**Title:**

Effects of mTORC1 Hyperactivation on Mammary Gland Function, Milk Composition, and Offspring Outcome

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**Declaration of Interests**

The authors have no competing interests to declare.

**Abstract:**

Changes in the mammary gland biology during pregnancy and lactation remain largely unknown. Specifically, the interplay between mammary gland adipocytes and epithelial cells remains elusive. Mammary adipocytes are thought to "disappear" during lactation, and their contribution to milk composition is still unknown. Additionally, the role of mammary gland in sensing maternal nutritional status is understudied. A major nutrient sensor in most tissues is the mechanistic target of rapamycin 1 (mTORC1). mTORC1 regulates lipid metabolism and protein synthesis in response to nutrient availability. Our aim is to understand the effect of adipocyte mTORC1 hyperactivation on mammary gland function, milk composition, and offspring outcome in an adiponectin-Cre *Tsc1/2* knockout mouse model. Knockout (KO) and wild type (WT) C57BL/J6 female mice were mated with KO or WT males at 6-8 weeks of age. Data on maternal body mass composition during pregnancy and lactation, pup survival and weight, litter numbers, milk volume production, milk composition, and mammary gland weights were collected. Our results show that female pups born to KO dams are heavier, milk composition of KO dams has higher fat and protein percentages, and KO dams have lower mammary gland weights. Our data suggests a key role of mammary adipocyte mTORC1 in mammary gland function, milk composition, and offspring health.

**Key words**: Mammary glands, Milk composition, Adipocytes, mTORC1, Obesity

**1.0 Introduction**

Maternal obesity is on the rise and has reached 38% in 2013 (Chen *et al.*, 2018). Obesity can impact offspring health during *in utero* development and during early postnatal life through lactation. According to the Developmental Origins of Health and Disease theory, the health of the offspring is highly influenced by intrauterine and early postnatal exposures (Barker, 2007). Furthermore, obesity can affect milk composition and lactational capacity. However, how maternal obesity affects lactation and offspring health during the postnatal critical window of development and the mechanisms that mediate those effects remain less clear.

Maternal obesity can influence early postnatal development through its impact on mammary gland function. Maternal weight has been positively correlated with milk protein and fat contents and overall caloric value (Bzikowska-Jura *et al.*, 2018). 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).

The macronutrients present in mammalian milk are lactose, protein, and lipids. Lactose, the main carbohydrate in milk, is synthesized in the Golgi of the alveolar epithelial cells (Anderson *et al.*, 2007; Rezaei *et al.*, 2016). Milk 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 these triglycerides are packaged and transported into the milk remain elusive (McManaman, 2009, 2014). The mammary gland is composed of adipocytes and alveolar cells. Mammary 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). During pregnancy and lactation, the mammary adipocytes undergo transformation and almost disappear to support lactation and epithelial cell expansion and then revert to their original state at weaning throughout the involution process (Zwick *et al.*, 2018). The mammary adipocytes within closer proximity to the alveolar epithelial cells are thought to provide a primary source of lipids for milk production (Zwick *et al.*, 2018). Although the mammary fat pad undergoes restructuring during pregnancy and lactation, the exact fate of these fat cells and the mechanisms regulating their transformation are less understood.

Mechanistic Target of Rapamycin Complex 1 (mTORC1) activity is higher in obese subjects that have excess fat mass (Catania *et al.*, 2011). mTORC1 is a critical nutrient sensor and a main regulator of protein and lipid synthesis (Wang & Proud, 2006; Cai *et al.*, 2016). In the presence of anabolic signals like insulin, energy abundance, and amino acid availability, mTORC1 function is upregulated via the Akt pathway (Catania *et al.*, 2011). mTORC1 promotes lipogenesis via SREBP1, promotes adipogenesis, and inhibits lipolysis (Laplante & Sabatini, 2009; Cai *et al.*, 2016). mTORC1 hyperactivation in mammary glands has been studied in the context of breast cancer (Chen *et al.*, 2014), but little is known about the role of mTORC1 in macronutrient synthesis in the mammary gland (Rezaei *et al.*, 2016).the specific role of mammary adipocyte mTORC1 in maternal obesity remains largely unknown. We show that chronic mTORC1 activation in maternal adipocytes via deletion of its upstream negative regulator, *Tsc1*, causes increased milk macronutrient composition and increased weight gain of lactating offspring. Our model will focus on mTORC1 activation in differentiated adipocytes after a first pregnancy, not during the process of adipogenesis.

**2.0 Materials and Methods**

**2.1 Animals**

All mice were purchased from the Jackson Laboratory. Unless otherwise stated, all mice were fed a normal chow diet with *ad libitum* access to food and water.

