D19, respectively Placental weight was reduced at both points Number of viable pups was unaffected At D19, materno-fetal clearance and fetal accumulation of glucose tracer was lower than controls at E19. No difference in clearance or accumulation at D16 Placental *Slc2a1&3* (GLUT 1 and 3) mRNA expression increased at E16, no change in expression on E19 *Redd1* expression increased on D19 but not D16 with cort and was in sync with the reduced transplacental glucose transport at D19 No change in placental *Igf2* expression On D16, pAkt was reduced → less active Akt
(Vaughan *et al.*, 2012)	2 Mice cohorts given corticosterone in drinking water at two intervals:	Fetal weight reduced in both At D16, no effect on materno-fetal transfer of labeled amino acid

	1. E11-E16 2. E14-E19	Fetal and placental weight reduced by 7% on D16 On D19, fetus weight decreased by 16% and placental weight was 11% smaller Fetal weight negatively correlated with maternal corticosteroid levels at E19 but not E16 Number of viable pups per litter was unchanged with maternal cort Fetal accumulation of MeAIB was not changed at E16, but placental accumulation was 35% more (expression of placental transporters was up as well, mentioned below) At E19, placental and fetal MeAIB accumulation was reduced by 40-50%, after tx from E14-E19 (although placental transporter snat1 increased and others did not change) Oppositely at E19, from dams treated E11-E16 (3 days post tx), fetal accumulation and clearance were 38% higher but placental accumulation was unchanged → longer term effects of GC tx after cessation of tx At E16, *Slc38a1 and 2* expression in placenta was increased, *Slc38a4* was unchanged At E19, *Slc38a1* expression increased, but no change in *Slc38a2 or 4* Placentas weighed less at E16 but volume of zones did not differ. No difference in zone at E19 Reduced vascularity shown by less fetal capillaries in the labyrinthine zone by 55% at E16
(Audette *et al.*, 2010)	Human placental explants from term-pregnancies in healthy women Placental explants incubated with radiolabeled 14C-MeAIB for different periods Dex added at 10^{-6} M	Dex treatment increased placental uptake of MeAIB at 10^{-6}M but not at 10^{-8}M → stimulated system A activity at 10^{-6}M with 30% increase of MeAIB uptake No change in mRNA expression of SNAT1,2 or 4 with Dex. No effect on placental apoptosis
(Jones *et al.*, 2006)	BeWo choriocarcinoma cell line used with 14CMeAIB infusion to assess transport of system A aa Cortisol was added to incubated cells at concentrations 5nM-2.5uM for up to 24 hours	BeWo cells incubated with 1000nM cortisol had higher MeAIB transfer from apical to basolateral chambers over 20 minutes SNAT1 mRNA was unchanged with cortisol at multiple concentrations

		SNAT2 mRNA levels increased by 21% at 24h incubation of 1uM cortisol. Cort exposure of 2.5uM for 24 hours increase SNAT2 mRNA expression by 30%
		Protein expression of SNAT1 was not assessed Protein expression of SNAT2 showed increased expression with 1uM of cortisol for 24 hours by 11%
(Audette *et al.*, 2011*b*)	Pregnant mice treated with 0.1mg/kg dex injected at E13.5 and E14.5 (midgestation exposure) Transfer studies done at E12.5, E15.5 (24hr after tx) , E17.5 (72h after tx) and E18.5 (96h after tx) Subset of dams were allowed to deliver their pups	In saline injected controls, placental and fetal weights increased from E12.5 to E15.5 to E18.5. Placental 14CMeAIB transfer also increased which was consistent with increases in system A gene expression of SNAT1, 2 and 4 as pregnancy progressed. Effects of Dex: Treatment from E13.5 and E14.5 did not alter 14CMeAIB transfer at E15.5 or E17.5, but transfer was reduced at E18.5 in male and female placentas (long-term after treatment cessation). SNAT1,2 and 4 mRNA expression was unchanged with Dex in male and female placentas at E15.5, 17.5 and 18.5 (despite reduced transfer at E18.5) Fetal weights at E15.5, E18.5 or at birth was unchanged. No change on placental weight at E15.5, E17.5 and E18.5 in males. In females there was no change at E15.5 or E17.5, but placental weight was reduced at E18.5 → the reduced female placental weight at E18.5 increased the fetal:placental ratio at E18.5 No change in placental labyrinth or junctional zone proportions w.r.t. total placental area No difference in maternal or fetal plasma corticosterone concentrations at E18.5
(Cuffe *et al.*, 2011*b*)	Pregnant mice treated with Dex 1ug/kg/h minipump for 60 hours (2.5 days) via a minipump starting at E12.5 Placentas collected at E14.5 (2 days- 48 hours) and at E17.5 (after 5 days of initial exposure, after 2.5 days from end of exposure)	Reduced fetal body weight at E14.5 in males and females, but not at E17.5. Reduced female placental weight at E14.5 but not E17.5. Male placental weight was unchanged in both days. *Igf2* expression not affected by Dex at either age. GLUT1, GLUT3, SNAT 1, SNAT2 and SNAT4 gene expression was unaltered after Dex at E14.5 and E17.5 No differences in placental areas or gross morphology Female junctional zone cross sectional area was smaller at E14.5.

		Whole placental cross sectional area was smaller.
(Audette *et al.*, 2014)	Used placental extracts from pregnancies treated with GC who delivered at various times during gestation. Women recruited if they received 2 doses of celestone (betamethasone 12 mg intramuscular ~12 hours apart) at 23.6 and 33.9 weeks of gestation Groups: 1. mom who delivered preterm 24h-14 days after tx 2. who delivered 14d-after treatment but still delivered before term 3.who received GC but delivered at term	Fetuses born 24hours-14 days after the GC treatment (preterm delivery) had reduced birth weight compared to fetuses born 14days post treatment until term (term pregnancies). No difference between birth weight of GC treated fetuses at term and term controls (not treated with GC) Placentas of fetuses delivered between 24h-14d after the tx had significantly lower weights compared to placentas from 14d-term deliveries with GC. Uptake of 14CMeAIB by placental explants from GC treated moms who delivered 14d-term or at term after the treatment had reduced system A activity compared to placentas from preterm delivery. Placentas from preterm delivery (24h-14d post GC) had no change in MeAIB uptake compared to control term placentas Placentas of GC treated moms who delivered at term had significantly reduced system A transport compared to control term placentas. Expression of placental AA transporters: No effect of SNAT 1 or SNAT2 SNAT4 gene expression was reduced in placentas of GC treated moms at term compared to GC treated placentas of fetuses born 14d-term after GC treatment
(Mateos *et al.*, 2018)	Placentas obtained from healthy women who delivered at term. Placental explants cultured with or without GC hydrocortisone 1mg/ml (2.75 mM)	Placentas incubated with 1mg/ml hydrocortisone had unchanged 3H-2DOG uptake, but higher concentrations of 2mg/ml and 20mg/ml showed reduced DOG uptake by 30-40% Expression of GLUT1 was not changed with all concentrations GLUT3 mRNA expression was increased with 2mg/ml incubation only GLUT1 protein expression was increased at 1mg/ml, 2mg/ml and 20mg/ml of hydrocortisone Fatty acid oxidation was reduced by 25%, 50% and 75% in explants treated with 1, 2 and 20 mg/ml, respectively Fatty acid esterification (to make TG or to undergo oxidation) was also reduced at all concentrations, consistent with the fact that there was less oxidation.

		Lipoprotein lipase activity was reduced significantly by 40% and 80% at 2 and 20 mg/ml doses, respectively (LPL is needed to allow uptake of fatty acids that will then become esterified and undergo oxidation or become TG)
		Mitochondrial activity in placental explants was significantly reduced at 20mg/ml only, but TUNEL analysis showed no differences in apoptosis
		Hence, glucose and lipid uptake were reduced in placentas despite available nutrients
(Baisden *et al.*, 2007*b*)	Pregnant mice injected with 0.5mg/kg intraperitoneal dexamethasone on E15, E16 and E17 to mimic multiple course of antenatal GC treatment	At E20, dex placentas were pale and weighed less.
		Dex treatment was not associated with fetal death.
		Trophoblasts in labyrinth and junctional zones were swollen with loss of TB in junctional zone (marked by empty space in H&E stain)
		Downregulation of 1212 genes and up-regulation of 1382 genes → decreased expression of genes involved in cell division with mixed responses on genes regulating glucose, cholesterol and steroid metabolism
		No difference in gene expression of *Igf1 or 2*
(Braun *et al.*, 2013*b*)	Mothers who received a single course of betamethasone treatment during pregnancy Single course is 2 x 12 mg betamethasone in 2 consecutive days given intramuscularly Collected maternal plasma at 4 timepoints: 1.prior to first GC administration 2. 24 hours after first GC administration and right before the second dose of 12mg betamethasone 3. 48 hours after the first GC tx (24h after second dose) 4. Finally, one sample collected during delivery at 4-5cm cervical dilation	Single Betamethasone treatment was associated with reduced fetal growth and reduced head circumference. Birth weight was reduced by 18.2% after betamethasone. Placental width was reduced by 5.5% with insignificant but reduced surface area by 14.7% Birth weight was positively associated with placental surface area. Betamethasone increased STB cell circumference and cell surface.

(Langdown & Sugden, 2001)	Pregnant rats given dexamethasone by subcutaneous infusion at E15 via a pump at a dose of 100 or 200 ug/kg body wt/day Sac at E21	Reduced fetal and placental weights that was dose-dependent, the 200 dex dose had a larger impact on weight reduction No effect of dex on gestation length or offspring number or viability Maternal blood showed higher but insignificant blood glucose when dex treated at 200 dose. Fetal hypoglycemia was evident and showed 36% and 49% reduction in fetal plasma glucose at 100 and 200 ug dex, respectively. Increase in placental GLUT1 protein expression by 1.6 and 1.9 fold at 100 and 200 ug/kg/day dex doses, respectively. Increased GLUT3 protein expression by 2.3 fold only with the 200 ug dex dose.
(Dupouy et al., 1987)	Dex treated rats at E15 till E21 with dexamethasone acetate in drinking water at 10ug/ml dose	21 day old rats offspring from stressed dams showed reduced headless body weight (- 66%) Lower offspring hypothalamic Corticotropin releasing factor content and concentration from 21-day old rats of stressed dams (-57 and -67%, respectively) Lower pituitary ACTH content (-93 %) and lower plasma ACTH levels. Lower adrenal corticosterone concentrations (-74%) and lower plasma corticosterone levels. Severe atrophy of adrenals with reduced absolute adrenal weight (- 83%) and reduced relative adrenal to body weight
(Ain et al., 2005b)	Rats injected subcutaneously with 100ug dexamethasone acetate in 0.1% ethanol at E13. Pump was then implanted to release 200ug dex acetate/kg body wt/day Sac on E20	Dex did not affect litter size or fetal viability. Significant reduction of fetal and placental weights. Decrease of junctional zone. *Igf2* mRNA expression but no effect on it in labyrinth zone → can be a contributor to placental growth restriction Decrease protein expression of phosphorylated/active Akt, but no effect on total Akt → attenuated Akt signaling
(Lesage et al., 2004)	Rats exposed to stress by being in a plastic cylinder in a lighted environment 3x/day for 45 minutes each during last week of gestation. Sac at E20	Fetuses of stressed dams had reduced body weights in males and females → IUGR Fetal plasma glucose and corticosterone levels were reduced but leptin was unchanged. Effect of antenatal stress on offspring:

| | | 24-month old male rats had unchanged weights after antenatal exposure of stress
Basal plasma corticosterone levels were higher but not significant
Plasma leptin was reduced
OGTT showed higher plasma glucose levels in antenatally stressed rats at all timepoints (0, 60 and 120 minutes tested), but insulin secretion was similar. |

Specific Aim 2

Identify the relationship between glucocorticoid exposure during lactation and mammary gland function. Postpartum corticosteroid prescription proportion was recorded at 2.4 per 1000 women in a Danish cohort from 1991-1996 (Olesen *et al.*, 1999). It is reported that postpartum corticosteroid use is low since late pregnancy use is linked with delayed lactation initiation depending on the course and timing of treatment (Anon, 2006*a*). Furthermore, lifestyle stressors can impact milk composition (Thibeau *et al.*, 2016). The specific mechanisms by which stress or glucocorticoid exposure at preconception, during pregnancy, or during lactation affects mammary gland function remain unclear. Current studies have not thoroughly assessed the effects of stress or glucocorticoid exposure on the development and function of mammary glands. Furthermore, many of the studies are conducted with ovine models and much less data is available on rodent models or humans. For humans, there are no contraindications to taking glucocorticoids during lactation or pregnancy. Data regarding potential side effects is lacking, and medical consensus is that the treatment benefits of critical conditions like asthma or irritable bowel syndrome outweigh potential harms. My hypothesis is that maternal glucocorticoid exposure during lactation will impair mammary gland development, reduce milk output and macronutrient composition, ultimately leading to reduced offspring growth prior to weaning and impaired adult metabolic health. To test this, I will examine a) how lactational dexamethasone exposure affects mammary gland size and development, b) how dexamethasone exposure affects milk output volume and milk carbohydrate, protein and fat composition, and c) the effect of the exposure on offspring health via assessing body composition and glucose tolerance.

Rationale and Background

Mammary Gland Development

Glucocorticoids are important for proper mammary gland development (Anderson & Turner, 1956). Primarily, prolactin is the main hormone that promotes the transcriptional activity of STAT5 and mediates mammogenesis- the development of the alveolar duct in preparation for lactation (Feng *et al.*, 1995; Yang & Friedl, 2015). Currently, there are no contraindications to using glucocorticoids during lactation for asthma, allergies, irritable bowel syndrome and other symptoms. Nevertheless, lactating women are advised to breastfeed 4 hours after treatment to minimize transfer of glucocorticoids to the newborn via milk[6]. Evidence regarding the effects of glucocorticoids on mammary gland development in preparation for lactogenesis II and during lactation remains very scarce (Anon, 2006*b*).

Role of Glucocorticoids in Mammary Gland Development

It appears that the effects of glucocorticoids may be more essential for normal growth at low doses during early pregnancy when the alveolar ducts are still developing with a negative effect at high doses. At midgestation, alveolar development seems to be almost complete as their capacity to produce milk seemed intact despite glucocorticoid treatment (Henderson *et al.*, 2009). Glucocorticoid exposure near preterm delivery had time-dependent effects on lactogenesis II initiation (Henderson *et al.*, 2007).

