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# 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 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 with initiation, weaning, and milk composition being altered (Rasmussen & Kjolhede, 2004; Panagos *et al.*, 2016; Castillo *et al.*, 2016). I will test the hypothesis 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

## 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; 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 PPARy 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 (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[[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 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 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; 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 twelve 6-8-week old virgin floxed-adipocyte *Tsc1* wildtype (WT) and knockout (KO) female and male mice bred in our facility (n=6WT and 6KO dams). 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 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 (see reference in grant), which is expressed in all adipocyte lineages (brown, white and maternal). 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 WT males and vice-versa to ensure that pups are a combination of wild-type and knockout adipocyte *Tsc1* knockout 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 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.

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



### Figure 2: Diagram representing the experimental design and respective timeline



# 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.

## 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. 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 branching and for ductal, alveolar and adipocyte 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. The size of alveolar and adipocyte cells will be measured in millimeters under a microscope using a representative section of the slides.

# Expected Results

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

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, 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. Histological examination is expected to reveal bigger alveolar cells indicating a hyperactive glandular function 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?

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 (Kim *et al.*, 2016). The growth trajectory of children at birth till the age of 2 or 4 years was highest in mothers who had pre-pregnancy obesity or overweight (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). Furthermore, since milk lipid and protein composition along with milk output volume are expected to 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.

# Potential Pitfalls and Alternate Approaches (Aims 4.1-4.3)

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 C57Bl6/J 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.

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