Author's response to reviews

Title: Diurnal Variation of the Human Adipose Transcriptome and the Link to Metabolic Disease

Authors:

Andrey Loboda (andrey_loboda@merck.com)
Walter K Kraft (Walter.Kraft@jefferson.edu)
Jeffrey Joseph (Jeffrey.Joseph@Jefferson.edu)
Bernard Fine (bfine@mit.alum.edu)
Michael Nebozhyn (michael_nebozhyn@merck.com)
Chunsheng Zhang (chunsheng_zhang@merck.com)
Yudong He (yudong_he@merck.com)
Xia Yang (xia_yang@merck.com)
Christopher Wright (christopher_wright2@merck.com)
Mark Morris (mark_morris@merck.com)
Ira Chalikonda (ira_chalikon@merck.com)
Mark Ferguson (mark_ferguson@merck.com)
Hongyue Dai (hongyue_dai@merck.com)
Valur Emilsson (valur_emilsson@merck.com)
Eric Schadt (eric_schadt@merck.com)
Howard E Greenberg (heg23@cornell.edu)
Pek Yee Lum (pek_lum@merck.com)

Version: 2 Date: 24 November 2008

Author's response to reviews: see over
November 24, 2008

Dear Editor and Reviewers,

Thank you very much for your comments and suggestions for this manuscript. We have responded to every comment to the best of our abilities. Please find below a point-by-point description of the changes made. We have engaged a senior medical editor for help with the copyediting of the manuscript to improve the style of written English. This is stated in the Acknowledgments. We have also edited the layout of the manuscript according to the journal's requirements.

Thank you again for your consideration.

Sincerely,

Pek Yee Lum, Ph.D.
Rosetta Inpharmatics
A wholly owned subsidiary of Merck & Co., Inc.
401 Terry Avenue N, Seattle WA 98109
Tel: 206-802-7371.
Fax: 206-802-6377
pek_lum@merck.com
Reviewer's report
Title: Diurnal Variation in Human Adipose Tissue: Link to Metabolic Diseases and mTOR Signaling
Version: 1 Date: 26 September 2008
Reviewer: John Hogenesch
Reviewer's report:
Diurnal variation in human adipose tissue: link to metabolic diseases and mTOR signaling
Loboda et al. 2008.
Dozens of studies have used microarrays to examine circadian transcription in a variety of different model organisms establishing reasonably comprehensive groups of genes under clock control. Less studied, however, is the circuitry that generates a lot of these gene expression changes such as locomotor activity, feeding and metabolism. Feeding and fasting have been shown to play a significant role in the overall circadian transcription in peripheral tissues and most of the work to date has been done in model organisms such as the mouse. Mouse models of circadian clock components display many characteristics of human metabolic syndrome. An important, but unanswered, question is do these studies relate to how human beings regulate gene expression?
This present study seeks to address this issue by studying fat tissue extracted in a minimally invasive way to define circadian transcriptional regulation in a clinical setting.
There are many things to like about the present study; to my knowledge, it’s the first attempt to profile the circadian transcriptome from human fat tissue. However, the presentation of the manuscript, including the organization of the text as well as the construction of the figures, needs to be overhauled for this manuscript to maximize its impact. More troubling, there are a number of major claims made by the authors do not appear supported by the available data. Addressing these issues of organization and clarity would make this manuscript a valuable contribution to literature.

Major concerns:
1. There are several major gaps in the methodological descriptions presented in this paper. How were correlations to Per1 determined? What is the variance criteria used? What microarray platform was used? How was enrichment of genes involved in RNA processing defined as significant (and other biology not considered significant)? How was the Connectivity Map queried and what statistical criteria were used? A tissue / treatment’s “signature” is never defined in the text. These issues need to be addressed before a reviewer can assess if they're done reasonably.