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[6] Specialist Pharmacy Service, Safety in Lactation: Corticosteroids, Published May 20, 2012. Retrieved from:https://www.sps.nhs.uk/articles/safety-in-lactation-corticosteroids/
Mother to Baby, Medications and More During Pregnancy and Breastfeeding, Published October 1, 2018. Retrieved from:https://mothertobaby.org/fact-sheets/prednisoneprednisolone-pregnancy/
The Breastfeeding Network, Prednisolone and Breastfeeding, Published May 2017. Retrieved from:https://breastfeedingnetwork.org.uk/wp-content/dibm/prednisolone%20and%20breastfeeding.pdf

Glucocorticoid exposure at weaning prolongs lactogenesis and inhibit mammary gland apoptosis (Feng *et al.*, 1995). The mechanisms by which glucocorticoids mediate their effect on mammary gland development remain poorly understood. Furthermore, milk output and macronutrient composition after glucocorticoid exposure during pregnancy and/or lactation is insufficient in rodents and humans. Prolactin is also responsible for milk synthesis, while oxytocin promotes milk ejection by alveolar contractions (Anon, 2009). Dexamethasone was found to work in collaboration with prolactin to coactivate the prolactin/STAT5 and GC/GR pathways that drive milk production in a synergistic manner (Kobayashi *et al.*, 2016). Dexamethasone alone was not found to promote milk synthesis but instead augmented prolactin-induced casein secretion (Kobayashi *et al.*, 2016). In adrenalectomized rats, mammary gland size was reduced, suggesting the importance of glucocorticoids (Anderson & Turner, 1956). Upon injection of prednisone to adrenalectomized-ovariectomized rats, mammary gland development was normalized to the size in ovariectomized rats (Anderson & Turner, 1956). In hypophysectomized-ovariectomized-adrenalectomized mice, cortisol acetate treatment improved mammary gland ductal branching (NANDI, 1958).

Glucocorticoid Excess Reduces Mammary Gland Development and Function

Treatment with deoxycorticosterone acetate at lower doses improved ductal branching but caused mammary gland regression at higher doses (NANDI, 1958). Despite the need of both prolactin and glucocorticoids for normal development of mammary glands, the effects of glucocorticoids on lactation remain conflicting and scarce. Dexamethasone administration in lactating rats after a short and prolonged period of pup separation showed inhibition of suckling-induced prolactin release that later normalized (BARTHA *et al.*, 1991). This indicates a potential direct inhibitory effect of cortisol on pituitary prolactin production. In concordance with this, adrenalectomized and dexamethasone-treated male rodents had reductions in prolactin levels (BARTHA *et al.*, 1991). As a drop in glucocorticoid level is necessary to promote involution, exogenous glucocorticoid exposure after suckling cessation has been shown to prevent mammary gland involution and was shown to preserve alveolar structure and increase alveolar size in mice (Feng *et al.*, 1995; Li *et al.*, 1997). Lactating PND6 rats that received dexamethasone just prior to nursing had impaired suckling (Vilela & Giusti-Paiva, 2011). In the dexamethasone group, oxytocin and prolactin levels were lower than the control groups that received saline. Furthermore, pups of dexamethasone-treated rats gained less weight after nursing, suggesting that milk volume was impaired (Vilela & Giusti-Paiva, 2011). Human studies conflict as to the effects of glucocorticoids on milk production. In a case study, a lactating woman who received local corticosteroid injection reported cessation of milk production 30 hours post injection with a spontaneous resumption of lactation within another day (Babwah *et al.*, 2013). On the contrary, in preterm deliveries, maternal betamethasone treatment had a time-dependent effect on milk volume but not composition (Henderson *et al.*, 2007). Women who delivered within 0-2 days of the treatment had increased milk volume, while women who delivered 3-9 days post-treatment had a reduced milk volume. This indicates an immediate postpartum effect of glucocorticoids on mammary gland function.

Glucocorticoid-Dependent Effects on Milk Composition

Glucocorticoids can also have an impact on milk composition. In most tissues, glucocorticoids reduce protein synthesis by inhibiting mTORC1 and activating FOXO1/3 (Sandri *et al.*, 2004; Wang *et al.*, 2006; Waddell *et al.*, 2008; Wolff *et al.*, 2014)*.* Dexamethasone exposure in cows reduced milk output volume to its lowest after one day of treatment, then gradually increased afterward (Shamay *et al.*, 2000). Lactose levels in milk were unaltered, while milk protein and fat percentages increased reaching a maximum after one day of treatment then gradually decreased to normal within 3 days with a prolonged reduced concentration of whey protein (Shamay *et al.*, 2000). Owing to the reductions in

total volume, the total protein and fat yield were reduced. Similarly, adrenocorticotropin injection in lactating cows reduced milk yield and protein yield after injection (Varner & Johnson, 1983). Lactose is thought to be the main regulator of milk output (Kronfeld & Hartmann, 1973). Hence, the reduced milk macronutrient yield was suggested to be due to mammary gland's reduced ability to utilize glucose for lactose synthesis after glucocorticoid treatment (Varner & Johnson, 1983).

Effects of Lactational Glucocorticoid Exposure on Offspring Health

The long-term effects of lactational glucocorticoid exposure on the offspring remain largely unknown. Children of mothers who used glucocorticoids during pregnancy had an elevated stress response and impaired neurodevelopment (Alexander *et al.*, 2012; Asztalos *et al.*, 2014). The effects of corticosteroid use further manifest in childhood where maternal third trimester cortisol levels were shown to influence childhood adiposity (Entringer *et al.*, 2016). In mice, studies have shown reduced placental weights after a short period preterm exposure to dexamethasone and potential fetal growth restriction (Cuffe *et al.*, 2011*a*).

In one study on lactating rats, the investigators used prolonged maternal dexamethasone exposure at a dose of 100ug/kg/day on PND1-7, 1-14, and 1-21 (Jeje & Raji, 2015). At PND14 and 21 and at 12 weeks of age, offspring of dams exposed to maternal glucocorticoids at PND1-7, 1-14, and 1-21 had significantly reduced body weights. Offspring lipid profile at 12 weeks of age showed increased liver cholesterol, low-density lipoproteins, and triglycerides with reduced liver high-density lipoprotein levels along with elevated fasting blood glucose (Jeje & Raji, 2015). The effect of this exposure on offspring kidneys at 12 weeks of age, showed signs of necrosis and increased oxidative stress (Jeje *et al.*, 2016).

Experimental Design

To assess the effects of glucocorticoids on milk production and milk volume, we will obtain a total of 56 female and 56 male mice (n=14 females and 14 males per group- 4 groups total)[7] that are 8-week old virgin C57BL6/J female and male mice from The Jackson Laboratory. Mice will be given two weeks to acclimatize with *ad libitum* access to normal chow diet and water. After acclimatization, dams will be assigned to one group of the following four (see Figure 1):

1. *Water PND0.5-16.5*: control group given water during lactation till PND16.5
2. *Dexamethasone PND0.5-16.5*: experimental group given dex during lactation till PND16.5
3. *Water PND0.5-21.5*: control group given water all throughout lactation until weaning
4. *Dexamethasone PND0.5-21.5*: experimental group given dex all throughout lactation until weaning

After being assigned a group, the dams will be mated with age-matched males and with *ad libitum* normal chow diet and water all throughout pregnancy and prior to delivery. Male breeders from both cohorts will be removed from the cage after 18 days of mating to avoid the occurrence of a second pregnancy. In all groups, the dams will undergo body mass assessment three times weekly throughout the experiment and immediately postpartum using magnetic resonance to assess body composition. We will measure dam food and water intake weekly. We will check for litters on a daily basis after 2.5 weeks of mating. After delivery (PND0.5), the dams will either receive water (controls) or dexamethasone (experimental) at a dose of 1mg/kg/day. Pups will be sexed then culled to four animals (ideally 2 females

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[7] Powered to see 1SD effect size in milk protein composition and in offspring weights, we need 14 female mice and 14 male mice per group (4 groups total) to get a power of 0.824.

and 2 males, if possible) per litter at PND2.5. The offspring will be weighed at PND0.5, PND7.5, 14.5, 16.5 (for PND0.5-16.5- groups 1 and 2only), and at 21.5 (for PND0.5-21.5- groups 3 and 4 only). Control and experimental groups of PND0.5-16.5 will receive water or dexamethasone throughout lactation and until PND16.5, where the dams and the pups will be sacrificed and maternal mammary glands will be weighed and collected for cryosectioning and molecular studies. Control and experimental groups of PND0.5-21.5 will be allowed to complete nursing fully and the pups will be weaned to evaluate effects on the offspring.

The pups of groups PND0.5-21.5 will be weaned into new cages based on sex and treatment group. 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. To assess glucose homeostasis, a glucose tolerance test (GTT) will be done at 6 weeks of age followed by sacrifice and tissue collection of fat pads 3 days later. Their fat pads (gWAT and iWAT) will be collected and weighed to determine adiposity.

Figure 1: Diagram representing the experimental design and respective timeline
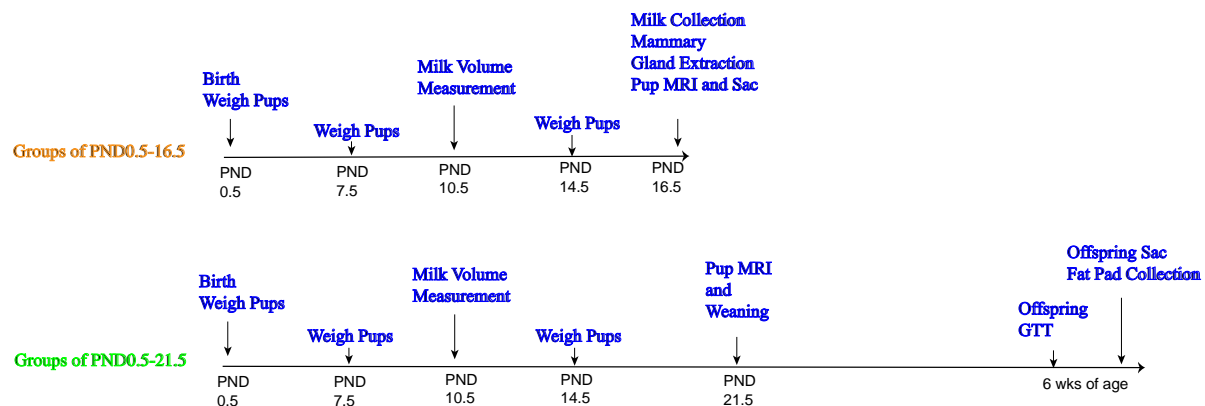
Methods

Dexamethasone Exposure
Water-soluble dexamethasone (Sigma) will be prepared at a concentration of 53 mg/L, which our previous work shown results in a dose of approximately 1 mg/kg/day in non-nursing mice.
If the dam is single housed or with nursing pups:

(the new added total water/dexamethasone- the last measurement's water/dexamethasone) / # of days between measurements

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

(the new added total water/dexamethasone - the last measurement's water/dexamethasone) * #of days between measurements / sum of days spent by each mouse in that cage between measurements

Food Intake

Food will be weighed when the breeding cages are set up for mating. 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

All animals will be sacrificed using anesthetic gas inhalation (5% isoflurane drop jar). Cervical dislocation will be done as a secondary method to confirm euthanasia. The mice will be pinned on a dissection board in a supine position. For dams from control and experimental groups PND0.5-16.5, we will dissect the mammary glands by a midline incision of the skin from the rectum to the diaphragm, extract thoracic, abdominal and inguinal mammary glands. The peritoneum will be pulled apart from the skin. The lower glands will be excised carefully then weighed. A portion of the upper and lower glands will be embedded in paraffin for histology, while the rest will be collected in 2ml tubes and snap frozen in liquid nitrogen then alter stored at -80C for possible future molecular studies. Offspring of dams from control and experimental groups PND0.5-21.5 will be sacrificed similarly at 6 weeks of age. For the offspring, fat pad collection will be done. 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 and weighed. Right gonadal white adipose tissue (gWAT) will be collected next by scraping the fat along the gonads (ovaries or testis) then will be weighed. Fat pads will be snap frozen in liquid nitrogen in 2ml tubes and will be stored at a temperature of -80C for later molecular studies.

Determining Milk Output Volume

At PND10.5, we will determine milk output volume for the control and experimental groups PND0.5-16.5. To determine milk volume, we will use the weigh-suckle-weigh technique (Boston *et al.*, 2001). Briefly, we will weigh the dam then determine the aggregate weight of the pups. The dam and pups will then be separated for two hours. During the two-hour separation, the pups will be placed in a new cage and will be kept warm using a heating pad. In the meantime, the dam will remain in its initial cage with *ad libitum* access to normal chow diet and water or dexamethasone-water based on its assigned group. After the two-hour separation period, the dam will be weighed again and the aggregate weight of the pups will be measured. The pups will then be returned to the dam's cage and will be allowed to nurse for one hour. At the end of the nursing timepoint, the dam will be weighed and the aggregate weight of the pups will be determined. After the one-hour nursing period, milk volume will be determined as the

weight change of the pups after nursing and after the 2-hour separation. The difference in the dam's weight after nursing and after the 2-hour separation will help further ascertain the dam's milk supply.

Determining Milk Composition

On PND16.5, we will collect milk samples (~0.5ml) from the nursing dams in groups control and experimental PND0.5-16.5. Briefly, we will separate the dam and pups for 2 hours. The pups will be weighed and will undergo body composition assessment using echoMRI. Afterwards, the pups will be sacrificed using isoflurane and a secondary measure of cervical dislocation. We will anesthetize the dam after two hours of separation by intraperitoneal injection of Ketamine (0.1275g/kg body weight). We will then perform an intraperitoneal injection of oxytocin into the forelimb (2U/dam) to induce milk production. The dam's nipples will be manually squeezed to promote milk letdown, and the milk will be collected into a 1.5 ml tube via suction. After milking is complete, the dam will immediately be sacrificed using isoflurane and a secondary measure of cervical dislocation. We will then dissect the dam by a midline incision of the skin, extract thoracic, abdominal and inguinal mammary glands. The lower mammary gland pads will be weighed. A small section of the lower mammary glands will be saved for paraffin embedding for histology while the rest will be snap frozen in liquid nitrogen and cryopreserved to later determine mTORC1 expression as previously discussed via Western blotting. Milk protein composition will be analyzed using SDS-PAGE gels and diluted milk samples.

Determining Milk Protein Concentrations

Milk samples collected from control and experimental groups of PND0.5-16.5 will be assessed for protein content. Milk will be diluted to a factor of 4 (1:3 in PBS+EDTA). Skimmed milk will be collected after centrifuging. Samples will be heated to ~95C and loading cocktail will be added onto the plastic plate with the gel along with a ladder. Gels will be stained by Coomassie blue and quantified by near-infra-red imaging. Imaging will be done using LiCOR Odyssey to determine levels protein of whey acidic protein, alpha casein, beta casein, lactose, and serum albumin identified based on known molecular weights.

Determining Milk Fat Content

Milk samples collected will be assessed for fat content by the creamatocrit method using a hematocrit centrifuge. Briefly, samples will be diluted to a factor of 3 (1:2 in PBS) into well-sealed capillary tubes. The tubes will be placed in CritSpin mini-creamatocrit spinner. Samples will be centrifuged for 8 cycles of 120 seconds. The capillary will form layers of white fat and non-fat milk. The distance of the fat layer will be measured in millimeters (mm) accurately. The total volume of milk (fat + non-fat milk) will be measured in mm. Percentage of fat will be determined with respect to the total volume.

