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# Specific Aim 1

**Determining the effects of chronic stress on placental** **transport of nutrients and endocrine function.** We will expose pregnant dams to the synthetic glucocorticoid dexamethasone and then collect pre-term placentae in order to evaluate nutrient transport and endocrine function.  In separate cohorts we will monitor how gestational dexamethasone exposure affects offspring metabolic health.

The mechanisms by which maternal corticosteroids influence fetal health and placental function remain understudied with conflicting results as reviewed by Kemp et al. (Kemp *et al.*, 2015). Some side effects like reduced birthweight, offspring hypertension and altered HPA axis remain controversial (Reynolds, 2013; Moisiadis & Matthews, 2014*b*). Corticosteroidsin pregnant murine models have not been thoroughly examined in context of placental function. Our hypothesis is that corticosteroid treatments prior to pregnancy and/or throughout conception cause altered placental transport and hormonal function in a time-dependent manner by which a longer and earlier exposure has more prominent side effects on the placenta and fetus. To test this hypothesis, we will explore the a) mechanisms by which corticosteroid treatment affects placental nutrient transport to the fetal compartment and b) placental growth hormone secretions in light of excess maternal cortisol.

# Rationale and Background

## Cortisol Levels in Pregnancy

During pregnancy, mean cortisol rises gradually as pregnancy progresses (Carr *et al.*, 1981). Mean cortisol levels increase 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) and/or by placental production of free cortisol into the maternal circulation (Jung *et al.*, 2011). Despite the natural increase in maternal circulating cortisol levels, the placenta is efficient at inactivating cortisol by hydroxysteroid 11-beta dehydrogenase 2 (Hsd11B2) activity allowing only 10-20% of maternal cortisol to cross to the fetus (Gitau *et al.*, 1998; Ellman *et al.*, 2008).

## Fetal HPA Axis Development

The 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). 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). In mice, the 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 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). 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; Singh *et al.*, 2012).

## Effect of In Utero Glucocorticoid Exposure on Placental Function

Multiple doses of dexamethasone treatment reduced placental and fetal weights (Hahn *et al.*, 1999; Baisden *et al.*, 2007) and increased trophoblast apoptosis thus impairing placental growth (Baisden *et al.*, 2007; Chan *et al.*, 2007).

Triamcinolone (TA) glucocorticoid treatments showed an increase in GLUT3 and GLUT1 mRNA expression in human placental endothelial cells (HPEC) suggesting that glucose flux to the fetus may be increased (Kipmen-Korgun *et al.*, 2012).

Furthermore, despite the placenta’s role in inactivating maternal free cortisol into cortisone, administering high doses of glucocorticoids overwhelms the placental HSD11B2 system in addition to the fact that synthetic cortisol readily passes to the fetus bypassing this enzymatic activity (Cuffe *et al.*, 2011; Singh *et al.*, 2012). Nutrient transport across the placenta and placental hormone secretions are understudied with glucocorticoid treatments.

Effects of Dex on mouse <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3723833/>

<https://academic.oup.com/edrv/article/18/3/378/2530781>

review with **figure** on placental hormone activity on fetus and mother

Glucose and AA transport with dex exposure in mice <https://academic.oup.com/edrv/article/34/6/885/2354710>

Link with mTORC! <https://academic.oup.com/edrv/article/34/6/885/2354710> Obesity increases mTORC activity but GC decrease it. “A connection between the mTOR pathway and glucocorticoid signaling has been established in cell lines (355), but the effect of glucocorticoids on placental mTOR signaling is not known. Elevated endogenous levels of glucocorticoid in the rat have been shown to suppress mTOR signaling in skeletal muscle (356). A recent study on human term placentas in women with self-reported high levels of environment-related stressful conditions during pregnancy showed a significant downregulation of Dishevelled, Egl-10, and Pleckstrin domain-domain-containing and mTOR-interacting protein (DEPTOR) as a modulator of mTOR signaling (357). There are currently no data regarding antenatal glucocorticoid exposure and placental mTOR signaling. Given the importance of antenatal administration of synthetic glucocorticoids in the management of preterm delivery, it is essential that future studies interrogate these pathways.”

Mouse studies:

Using a minipump on embryonic day 12.5,pregnant dams were given dexamethasone (dex) at a rate of 1ul/hr for 48 or 60 hours prior to sacrifice at E14.5 or E17.5, respectively. At E14.5, fetal weights were smaller for male and female dex-exposed pups. Placental weight was smaller at E14.5 for dex-exposed females only. At E17.5, no differences were noted in fetal or placental weight. HSD11b2 gene expression was higher in female placentas exposed to dex at E14.5, and protein expression was increased in female placentas at E17.5. Placental vasculogenesis was measured by genes of the vascular endothelial growth factor family (VEGFA) receptors: VEGFR1 (Flt1) and VEGFR2 (Kdr). Female placentas showed increased gene expression of Vegfa at E14.5 while protein expression as unchanged, and reduced placental growth factor (Pgf) expression at E17.5 after dex exposure. Kdr expression was significantly lower in male placentas at E14.5.