Pg 10-12: Additions to the Methods section under the subsections "Data analysis" and "In silico experiment: correlation between the diurnal signature and the Connectivity Map" were made to address all the above questions. In addition, a supplementary (Figure S4) was added to illustrate the methodology and results of the Connectivity Map query.
2. Microarray data permits many thousands of p-values to be calculated, but as far as I can tell, no effort was made to correct for multiple testing. Converting p-values into q-values based on the method described in Storey et al. would improve the manuscript. If these q-values do not meet significance criteria, some discussion of multiple testing and care in interpretation of p-values would be necessary.

PG 10: Additional analysis method described in Storey et al were performed and the results shown in a new supplementary figure (Figure S3) and described in the Methods section under the subsection "Data Analysis", first paragraph.

3. “We demonstrate that the majority of genes in peripheral tissues are under diurnal regulation…” Only adipose tissue was tested, and only ~5,000 of ~20,000 genes had p-values < 0.01. Once multiple testing is adjusted for, this number will be even lower. This claim in the abstract needs to be revised to "a significant minority".

Done

4. “…and demonstrate that the key processes for energy metabolism are compartmentalized in time.” This was never demonstrated. RNA expression levels may be compartmentalized, but the actual biological process has not been examined. Eliminate this contention entirely or soften to "suggest".

Done. We have eliminated this contention from the abstract and text.

5. “Finally we show that mTOR inhibitors significantly reversed the observed diurnal signature…” This was never directly tested. The line of argument is interesting and suitable for the discussion, but this section should most definitely be removed from the ‘Results’ section and the description in the abstract must be changed to reflect the fact that no actual experiments were performed to test this. The abstract is unsuitable for publication as currently written.

We agree with this reviewer that the statement does not appropriately reflect what was done. Together with the comments of the second reviewer, we have removed the original sentence and replaced it with the suggested sentence from the second reviewer with an added emphasis that this was an in silico experiment in both the abstract and throughout the text.

The modified sentence in the abstract now reads the following:

Finally, an in silico comparison of the diurnal signature with data from the publicly-available Connectivity Map demonstrated a significant association with transcripts that were repressed by mTOR inhibitors, suggesting a possible link between mTOR signaling, diurnal gene expression and metabolic regulation.

Done

6. Be very careful with kinetic statements: “gene levels reaching a steady state,” or “Per1 mRNA expression levels dropping rapidly.” Given the cost of arrays and the difficulties working with patients, the 3 time point design of this study is
permissible; however, extreme caution must be used when inferring rates of change from three unequally spaced time points.

We agree and have removed the statements.

7. The paper would be strengthened immensely by comparing the results in these human studies with those already performed in model organisms. How valid are these model organisms for the study of circadian control of metabolism? Are there significant similarities despite differences in species and / or tissue? Any significant commonalities between humans and rodents would suggest a number of interesting follow up studies.

We agree and have added a paragraph discussing this important point (pg 25-26)

8. This paper used over-weight individuals. Clock genes regulate obesity and obesity regulates circadian rhythms. Is this a confounding variable in this study? This is an essential methodological detail and should be found in the abstract, the results, and certainly should be handled in the discussion.

We agree with the reviewer that this is a critical point. The details have been added to the abstract and the following sentences added to the results and discussion sections.

We also discussed this point in the discussion in a newly added paragraph (pg 25-26)

Pg 2 in the abstract: "... gene expression in the subcutaneous adipose tissue of overweight to mildly obese, healthy individuals."

Pg 13: " Figure 1 illustrates a schematic of the study, where biopsies from 17 subjects (BMI 27-35; Age 21-45) were..."

Pg 21: "...... sensitive to diurnal regulation in the adipose of mildly obese subjects."

Pg 25: " However, compared with the present study conducted in overweight to mildly obese humans in the course of one day, many of the rodent studies were conducted in lean mice and the restricted feeding regimen was conducted over many days, potentially confounding the comparison between rodents and humans..."

