Table of Contents

[Specific Aim 4 2](#_Toc14280960)

[Rationale and Background 2](#_Toc14280961)

[Milk Macronutrient Synthesis 2](#_Toc14280962)

[Mammary Adipocytes and Mammary Function 2](#_Toc14280963)

[mTORC1 Activity in Obesity 3](#_Toc14280964)

[Role of mTORC1 in Lactation 3](#_Toc14280965)

[Maternal Obesity and Offspring Health 3](#_Toc14280966)

[Obesity and Lactation 3](#_Toc14280967)

[Experimental Design 4](#_Toc14280968)

[Methods 4](#_Toc14280969)

[Food Intake 4](#_Toc14280970)

[Body Composition 5](#_Toc14280971)

[Sacrifice and Tissue Collection 5](#_Toc14280972)

[Determining Milk Output Volume 5](#_Toc14280973)

[Determining Milk Composition 5](#_Toc14280974)

[Determining Milk Protein Concentrations 6](#_Toc14280975)

[Determining Milk Fat Content 6](#_Toc14280976)

[Western Blotting 6](#_Toc14280977)

[Histology 6](#_Toc14280978)

[Expected Results 6](#_Toc14280979)

[Aim 4.1 6](#_Toc14280980)

[Aim 4.2 6](#_Toc14280981)

[Aim 4.3 6](#_Toc14280982)

[Potential Pitfalls and Alternate Approaches (Aims 4.1-4.3) 6](#_Toc14280983)

# Specific Aim 4

**Detect the effects of nutritional stress on lactation.** Milk composition is important for xxx. 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. 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

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). 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 (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 function of the epithelial cells during lactation (Wang *et al.*, 2018; 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). MENTION HOW ADIPOYTES TAKE BACK FA FROM MILK IN EPITHELIAL CELLS,

MENTION HOW ADIPOCYTES RELEASE THEIR FFA TO THE EPITHELIAL CELLS THUS CONTRIBUTING TO THE MILK COMPOSITION OF FA (ZWICK SAYS THAT IN THE PAPER)

## mTORC1 Activity in Obesity

## 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 but higher lipid droplet composition and size in the mammary epithelial during mid and late gestation with persistent increases in lipid size during 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-POK, regulator of insulin-induced AKT and mTORC1 signaling, has been shown to affect milk production. Th-POK knockout mice had reduced pup weight, reduced milk lipid synthesis, redeuced milk volume, and precocious mammary epithelial involution (Zhang *et al.*, 2018) 🡪 use their discussion

Ain the mammary gland, AKT is thought to regulate lipid synthesis. This implies that mTORC

## 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 obese mothers are at higher risk of developing non-communicable diseases like hypertension, insulin resistance and diabetes later in life, and they have are 3.8 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 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 results in delayed lactogenesis and reduced mammary gland alveolar development (Rolls & Rowe, 1982; Flint *et al.*, 2005). 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).

<https://academic.oup.com/hmg/article/11/5/525/2901618> use this for aim 3 mtorc hyperactivation in placenta

# 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). 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.

# 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 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 4.1

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

## Aim 4.2

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

## Aim 4.3

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

# 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.

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