In females, Igf2 gene expression as higher in female placentas regardless of treatment at E14.5.MAPK1 protein expression was reduced in female placentas at E14.5 but gene expression was not changed. Selected glucose (GLUT1 and GLUT3) and amino acid (SNAT1,2 and 4) transporters were unaltered as an effect of treatment or sex.

## Effect of In Utero Glucocorticoid Exposure on Offspring

Limited studies have investigated the effect of antenatal glucocorticoid treatment on fetal hypothalamic-pituitary-adrenal axis showing potential blunted offspring HPA activity (Waffarn & Davis, 2012). Despite popular use of corticosteroids, offspring side effects have been understudied and largely unknown. Some studies have shown 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). 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). The mechanisms by which maternal corticosteroids influence fetal health and placental function remain understudied with conflicting results (Kemp *et al.*, 2015). The effects of prenatal glucocorticoid exposure remain controversial, and the exact mechanisms by which they are manifested remain poorly understood (Bandoli *et al.*, 2017).

# Experimental Design

To determine how corticosteroid exposure affects placental function, we will have 50 12 week-old C57BL/6 virgin mice on regular chow diet exposed to water (control group) or 1mg/kg/day dexamethasone in their drinking water (treatment group). We are interested in the time-dependent effects of dexamethasone on placental function and therefore we will conduct the exposure at various time points. To assess pre-conception effects on placental development, a group of mice will be exposed to dexamethasone a week prior to timed-mating and throughout gestation and lactation. To assess dexamethasone exposure during pregnancy, we will have early, mid and late exposure groups with dexamethasone introduced at E5.5, at E12.5, and at E17.5, respectively. Dams will undergo MRI weekly to monitor weight trends throughout pregnancy. Water and food intake will be recorded weekly. The dams from each group will be euthanized and the placentas and fetuses will be extracted as hitherto mentioned. Briefly, litter size will be accounted for as we predict fetal viability to differ per treatment arm. Maternal and fetal blood collection will be required to determine respective cortisol levels using cortisol ELISA kit (Gong *et al.*, 2015). Placental mRNA transporter expression will be performed along with H&E staining and morphological analysis. *In situ* hybridization to localize HSD11B2 mRNA in the placenta will be conducted as per Thompson et al. (Thompson *et al.*, 2002). *In vitro* nutrient uptake will be performed in BeWo and HUVEC cells as mentioned earlier using uptake assays and the cells will be treated with cortisol (0.01 to 1000uM) and dexamethasone (0.001 to 100uM) as conducted by Topor et al. for 8, 24, 48 and 72 hours (Topor *et al.*, 2011).

Using the above-mentioned model,we will measure placental expression of HSD11B2 mRNA using RT-qPCR. Placental cell lines described above will also be used to quantify HSD11B2 expression using western blotting and RT-qPCR to measure mRNA expression. HSD11B2 activity will be measured by determining the conversion of cortisol to cortisone as mentioned by Zhu et al. (Zhu *et al.*, 2016). We will further determine if there is a correlation between maternal, fetal and placental cortisol levels. In normal pregnancies, fetal cortisol levels are supposed to be less than the maternal serum cortisol, as the placenta efficiently inactivates 80-90% of the maternal cortisol (Duthie & Reynolds, 2013). Increased fetal levels in response to increased maternal cortisol following dexamethasone treatment will highlight the placenta’s failed ability to adequately inactivate the surplus of maternal cortisol and will further corroborate our previous results that may indicate altered placental HSD11B2 function and expression. We will further assess the exact flux of maternal cortisol by using radiolabeled corticosterone as per Li et al. (Li *et al.*, 2015) and further quantify placental and fetal expressions using the HSD11B2 assay kit.

**Aim 2.3: Is placental endocrine function altered with increased maternal cortisol levels?** As previously mentioned, multiple doses of dexamethasone treatment reduced placental and fetal weights (Hahn *et al.*, 1999; Baisden *et al.*, 2007), and small-for-gestational-age babies show reduced PGH levels (Männik *et al.*, 2010). As our preliminary data suggests that the pups of dexamethasone-treated dams are inviable, placental growth hormone and fetal GH secretions can be altered in a dose-dependent manner. We will conduct the previously mentioned studies in Aim 1.2 but using the cortisol exposure.

# 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, 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.

## Western Blotting

Using the fat tissues collected from offspring of groups PND0.5-21.5, gWAT and iWAT 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 branching and for ductal size. To assess branching, we will count the number of ramifications along portions of the main duct (Plante *et al.*, 2011). The length of the primary duct will also be measured in millimeters to determine the development of the gland.