Glucose Tolerance Test

Weaned offspring in groups control and experimental PND0.5-21.5 will undergo a glucose tolerance test (GTT) being challenged with 1g/kg of lean body mass (determined by echoMRI) after a 6-hour fast with *ad libitum* access to water. The effects of maternal glucocorticoid exposure during lactation on offspring adolescent glucose 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 glucose 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 GTT 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 lower mammary gland tissues collected from the dams, we will assess RNA expression of lipogenic genes. 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 the genes ACC1, SREBP1c, ACLY and FASN using primer pairs (forward and reverse). This will allow us to assess lipogenic activity of the mammary glands of KO and WT dams.

Western Blotting
Using the mammary gland tissues collected from dams of groups PND0.5-16.5, mammary glands will be assessed for mTORC1 activity. 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) will be used.

Histology
Mammary glands collected from control and experimental groups PND0.5-16.5 will be embedded in paraffin and stained at the Rogel Cancer Center's Tissue and Molecular Pathology. Slides will be blindly assessed for alveolar count and adipocyte size.

Expected Results

Aim 2.1: Is mammary gland development altered after maternal glucocorticoid exposure during lactation?
As glucocorticoid treatments show reductions in mammary gland development (Zhu *et al.*, 1998), and since high doses ultimately reduce mammary gland size (NANDI, 1958), I hypothesize that our prolonged dexamethasone exposure will cause reductions in mammary gland development at PND16.5. This will be evident in the reduced count of alveolar cells and reduced adipocyte size. Despite the need of low doses of glucocorticoids for normal development of mammary glands, administration of deoxycorticosterone acetate at high doses caused mammary gland regression in mice (NANDI, 1958).

Aim 2.2: How does maternal glucocorticoid exposure during lactation affect milk output and macronutrient composition?
As prior studies show that a short-term glucocorticoid exposure reduced macronutrient yield but increased percentage (Shamay *et al.*, 2000; Vilela & Giusti-Paiva, 2011), I hypothesize that our prolonged exposure will increase milk protein and fat macronutrient percentage. As lactose is the regulator of milk output, reductions in milk and lactose yield will be simultaneous (Varner & Johnson, 1983). Hence, I hypothesize that dexamethasone exposure will have unaltered lactose percentage despite the

reductions in the milk output volume. The hypothesized results are in agreement with the previous hypothesis suggesting reduced mammary gland development in Aim 2.1, as a less developed mammary gland will yield less milk with altered macronutrient composition.

Aim 2.3: Is offspring metabolic health altered after maternal glucocorticoid exposure during lactation?

Based on prior results showing that offspring of dams treated with dexamethasone at PND1-7, 1-14, and 1-21 showed reduced weights, had higher fasting blood glucose, and impaired lipid profile (Jeje & Raji, 2015), I predict that offspring will have lower body weights at PND14.5, 16.5 (from groups of PND0.5-16.5 only), 21.5 (for groups PND0.5-21.5 only), and weekly afterwards until week 6 of age when the offspring will be sacrificed (for groups PND0.5-21.5 only). Additionally, I hypothesize that fat mass in experimental offspring will be higher despite an overall reduced body weight. When offspring undergo the GTT at 6 weeks of age, I expect that experimental offspring to have higher fasting blood glucose with impaired glucose tolerance. Since glucocorticoids pass through the milk to the offspring (Hollanders *et al.*, 2017) and since they promote adipogenesis (Patel *et al.*, 2014), I hypothesize that fat pads collected from offspring of experimental group PND0.5-21.5 will have higher mass than controls. Future studies will explore the molecular underpinnings of these phenotypes.

Potential Pitfalls and Alternate Approaches (Aims 2.1-2.3)

It is possible that the chronic dexamethasone exposure will drastically reduce mammary gland capacity to produce milk, making it impossible to nurse pups and ultimately leading to pup death. In that case, we will alter dexamethasone exposures in new cohorts with treatment windows as follows: PND1-7, PND7-14, and PND14-21. This will allow us to determine more accurately when the effects of dexamethasone are most critical on the offspring.

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), and it is highly regulated to ensure adequate growth of the fetus in normal pregnancies (Napso *et al.*, 2018*a*). 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[8] (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 fetal and offspring metabolic health. My hypothesis is that placental 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,

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[8] 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

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)
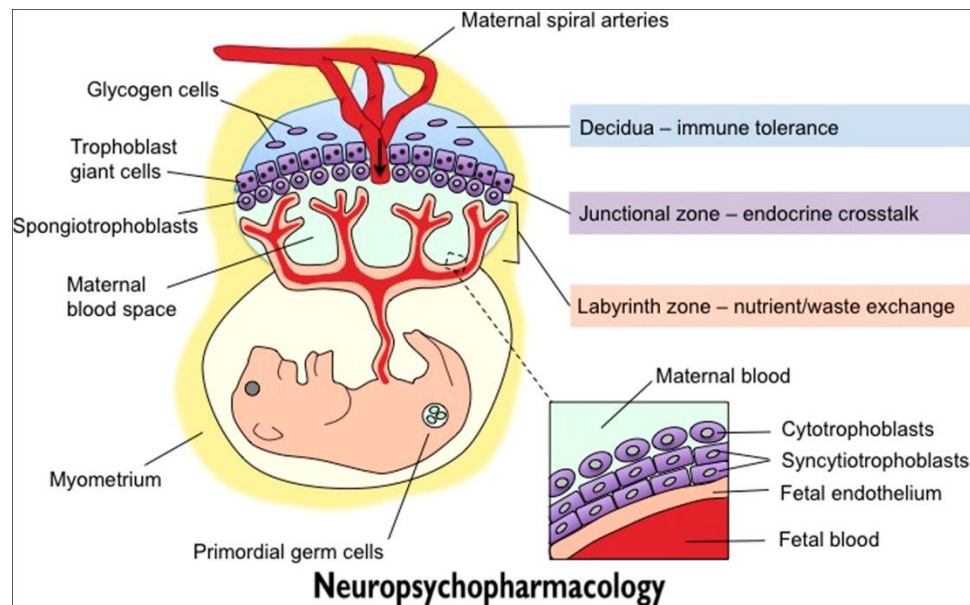
Neuropsychopharmacology Reviews (2016) **41**, 207-218;
doi:10.1038/npp.2015.231

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*a*). 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*a*).

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-Cre*$^{Tg/+}$ (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 (KO) *Tsc1*$^{fl/fl}$;*Cyp19a1-Cre*$^{Tg/+}$, conditionally heterozygous *Tsc1*$^{fl/+}$; *Cyp19a1-Cre*$^{Tg/+}$, 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 a total of 44 WT and KO female and 44 WT and KO male mice (n=11 females and 11 males per group- 4 groups total)[9] that are WT and KO at a ratio of 1:1 and that are 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 (at 8 weeks of age) 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:
5. *WT till E14.5:* WT female mated with WT male on water and normal chow diet until midgestation at embryonic day 14.5
6. *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 while having *ad libitum* access to water and normal chow diet. A copulatory plug will be checked daily to identify E0.5 day. 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 be allowed to complete their pregnancy and deliver their pups at around E21.5, survival and birth rates will be noted. Pups will be sexed and culled to four (ideally 2 males and 2 females) 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

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[9] Powered to see 1.1SD effect size in placental and offspring weights, we need 11 female mice and 11 male mice per group (4 groups total) to get a power of 0.801.

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

♀ **Tsc1$^{fl/fl}$;Cyp19a1-Cre$^{+/+}$**   x   ♂ **Tsc1$^{+/+}$;Cyp19a1-Cre$^{Tg/+}$**

9-12 weeks expected timeline

Tsc1$^{fl/+}$;Cyp19a1-Cre$^{+/+}$ &   Tsc1$^{fl/+}$;Cyp19a1-Cre$^{Tg/+}$
1WT : 1Het

♀ **Tsc1$^{fl/+}$;Cyp19a1-Cre$^{+/+}$**   x   ♂ **Tsc1$^{fl/+}$;Cyp19a1-Cre$^{Tg/+}$**

9-12 weeks expected timeline

Tsc1$^{fl/fl}$; Cyp19a1-Cre$^{Tg/+}$	Tsc1$^{fl/fl}$; Cyp19a1-Cre$^{+/+}$	Tsc1$^{fl/+}$; Cyp19a1-Cre$^{Tg/+}$	Tsc1$^{fl/+}$; Cyp19a1-Cre$^{+/+}$	Tsc1$^{+/+}$; Cyp19a1-Cre$^{Tg/+}$	Tsc1$^{+/+}$; Cyp19a1-Cre$^{+/+}$
1	1	2	2	1	1

1KO : 2Het : 5WT

♀ **Tsc1$^{fl/fl}$;Cyp19a1-Cre$^{+/+}$**   x   ♂ **Tsc1$^{fl/fl}$;Cyp19a1-Cre$^{Tg/+}$**

2-12 weeks expected timeline

Tsc1$^{fl/fl}$;Cyp19a1-Cre$^{+/+}$ &   Tsc1$^{fl/fl}$;Cyp19a1-Cre$^{Tg/+}$
1WT : 1KO

Figure 4: Diagram representing the experimental design and respective timeline

Cohort A

Group 1 and 2 with WT dam and WT or KO male

Mating

Sac dams
Weigh/extract placentas
Weigh Fetuses

0   E14.5

Cohort B

Group 1 and 2 with WT dam and WT or KO male

Mating

Weigh Pups

Pup MRI and Weaning

Offspring ITT

Offspring Sac
Fat Pad Collection

0   Delivery   PND 21.5   6 wks of age

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 *IgfII* 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 *IgfII*(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.

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* placental 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.

Specific Aim 4

Detect the effects of nutritional stress on lactation. Milk composition is important to provide essential nutrients for optimal offspring growth (Eriksen *et al.*, 2018). Given the links between maternal obesity and offspring health, it is plausible that obesity or overnutrition may alter lactation, with important effects for the offspring. The mechanisms by which some micro and macronutrients are metabolized, transported, and incorporated into the secreted milk during lactation are not well understood, nor is their regulation by nutrient sensing pathways. mTORC1 is a critical nutrient sensing pathway in most tissues and is activated under conditions of nutrient excess, including obesity. We will use mTORC1 activation as a model of excessive nutrient signaling in mammary adipocytes. In humans, maternal obesity affects lactation whereby lactation initiation, weaning, and milk composition are altered (Rasmussen & Kjolhede, 2004; Panagos *et al.*, 2016; Castillo *et al.*, 2016). My hypothesis is that maternal adipocyte mTORC1 hyperactivation as a model of obesity will increase mammary gland function and increase milk output and milk macronutrient composition, ultimately leading to increased offspring growth prior to weaning. To test this, I will identify how a) adipocyte mTORC1 hyperactivation affects mammary gland size and development, b) how mTORC1 hyperactivation affects milk output volume and milk carbohydrate, protein and fat composition, and c) the effect of mTORC1 hyperactivation on offspring health via assessing body composition.

Rationale and Background

Milk Macronutrient Synthesis

Lactation requires successful milk secretion, a process referred to as lactogenesis. To achieve that, lactogenesis occurs in two stages. Lactogenesis I encompasses the differentiation of mammary glands and is evident mid-gestation through term in humans. Lactogenesis II, the phase where milk production is initiated occurs prior to delivery in most animals, but in humans, lactogenesis II is initiated post-delivery due to placental removal and a gradual drop in progesterone levels (Neville *et al.*, 2001, 2002; Soares, 2004; Ben-Jonathan *et al.*, 2008; Napso *et al.*, 2018*b*; Pillay & Davis, 2019). The critical macronutrients in mammalian milk are fat, protein and lactose. Mouse milk showed the highest fat and protein content on PND14 with 12.5% crude protein, 29.8% crude fat, and 1.58% lactose (Görs *et al.*, 2009). Highest lactose content of 2.41% was evident on PND18 (Görs *et al.*, 2009). Proteins are synthesized in the rough endoplasmic reticulum of the alveolar epithelial cells (Anderson *et al.*, 2007; Rezaei *et al.*, 2016). Lipids, almost exclusively in the form of triglycerides, are synthesized in the smooth endoplasmic reticulum by de novo synthesis from available glucose, or they are derived from maternal diet or fatty acids from adipose tissue stores (Anderson *et al.*, 2007; McManaman, 2009; Rezaei *et al.*, 2016). The mechanisms by which lipids are packaged and transported into the milk remain elusive (McManaman, 2009). Lactose is synthesized in the Golgi of the alveolar epithelial cells (Anderson *et al.*, 2007; Rezaei *et al.*, 2016).

Mammary Adipocytes and Mammary Function

Adipocytes form a major proportion of the mammary gland and are necessary for proper gland development and proliferation (Machino, 1976; Landskroner-Eiger *et al.*, 2010). At puberty, alveolar ducts expand at the expense of the fat pad in the mammary gland (Hovey & Aimo, 2010; Macias & Hinck, 2012). A case study of a female with progressive lipodystrophy showed suboptimal lactation and early cessation of lactation due to ceased milk production 3 weeks postpartum (RUSSELL, 1958). Two females with familial lipodystrophy had reduced mammary adipocytes despite normal mammary tissue

size (Garg *et al.*, 1999). A mouse model of lipodystrophy with underdeveloped fat tissues was developed to determine its effects on mammary gland development (Li *et al.*, 2015). The knockout mice had smaller mammary adipocytes, accelerated ductal growth, and potential sloughing of the ductal epithelial cells into the lumen indicating suboptimal mammary gland function and growth compared to controls (Li *et al.*, 2015). A PPARγ knockout mouse model of impaired adipocyte function showed reduced expansion of the ducts at the expense of the fat pad along with prepubertal cessation of ductal growth (Wang *et al.*, 2013). During pregnancy and lactation, adipocytes have a unique supportive function. Recently, it has been determined that mammary adipocytes de-differentiate gradually during gestation and almost disappear entirely during lactation allowing more space for milk production by the mammary alveolar epithelial cells (Wang *et al.*, 2018; Zwick *et al.*, 2018). Adipocytes closest to the mammary epithelial cells de-differentiate quicker than those farther away in the cleared fat pad (Hovey & Aimo, 2010; Lawson *et al.*, 2015). The alveoli expand at the expense of the fat pad almost entirely covering its area (Richert *et al.*, 2000). It is hypothesized that the adipocytes in the body mobilize their fat stores and provide for the mammary epithelial milk lipid production, which explains the reduction in size of the adipocytes during lactation (Flint & Vernon, 1998; Richert *et al.*, 2000; Cinti, 2018). The exact fate of adipocytes during the de-differentiation phase of lactation remains unknown (Wang *et al.*, 2018). It is shown that the adipocytes do not transdifferentiate into epithelial cells unlike what was previously shown (Morroni *et al.*, 2004; Prokesch *et al.*, 2014), indicating that the adipocytes do not contribute directly to the milk production function of the epithelial cells during lactation (Wang *et al.*, 2018; Zwick *et al.*, 2018). As milk production gradually decreases at weaning, adipocytes later grow rapidly in size by taking up excess milk lipids from the alveolar lumen and alveolar epithelial cells (Zwick *et al.*, 2018). This is referred to as a "refilling" process for the mammary gland adipocytes and it simultaneously occurs along epithelial cell regression (Zwick *et al.*, 2018). The role of the adipocytes and the mechanisms regulating their regression and fate warrant further studies. Our model will focus on mTORC1 activation in differentiated adipocytes after a first pregnancy, not during the process of adipogenesis. Little is known about the role of mTORC1 in macronutrient synthesis in the mammary gland (Rezaei *et al.*, 2016).

mTORC1 Activity in Obesity

mTORC1 is a main regulator of protein and lipid synthesis (Wang & Proud, 2006; Cai *et al.*, 2016). In the presence of insulin, an anabolic signal, mTORC1 function is upregulated via the Akt pathway (Catania *et al.*, 2011). mTORC1 promotes lipogenesis via SREBP1 and promotes adipogenesis while inhibiting lipolysis (Laplante & Sabatini, 2009; Cai *et al.*, 2016). Obesity, identified by having excess fat mass, promotes mTORC1 activity (Catania *et al.*, 2011). In obese subjects, gene expression of mTORC1 and pS6K was upregulated in the visceral fat compartments (Catalán *et al.*, 2015). Mice deficient in S6K are resistant to obesity by which they have higher lipolytic rate and fewer fat mass (Um *et al.*, 2004; Dann *et al.*, 2007). This suggests the important and active role of mTORC1 in promoting an obese phenotype.

