Response to Initial Reviews

Manuscript Number: JLR-D-21-00266     
  
Activation of Adipocyte mTORC1 Increases Milk Lipids in a Mouse Model of Lactation   

**Thank you to the editors and reviewers for your thoughtful consideration of our initial manuscript and your patience while we completed the requested experiments to revise this manuscript. Please find below our point-by-point responses to the reviewer critiques in bold. Specific sections in the revised manuscript are noted in bold red font with line numbers.**

Reviewer 1: In this study, El Habbal and colleagues investigated the effects of constitutive mTORC1 activation in adipocytes induced by Tsc1 deletion via adiponectin Cre on mammary gland structure, function, milk composition, and offspring weights. Their main findings were that constitutive mTORC1 activation in adipocytes increased milk fat content mainly by increasing monounsaturated fatty acids and DHA and this leads to a higher milk caloric density and heavier offspring weight during lactation. The mechanisms by which constitutive mTORC1 activation in adipocytes promotes these actions still remain elusive. Please find below some suggestions that may strengthen manuscript message.  
  
1- It would be important to characterize mTORC1 physiology in mammary gland development and function in lactating dams. Is mTORC1 activated in mammary gland alveolar cells and adipocytes during lactation? What is the impact of prolactin on mTORC1 signaling in these cells?

**This is an interesting question, specifically**   
  
2- Tsc1fl/fl; Cre+/+. For clarity, please use the term Tsc1fl/fl for wild-type mice. The term Tsc1fl/fl; Cre+/+ may be confusing as it may be interpreted as the mice are positive for Cre, which is not the case.

**Update**

In addition, it would be important to measure mTORC1 activity in the adipocytes at mammary gland to show that the deletion of Tsc1 using the Cre-lox system was successful. These adipocytes are different from regular white adipose tissue residing adipocytes and there is no clear indication that adiponectin Cre works the same in these cells.

**This is an excellent point, and extensive efforts were undertaken to clarify this point. As shown in our histology (Figure 1G and H) adipocytes are a relatively minor cell type in the mammary gland, so whole mammary gland western blotting (wherein only the adipocytes would be predicted affected) did not clearly demonstrate effective knockout. In lieu of these data, we instead performed immunohistochemistry using antibodies raised against the mTORC1 downstream product phospho-S6 which is not presented in the revised manuscript as Figure XX. These data show that mammary adipocytes have elevated pS6 staining, which is consistent with mTORC1 activation and *Tsc1* deletion in these cell types**  
  
3- Recent studies from Phil Scherer group have shown that mammary gland adipocytes de-differentiate upon lactation losing lipid droplets. So for some unknown reason constitutive mTORC1 activation in adipocytes is preventing this de-differentiation. Authors should discuss this.  
  
4- Please provide a brief explanation or a citation for the method for fatty acid extraction, semi-purification and derivatization used in the GC analysis. This is not a lipidomic analysis as mentioned in the Methods. This is only a simple analysis of milk fatty acid profile. In fact, a milk lipidomic analysis would be very informative to reveal the lipid species in milk.

**We apologize for the lack of clarity in terminology. Indeed, we only present data on fatty acid profiles, from fatty acids extracted from all the lipids in breastmilk (the majority of which are triglycerides). We agree that a total lipidomic analysis would be fascinating, but this was not performed, and we look forward to a more comprehensive lipidomic analysis in future studies. We clarified this in the methods section to read XX and provided additional data (see point 6) and details on this methodology.**  
5- Please improve graphics presentation and readability. Gray lines are almost impossible to see in the gray background in Figure 1.

**Fixed**  
  
6- Please provide the milk fatty acid content in absolute values, not in percentage. In my opinion, a Table containing the most predominant fatty acids in milk in both genotypes should be included in the manuscript. Please, it is important to detail the main fatty acids in each class that are being modified in milk between genotypes.

**We agree have added this as a table (Table 2) in the revised manuscript and described these data in the revised manuscript on lines XX:**

The increase in DHA is unexpected and interesting. DHA is mainly oxidized at peroxisomes and/or serve as precursor for the synthesis of D-series resolvins. Any changes in the expression of enzymes involved in these processes?

**Excellent question, and we had not considered the signaling role of the DHA-derived eicosanoids within the mammary gland. This is the subject of a separate study we have started, and hope to share these data soon. To understand if any resolvin-related enzymes are modified, we evaluated the expression of several enzymes involved in resolvin metabolism and provide those below:**

7- Changes in gene expression may not be the main mechanism by which mTORC1 regulates fatty acid and triacylglycerol synthesis. Other possible mechanisms should be explored. In this sense, authors should look at adiponectin, whose serum levels are markedly increased upon constitutive mTORC1 activation in adipocytes.

**This is an excellent point, and indeed adiponectin expression in the mammary gland is XXX. Given the role of mTORC1 in protein translation, and co-ordination of multiple other signaling pathways within and outside of the mammary gland we agree that there could be multiple mechanisms by which mTORC1 in adipocytes (in the mammary gland or not) could affect breastmilk lipid composition. At the moment, we are not aware of technologies that could modify adipocyte mTORC1 only in the mammary gland, and agree that profiling of endocrine and paracrine signaling, as well as proteomic analyses could shed more light into the mechanism, but this is beyond the scope of this initial report. We have added these limitations in the revised discussion.**  
  
Reviewer 2: In the current manuscript, the authors showed that adipocyte-specific hyper-activation of mTORC1 increase milk lipids and alters mammary gland adipocyte histology. The work described here is straightforward and the results in general support the conclusion.

**We thank the second reviewer for their review.**