# Expected Results

## **Aim 1.1:**How does maternal GC exposure affect placental, fetal IUGR, and offspring survival?

## **Aim 1.2:** How does maternal GC exposure affect placental endocrine function (specific hormones: lactogen,IGF2 , GDF15…) look at qPCR mRNA expression – will not use ELISA yet since ELISA is expensive and we may not see a difference in qPCR/mRNA expression initially

The experiments conducted above will determine the placental efficiency at inactivating maternal cortisol and will shed light on potential side effects of the treatment dose and timing. We expect that placental expression of HSD11B2 will be upregulated in a time-dependent manner by which an earlier and more prolonged dexamethasone exposure will manifest this placental change in gene expression. Despite the increased placental expression of HSD11B2, we expect the placenta to fail to overcompensate for the excess maternal cortisol thus allowing the increased passage of cortisol to the fetus. We expect the fetal cortisol levels to increase and correlate with maternal levels. We expect that fetal weight will be reduced and thus placental growth hormone secretion will be decreased in a corticosteroid dose-dependent manner.

## **Aim 1.3:** Is placental mTORC1 signaling altered after maternal GC exposure?  Western blot for 4EBP, S6, PS6, AKT

## **Aim 1.4:** How does maternal time-dependent GC exposure affect the expression of placental nutrient transporters? qPCR of transporters, not flux until we see a change in nutrient transporters

The experiments conducted in this aim will examine the effect of timed corticosteroid treatment on placental transport and transporters. We predict that placental glucose transporters (see Table 1) will have increased expression (Kipmen-Korgun *et al.*, 2012) and glucose flux across the placenta to the fetus will be increased . We further expect increased placental lipid transporters and flux but reduced amino acid transporters and flux. We predict maternal corticosteroid treatment will alter placental glucose, lipid, and amino acid transporters and nutrient flux and will influence fetal and placental viability in a time-dependent manner. Earlier exposure at E5.5 and E12.5 will have the most prominent effect on placental transport and may lead to reduced nutrient flux overall, while later exposure at E17.5 will only increase glucose and lipid transporters and flux.

## **Aim 1.5:** Is offspring metabolic health survival, wt, mri, if they survive after Dex exposure during gestation only (no 1 week preconception)

## **Aim 1.6:**Does a placental GR-KO model rescue the placental and fetal effects of GC exposure?

# Potential Pitfalls and alternate Approaches (Aims 1.1-1.6)

**Potential Pitfalls and Alternate Approaches:** It might be that we are unable todetermine *in vitro* uptake since cortisol and dexamethasone treatments may alter placental cell differentiation and function, in which case we will resort to looking at different cell lines that may be less influenced in regards to differentiation by cortisol and dexamethasone. Another potential problem that may arise is fetal resorption with early dexamethasone exposures. Our pilot study has shown that some dams on dexamethasone who were pregnant did not deliver pups which highlights the possibility of fetal death. In this case, we will have to resort to testing later dexamethasone exposures at 1-6 days prior to conception, instead of starting one full week prior to conception, to determine which treatment leads to the highest birth rate and least fetal resorption rate. We may also need to limit our exposure time to better mimic the human one-course treatment of corticosteroids and to ensure the viability of the pups.

**Potential Pitfalls and Alternate Approaches:**If our hypothesis is correct and fetal cortisol is upregulated, then the change in fetal and placental weights may be due to the cortisol and not the nutrient acquisition. Using results from Aim 2.1, we need to distinguish the effects of fetal nutrient uptake and fetal cortisol uptake to prevent misinterpreting our findings. Although both effects synergistically will yield the final phenotype, we need to be diligent about interpreting our data keeping the full results in mind.Corticosteroid treatment may cause perinatal death and thus placental collection to determine placental function may be difficult. If this is the case, we will collect the placentas at an earlier time point prior to E18.5 when perinatal death may have already occurred. Finally, quantification of radiolabeled corticosterone is uncommon, and so we may need to develop our own protocol using the available references.

Our preliminary data show that dams on dexamethasone (1 mg/kg/day) one week prior to conception and throughout pregnancy are fertile but their offspring are inviable and die either at postnatal day one or prior to delivery suggesting that the placental transport of nutrient, placental endocrine function, or both were impaired or that the fetuses were small for gestational age and thus inviable. Dams on dexamethasone do not gain as much weight during pregnancy compared to the control dams on water, and they mainly lose lean mass consistent with other studies on chronic dexamethasone treatment, while maintaining a constant fat mass. This further directs our future investigation to determine the underlying mechanisms altering placental function and leading to perinatal fetal death during dexamethasone-treated pregnancy compared to a normal pregnancy. This aim will help determine the effect of corticosteroids on placental nutrient transport and placental hormone-secreting capacity in a stress-induced environment.

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