Role of mTORC1 on Mammary Gland Function

mTORC1 is a nutrient sensor and is crucial for proliferation and growth. Mice treated with rapamycin for 12 days starting at gestational day 19 had reduced mammary gland size and reduced epithelial tissue (Jankiewicz *et al.*, 2006). Furthermore, milk beta-casein protein composition was reduced by half in the rapamycin treated group (Jankiewicz *et al.*, 2006). This indicates the important role of mTORC1 In mammary gland proliferation and protein synthesis. In bovine mammary epithelial cells, mTORC1 signaling was upregulated in response to lactogenic stimulus via insulin and prolactin (Li *et al.*, 2017). The mechanisms by which mTORC1 promotes protein synthesis has been linked to downregulation of Menin protein, an inhibitor of AKT activity upstream of mTORC1 (Li *et al.*, 2017). Transgenic pregnant

mice with activated AKT in the mammary epithelial cells had comparable mammary gland development during pregnancy, but showed distended alveoli during lactation and a higher lipid droplet composition and size in the mammary epithelial during gestation and lactation (Schwertfeger *et al.*, 2003). Milk composition from the transgenic mice revealed higher fat percentage and a higher protein concentration compared to controls (Schwertfeger *et al.*, 2003). AKT, upstream of mTORC1, may play a significant role in regulating mammary gland differentiation and lipid and protein synthesis (Schwertfeger *et al.*, 2003). Furthermore, Th-inducing POK, a transcription factor, was found to be correlated with mTORC1 and a potential feed-forward regulator of insulin signaling via IRS1/Akt/mTORC1 pathway in the mammary gland (Zhang *et al.*, 2018). Mice lacking Th-POK had lower pup survival rate that was attributed to lactation. Knockout mice further had reduced milk triglycerides and increased milk non-esterified fatty acids. This was due to large lipid droplet accumulation in the mammary alveolar cells. Th-POK knockout mice further showed signs of precocious mammary epithelial involution (Zhang *et al.*, 2018). This implies the important role of mTORC1 in modulating lipid synthesis in the mammary alveolar cells.

Maternal Obesity and Offspring Health

Maternal obesity can influence the offspring health via pre-gestational, gestational and lactational exposures. Children of mothers with class III obesity are at 2.3 times higher risk of being large for gestational age (Kim *et al.*, 2016). Children of overweight or obese mothers had increased weight gain at age 0-4 years and a higher BMI z-score compared to children of lean mothers (Hu *et al.*, 2019). Another study found no effect on offspring weight. Pre-pregnancy obesity was positively associated with higher weight gain and obesity risk in early childhood. This association was unaltered when breastfeeding was accounted for (Hu *et al.*, 2019). A systematic review revealed benefits of breastfeeding that were attenuated when accounting for maternal BMI, suggesting an interplay between maternal weight and benefits of lactation (Bider-Canfield *et al.*, 2017). Furthermore, breastfeeding was positively associated with childhood obesity in mothers who had a higher-than-expected gestational weight gain, suggesting that maternal pre-pregnancy weight and gestational weight gain are the main predictors of childhood obesity risk (Ohlendorf *et al.*, 2019).This implies the effects of maternal weight on reducing benefits of lactation (Ohlendorf *et al.*, 2019). Alarmingly, data collected in the United States show that more than 50% of pregnant women were either obese or overweight in 2014[10] (Branum *et al.*, 2014). The exact mechanisms by which the offspring health is affected in response to early life exposures remain elusive due to the multiple critical developmental windows that can be influenced. This aim will focus on the developmental window of lactation in maternal obesity, as a lot of evidence points to the importance of lactation on offspring health (Neri & Edlow, 2015).

Obesity and Lactation

Maternal obesity can influence early postnatal development through its impact on mammary gland function. Maternal weight has been positively correlated with milk protein content and caloric value (kilocalories from protein, lipids and carbohydrates per 100 ml milk) on the third month of lactation postpartum (Bzikowska-Jura *et al.*, 2018). Milk fat content was positively correlated with maternal weight at six months postpartum (Bzikowska-Jura *et al.*, 2018). An altered milk lipid composition was found in milk of obese mothers with a higher omega 6:omega3 ratio (Panagos *et al.*, 2016). Initiation of lactation was also affected by maternal weight, by which pre-pregnancy obesity or overweight reduced

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[10] 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

the suckling-induced prolactin secretion at 48 hours postpartum (Rasmussen & Kjolhede, 2004). Furthermore, breastfeeding duration for 6 months or more was lower in mothers who were overweight or obese (Bider-Canfield *et al.*, 2017). The probability of early weaning at 3 months postpartum was highest for infants of obese mothers (Castillo *et al.*, 2016).

In rats, obesity induced by high-energy diet doubled fat content in milk (Rolls & Rowe, 1982). Mice fed a high fat diet had delayed lactogenesis which was evident by reduced litter weight gain on the first day of lactation which later normalized (Flint *et al.*, 2005). The mice further had impaired alveolar development with abnormal reduced branching at gestational day 14 (Flint *et al.*, 2005).

Experimental Design

TSC1 is a negative regulator of mTORC1 as shown in Figure 1 (Kwiatkowski *et al.*, 2002*b*; Garami *et al.*, 2003; Zhang *et al.*, 2003; Harrington *et al.*, 2004), and when it is deleted, mTORC1 is hyperactivated. To assess the effects of maternal mTORC1 adipocyte hyperactivation on milk production and offspring health, we will use a total of 16 WT and KO female and 16 WT and KO male mice (n=8 females and 8 males per group- 2 groups total)[11] that are WT and KO at a ratio of 1:1 and that are 6-8-week old virgin floxed-adipocyte *Tsc1* wildtype (WT) and knockout (KO) female and male mice bred in our facility. To hyperactivate mTORC1, we used the Cre-loxP recombination technology. *Tsc1* ^fl/fl^ mice with flanked *Tsc1* gene exons 17 and 18 were crossed with *Adipoq*-Cre mice expressing the adipocyte-specific constitutive Cre recombinase controlled by adiponectin gene promoter. The parental strains for this experiment will be male *Tsc1* ^fl/fl^;Tg/+ or *Tsc1* ^fl/fl^;+/+ crossed with female *Tsc1* ^fl/fl^;+/+ or *Tsc1* ^fl/fl^;Tg/+, respectively. The offspring will be a combination of knockout (fl/fl;Tg/+) and phenotypically wild-type (fl/fl;+/+) at an expected ratio of 1:1. The knockout of the floxed alleles are driven by Adiponectin-Cre (Kwiatkowski *et al.*, 2002*b*; Eguchi *et al.*, 2008), which is expressed in all adipocyte lineages- brown, white and mammary adipocytes as shown in Wang et al. (Wang *et al.*, 2013). As such, one limitation of this approach is that all adipocytes are affected, not just mammary adipocytes (for which there is no known specific Cre driver). KO females will be crossed with WT males and vice-versa to ensure that pups are a combination of wild-type and knockout adipocyte *Tsc1* mice. Mice will be given *ad libitum* access to normal chow diet and water. Male breeders will be removed from the cage after 16 days of mating to avoid the occurrence of a second pregnancy, which may bias our results due to changes in the maternal hormonal milieu. In all groups, the dams will undergo body mass assessment three times weekly throughout the experiment and immediately postpartum using magnetic resonance to assess body composition. We will measure dam food intake 3 times a week. We will check for litters on a daily basis after 2.5 weeks of mating. The number of pups born will be recorded to determine maternal fertility and pup viability. After delivery (PND0.5), the dams will continue to have *ad libitum* access to food and water. Milk volume will be determined on PND10, when the milk output is at its peak. On PND16.5, the dams and the pups will be sacrificed and maternal mammary glands will be weighed and collected for cryosectioning and molecular studies, as mammary gland development is at its peak on PND16.5. Pups will be sexed then culled to four animals (2 females and 2 males, if possible) per litter at PND2.5. The offspring will be weighed at PND0.5, PND7.5, 14.5, 16.5. The pups will undergo body composition analysis by echoMRI at PND16.5 prior to sacrifice.

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[11] Powered to see 1.4SD effect size in milk protein composition and in offspring weights, we need 8 female mice and 8 male mice per group (2 groups total, WT and KO) to get a power of 0.845.

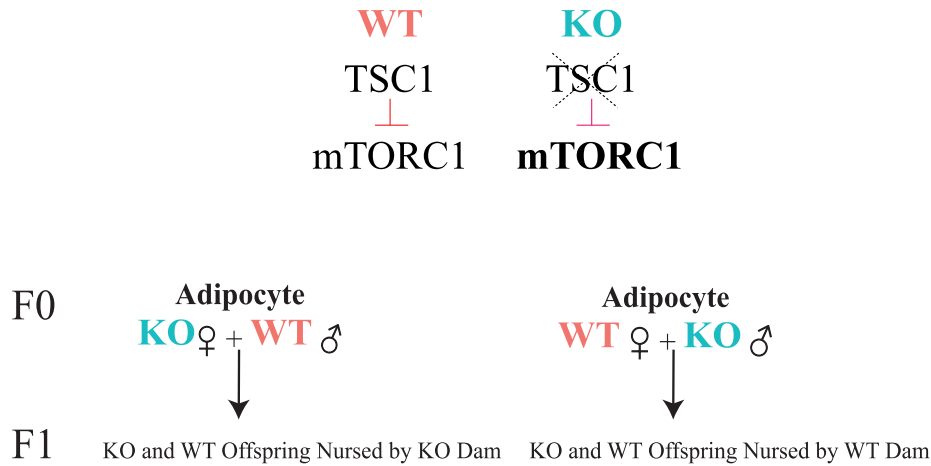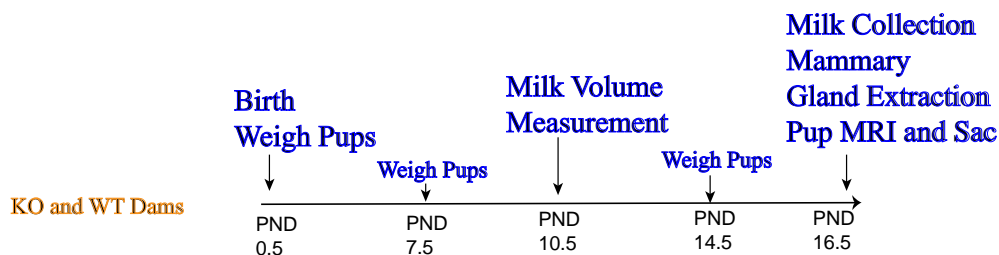*Figure 1: Schematic diagram representing TSC1/mTORC1 pathway in KO and WT model*

WT   **KO**

TSC1   TSC1

⊥   ⊥

mTORC1   **mTORC1**

F0   **Adipocyte**   **Adipocyte**

KO♀ + **WT** ♂   **WT** ♀ + **KO** ♂

↓   ↓

F1   KO and WT Offspring Nursed by KO Dam   KO and WT Offspring Nursed by WT Dam

Figure 2: Diagram representing the experimental design and respective timeline

Milk Collection
Mammary
Milk Volume   **Gland Extraction**
Measurement   **Pup MRI and Sac**

Birth
Weigh Pups   Weigh Pups   Weigh Pups

KO and WT Dams   ↓   ↓   ↓   ↓   ↓

PND   PND   PND   PND   PND
0.5   7.5   10.5   14.5   16.5

Methods

Food Intake

Food will be weighed when the breeding cages are set up for mating. 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), 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

All animals will be sacrificed using anesthetic gas inhalation (5% isoflurane drop jar). Cervical dislocation will be done as a secondary method to confirm euthanasia. The mice will be pinned on a dissection board in a supine position. We will dissect KO and WT dams by a midline incision of the skin from the rectum to the diaphragm, extract thoracic, abdominal and inguinal mammary glands. The peritoneum will be pulled apart from the skin. The lower glands will be excised carefully then weighed. A portion of the upper and lower glands will be embedded in paraffin for histology, while the rest will be collected in 2ml tubes and snap frozen in liquid nitrogen then alter stored at -80C for molecular studies. Offspring of dams will be sacrificed without tissue extraction.

Determining Milk Output Volume

At PND10.5, we will determine milk output volume for the WT and KO dams. To determine milk volume, we will use the weigh-suckle-weigh technique (Boston *et al.*, 2001).Briefly, we will weigh the dam then determine the aggregate weight of the pups. The dam and pups will then be separated for two hours. During the two-hour separation, the pups will be placed in a new cage and will be kept warm using a heating pad. In the meantime, the dam will remain in its initial cage with *ad libitum* access to normal chow diet and water. After the two-hour separation period, the dam will be weighed again and the aggregate weight of the pups will be measured. The pups will then be returned to the dam's cage and will be allowed to nurse for one hour. At the end of the nursing timepoint, the dam will be weighed and the aggregate weight of the pups will be determined. After the one-hour nursing period, milk volume will be determined as the weight change of the pups after nursing and after the 2-hour separation. The difference in the dam's weight after nursing and after the 2-hour separation will help further ascertain the dam's milk supply.