To hyperactivate adipocyte mTORC1 and generate an adipose-specific *Tsc1* knockout, 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 (F0) for this experiment were 6-8 week old male *Tsc1* fl/fl ;Tg/+ or *Tsc1* fl/fl ;+/+ crossed with 6-8 week old female *Tsc1* fl/fl ;+/+ or *Tsc1* fl/fl ;Tg/+, respectively. The offspring (F1) were a combination of knockout (KO, fl/fl;Tg/+) and phenotypically wild-type (WT, fl/fl;+/+) at an expected ratio of 1:1. The knockout of the floxed alleles are driven by Adiponectin-Cre (Kwiatkowski *et al.*, 2002; 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). All mice were bred in our facility at the University of Michigan. All animal procedures were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals and was approved by the University of Michigan Institutional Animal Care and Use Committee prior to the work being performed.

Mice had *ad libitum* access to normal chow diet and water. Male breeders were removed from the cage after 16 days of mating to avoid the occurrence of a second pregnancy.We checked for litters on a daily basis after 2.5 weeks of mating. The number of pups born was recorded to determine maternal fertility and pup viability. After delivery (PND0.5), the dams continued to have *ad libitum* access to food and water.

Pups were sexed then culled to four animals (2 females and 2 males, when possible) per litter at PND2.5. The offspring were weighed at PND0.5, PND7.5, 14.5, 16.5. The pups underwent body composition analysis by echoMRI at PND16.5 prior to sacrifice.

**2.2 Body Composition and Indirect Calorimetry**

In all groups, the dams underwent body mass assessment three times a week during pregnancy and lactation and on the day of delivery using magnetic resonance (EchoMRI 1100, EchoMRI, Houston, TX) to assess body composition. Mice were weighed by dynamic weighing to capture accurate weight using a digital scale. The weight was recorded along with the mouse ear tag number. The mouse was then gently placed in the MRI tube with the plunger slightly compressing along the mouse’s body to ensure restrained movement during the measurement. Fat, lean, free water and total water mass (g) were recorded for each animal. We weighed offspring and performed body mass assessment using MRI at PND16.5.

## **2.3 Sacrifice and Tissue Collection**

All animals were sacrificed using anesthetic gas inhalation (5% isoflurane drop jar) at PND16. Cervical dislocation was conducted as a secondary method to confirm euthanasia. The mice were pinned on a dissection board in a supine position. We dissected KO and WT dams by a midline incision of the skin from the rectum to the diaphragm and extracted thoracic, abdominal and inguinal mammary glands. Briefly, the peritoneum was pulled apart from the skin. The lower glands were excised carefully then weighed. Portions of the upper and lower glands were embedded in paraffin for histology, while the remaining tissue from the lower left and right mammary glands were collected in 2ml tubes and snap frozen in liquid nitrogen and later stored at -80C for molecular studies. Offspring of dams were sacrificed without tissue extraction at PND16 after body assessment measurements.

## **2.4 Determining Milk Output Volume**

At PND10.5, considered the peak of lactation (Wang *et al.*, 2018), we determined milk output volume for the WT and KO dams. To determine milk volume, we used the weigh-suckle-weigh technique (Boston *et al.*, 2001). Briefly, we weighed the dam separately then determined the aggregate weight of the pups. The dam and pups were then separated for two hours. During the two-hour separation, the pups were placed in a new cage and were kept warm using a heating pad. In the meantime, the dam remained in its initial cage with *ad libitum* access to normal chow diet and water. After the two-hour separation period, the dam was weighed again and the aggregate weight of the pups was measured. The pups were then returned to the dam’s cage and were allowed to nurse for one hour undisturbed. At the end of the nursing timepoint, the dam was weighed again and the aggregate weight of the pups was determined. After this one-hour nursing period, milk volume was 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 was calculated to further ascertain the dam’s milk supply.

## **2.5 Determining Milk Composition**

On PND16.5, we collected milk samples (~0.5ml) from the nursing dams. Briefly, we anesthetized the dam after two hours of separation from the pups by intraperitoneal injection of Ketamine (0.1275g/kg body weight). We then performed an intraperitoneal injection of oxytocin into the forelimb (2U/dam) to induce milk production. The dam’s nipples were manually squeezed to promote milk letdown, and the milk was collected into a 1.5 ml tube via suction. After milking was complete, the dam was immediately sacrificed using isoflurane and a secondary measure of cervical dislocation. We then dissected the dam by a midline incision of the skin, extracted thoracic, abdominal and inguinal mammary glands. The lower mammary gland pads were weighed. A small section of the lower mammary glands was saved for paraffin embedding for histology while the rest was snap frozen in liquid nitrogen and cryopreserved. Milk protein composition was analyzed using SDS-PAGE gels and diluted milk samples (4-fold dilution).