Text issues:

1. “impacted” is not a verb, it’s an adjective used to describe wisdom teeth, bone fractures, and feces (Oxford / Merriam-Webster). I recommend in the strongest terms that this choice of words be reconsidered for each one of its dozen or so uses in the text.

Done. We have removed all "impacted by" statements and replaced them with other more appropriate terms.

2. Pg 2: “We demonstrate … and demonstrating that…” These verbs should agree with each other.

Done.

3. Pg 4: “involves dozens to up to several hundred genes…” this is an awkward construction. Is there a citation suggesting that several hundred genes are involved in the core pacemaker? My last count was at ~ 15.
4. Pg 4: “The genes that show oscillatory behavior ... can be much larger.” The number of genes can be much larger, but the genes themselves are all different sizes.

Done. Sentence is corrected.

5. Comma errors in a number of places.
6. Pg 5: Turek et al.

Corrected

7. Pg 7: “Each participant...” This sentence needs to be re-phrased.

Corrected. The sentence and the sentence after that were changed to the following: Each subject was admitted to the Phase 1 clinical unit the evening before the start of the trial so that food intake prior to the first biopsy can be properly controlled. The subject then stayed in the clinic throughout the day until all three biopsies were completed.

8. Pg 8: “Figure 2B also shows...”

Corrected

9. Pg 8: “ZNF145 is a...”

Corrected

10. Pg 8: “Diurnal regulated genes ... were...”

Corrected

11. Pg 9: “a modest number of genes...” how many?

Sentence modified to "...nevertheless, there were ~500 genes with significantly different correlations to PER1 among the three states."

12. Pg 15: “growth factor inhibitors”

Corrected

13. Pg 18: “similar to what has been found...”

Corrected

14. Pg 18: “Zvonich et al. have observed... and have shown...”

Corrected

Figure Issues:
1. 2A: The clustergram on the top of the panel isn’t useful without knowing which genes are which; eliminate it. The heatmap color code is presumably a log-scale, but that’s not made clear in the figure or the legend. Label both axes; their
identity can be inferred from the text, but it’s best to be explicit.

**Done. All axes are labeled clearly and the log_{10} scale explicitly stated in the legend.**

2. Figure 3: Green and Red aren’t explained in panel A, likewise –ve and +ve are unclear. + = “positive”, does that make it “positiveve”? Titles for the heatmaps in B are too long, and cut off by the image besides. Clustergrams should be eliminated, they’re un-interpretable. Labels for both axes of the heatmaps are unreadable. Panel C is unnecessary, the figure would be improved by its removal.

**Titles of heatmaps are shortened. The cluster trees are removed. We have clarified all the labeling. We do feel that Panel C serves as a schematic to make Panels A and B clearer, so we would like to leave it in there.**

3. Figure 4: Axis labels are required even for histograms, but especially when similar graphs with different y-axes are presented together.\n
**Done. All axes are properly labeled.**

4. Figure 5, right panel is unnecessary and entirely speculative. It’s awfully confusing besides, it would be best to remove it.

**Done. Right panel has been removed.**

Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Not suitable for publication unless extensively edited

**Done. We have extensively edited the manuscript for clarity and grammatical errors with the help of a senior medical editor as stated in the Acknowledgements.**

Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests:
I declare that I have no competing interests.
Reviewer's report
Title: Diurnal Variation in Human Adipose Tissue: Link to Metabolic Diseases and mTOR Signaling
Version: 1 Date: 14 September 2008
Reviewer: Jeffrey Gimble
Reviewer's report:
This study extends prior circadian studies on murine adipose tissue to human subjects. It represents the first clinical study analyzing human adipose tissue diurnal biology at the transcriptomic level. The findings are correlated relative to a robust circadian gene marker (Per) in the context of the fed, fasting, and sibutramine treated conditions.