Determining Milk Composition

On PND16.5, we will collect milk samples (~0.5ml) from the nursing dams. Briefly, we will separate the dam and pups for 2 hours. The pups will be weighed and will undergo body composition assessment using echoMRI. Afterwards, the pups will be sacrificed using isoflurane and a secondary measure of cervical dislocation. We will anesthetize the dam after two hours of separation by intraperitoneal injection of Ketamine (0.1275g/kg body weight). We will then perform an intraperitoneal injection of oxytocin into the forelimb (2U/dam) to induce milk production. The dam's nipples will be manually squeezed to promote milk letdown, and the milk will be collected into a 1.5 ml tube via suction. After milking is complete, the dam will immediately be sacrificed using isoflurane and a secondary measure of cervical dislocation. We will then dissect the dam by a midline incision of the skin, extract thoracic, abdominal and inguinal mammary glands. The lower mammary gland pads will be weighed. A small section of the lower mammary glands will be saved for paraffin embedding for histology while the rest will be snap frozen in liquid nitrogen and cryopreserved to later determine mTORC1 expression as previously discussed via Western blotting. Milk protein composition will be analyzed using SDS-PAGE gels and diluted milk samples.

Determining Milk Protein Concentrations

Milk samples collected from WT and KO dams will be assessed for protein content. Milk will be diluted to a factor of 4 (1:3 in PBS+EDTA). Skimmed milk will be collected after centrifuging. Samples will be heated to ~95C and loading cocktail will be added onto the plastic plate with the gel along with a ladder. Gels will be stained by Coomassie blue and quantified by near-infra-red imaging. Imaging will be done using LiCOR Odyssey to determine protein levels of whey acidic protein, alpha casein, beta casein, lactose, and serum albumin that will be identified based on known molecular weights.

Determining Milk Fat Content

Milk samples collected from WT and KO dams will be assessed for fat content by the creamatocrit method using a hematocrit centrifuge. Briefly, samples will be diluted to a factor of 3 (1:2 in PBS) into well-sealed capillary tubes. The tubes will be placed in CritSpin mini-creamatocrit spinner. Samples will be centrifuged for 8 cycles of 120 seconds. The capillary will form layers of white fat and non-fat milk. The distance of the fat layer will be measured in millimeters (mm) accurately. The total volume of milk (fat + non-fat milk) will be measured in mm. Percentage of fat will be determined with respect to the total volume.

Real time qPCR

Using the lower mammary gland tissues collected from the dams, we will assess RNA expression of lipogenic genes. 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 the genes ACC1, SREBP1c, ACLY, FASN, using primer pairs (forward and reverse). This will allow us to assess lipogenic activity of the mammary glands of KO and WT.

Western Blotting

Using the lower mammary gland tissues collected from the dams, we will assess TSC1/2 protein levels and mTORC1 activity to confirm knockout in mammary glands. 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 TSC1/2, total and phosphorylated mTORC1 targets (S6K, 4EBP1, S6) and regulators (Akt, IRS) will be used.

Histology

Mammary glands collected from WT and KO dams will be embedded in paraffin and stained at the Rogel Cancer Center's Tissue and Molecular Pathology. Slides will be blindly assessed for alveolar count and adipocyte size.

Expected Results

Aim 4.1: Is mammary gland development altered with maternal adipocyte mTORC1 hyperactivation?

Since it is evident that mTORC1 is crucial for proper mammary gland development and function during lactation, I suggest that our model of hyperactivation will reduce mammary gland size in KO dams. Akt overexpression in mammary glands caused no change in mammary gland size during gestation but enlarged alveoli during lactation (Schwertfeger *et al.*, 2003). In overweight or obese mothers, lactation duration was shorter than that of lean mothers (Bider-Canfield *et al.*, 2017), which may indicate reduced lactational capacity. Mammary alveolar development was decreased in rodent models of obesity (Flint *et al.*, 2005). Hence, I predict histological examination to reveal bigger alveolar cells and reduced lipid size indicating a hyperactive glandular function and mammary adipocytes due to mTORC1 hyperactivation.

Aim 4.2: How does adipocyte mTORC1 hyperactivation affect milk output and composition?

Rapamycin treatment for 12 days starting at day 19 of gestation caused reduced mammary gland size and reduced protein composition in the milk, suggesting the important role of mTORC1 in milk composition (Jankiewicz *et al.*, 2006). Additionally, Akt overexpression in the mammary gland caused an increase in milk lipid and protein composition (Schwertfeger *et al.*, 2003). Given the important role of mTORC1 in lipid and protein composition of milk, I expect total protein and lipid percentages in milk from KO dams to be increased compared to WT dams. Th-POK is suggested to work via the mTORC1/SREBP1 pathway, and its deletion showed reduced milk triglyceride composition (Zhang *et al.*, 2018). Furthermore, since Th-POK knockout caused reduced milk volume, I expect mTORC1 hyperactivation to act in the opposite manner and to increase milk volume from KO dams. This will be demonstrated by an increased pup gained weight after nursing at PND10.

Aim 4.3: Is offspring body composition altered with maternal adipocyte mTORC1 hyperactivation?

As I predict that milk lipid and protein composition along with milk output volume will be increased in KO dams (Expected Results from *Aim 4.2*), then I hypothesize that mTORC1 hyperactivation will increase weights of pups at PND 0.5, 7.5, 14.5 and 16.5 with higher fat body composition at PND16.5 for pups of KO dams. This data is supported as offspring weight was reduced with Th-POK KO at birth and during lactation (Zhang *et al.*, 2018). In humans, newborns of obese mothers were at higher risk of being heavier at birth and had higher growth trajectories (Kim *et al.*, 2016; Bider-Canfield *et al.*, 2017; Hu *et al.*, 2019). Interestingly, breastfeeding was associated with higher weight gain in children of mothers who had excess weight gain during pregnancy, indicating a potential interplay between maternal weight and lactation (Ohlendorf *et al.*, 2019).

Aim 4.4: How does adipocyte mTORC1 hyperactivation alter lipogenesis in mammary glands?

Since mammary gland development is expected to be reduced along with reduced mammary adipocyte size (Expected Results from *Aim 4.1*), and since offspring weight is expected to be higher in KO dams (Expected Results from *Aim 4.3*), I hypothesize that mammary gland lipogenic activity will be increased thus contributing to the increased milk fat percentage (Expected Results from *Aim 4.2*). I expect RNA expression of ACC1, SREBP1c, ACLY and FASN to be increased in mammary glands of KO dams. This will explain the offspring phenotype and the altered mammary gland size and altered milk composition.

Potential Pitfalls and Alternate Approaches (Aims 4.1-4.4)

It is possible that the dams may cannibalize the litters. In that case, virgin mice will be bred again for consistency. It is also likely, since this is the first parity, that mammary gland development may not be fully mature yet. In this case, milk collection yield may be low at PND16.5. If that is the case, we may consider repeating the experiment again and collecting milk after a second parity to allow further mammary gland development. It is also possible that our model which only targets mammary adipocytes, may not cause an altered offspring phenotype which is often seen in maternal obesity. In that case, we will repeat this experiment using virgin C57BL/6J mice that will be placed on a high fat diet to establish obesity then they will be mated with lean males and will be maintained on a high fat diet during pregnancy and lactation. We will then determine maternal, offspring and milk characteristics and compare that to our model to verify if obesity causes hyperactivation of mTORC1 in mammary adipocytes.

Acosta O, Ramirez VI, Lager S, Gaccioli F, Dudley DJ, Powell TL & Jansson T (2015). Increased glucose and placental GLUT-1 in large infants of obese nondiabetic mothers. *Am J Obstet Gynecol* **212,** 227.e1-227.e7.

Ain R, Canham LN & Soares MJ (2005*a*). Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway. *J Endocrinol* **185,** 253–263.

Ain R, Canham LN & Soares MJ (2005*b*). Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway. *J Endocrinol* **185,** 253–263.

Alexander N, Rosenlöcher F, Stalder T, Linke J, Distler W, Morgner J & Kirschbaum C (2012). Impact of Antenatal Synthetic Glucocorticoid Exposure on Endocrine Stress Reactivity in Term-Born Children. *J Clin Endocrinol Metab* **97,** 3538–3544.

Anderson RR & Turner CW (1956). *Progress in Medical Virology*. Available at: https://journals.sagepub.com/doi/pdf/10.3181/00379727-109-27111 [Accessed June 24, 2019].

Anderson SM, Rudolph MC, McManaman JL & Neville MC (2007). Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis! *Breast Cancer Res* **9,** 204.

Andrade SE, Gurwitz JH, Davis RL, Chan KA, Finkelstein JA, Fortman K, Mcphillips H, Raebel MA, Roblin D, Smith DH, Yood MU, Morse AN & Platt R (2004). Prescription drug use in pregnancy. *Am J Obstet Gynecol* **191,** 398–407.

Anon (2006*a*). *Prednisone*. National Library of Medicine (US). Available at: http://www.ncbi.nlm.nih.gov/pubmed/30000136 [Accessed May 24, 2019].

Anon (2006*b*). *Cortisone*. National Library of Medicine (US). Available at: http://www.ncbi.nlm.nih.gov/pubmed/30000722 [Accessed June 24, 2019].

Anon (2009). The physiological basis of breastfeeding. Available at: https://www.ncbi.nlm.nih.gov/books/NBK148970/ [Accessed July 1, 2019].

Asztalos E, Willan A, Murphy K, Matthews S, Ohlsson A, Saigal S, Armson A, Kelly E, Delisle M-F, Gafni A, Lee S, Sananes R, Rovet J, Guselle P, Amankwah K & MACS-5 Collaborative Group (2014). Association between gestational age at birth, antenatal corticosteroids, and outcomes at 5 years: multiple courses of antenatal corticosteroids for preterm birth study at 5 years of age (MACS-5). *BMC Pregnancy Childbirth* **14,** 272.

Audette MC, Challis JRG, Jones RL, Sibley CP & Matthews SG (2011*a*). Antenatal Dexamethasone Treatment in Midgestation Reduces System A-Mediated Transport in the Late-Gestation Murine Placenta. ; DOI: 10.1210/en.2011-0104.

Audette MC, Challis JRG, Jones RL, Sibley CP & Matthews SG (2011*b*). Antenatal Dexamethasone Treatment in Midgestation Reduces System A-Mediated Transport in the Late-Gestation Murine Placenta. *Endocrinology* **152,** 3561–3570.

Audette MC, Challis JRG, Jones RL, Sibley CP & Matthews SG (2014). Synthetic Glucocorticoid Reduces Human Placental System A Transport in Women Treated With Antenatal Therapy. *J Clin Endocrinol Metab* **99,** E2226–E2233.

Audette MC, Greenwood SL, Sibley CP, Jones CJP, Challis JRG, Matthews SG & Jones RL (2010). Dexamethasone stimulates placental system A transport and trophoblast differentiation in term villous explants. *Placenta* **31,** 97–105.

Aune D, Saugstad OD, Henriksen T & Tonstad S (2014). Maternal Body Mass Index and the Risk of Fetal Death, Stillbirth, and Infant Death. *JAMA* **311,** 1536.

Babwah TJ, Nunes P & Maharaj RG (2013). The European Journal of General Practice An unexpected temporary suppression of lactation after a local corticosteroid injection for tenosynovitis Clinical Lesson An unexpected temporary suppression of lactation after a local corticosteroid injection for tenosynovitis. ; DOI: 10.3109/13814788.2013.805198.

Baeten JM, Bukusi EA & Lambe M (2001). Pregnancy complications and outcomes among overweight and obese nulliparous women. *Am J Public Health* **91,** 436–440.

Baisden B, Sonne S, Joshi RM, Ganapathy V & Shekhawat PS (2007*a*). Antenatal dexamethasone treatment leads to changes in gene expression in a murine late placenta. *Placenta* **28,** 1082–1090.

Baisden B, Sonne S, Joshi RM, Ganapathy V & Shekhawat PS (2007*b*). Antenatal dexamethasone treatment leads to changes in gene expression in a murine late placenta. *Placenta* **28,** 1082–1090.

Bakker JM, Schmidt ED, Kroes H, Kavelaars A, Heijnen CJ, Tilders FJH & van Rees EP (1995). Effects of short-term

dexamethasone treatment during pregnancy on the development of the immune system and the hypothalamo-pituitary adrenal axis in the rat. *J Neuroimmunol* **63,** 183–191.

Barker DJP (2007). The origins of the developmental origins theory. *J Intern Med* **261,** 412–417.

Barlow SM, Morrison PJ & Sullivan FM (1973). Plasma corticosterone levels during pregnancy in the mouse. *Br J Pharmacol* **48,** 346P.

BARTHA L, NAGY GM, KIEM DT, FEKETE MIK & MAKARA GB (1991). Inhibition of Suckling-Induced Prolactin Release by Dexamethasone. *Endocrinology* **129,** 635–640.

Bayliss RIS, Browne JCM, Round B & Steinbeck AW (1955). PLASMA-17-HYDROXYCORTICOSTEROIDS IN PREGNANCY. *Lancet* **265,** 62–64.

Ben-Jonathan N, LaPensee CR & LaPensee EW (2008). What can we learn from rodents about prolactin in humans? *Endocr Rev* **29,** 1–41.

Bider-Canfield Z, Martinez MP, Wang X, Yu W, Bautista MP, Brookey J, Page KA, Buchanan TA & Xiang AH (2017). Maternal obesity, gestational diabetes, breastfeeding and childhood overweight at age 2 years. *Pediatr Obes* **12,** 171–178.

Boney CM, Verma A, Tucker R & Vohr BR (2005). Metabolic Syndrome in Childhood: Association With Birth Weight, Maternal Obesity, and Gestational Diabetes Mellitus. *Pediatrics* **115,** e290–e296.

Boston WS, Bleck GT, Conroy JC, Wheeler MB & Miller DJ (2001). *Short Communication: Effects of Increased Expression of α-Lactalbumin In Transgenic Mice on Milk Yield and Pup Growth*. American Dairy Science Association. Available at: https://www.journalofdairyscience.org/article/S0022-0302(01)74516-X/pdf [Accessed June 19, 2019].

Branum AM, Kirmeyer SE & Gregory ECW (2014). National Vital Statistics Reports Prepregnancy Body Mass Index by Maternal Characteristics and State: Data From the Birth Certificate, 2014. Available at: https://www.cdc.gov/nchs/data/nvsr/nvsr65/nvsr65_06.pdf [Accessed December 8, 2017].

Braun T, Challis JR, Newnham JP & Sloboda DM (2013*a*). Early-Life Glucocorticoid Exposure: The Hypothalamic-Pituitary-Adrenal Axis, Placental Function, and Long-term Disease Risk. *Endocr Rev* **34,** 885–916.

Braun T, Husar A, Challis JRG, Dudenhausen JW, Henrich W, Plagemann A & Sloboda DM (2013*b*). Growth restricting effects of a single course of antenatal betamethasone treatment and the role of human placental lactogen. *Placenta* **34,** 407–415.