## **2.6 Determining Milk Protein Concentrations**

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

## **2.7 Determining Milk Fat Content**

Milk samples collected from WT and KO dams were assessed for fat content by the creamatocrit method using a hematocrit centrifuge (Collares *et al.*, 1997). Briefly, samples were diluted to a factor of 4 (1:3 in PBS). Diluted samples were transferred into plain micro-hematocrit glass capillary tubes. The tubes were sealed from one end using Critoseal. The tubes were later placed in CritSpin mini-creamatocrit spinner. Samples were centrifuged for 8 cycles of 120 seconds per cycle for a total spin time of 16 minutes. The capillary formed layers of white fat and non-fat milk. The distance of the fat layer was measured in millimeters (mm) accurately using a 150 mm dial caliper (General Tools and Instruments 6” Dial Caliper, 2011). The total volume of milk (fat + non-fat milk) was also measured in mm. Percentage of fat was determined with respect to the total milk volume.

## **2.8 Real time qPCR**

Using the lower right mammary gland tissues collected from the dams, we assessed RNA expression of lipogenic genes. RNA samples were prepared from the mouse tissues using the PureLink RNA Mini Kit. Briefly, tissues were cut on dry ice to ~50mg samples then homogenized and treated to collect the purified RNA. The RNA was quantified using a nanodrop. Later, first strand cDNA was synthesized from the purified RNA samples using High Capacity cDNA Reverse Transcription Kit. The cDNA samples were diluted and added to the clear 384 well plate in triplicates. A Primer/SYBR Green mix was prepared for each primer. Briefly, we used sequence-specific primers to amplify the genes ACC1, SREBP1c, ACLY, FASN, using primer pairs (forward and reverse). This allowed us to assess lipogenic activity of the mammary glands of KO and WT.

## **2.9 Western Blotting**

Using the lower right mammary gland tissues collected from the dams, we assessed TSC1/2 protein levels and mTORC1 activity to confirm knockout in mammary glands as a whole tissue and specifically in mammary adipocytes. To better determine the site of the knockout, a separate cohort of KO and WT dams were bred for this purpose. The mammary gland fat and stromal vascular fraction (SVF) were separated. Genotyping was separately done on the glandular and SVF compartments to verify knockout specificity in the mammary gland. Briefly, a portion of the whole lower right mammary gland, the SVF, and the fat samples was boiled and loaded into different wells with a ladder control. Proteins were transferred to nitrocellulose overnight. The matrix was stained for total protein using Revert total protein and scanned by LiCOR to normalize against total protein. Samples were 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) were used.

## **2.10 Histology**

Mammary glands collected from WT and KO dams were embedded in paraffin and Hematoxylin and Eosin (H&E) stained at the Rogel Cancer Center’s Tissue and Molecular Pathology. Slides were blindly assessed for alveolar count and adipocyte size and count.

## **2.11 Statistical Analysis**

Pairwise testing, two way anova, linear modeling

**3.0 Results**

To understand how activation of mTORC1 in adipocytes affects lactation we evaluated pregnant mice that were either wild-type (*Tsc1fl/fl; Adipoq-CreTg/+*) *or* knockout(*Tsc1fl/fl; Adipoq-CreTg/+*). In this model all adipocytes, including all white and brown adipocyte depots including mammary adipocytes are ablated for TSC1 and are predicted to have activation of mTORC1. These mice were mated with a male and dams and their offspring were monitored through lactation (See Figure 1A).

## **3.1 Maternal Body Composition Was Similar during Pregnancy and Lactation in Adipocyte *Tsc1* Knockout Mice**

Dam body composition was measured every Monday, Wednesday, and Friday during pregnancy and lactation an on the day of delivery. Body weight were comparable between dams (Figure 2B). Lean mass was also comparable between KO and WT dams (Figure 2C). KO dams had a slightly lower fat mass during pregnancy and during lactation (Figure 2D). While WT dams lost fat mass gradually during lactation, KO dams gained fat mass (Figure 2E). Consistent with this, KO and WT dams had similar food intake during pregnancy, however during lactation KO dams had higher food intake compared to WT dams (Figure 2F). The average litter size from KO and WT dams was similar (Figure 2G). Pups were culled to four pups per dam to normalize milk supply.

## **3.2 Pups Born to Adipocyte *Tsc1* Dams are Heavier During Peak Lactation.**

To evaluate effects on the offspring we monitored growth of pups during lactation (Figure 2) There was no significant differences in pup weight at birth (PND0.5). At PND7.5, females born to KO dams were heavier than females born to WT dams (p=0.047), but weights of males born to KO or WT mothers were not significantly different. At PND14.5 and PND16.5, there were no weight differences between groups or sexes. We hypothesize that this is because at later time points mice are eating more chow-based food and relying less on maternal lactation.

