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# Specific Aim 4

**Detect the effects of nutritional stress on lactation.** Little is known about the mechanisms that regulate the function and variation of mammary epithelial cell and mammary adipocyte during pregnancy, lactation, and weaning. Furthermore, the exact mechanisms by which macronutrients are synthesized or incorporated into the secreted milk are not yet fully elucidated. With little being known about the mammary gland function, the effect of maternal obesity on lactation further presents a less explored field of study. In humans, maternal obesity affects lactation by which lactation initiation, weaning, and milk composition are altered. The effect of maternal obesity on lactation further manifests in offspring health in childhood and adulthood. My hypothesis is that maternal adipocyte mTORC1 hyperactivation as a model of obesity will enhance mammary gland function and increase milk output and milk macronutrient composition ultimately leading to increased offspring growth prior to weaning. To test this, we will identify how a) adipocyte mTORC1 hyperactivation affects mammary gland size and development, b) how mTORC1 hyperactivation affects milk output volume and carbohydrate, protein and fat composition, and c) the effect of mTORC1 hyperactivation on offspring health via assessing body composition.

# Rationale and Background

## Maternal Obesity and Offspring Health

Pregnancy is associated with increased maternal weight (Abrams *et al.*, 1995; Jung *et al.*, 2011). Of concern, pre-pregnancy maternal obesity can influence the offspring health gravely. Children of mothers with class III obesity are at 2.32 times higher risk of being large for gestational age (Kim *et al.*, 2016). Children of obese mothers are at higher risk of developing non-communicable diseases like hypertension, insulin resistance and diabetes later in life, and they have are 3.84 and 3.0 times more likely to develop childhood overweight and cardiometabolic profile, respectively, as early as six years of age (Leddy *et al.*, 2008; Samuelsson *et al.*, 2008; Mingrone *et al.*, 2008; Gaillard *et al.*, 2014; Williams *et al.*, 2014; Stubert *et al.*, 2018). Alarmingly, data collected from 47 states in the United States show that more than 50% of pregnant women were either obese or overweight in 2014[[1]](#footnote-1) (Branum *et al.*, 2014). The exact mechanisms by which the offspring health is affected in response to 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, 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 energy value on the third month of lactation postpartum (Bzikowska-Jura *et al.*, 2018). In rodents, nutritional stress established by obesity shows delayed lactogenesis and reduced mammary gland alveolar development (Rolls & Rowe, 1982; Flint *et al.*, 2005). Of interest, cortisol and prolactin are necessary hormones for inducing milk production, but it is thought that maternal obesity can blunt endocrine function thus affecting lactogenesis (Rasmussen & Kjolhede, 2004). Maternal nutritional stressors can have an effect on lactogenesis, ultimately impairing lactogenesis II initiation, milk volume, composition, and time of weaning (Thibeau *et al.*, 2016; Panagos *et al.*, 2016; Castillo *et al.*, 2016).

This hypothesis is also supported by data demonstrating that maternal nutritional stress in obesity reduces placental growth hormone and amino acid transporter expressions, is associated with intrauterine growth restriction, delayed initiation of lactation, earlier weaning and proinflammatory milk composition (Koutsaki *et al.*, 2011; Gaccioli *et al.*, 2013; Panagos *et al.*, 2016; Castillo *et al.*, 2016).

## Role of Mammary Adipocytes in Lactation

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). 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). Interestingly, the fate of adipocytes during the de-differentiation phase of lactation remains unknown. It is shown that the adipocytes do not transdifferentiate into epithelial cells, indicating that the adipocytes do not contribute directly to the function of the epithelial cells during lactation (Zwick *et al.*, 2018). The role of the adipocytes and the mechanisms regulating their regression and fate warrant further studies. Hence, it is necessary to note that our adipocyte mTORC1 hyperactivation model may modulate its effect on mammary gland function indirectly by increasing the available maternal pool of proteins and lipids that can be utilized for milk synthesis.

## Milk Macronutrient Synthesis

Mouse milk is composed of 12% total protein, 30% lipids, and 5% lactose (Anderson *et al.*, 2007). Proteins are synthesized in the rough endoplasmic reticulum of the alveolar epithelial cells (Anderson *et al.*, 2007; Rezaei *et al.*, 2016). Lipids are synthesized in the smooth endoplasmic reticulum by de novo from available glucose or derived from plasma fatty acids (Anderson *et al.*, 2007; Rezaei *et al.*, 2016). Lactose is synthesized in the Golgi of the alveolar epithelial cells (Anderson *et al.*, 2007; Rezaei *et al.*, 2016).

# Experimental Design

To assess the effects of maternal mTORC1 adipocyte hyperactivation on milk production and offspring health, we will use twelve 6-8-week old virgin floxed-adiposeTSC1 wildtype (WT) and knockout (KO) female and male mice bred in our facility (n=6WT and 6KO dams). KO females will be crossed WT males and vice-versa. 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 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. 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.

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.

# Methods

## Food Intake

Food will be weighed using a digital balance scale 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

## MRI

Mice will be weighed by using dynamic weighing to capture accurate weight using a digital balance 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. Body 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 using a metric scale. 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 milk gels and diluted milk samples (by a factor of 4, 1:3 ratio).

## Determining Milk Proteins

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). Skim 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. Coomasiee Destaining Solution will be added to the gel and fixed. On the next day, the destaining solution will be added every 30 minutes to the gel until the solution runs clear. The gel will be placed in 5% acetic acid until imaging. Imaging will be done using Licor machine to determine protein expression. Briefly, we are interested in whey acidic protein, alpha casein, beta casein, lactose, and serum albumin.

## 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 by formulas on Excel software.

## Western Blotting

Using the lower mammary gland tissues collected from the dams, we will assess mTORC1 activity to confirm our model. Briefly, a portion of the sample will be boiled and loaded into different wells with a ladder control. Proteins will transfer to a second matrix 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 mTORC, phoshph-mTOR, S6K, phosphorylated S6K, AKT, phosphorylated AKT, S6, phosphorylated S6, 4E-BP1, and phosphorylated 4E-BP1 will be used.

## Histology

Mammary glands collected from WT and KO dams will be embedded in paraffin. Slides will be assessed for branching and for ductal size. As described by Plante et al, 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

## Expected Results (Aim 4.1)

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

## Expected Results (Aim 4.2)

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

## Expected Results (Aim 4.3)

*Aim 4.3: Is offspring metabolic health altered with maternal adipocyte mTORC1 hyperactivation?*

# 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.

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