Brett K, Ferraro Z, Yockell-Lelievre J, Gruslin A & Adamo K (2014). Maternal–Fetal Nutrient Transport in Pregnancy Pathologies: The Role of the Placenta. *Int J Mol Sci* **15,** 16153–16185.

Bronson SL & Bale TL (2016). The Placenta as a Mediator of Stress Effects on Neurodevelopmental Reprogramming. *Neuropsychopharmacology* **41,** 207–218.

Buss C, Davis EP, Shahbaba B, Pruessner JC, Head K & Sandman CA (2012). Maternal cortisol over the course of pregnancy and subsequent child amygdala and hippocampus volumes and affective problems. *Proc Natl Acad Sci U S A* **109,** E1312-9.

Bzikowska-Jura A, Czerwonogrodzka-Senczyna A, Olędzka G, Szostak-Węgierek D, Weker H & Wesołowska A (2018). Maternal Nutrition and Body Composition During Breastfeeding: Association with Human Milk Composition. *Nutrients*; DOI: 10.3390/nu10101379.

Cai H, Dong LQ & Liu F (2016). Recent Advances in Adipose mTOR Signaling and Function: Therapeutic Prospects. *Trends Pharmacol Sci* **37,** 303–317.

Campbell FM, Bush PG, Veerkamp JH & Dutta-Roy AK (1998). Detection and cellular localization of plasma membrane-associated and cytoplasmic fatty acid-binding proteins in human placenta. *Placenta* **19,** 409–415.

Carr BR, Parker CR, Madden JD, MacDonald PC & Porter JC (1981). Maternal plasma adrenocorticotropin and cortisol relationships throughout human pregnancy. *Am J Obstet Gynecol* **139,** 416–422.

Castillo H, Santos IS & Matijasevich A (2016). Maternal pre-pregnancy BMI, gestational weight gain and breastfeeding. *Eur J Clin Nutr* **70,** 431–436.

Catalán V, Gómez-Ambrosi J, Rodríguez A, Ramírez B, Andrada P, Rotellar F, Valentí V, Moncada R, Martí P, Silva C, Salvador J & Frühbeck G (2015). Expression of S6K1 in human visceral adipose tissue is upregulated in obesity and related to insulin resistance and inflammation. *Acta Diabetol* **52,** 257–266.

Catalano PM, Farrell K, Thomas A, Huston-Presley L, Mencin P, de Mouzon SH & Amini SB (2009*a*). Perinatal risk factors for childhood obesity and metabolic dysregulation. *Am J Clin Nutr* **90,** 1303–1313.

Catalano PM, Presley L, Minium J & Hauguel-de Mouzon S (2009*b*). Fetuses of Obese Mothers Develop Insulin Resistance in Utero. *Diabetes Care* **32,** 1076–1080.

Catania C, Binder E & Cota D (2011). mTORC1 signaling in energy balance and metabolic disease. *Int J Obes* **35,** 751–761.

Cetin I, Ronzoni S, Marconi AM, Perugino G, Corbetta C, Battaglia FC & Pardi G (1996). Maternal concentrations and fetal-maternal concentration differences of plasma amino acids in normal and intrauterine growth-restricted pregnancies. *Am J Obstet Gynecol* **174,** 1575–1583.

Chen C, Xu X & Yan Y (2018). Estimated global overweight and obesity burden in pregnant women based on panel data model. *PLoS One*; DOI: 10.1371/journal.pone.0202183.

Chen Q, Wang Y, Zhao M, Hyett J, da Silva Costa F & Nie G (2016). Serum levels of GDF15 are reduced in preeclampsia and the reduction is more profound in late-onset than early-onset cases. *Cytokine* **83,** 226–230.

Cinti S (2018). Pink Adipocytes. *Trends Endocrinol Metab* **29,** 651–666.

Collins JW, David RJ, Handler A, Wall S, Andes S & Andes S (2004). Very low birthweight in African American infants: the role of maternal exposure to interpersonal racial discrimination. *Am J Public Health* **94,** 2132–2138.

Cuffe JSM, Dickinson H, Simmons DG & Moritz KM (2011*a*). Sex specific changes in placental growth and MAPK following short term maternal dexamethasone exposure in the mouse. *Placenta* **32,** 981–989.

Cuffe JSM, Dickinson H, Simmons DG & Moritz KM (2011*b*). Sex specific changes in placental growth and MAPK following short term maternal dexamethasone exposure in the mouse. *Placenta* **32,** 981–989.

Dalziel SR, Walker NK, Parag V, Mantell C, Rea HH, Rodgers A & Harding JE (2005). Cardiovascular risk factors after antenatal exposure to betamethasone: 30-year follow-up of a randomised controlled trial. *Lancet* **365,** 1856–1862.

Dann SG, Selvaraj A & Thomas G (2007). mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol Med* **13,** 252–259.

Davis EP, Townsend EL, Gunnar MR, Georgieff MK, Guiang SF, Ciffuentes RF & Lussky RC (2004). Effects of prenatal betamethasone exposure on regulation of stress physiology in healthy premature infants. *Psychoneuroendocrinology* **29,** 1028–1036.

Van Dijk AE, Van Eijsden M, Stronks K, Gemke RJBJ & Vrijkotte TGM (2012). The relation of maternal job strain and cortisol levels during early pregnancy with body composition later in the 5-year-old child: The ABCD study. *Early Hum Dev* **88,** 351–356.

Doyle LW, Ford GW, Rickards AL, Kelly EA, Davis NM, Callanan C & Olinsky A (2000). Antenatal corticosteroids and outcome at 14 years of age in children with birth weight less than 1501 grams. *Pediatrics* **106,** E2.

Dubé E, Gravel A, Martin C, Desparois G, Moussa I, Ethier-Chiasson M, Forest J-C, Giguère Y, Masse A & Lafond J (2012). Modulation of Fatty Acid Transport and Metabolism by Maternal Obesity in the Human Full-Term Placenta1. *Biol Reprod*; DOI: 10.1095/biolreprod.111.098095.

Dupouy J-P, Chatelain A, Boudouresque F, Conte-Devolx B & Oliver C (1987). Effects of Chronic Maternal Dexamethasone Treatment on the Hormones of the Hypothalamo-Pituitary-Adrenal Axis in the Rat Fetus. *Neonatology* **52,** 216–222.

Duthie L & Reynolds RM (2013). Changes in the maternal hypothalamic-pituitary-adrenal axis in pregnancy and postpartum: influences on maternal and fetal outcomes. *Neuroendocrinology* **98,** 106–115.

Edwards CR., Benediktsson R, Lindsay R. & Seckl J. (1993). Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet* **341,** 355–357.

Eguchi J, Yan Q-W, Schones DE, Kamal M, Hsu C-H, Zhang MQ, Crawford GE & Rosen ED (2008). Interferon Regulatory Factors Are Transcriptional Regulators of Adipogenesis. *Cell Metab* **7,** 86–94.

Ehrenberg HM, Mercer BM & Catalano PM (2004). The influence of obesity and diabetes on the prevalence of macrosomia. *Am J Obstet Gynecol* **191,** 964–968.

Entringer S, Buss C, Rasmussen JM, Lindsay K, Gillen DL, Cooper DM & Wadhwa PD (2016). Maternal cortisol during pregnancy and infant adiposity: a prospective investigation. *J Clin Endocrinol Metab* **102,** jc.2016-3025.

Erbay E, Park I-H, Nuzzi PD, Schoenherr CJ & Chen J (2003). IGF-II transcription in skeletal myogenesis is controlled by mTOR and nutrients. *J Cell Biol JCB J Cell Biol* **163,** 931–936.

Eriksen KG, Christensen SH, Lind M V. & Michaelsen KF (2018). Human milk composition and infant growth. *Curr Opin Clin Nutr Metab Care* **21,** 1.

Eskenazi B, Marks AR, Catalano R, Bruckner T & Toniolo PG (2007). Low birthweight in New York city and upstate New York following the events of September 11th. *Hum Reprod* **22,** 3013–3020.

Feng Z, Marti A, Jehn B, Altermatt HJ, Chicaiza G & Jaggi R (1995). Glucocorticoid and progesterone inhibit

involution and programmed cell death in the mouse mammary gland. *J Cell Biol* **131,** 1095–1103.

Flegal KM, Kruszon-Moran D, Carroll MD, Fryar CD & Ogden CL (2016). Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA* **315,** 2284.

Flint D & Vernon R (1998). Effects of food restriction on the responses of the mammary gland and adipose tissue to prolactin and growth hormone in the lactating rat. *J Endocrinol* **156,** 299–305.

Flint DJ, Travers MT, Barber MC, Binart N & Kelly PA (2005). Diet-induced obesity impairs mammary development and lactogenesis in murine mammary gland. *Am J Physiol Metab* **288,** E1179–E1187.

Gaccioli F, Aye ILMH, Roos S, Lager S, Ramirez VI, Kanai Y, Powell TL & Jansson T (2015). Expression and functional characterisation of System L amino acid transporters in the human term placenta. *Reprod Biol Endocrinol* **13,** 57.

Gaccioli F, Lager S, Powell TL & Jansson T (2013*a*). Placental transport in response to altered maternal nutrition. *J Dev Orig Health Dis* **4,** 101–115.

Gaccioli F, White V, Capobianco E, Powell TL, Jawerbaum A & Jansson T (2013*b*). Maternal Overweight Induced by a Diet with High Content of Saturated Fat Activates Placental mTOR and eIF2alpha Signaling and Increases Fetal Growth in Rats1. *Biol Reprod* **89,** 96.

Gaillard R, Steegers EAP, Duijts L, Felix JF, Hofman A, Franco OH & Jaddoe VWV (2014). Childhood Cardiometabolic Outcomes of Maternal Obesity During Pregnancy. *Hypertension* **63,** 683–691.

Gangloff Y-G, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz J-F, Um SH, Brown EJ, Cereghini S, Thomas G & Kozma SC (2004). Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. *Mol Cell Biol* **24,** 9508–9516.

Garami A, Zwartkruis FJT, Nobukuni T, Joaquin M, Roccio M, Stocker H, Kozma SC, Hafen E, Bos JL & Thomas G (2003). Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* **11,** 1457–1466.

Garg A, Peshock RM & Fleckenstein JL (1999). Adipose Tissue Distribution Pattern in Patients with Familial Partial Lipodystrophy (Dunnigan Variety) [1]. *J Clin Endocrinol Metab* **84,** 170–174.

Georgiades P, Ferguson-Smith AC & Burton GJ (2002). Comparative Developmental Anatomy of the Murine and Human Definitive Placentae. *Placenta* **23,** 3–19.

Glazier JD, Cetin I, Perugino G, Ronzoni S, Grey AM, Mahendran D, Marconi AM, Pardi G & Sibley CP (1997). Association between the Activity of the System A Amino Acid Transporter in the Microvillous Plasma Membrane of the Human Placenta and Severity of Fetal Compromise in Intrauterine Growth Restriction. *Pediatr Res* **42,** 514–519.

Görs S, Kucia M, Langhammer M, Junghans P & Metges CC (2009). Technical note: Milk composition in mice—Methodological aspects and effects of mouse strain and lactation day. *J Dairy Sci* **92,** 632–637.

Gupta MB & Jansson T (2019). Novel roles of mechanistic target of rapamycin signaling in regulating fetal growth†. *Biol Reprod* **100,** 872–884.

Hahn T, Barth S, Graf R, Engelmann M, Beslagic D, Reul JMHM, Holsboer F, Dohr G & Desoye G (1999*a*). Placental Glucose Transporter Expression Is Regulated by Glucocorticoids [1]. *J Clin Endocrinol Metab* **84,** 1445–1452.

Hahn T, Barth S, Graf R, Engelmann M, Beslagic D, Reul JMHM, Holsboer F, Dohr G & Desoye G (1999*b*). Placental Glucose Transporter Expression Is Regulated by Glucocorticoids [1]. *J Clin Endocrinol Metab* **84,** 1445–1452.

Hahn T & Desoye G (1996). Ontogeny of glucose transport systems in the placenta and its progenitor tissues. *Early Pregnancy* **2,** 168–182.

Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP & Lamb RF (2004). The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J Cell Biol* **166,** 213–223.

Hayes EK (2012). *THE EFFECT OF LIFELONG MATERNAL OBESITY ON PREGNANCY OUTCOMES AND PLACENTAL DEVELOPMENT*. Available at: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.1004.8647&rep=rep1&type=pdf [Accessed August 15, 2019].

Henderson JJ, Hartmann PE, Newnham JP & Simmer K (2007). Effect of Preterm Birth and Antenatal Corticosteroid Treatment on Lactogenesis II in Women. ; DOI: 10.1542/peds.2007-1107.

Henderson JJ, Newnham JP, Simmer K & Hartmann PE (2009). Effects of Antenatal Corticosteroids on Urinary Markers of the Initiation of Lactation in Pregnant Women. *Breastfeed Med* **4,** 201–206.

Hennig M, Fiedler S, Jux C, Thierfelder L & Drenckhahn J-D (2017). Prenatal Mechanistic Target of Rapamycin

Complex 1 (m TORC1) Inhibition by Rapamycin Treatment of Pregnant Mice Causes Intrauterine Growth Restriction and Alters Postnatal Cardiac Growth, Morphology, and Function. *J Am Heart Assoc*; DOI: 10.1161/JAHA.117.005506.

Hochberg I, Tran QT, Barkan AL, Saltiel AR, Chandler WF & Bridges D (2015). Gene Expression Signature in Adipose Tissue of Acromegaly Patients. *PLoS One* **10,** e0129359.

Hollanders JJ, Heijboer AC, van der Voorn B, Rotteveel J & Finken MJJ (2017). Nutritional programming by glucocorticoids in breast milk: Targets, mechanisms and possible implications. *Best Pract Res Clin Endocrinol Metab* **31,** 397–408.

Hovey RC & Aimo L (2010). Diverse and active roles for adipocytes during mammary gland growth and function. *J Mammary Gland Biol Neoplasia* **15,** 279–290.

Hu Z, Tylavsky FA, Han JC, Kocak M, Fowke JH, Davis RL, Lewinn K, Bush NR & Zhao • Qi (2019). Maternal metabolic factors during pregnancy predict early childhood growth trajectories and obesity risk: the CANDLE Study. *Int J Obes*; DOI: 10.1038/s41366-019-0326-z.

Huter O, Wolf HJ, Schnetzer A & Pfaller K (1997). Lipoprotein lipase, LDL receptors and apo-lipoproteins in human fetal membranes at term. *Placenta* **18,** 707–715.

Hviid A & Mølgaard-Nielsen D (2011). Corticosteroid use during pregnancy and risk of orofacial clefts. *CMAJ* **183,** 796–804.

Inder WJ, Prickett TCR, Ellis MJ, Hull L, Reid R, Benny PS, Livesey JH & Donald RA (2001). The Utility of Plasma CRH as a Predictor of Preterm Delivery. *J Clin Endocrinol Metab* **86,** 5706–5710.