Major Compulsory Revisions:
1. In the context of references to papers by Ptitsyn or Zvonic, it would be appropriate to include the work by Ando H et al Endocrinology 146:5631, 2005. This study was the first to report the oscillatory expression of the circadian genes in murine adipose tissue by RT-PCR.

Done.

2. Pg16. The authors introduce the concept of HDAC drugs in their discussion as a future direction predicted by their analyses. The authors need to cite the following papers:
These and related publications have documented that deacetylases act on the components of the core or common circadian regulatory apparatus and impact oscillatory expression profiles. This prior work needs to be acknowledged and incorporated into the discussion.

The references are now incorporated and a new paragraph (pg 23) has been added in the discussion around SIRT1.

3. Pg 16. The authors point to a relationship of diurnal patterns in adipose tissue and the mTOR/PI3K/AKT pathways. This association is one that is logical, and to a degree, predictable since the next step in the progression is glycogen synthase kinase 3 beta. Most biochemical pathways describing the core or common circadian oscillatory apparatus will include GSK3B and/or casein kinase 1 epsilon as components. Their role is to phosphorylate the Per protein, which is then targeted to the proteasomal pathway for degradation. Again, this concept with appropriate references should be acknowledged in the Discussion.

The appropriate references are now incorporated and GSK3B is now added to the discussion (pg 22).
4. Pg 17, end of first paragraph. In their comments associating glucose metabolism to the diurnal gene expression profile, the authors are encouraged to acknowledge the publication by Rudic RD et al PLOS Biology 2:e377, 2005 "Bmal1 and clock, two essential components of the circadian clock, are involved in glucose homeostasis".

Done.

Minor Essential Revisions:
1. Table 1. The sixth most enriched pathway identified is the PPAR signal pathway. In light of its important role in adipogenesis and lipogenesis, it would be appropriate to include it for comment in the Results and/or Discussion.

Done. We have noted it in the results (pg 15)

2. Figure 4. Identify the X axis in the figure itself. Identify the Y axis directly as "fold change".

Done. The figure is now more clearly annotated.

3. Figure 5. Specify the identity of the Y axis. As presented, it could be easily understood as the "fold change" or the "number of genes".

Done. The figure is now more clearly annotated.

Discretionary Revisions:
1. Consider:
Reporting the mean BMI and age of the study subjects on pg 7.
Including any physiological measures of circadian biology (body temperature, blood pressure measures, serum corticosterone levels) that might have been evaluated in the study in addition to the transcriptome itself.

Done. BMI and age are now reported earlier (Pg 7 in methods and Pg 13 in results)

Identifying the biopsy site on pg 20 rather than waiting solely to doing so in the methods.

Done. Biopsy site is now reported in Pg 13 in the results and Pg 8 in the methods

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
Yes. I have applied for a patent relating to circadian mechanisms regulating adipogenesis and obesity.
Reviewer's report
Title: Diurnal Variation in Human Adipose Tissue: Link to Metabolic Diseases and mTOR Signaling
Version: 1 Date: 17 October 2008
Reviewer: Ronald Evans
Reviewer's report:
In “Diurnal Variation in Human Adipose Tissue: Link to Metabolic Diseases and mTOR Signaling”, Loboda et. al. examine global gene expression patterns in adipose tissue samples collected from human patient biopsies at three different times of day under fed, fasted or sibutramine-treated conditions. They convincingly demonstrate that time of day has a greater effect on gene expression in human adipose tissue than does either fasting or the weight loss drug sibutramine. Indeed, approximately 25% of the expressed transcripts displayed significant diurnal expression, which is consistent with previous studies of circadian gene expression in liver, heart and adipose tissue in mice. Consistent with reports demonstrating the dependence of circadian clock timing in mouse peripheral tissues on feeding patterns, the authors found that fasting altered the timing of expression of circadian clock genes and other diurnally expressed genes in a manner suggestive of a fasting-induced delay of circadian rhythm in human adipose tissue. Sibutramine treatment mimicked or increased the effect of fasting on diurnal gene expression. Also consistent with previous reports from studies in rodents, transcripts central to cellular and organismal metabolism were significantly overrepresented among the diurnally expressed transcripts. Finally, the authors compared their diurnal gene signature to gene expression changes elicited by various pharmacological treatments in cultured cells and found that the most significantly associated gene signatures were those induced by inhibitors of the PI 3-kinase and mTOR signaling pathway. This association seems to be a weak point of the paper; therefore, the reference to mTOR signaling should be removed from the title.