## **3.3 Adipocyte *Tsc1* Dams Produce Similar Volumes of Milk, but with Higher Milk Fat.**

Based on the changes in offspring weight trajectories, we calculated the mass of milk produced per dam. This was calculated by a two-hour separation period and then as the difference between pup weight after nursing and pup weight before nursing. As shown in Figure 3A, this was not significantly different between groups. Similar data were obtained by measuring the dams pre- and post- lactation. To test milk composition, milk was extracted from dams at PND 16.5. Creamatocrit fat analysis revealed that milk of KO dams had higher fat percentage than milk of WT dams (Figure 3B; p=XXX).To assess total milk protein, we XXX. At an individual milk protein level, milk proteins were separated by electrophoresis and quantified by densitometry. The milk of adipocyte *Tsc1* dams had higher whey alpha protein (WAP) (p=0.033) and alpha-Casein protein (p=0.045) percentages compared to milk of WT dams. Other milk proteins including beta-Casein, lactoferrin, and albumin were similar between milk of KO and WT dams. In terms of milk lactose XXX.

## **3.7 Mammary Gland Weights of KO Dams were Lighter**

Based on these changes in milk composition, we next examined the mammary glands from the adipocyte *Tsc1* knockout dams. At PND16.5, the lower abdominal and inguinal mammary glands were collected and weighed from KO and WT dams. The right lower mammary glands of KO dams were significantly lighter than those of WT dams (p=0.042). Left lower mammary glands of KO dams significantly weighed less than those of WT dams (p=0.001).

## **3.11 Adipocyte Size and Count**

So far, the KO dams have more smaller-sized adipocytes compared to the WT. Additionally, the KO dams have fewer number of adipocytes in the lower right mammary gland.

## **3.12 Gene and Protein Expression in Whole Mammary Glands, Mammary Epithelial, and Mammry SVF Compartments**

Western to confirm KO in SVF and not epithelial portion pending

qPCR for lipogenic activity in whole mammary gland and expression of milk protein pending

**4.0 Discussion**

Here, we show that hyperactivation of mTORC1 in adipocytes increases weight of female offspring at PND7.5 and increases milk macronutrient composition in KO dams. We also demonstrate that mTORC1 hyperactivation in adipocytes causes reductions in mammary gland weight, number of mammary adipocytes, and size of mammary adipocytes in KO dams.

Mammary adipocytes play a critical role in the development and successful functioning of mammary glands. At puberty, alveolar ducts expand at the expense of the fat pad in the mammary gland (Hovey & Aimo, 2010; Macias & Hinck, 2012). A mouse model of lipodystrophy with underdeveloped fat tissues reveals 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). 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). In our KO model, we observed increased emptying of mammary adipocyte content as evident by the smaller size of adipocytes compared to the WT. We also observed a higher fat content in the milk produced from KO dams. This is consistent with the idea that these adipocytes could be emptying their content into the milk in KO dams at a higher rate than that of WT dams. Additionally, and supporting our finding, transgenic pregnant mice with activated AKT in the mammary epithelial cells 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 these 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) which is concordant with our findings that milk from dams with mTORC1 hyperactivation had increased milk fat and protein composition.

In addition to differences in milk composition and mammary adipocyte count and size, 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. Our data revealed that mTORC1 hyperactivation in adipocytes caused reductions in mammary gland weight. This could be explained by the increased fat content in milk from KO dams suggesting that mammary gland weights could be reduced due to the increased emptying of their content into the milk in KO dams. Despite our findings being contradictory to the expected findings based on previous research, this finding is consistent with the idea that mTORC1 increases lipid and protein synthesis which are incorporated into milk composition and thus causing reduced mammary gland weights.

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). 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). Our data revealed increased offspring weight of females at PND7.5 which is consistent with the human findings from previous studies.

**5.0 Conclusions**

We have shown that hyperactivation of mTORC1 activity in adipocytes of pregnant and lactating dams increases milk macronutrient composition in KO dams which is demonstrated by the heavier pup weight of female offspring at PND7.5 when the pups are only reliant on lactation and milk being their primary and only source of nutrition. The mammary gland weights and morphology revealed smaller mammary gland weight, less adipocyte count and smaller adipocytes in KO mice supporting our hypothesis that mTORC1 hyperactivation in adipocytes increases mammary adipocyte capacity to produce fat and secrete it into the produced milk. Whether the hyperactivation of mTORC1 is affecting protein synthesis in the alveolar epithelial cells and is causing the increased milk protein composition in KO dams remains elusive. The mechanisms by which mTORC1 could be influencing mammary gland function and milk secretion is insightful for future research addressing the effects of maternal obesity on offspring health. Future studies are warranted to address potential therapeutic interventions to minimize the negative effects associated with lactation in cases of maternal obesity.

**6.0 Acknowledgements**

This work was supported by funding from the NIH (R01DK107535 to DB and K01DK102526 to BG) and supported by core facilities at the Rogel Cancer Center (NIH P30CA046592).

**7.0 Author Contributions**

**8.0 References**

**Figure Legends**