Jafari Z, Mehla J, Afrashteh N, Kolb BE & Mohajerani MH (2017). Corticosterone response to gestational stress and postpartum memory function in mice ed. Pawluski J. *PLoS One* **12,** e0180306.

Jankiewicz M, Groner B & Desrivières S (2006). Mammalian Target of Rapamycin Regulates the Growth of Mammary Epithelial Cells through the Inhibitor of Deoxyribonucleic Acid Binding Id1 and Their Functional Differentiation through Id2. *Mol Endocrinol* **20,** 2369–2381.

Jansson N, Rosario FJ, Gaccioli F, Lager S, Jones HN, Roos S, Jansson T & Powell TL (2013). Activation of Placental mTOR Signaling and Amino Acid Transporters in Obese Women Giving Birth to Large Babies. *J Clin Endocrinol Metab* **98,** 105–113.

Jansson T & Powell TL (2013). Role of placental nutrient sensing in developmental programming. *Clin Obstet Gynecol* **56,** 591–601.

Jansson T, Scholtbach V & Powell TL (1998). Placental Transport of Leucine and Lysine Is Reduced in Intrauterine Growth Restriction1. *Pediatr Res* **44,** 532–537.

Jeje SO, Akindele OO, Ushie G & Rajil Y (2016). Changes in kidney function and oxidative stress biomarkers in offspring from dams treated with dexamethasone during lactation in Wistar rats. *Afr J Med Med Sci* **45,** 237–242.

Jeje SO & Raji Y (2015). Effects of Maternal Dexamethasone Exposure During Lactation on Metabolic Imbalance and Oxidative Stress in the Liver of Male Offsprings of Wistar Rats. *Niger J Physiol Sci* **30,** 131–137.

Jones HN, Ashworth CJ, Page KR & McArdle HJ (2006). Cortisol stimulates system A amino acid transport and SNAT2 expression in a human placental cell line (BeWo). *Am J Physiol Metab* **291,** E596–E603.

Jones HN, Woollett LA, Barbour N, Prasad PD, Powell TL & Jansson T (2009). High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. *FASEB J* **23,** 271–278.

Jung C, Ho JT, Torpy DJ, Rogers A, Doogue M, Lewis JG, Czajko RJ & Inder WJ (2011). A Longitudinal Study of Plasma and Urinary Cortisol in Pregnancy and Postpartum. *J Clin Endocrinol Metab* **96,** 1533–1540.

Kaur G & Dufour JM (2012). Cell lines: Valuable tools or useless artifacts. *Spermatogenesis* **2,** 1–5.

Kemp MW, Newnham JP, Challis JG, Jobe AH & Stock SJ (2015). The clinical use of corticosteroids in pregnancy. *Hum Reprod Update* **22,** dmv047.

Khan NA, Nikkanen J, Yatsuga S, Jackson C, Wang L, Pradhan S, Kivelä R, Pessia A, Velagapudi V & Suomalainen A (2017). mTORC1 Regulates Mitochondrial Integrated Stress Response and Mitochondrial Myopathy Progression. *Cell Metab* **26,** 419-428.e5.

Kim SS, Zhu Y, Grantz KL, Hinkle SN, Chen Z, Wallace ME, Smarr MM, Epps NM & Mendola P (2016). Obstetric and Neonatal Risks Among Obese Women Without Chronic Disease. *Obstet Gynecol* **128,** 104–112.

Kipmen-Korgun D, Ozmen A, Unek G, Simsek M, Demir R & Korgun ET (2012). Triamcinolone up-regulates GLUT 1 and GLUT 3 expression in cultured human placental endothelial cells. *Cell Biochem Funct* **30,** 47–53.

Kobayashi K, Tsugami Y, Matsunaga K, Oyama S, Kuki C & Kumura H (2016). Prolactin and glucocorticoid signaling induces lactation-specific tight junctions concurrent with β-casein expression in mammary epithelial cells. *Biochim Biophys Acta - Mol Cell Res* **1863,** 2006–2016.

Kovo M, Zion-Saukhanov E, Schreiber L, Mevorach N, Divon M, Ben-Haroush A & Bar J (2015). The Effect of Maternal Obesity on Pregnancy Outcome in Correlation With Placental Pathology. *Reprod Sci* **22,** 1643–1648.

Kronfeld DS & Hartmann PE (1973). Glucose Redistribution in Lactating Cows Given Dexamethasone. *J Dairy Sci* **56,** 903–908.

Kwiatkowski DJ, Zhang H, Bandura JL, Heiberger KM, Glogauer M, el-Hashemite N & Onda H (2002*a*). A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum Mol Genet* **11,** 525–534.

Kwiatkowski DJ, Zhang H, Bandura JL, Heiberger KM, Glogauer M, el-Hashemite N & Onda H (2002*b*). A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum Mol Genet* **11,** 525–534.

Lager S, Jansson T & Powell TL (2014). Differential regulation of placental amino acid transport by saturated and unsaturated fatty acids. *Am J Physiol Cell Physiol* **307,** C738-44.

Landskroner-Eiger S, Park J, Israel D, Pollard JW & Scherer PE (2010). Morphogenesis of the developing mammary gland: stage-dependent impact of adipocytes. *Dev Biol* **344,** 968–978.

Langdown ML & Sugden MC (2001). Enhanced placental GLUT1 and GLUT3 expression in dexamethasone-induced fetal growth retardation. *Mol Cell Endocrinol* **185,** 109–117.

Laplante M & Sabatini DM (2009). An Emerging Role of mTOR in Lipid Biosynthesis. *Curr Biol* **19,** R1046–R1052.

Lawson DA, Werb Z, Zong Y & Goldstein AS (2015). The Cleared Mammary Fat Pad Transplantation Assay for Mammary Epithelial Organogenesis. *Cold Spring Harb Protoc* **2015,** pdb.prot078071.

Leddy MA, Power ML & Schulkin J (2008). The impact of maternal obesity on maternal and fetal health. *Rev Obstet Gynecol* **1,** 170–178.

Leon-Garcia SM, Roeder HA, Nelson KK, Liao X, Pizzo DP, Laurent LC, Parast MM & LaCoursiere DY (2016). Maternal obesity and sex-specific differences in placental pathology. *Placenta* **38,** 33–40.

Lesage J, Del-Favero F, Leonhardt M, Louvart H, Maccari S, Vieau D & Darnaudery M (2004). Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. *J Endocrinol* **181,** 291–296.

Levine S (1967). Maternal and environmental influences on the adrenocortical response to stress in weanling rats. *Science* **156,** 258–260.

Li H, Liu X, Wang Z, Lin X, Yan Z, Cao Q, Zhao M & Shi K (2017). MEN1/Menin regulates milk protein synthesis through mTOR signaling in mammary epithelial cells. *Sci Rep* **7,** 5479.

Li J, Kendall G, Henderson S, Downie J, Landsborough L & Oddy W (2008). Maternal psychosocial well-being in pregnancy and breastfeeding duration. *Acta Paediatr* **97,** 221–225.

Li M, Liu X, Robinson G, Bar-Peled U, Wagner K-U, Young WS, Hennighausen L & Furth PA (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proc Natl Acad Sci U S A* **94,** 3425.

Li R, Zowalaty AE El, Chen W, Dudley EA & Ye X (2015). Segregated responses of mammary gland development and vaginal opening to prepubertal genistein exposure in Bscl2–/– female mice with lipodystrophy. *Reprod Toxicol* **54,** 76.

Lindsay JR & Nieman LK (2005). The Hypothalamic-Pituitary-Adrenal Axis in Pregnancy: Challenges in Disease Detection and Treatment. *Endocr Rev* **26,** 775–799.

Lu B, Bridges D, Yang Y, Fisher K, Cheng A, Chang L, Meng Z-X, Lin JD, Downes M, Yu RT, Liddle C, Evans RM & Saltiel AR (2014). Metabolic Crosstalk: Molecular Links Between Glycogen and Lipid Metabolism in Obesity. *Diabetes* **63,** 2935–2948.

Lunghi L, Pavan B, Biondi C, Paolillo R, Valerio A, Vesce F & Patella A (2010). Use of Glucocorticoids in Pregnancy. *Curr Pharm Des* **16,** 3616–3637.

Machino M (1976). *Growth and Differentiation, Vo1*. Available at: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1440-169X.1976.00079.x [Accessed July 17, 2019].

Macias H & Hinck L (2012). Mammary gland development. *Wiley Interdiscip Rev Dev Biol* **1,** 533–557.

Magnusson AL, Waterman IJ, Wennergren M, Jansson T & Powell TL (2004). Triglyceride Hydrolase Activities and Expression of Fatty Acid Binding Proteins in the Human Placenta in Pregnancies Complicated by Intrauterine

Growth Restriction and Diabetes. *J Clin Endocrinol Metab* **89,** 4607–4614.

Malassine A, Frendo J-L & Evain-Brion D (2003). A comparison of placental development and endocrine functions between the human and mouse model. *Hum Reprod Update* **9,** 531–539.

Mateos RM, Jiménez G, Álvarez-Gil C, Visiedo F, Rivera-Rodríguez F, Santos-Rosendo C, Rodriguez-Pareja A, Perdomo G & Lechuga-Sancho AM (2018). Excess Hydrocortisone Hampers Placental Nutrient Uptake Disrupting Cellular Metabolism. *Biomed Res Int* **2018,** 1–11.

Matthews SG (2000). Antenatal Glucocorticoids and Programming of the Developing CNS. *Pediatr Res* **47,** 291–300.

McManaman JL (2009). Formation of milk lipids: a molecular perspective. *Clin Lipidol* **4,** 391–401.

Mingrone G, Manco M, Mora MEV, Guidone C, Iaconelli A, Gniuli D, Leccesi L, Chiellini C & Ghirlanda G (2008). Influence of maternal obesity on insulin sensitivity and secretion in offspring. *Diabetes Care* **31,** 1872–1876.

Moisiadis VG & Matthews SG (2014*a*). Glucocorticoids and fetal programming part 1: outcomes. *Nat Rev Endocrinol* **10,** 391–402.

Moisiadis VG & Matthews SG (2014*b*). Glucocorticoids and fetal programming part 1: outcomes. *Nat Rev Endocrinol* **10,** 391–402.

Morroni M, Giordano A, Zingaretti MC, Boiani R, De Matteis R, Kahn BB, Nisoli E, Tonello C, Pisoschi C, Luchetti MM, Marelli M & Cinti S (2004). Reversible transdifferentiation of secretory epithelial cells into adipocytes in the mammary gland. *Proc Natl Acad Sci U S A* **101,** 16801–16806.

Mparmpakas D, Zachariades E, Goumenou A, Gidron Y & Karteris E (2012). Placental DEPTOR as a stress sensor during pregnancy. *Clin Sci (Lond)* **122,** 349–359.

Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofer F, Kiyama H, Yonezawa K & Yamanaka S (2004). mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. *Mol Cell Biol* **24,** 6710–6718.

NANDI S (1958). Endocrine control of mammarygland development and function in the C3H/ He Crgl mouse. *J Natl Cancer Inst* **21,** 1039–1063.

Napso T, Yong HEJ, Lopez-Tello J & Sferruzzi-Perri AN (2018*a*). The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. *Front Physiol* **9,** 1091.

Napso T, Yong HEJ, Lopez-Tello J & Sferruzzi-Perri AN (2018*b*). The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. *Front Physiol* **9,** 1091.

Neri C & Edlow AG (2015). Effects of Maternal Obesity on Fetal Programming: Molecular Approaches. *Cold Spring Harb Perspect Med* **6,** a026591.

Neville MC, Mcfadden TB & Forsyth I (2002). *Hormonal Regulation of Mammary Differentiation and Milk Secretion*. Available at: https://link.springer.com/content/pdf/10.1023%2FA%3A1015770423167.pdf [Accessed May 24, 2019].

Neville MC, Morton J & Umemura S (2001). Lactogenesis: The Transition from Pregnancy to Lactation. *Pediatr Clin North Am* **48,** 35–52.

Ng PC (2000). The fetal and neonatal hypothalamic-pituitary-adrenal axis. *Arch Dis Child Fetal Neonatal Ed* **82,** F250-4.

Norberg S, Powell TL & Jansson T (1998). Intrauterine Growth Restriction Is Associated with a Reduced Activity of Placental Taurine Transporters. *Pediatr Res* **44,** 233–238.

O'Sullivan L, Cuffe JSM, Paravicini TM, Campbell S, Dickinson H, Singh RR, Gezmish O, Black MJ & Moritz KM (2013). Prenatal exposure to dexamethasone in the mouse alters cardiac growth patterns and increases pulse pressure in aged male offspring. *PLoS One* **8,** e69149.

Ochsner S et al. (2019). The Signaling Pathways Project: an integrated 'omics knowledgebase for mammalian cellular signaling pathways. *bioRxiv*401729.

Ohlendorf JM, Robinson K & Garnier-Villarreal M (2019). The impact of maternal BMI, gestational weight gain, and breastfeeding on early childhood weight: Analysis of a statewide WIC dataset. *Prev Med (Baltim)* **118,** 210–215.

Olesen C, Hald Steffensen F, Lauge Nielsen G, Jong-van den Berg L, Olsen J, Toft Sørensen H & group TE (1999). Drug use in first pregnancy and lactation: a population-based survey among Danish women. *Eur J Clin Pharmacol* **55,** 139–144.

Ouyang F, Parker M, Cerda S, Pearson C, Fu L, Gillman MW, Zuckerman B & Wang X (2013). Placental weight mediates the effects of prenatal factors on fetal growth: the extent differs by preterm status. *Obesity* **21,** 609–620.

Owens LA, O'Sullivan EP, Kirwan B, Avalos G, Gaffney G, Dunne F & ATLANTIC DIP Collaborators for the AD (2010). ATLANTIC DIP: the impact of obesity on pregnancy outcome in glucose-tolerant women. *Diabetes Care* **33,** 577–579.

Padoan A, Rigano S, Ferrazzi E, Beaty BL, Battaglia FC & Galan HL (2004). Differences in fat and lean mass proportions in normal and growth-restricted fetuses. *Am J Obstet Gynecol* **191,** 1459–1464.

Panagos PG, Vishwanathan R, Penfield-Cyr A, Matthan NR, Shivappa N, Wirth MD, Hebert JR & Sen S (2016). Breastmilk from obese mothers has pro-inflammatory properties and decreased neuroprotective factors. *J Perinatol* **36,** 284–290.

Patel R, Williams-Dautovich J & Cummins CL (2014). Minireview: New Molecular Mediators of Glucocorticoid Receptor Activity in Metabolic Tissues. *Mol Endocrinol* **28,** 999–1011.

Petry CJ, Ong KK, Burling KA, Barker P, Goodburn SF, Perry JRB, Acerini CL, Hughes IA, Painter RC, Afink GB, Dunger DB & O'Rahilly S (2018). Associations of vomiting and antiemetic use in pregnancy with levels of circulating GDF15 early in the second trimester: A nested case-control study. *Wellcome open Res* **3,** 123.