The dominance of diurnal regulation is not entirely unexpected because previous studies have shown that a large fraction of the mouse transcriptome is rhythmically expressed in various peripheral organs; however, this seems to be the first report demonstrating the importance of diurnal regulation of gene expression in biopsies from a human metabolically important organ. The study design is well controlled and the results emphasize the importance of carefully controlling for the time of day in studies of tissue gene expression. Furthermore, this clear demonstration of diurnal transcription of many metabolically important enzymes in human adipose tissue underlines the probable role of circadian compartmentalization of metabolic processes in humans, which may have implications for the development and treatment of metabolic disease.

Major Compulsory Revisions:
None.

Minor Essential Revisions:
1. As explained above, “…and mTOR Signaling” should be deleted from the title.
Done.
2. In the abstract, “We demonstrate that the majority of genes in peripheral tissues are under diurnal regulation and demonstrating that the key processes…” should be changed to “We demonstrate that a large fraction of genes in peripheral tissues are under diurnal regulation and that the key processes…” because 25% is not a “majority” and to fix the grammar.
Done. Abstract has been modified significantly reflect this.

3. In the abstract, the claim that “Finally, we show that mTOR inhibitors significantly reversed the observed diurnal signature, consistent with the key role of mTOR in energy storage and the dyslipidemia observed in patients treated with mTOR inhibitors” seems overstated as it is my understanding that the authors did not themselves do any experiments using mTOR inhibitors but merely found an inverse correlation between their diurnal gene signature and the gene expression changes induced by mTOR inhibitors in cultured adipocytes in publicly available datasets. This sentence could be changed to “Finally, we find a significant association between transcripts that are diurnally regulated in our study and transcripts that are repressed by mTOR inhibitors, suggesting a possible link between mTOR signaling, diurnal gene expression and metabolic regulation.”
Done. We have combined this suggestion with another reviewer’s comments and edited the abstract.

4. On page 6, the authors state: “We also report that the both the core clock genes and diurnal output genes were impacted with fasting and sibutramine. However, the effect was very subtle, hence very different from what had been observed in rodents, where the peripheral clock was profoundly affected by restricted feeding.” The comparison to the observations in rodents is not entirely fair because the experiment in rodents involved many days of restricted feeding while Loboda et. al. measured gene expression changes during a single day of altered feeding. The wording should be changed to reflect this. Also delete the unnecessary article “the” as indicated above by a strikethrough.
Done. The sentence comparing rodents and humans has been removed and the subject is further discussed in the discussion section (Pg 25)

5. On page 13, the authors state that the correlation between their diurnal gene signature and the gene signatures elicited by PI 3-kinase and mTOR inhibitors in human cell lines “…indicates that the growth factor pathway (AKT/PI3K/mTOR) in the adipose is diurnally regulated and thus, growth inhibitors would be expected to reverse those changes” is inaccurate. The inverse relationship between genes that are induced by inhibitors of PI3K or mTOR and genes that are diurnally regulated in the Loboda et. al. study does not indicate that the AKT/PI3K/mTOR pathway is diurnally regulated. The last 2 sentences of the paragraph could be changed to “The results show that diurnal genes were significantly but negatively impacted by these compounds, suggesting that PI3K-mTOR inhibitors may alter diurnal patterns of gene expression, perhaps by regulating circadian clocks.”
Sentence was removed from the results section.

Discretionary Revisions: None.
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests.