**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 has increased by about 10% reaching 38% in 2013 (Chen *et al.*, 2018). 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). Obesity during pregnancy and lactation can have an impact on offspring development. However, it remains unknown how maternal obesity affects offspring health during the critical window of lactation and the mechanisms that mediate those effects.

## 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 lactose, protein, and fat. Lactose, the main carbohydrate in milk, is synthesized in the Golgi of the alveolar epithelial cells (Anderson *et al.*, 2007; Rezaei *et al.*, 2016).

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). Despite the presence of fat cells surrounding the mammary epithelium, the function of these fat cells during lactogenesis is still less understood. However, 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).

## Mammary Adipocytes and Mammary Function

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 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).

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). The mammary adipocytes do not transdifferentiate into epithelial tissue 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 (Laplante & Sabatini, 2009; 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). This suggests the important and active role of mTORC1 in 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).

## 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 that benefits of breastfeeding 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).

**2.0 Materials and Methods**

**2.1 Animals**

C57Bl/J6 mice were bred in our facility at the University of Michigan, Ann Arbor, Michigan. **Refer to one of Dave’s older papers to show how the genotype was made.** WT and KO at a ratio of 1:1 and that are 6-8-week old virgin floxed-adipocyte *Tsc1* wildtype (WT) and knockout (KO) female and male mice bred in our facility. 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 adipocyte-specific constitutive Cre recombinase controlled by adiponectin gene promoter. The parental strains (F0) for this experiment were male *Tsc1* fl/fl ;Tg/+ or *Tsc1* fl/fl ;+/+ crossed with female *Tsc1* fl/fl ;+/+ or *Tsc1* fl/fl ;Tg/+, respectively. The offspring (F1) were 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 (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).

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.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 to assess body composition. 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**

Mice were weighed by using 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 body to ensure it cannot move during the measurement. Fat, lean, free water and total water mass (g) will be recorded for each animal.

## **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, extracted thoracic, abdominal and inguinal mammary glands. Briefly, the peritoneum was pulled apart from the skin. The lower glands were excised carefully then weighed. A portion of the upper and lower glands was embedded in paraffin for histology, while the remaining tissue was collected in 2ml tubes and snap frozen in liquid nitrogen then stored at -80C for molecular studies. Offspring of dams were sacrificed without tissue extraction at PND16.

## **2.4 Determining Milk Output Volume**

At PND10.5, 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 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 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 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 will be analyzed using SDS-PAGE gels and diluted milk samples.

## **2.6 Determining Milk Protein Concentrations**

Milk samples collected from WT and KO dams were assessed for protein content. Milk will be 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. Briefly, samples were 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 were centrifuged for 8 cycles of 120 seconds. The capillary formed layers of white fat and non-fat milk. The distance of the fat layer was measured in millimeters (mm) accurately. 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 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 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. Do we want to do qpcr per section fat and SVF as well to confirm a cell-specific difference in functionality? I guess yes!

## **2.9 Western Blotting**

Using the lower 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, another 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 two compartments to verify knockout specifity in the mammary gland. Briefly, a portion of the whole 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**

## Genotyping to confirm KO in Mammary Adipocytes and not in SVF Compartment

## **3.1 Maternal Weights were Comparable during Pregnancy and Lactation**

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 1). Lean mass was also comparable between KO and WT dams (Figure 2). KO dams had a slightly lower fat mass during pregnancy and during lactation (Figure 3a). While WT dams lost fat mass gradually during lactation, KO dams gained fat mass (Figure 3b).

## **3.2 Litter Size and Pup Survival Rate were Similar**

Average litter size from KO and WT dams was similar. Survival rates of pups born to KO and WT dams was not significantly different between groups.

## **3.3 Pup Weight at PND 0.5, 7.5, 14.5 and 16.5**

There were no significant differences in pup weight at PND0.5 (at birth). 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 different. At PND14.5 and PND16.5, there were no weight differences between groups or sexes.

## **3.4 Pup Body Composition at PND16.5 were Similar Across Groups**

Pups underwent body composition analysis at PND16.5. There were no differences in body weight, lean mass, or fat mass between males of KO and WT dams. No differences in body weight, lean mass, or fat mass were detected between females of KO and WT dams.

## **3.5 Milk Volume Production and Pup Weight Change After Nursing is Similar**

The difference between pup weight after nursing and pup weight before nursing (and after a two-hour separation period) was not significantly different between groups. The delta weight change per pup after nursing was similar between pups of KO and WT dams. The weight change of KO and WT dams before and after nursing was not significantly different between dams.

## **3.6 Mammary Gland Weights of KO Dams are Lighter**

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).

## **3.7 Milk Fat Composition is Higher in KO Dams**

Creamatocrit fat analysis revealed that milk of KO dams had higher fat percentage than milk of WT dams (p=XXX).

## **3.8 Milk Protein Composition is Higher in KO Dams**

Milk of KO 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.

## **3.9 Gene and Protein Expression in Mammary Glands and Fat and SVF Compartments**

## **3.10 Adipocyte Size and Count**

**4.0 Discussion**

The interplay between mammary gland.

We hypothesize that mammary adipocytes are “emptying” their contents into the milk and adipocyte interplay

Mammary gland function during pregnancy and lactation

Milk production

Mechanisms at play

**5.0 Conclusions**

**6.0 Acknowledgements**

**7.0 Author Contributions**

**8.0 References**

**Figure Legends**

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)