Pillay J & Davis TJ (2019). *Physiology, Lactation*. StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29763156 [Accessed May 24, 2019].

Ponnusamy S, Tran QT, Harvey I, Smallwood HS, Thiyagarajan T, Banerjee S, Johnson DL, Dalton JT, Sullivan RD, Miller DD, Bridges D & Narayanan R (2017). Pharmacologic activation of estrogen receptor β increases mitochondrial function, energy expenditure, and brown adipose tissue. *FASEB J* **31,** 266–281.

Prokesch A, Smorlesi A, Perugini J, Manieri M, Ciarmela P, Mondini E, Trajanoski Z, Kristiansen K, Giordano A, Bogner-Strauss JG & Cinti S (2014). Molecular Aspects of Adipoepithelial Transdifferentiation in Mouse Mammary Gland. *Stem Cells* **32,** 2756–2766.

Rangarajan A & Weinberg RA (2003). Comparative biology of mouse versus human cells: modelling human cancer in mice. *Nat Rev Cancer* **3,** 952–959.

Rasmussen KM & Kjolhede CL (2004). Prepregnant overweight and obesity diminish the prolactin response to suckling in the first week postpartum. *Pediatrics* **113,** e465-71.

Regnault TRH, Orbus RJ, de Vrijer B, Davidsen ML, Galan HL, Wilkening RB & Anthony RV (2002). Placental Expression of VEGF, PlGF and their Receptors in a Model of Placental Insufficiency—Intrauterine Growth Restriction (PI-IUGR). *Placenta* **23,** 132–144.

Rennebeck G, Kleymenova E V, Anderson R, Yeung RS, Artzt K & Walker CL (1998). Loss of function of the tuberous sclerosis 2 tumor suppressor gene results in embryonic lethality characterized by disrupted neuroepithelial growth and development. *Proc Natl Acad Sci U S A* **95,** 15629–15634.

Reynolds RM (2013). Glucocorticoid excess and the developmental origins of disease: Two decades of testing the hypothesis – 2012 Curt Richter Award Winner. *Psychoneuroendocrinology* **38,** 1–11.

Rezaei R, Wu Z, Hou Y, Bazer FW & Wu G (2016). Amino acids and mammary gland development: nutritional implications for milk production and neonatal growth. *J Anim Sci Biotechnol* **7,** 20.

Richert MM, Schwertfeger KL, Ryder JW & Anderson SM (2000). *An Atlas of Mouse Mammary Gland Development*. Available at: https://link.springer.com/content/pdf/10.1023%2FA%3A1026499523505.pdf [Accessed July 19, 2019].

Rolls BJ & Rowe EA (1982). Pregnancy and lactation in the obese rat: effects on maternal and pup weights. *Physiol Behav* **28,** 393–400.

Roos S, Jansson N, Palmberg I, Säljö K, Powell TL & Jansson T (2007). Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. *J Physiol* **582,** 449–459.

Roos S, Kanai Y, Prasad PD, Powell TL & Jansson T (2009*a*). Regulation of placental amino acid transporter activity by mammalian target of rapamycin. *Am J Physiol Physiol* **296,** C142–C150.

Roos S, Lagerlöf O, Wennergren M, Powell TL & Jansson T (2009*b*). Regulation of amino acid transporters by glucose and growth factors in cultured primary human trophoblast cells is mediated by mTOR signaling. *Am J Physiol Physiol* **297,** C723–C731.

Rosado-Yépez PI, Chávez-Corral D V., Reza-López SA, Leal-Berumen I, Fierro-Murga R, Caballero-Cummings S & Levario-Carrillo M (2019). Relation between pregestational obesity and characteristics of the placenta. *J Matern Neonatal Med*1–6.

Rosario FJ, Kanai Y, Powell TL & Jansson T (2015). Increased placental nutrient transport in a novel mouse model of maternal obesity with fetal overgrowth. *Obesity* **23,** 1663–1670.

Rosario FJ, Powell TL & Jansson T (2016). Activation of placental insulin and mTOR signaling in a mouse model of

maternal obesity associated with fetal overgrowth. *Am J Physiol Regul Integr Comp Physiol* **310,** R87-93.

RUSSELL JG (1958). Lipodystrophy progressiva and pregnancy. *Postgrad Med J* **34,** 530 passim.

Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH & Goldberg AL (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117,** 399–412.

Schmidt M, Enthoven L, van der Mark M, Levine S, de Kloet ER & Oitzl MS (2003). The postnatal development of the hypothalamic–pituitary–adrenal axis in the mouse. *Int J Dev Neurosci* **21,** 125–132.

Schwertfeger KL, McManaman JL, Palmer CA, Neville MC & Anderson SM (2003). Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation. *J Lipid Res* **44,** 1100–1112.

Scifres CM, Chen B, Nelson DM & Sadovsky Y (2011). Fatty Acid Binding Protein 4 Regulates Intracellular Lipid Accumulation in Human Trophoblasts. *J Clin Endocrinol Metab* **96,** E1083–E1091.

Sebire N, Jolly M, Harris J, Wadsworth J, Joffe M, Beard R, Regan L & Robinson S (2001). Maternal obesity and pregnancy outcome: a study of 287 213 pregnancies in London. *Int J Obes* **25,** 1175–1182.

Shamay A, Shapiro F, Barash H, Bruckental I, Silanikove N & Shamay A (2000). Effect of dexamethasone on milk yield and composition in dairy cows. *INRA/EDP Sci*; DOI: 10.1051/animres:2000125ï.

Shibata E, Hubel CA, Powers RW, von Versen-Hoeynck F, Gammill H, Rajakumar A & Roberts JM (2008). Placental system A amino acid transport is reduced in pregnancies with small for gestational age (SGA) infants but not in preeclampsia with SGA infants. *Placenta* **29,** 879–882.

Singh RR, Cuffe JS & Moritz KM (2012). Short- and long-term effects of exposure to natural and synthetic glucocorticoids during development. *Clin Exp Pharmacol Physiol* **39,** 979–989.

Soares MJ (2004). The prolactin and growth hormone families: pregnancy-specific hormones/cytokines at the maternal-fetal interface. *Reprod Biol Endocrinol* **2,** 51.

Song L, Sun B, Boersma GJ, Cordner ZA, Yan J, Moran TH & Tamashiro KLK (2017). Prenatal high-fat diet alters placental morphology, nutrient transporter expression, and mtorc1 signaling in rat. *Obesity* **25,** 909–919.

De Souza SW, F Adlard BP & Souza D (1973). *Growth of suckling rats after treatment with dexamethasone or cortisol Implications for steroid therapy in human infants*. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1648459/pdf/archdisch00863-0029.pdf [Accessed August 18, 2019].

Stephens MAC & Wand G (2012). Stress and the HPA axis: role of glucocorticoids in alcohol dependence. *Alcohol Res* **34,** 468–483.

Stephenson EJ, Redd JR, Snyder D, Tran QT, Lu B, Peloquin MJ, Mulcahy MC, Harvey I, Fisher K, Han JC, Qi N, Saltiel AR & Bridges D (2019). Skeletal Muscle mTORC1 Activation Increases Energy Expenditure and Reduces Longevity in Mice. *bioRxiv*720540.

Stubert J, Reister F, Hartmann S & Janni W (2018). The Risks Associated With Obesity in Pregnancy. *Dtsch Arztebl Int* **115,** 276–283.

Sugulle M, Dechend R, Herse F, Weedon-Fekjaer MS, Johnsen GM, Brosnihan KB, Anton L, Luft FC, Wollert KC, Kempf T & Staff AC (2009). Circulating and placental growth-differentiation factor 15 in preeclampsia and in pregnancy complicated by diabetes mellitus. *Hypertens (Dallas, Tex 1979)* **54,** 106–112.

Tang H et al. (2019). mTORC1 underlies age-related muscle fiber damage and loss by inducing oxidative stress and catabolism. *Aging Cell* **18,** e12943.

Thibeau S, D'Apolito K, Minnick AF, Dietrich MS, Kane B, Cooley S & Groer M (2016). Relationships of Maternal Stress with Milk Immune Components in African American Mothers of Healthy Term Infants. *Breastfeed Med* **11,** 6–14.

Tong S, Marjono B, Brown DA, Mulvey S, Breit SN, Manuelpillai U & Wallace EM (2004). Serum concentrations of macrophage inhibitory cytokine 1 (MIC 1) as a predictor of miscarriage. *Lancet* **363,** 129–130.

Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J & Thomas G (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431,** 200–205.

Urraca N, Memon R, El-Iyachi I, Goorha S, Valdez C, Tran QT, Scroggs R, Miranda-Carboni GA, Donaldson M, Bridges D & Reiter LT (2015). Characterization of neurons from immortalized dental pulp stem cells for the study of neurogenetic disorders. *Stem Cell Res* **15,** 722–730.

Varner MA & Johnson BH (1983). Influence of Adrenocorticotropin upon Milk Production, Milk Constituents, and

Endocrine Measures of Dairy Cows. *J Dairy Sci* **66,** 458–465.

Vaughan OR, Fisher HM, Dionelis KN, Jefferies EC, Higgins JS, Musial B, Sferruzzi-Perri AN & Fowden AL (2015). Corticosterone alters materno-fetal glucose partitioning and insulin signalling in pregnant mice. *J Physiol* **593,** 1307–1321.

Vaughan OR, Rosario FJ, Powell TL & Jansson T (2017). Regulation of Placental Amino Acid Transport and Fetal Growth. *Prog Mol Biol Transl Sci* **145,** 217–251.

Vaughan OR, Sferruzzi-Perri AN & Fowden AL (2012). Maternal corticosterone regulates nutrient allocation to fetal growth in mice. *J Physiol* **590,** 5529–5540.

Vilela FC & Giusti-Paiva A (2011). Glucocorticoids Disrupt Neuroendocrine and Behavioral Responses during Lactation. *Endocrinology* **152,** 4838–4845.

Waddell DS, Baehr LM, van den Brandt J, Johnsen SA, Reichardt HM, Furlow JD & Bodine SC (2008). The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. *Am J Physiol Metab* **295,** E785–E797.

Wadsack C, Tabano S, Maier A, Hiden U, Alvino G, Cozzi V, Hüttinger M, Schneider WJ, Lang U, Cetin I & Desoye G (2007). Intrauterine growth restriction is associated with alterations in placental lipoprotein receptors and maternal lipoprotein composition. *Am J Physiol Metab* **292,** E476–E484.

Waffarn F & Davis EP (2012). Effects of antenatal corticosteroids on the hypothalamic-pituitary-adrenocortical axis of the fetus and newborn: experimental findings and clinical considerations. *Am J Obstet Gynecol* **207,** 446–454.

Wang F, Mullican SE, DiSpirito JR, Peed LC & Lazar MA (2013). Lipoatrophy and severe metabolic disturbance in mice with fat-specific deletion of PPARγ. *Proc Natl Acad Sci U S A* **110,** 18656–18661.

Wang H, Kubica N, Ellisen LW, Jefferson LS & Kimball SR (2006). Dexamethasone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1. *J Biol Chem* **281,** 39128–39134.

Wang QA, Song A, Gupta RK, Deplancke B & Scherer PE (2018). Reversible De-differentiation of Mature White Adipocytes into Preadipocyte-like Precursors during Lactation. *Cell Metab* **28,** 282-288.e3.

Wang X & Proud CG (2006). The mTOR Pathway in the Control of Protein Synthesis. *Physiology* **21,** 362–369.

Wen HY, Abbasi S, Kellems RE & Xia Y (2005). mTOR: A placental growth signaling sensor. *Placenta* **26,** S63–S69.

Wenzel PL & Leone G (2007). Expression of Cre recombinase in early diploid trophoblast cells of the mouse placenta. *genesis* **45,** 129–134.

Whitaker RC (2004). Predicting Preschooler Obesity at Birth: The Role of Maternal Obesity in Early Pregnancy. *Pediatrics* **114,** e29–e36.

Wieczorek A, Perani C V., Nixon M, Constancia M, Sandovici I, Zazara DE, Leone G, Zhang M-Z, Arck PC & Solano ME (2019). Sex-specific regulation of stress-induced fetal glucocorticoid surge by the mouse placenta. *Am J Physiol Metab* **317,** E109–E120.

Williams CB, Mackenzie KC & Gahagan S (2014). The effect of maternal obesity on the offspring. *Clin Obstet Gynecol* **57,** 508–515.

Winterhager E & Gellhaus A (2017). Transplacental Nutrient Transport Mechanisms of Intrauterine Growth Restriction in Rodent Models and Humans. *Front Physiol* **8,** 951.

Wolff NC, McKay RM & Brugarolas J (2014). REDD1/DDIT4-Independent mTORC1 Inhibition and Apoptosis by Glucocorticoids in Thymocytes. *Mol Cancer Res* **12,** 867–877.

Woods L, Perez-Garcia V & Hemberger M (2018). Regulation of Placental Development and Its Impact on Fetal Growth-New Insights From Mouse Models. *Front Endocrinol (Lausanne)* **9,** 570.

Xu J, Lu C, Wang J, Zhang R, Qian X & Zhu H (2015). Regulation of Human Trophoblast GLUT3 Glucose Transporter by Mammalian Target of Rapamycin Signaling. *Int J Mol Sci* **16,** 13815–13828.

Yagi T, Ikawa Y, Yoshida K, Shigetani Y, Takeda N, Mabuchi I, Yamamoto T, Aizawa S & Knudson AG (1990). Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. *Proc Natl Acad Sci U S A* **87,** 9918–9922.

Yang X & Friedl A (2015). A Positive Feedback Loop Between Prolactin and Stat5 Promotes Angiogenesis. In *Advances in experimental medicine and biology*, pp. 265–280. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25472543 [Accessed June 24, 2019].

Zhang H, Cicchetti G, Onda H, Koon HB, Asrican K, Bajraszewski N, Vazquez F, Carpenter CL & Kwiatkowski DJ (2003). Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR.

J Clin Invest **112,** 1223–1233.

Zhang R, Ma H, Gao Y, Wu Y, Qiao Y, Geng A, Cai C, Han Y, Arial Zeng Y, Liu X & Ge G (2018). Th-POK regulates mammary gland lactation through mTOR-SREBP pathway. ; DOI: 10.1371/journal.pgen.1007211.

Zhu Z, Jiang W & Thompson HJ (1998). Effect of corticosterone administration on mammary gland development and p27 expression and their relationship to the effects of energy restriction on mammary carcinogenesis. *Carcinogenesis* **19,** 2101–2106.

Zwick RK, Rudolph MC, Shook BA, Holtrup B, Roth E, Lei V, Van Keymeulen A, Seewaldt V, Kwei S, Wysolmerski J, Rodeheffer MS & Horsley V (2018). Adipocyte hypertrophy and lipid dynamics underlie mammary gland remodeling after lactation. *Nat Commun* **9,** 3592.