Master's Thesis Review

STUDENT NAME: Bc. Valeriy Kutsyna

THESIS TITLE: "Assaying mitochondrial cell number, morphology and respiration status in the emerging three lineages of the preimplantation stage mouse embryo"

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SUMMARY:

The big question being addressed by Valeriy Kutsyna's master's thesis is how mitochondrial physiology (*e.g.* bioenergetic state and gross morphology of the network) affects development of pre-implantation mouse embryo. Toward this goal, he used two tools. The first was the inhibition of p38 mitogen-activated protein kinases (p38-MAPK), which are important effectors of an eponymous signaling pathway. Previous results by Valeriy's colleagues had suggested p38-MAPK may have role in regulating mitochondrial biogenesis on a transcript level and perhaps even post-translationally during pre-implantation mouse embryo development. This hypothesis is supported by a mentioned study on p38-MAPK's direct phosphorylation of DRP1, which catalyzes mitochondrial fission, in mouse macrophages. The second tool was established by Valeriy himself: the implementation of image analysis tools to assess the mitochondrial network characteristics and the bioenergetic state of the organelle in wild-type and p38-MAPK inhibited (p38-MAPKi) embryos. By applying these tools, Valeriy was able to successfully accomplish his two immediate aims: characterize mitochondria in the late blastocyst stage of preimplantation embryo development and provide evidence of p38-MAPK's involvement in this process, both quantitatively. As far as I know, his accomplishment of both aims represent novel methodical advances and results in pre-implantation embryo development. The thesis itself is long, comprising 72 pages, 34 Figures (including 7 supplemental), and 124 citations and was obviously written by Valeriy himself.

EVALUATION:

I will write the important part first: Valeriy's master's thesis in my estimation is of high scientific value and fulfills the requirements of a master's project. However, after reading the thesis, I feel I have to evaluate two aspects of the dissertation separately: 1) Valeriy's exceptional scientific output as represented by the thesis's content and (2) the suboptimal way the thesis was written to communicate Valery's impressive results. While it is regrettable that the second aspect casts a shadow over the first, Valeriy's scientific potential is clear and thus it is worth the space here to point out why the dissertation failed so that he can learn from this experience.

1) Scientific output

The strongest aspect of the thesis is the amount of data generated by Valeriy. He used an impressive array of sample preparation, microscopy and image analysis methods to achieve his aims, as described in the Materials and Methods section. Furthermore, he performed the whole RT-qPCR pipeline from RNA isolation to the qPCR procedure (although not clear if he designed the primers listed in Sup. Fig 6), showing he is a versatile in performing various methods in the lab.

I was also impressed by Valeriy's demonstrated ability to analyze and process his data, a vital skill that complements–and these days even outweighs–data acquisition. The presented data is

comprised of high-quality confocal images, further processed skeletonized images, assorted graphs (*e.g.* scatter and bar plots) quantifying traits from the aforementioned images. These data were acquired from various cell lineages in wild-type and p38-MAPKi to reveal their intrinsic mitochondrial properties as well as how these change in the presence of the inhibitor. Any claims about differences are backed up by robust statistical analysis of the data.

Another valuable skill for a life scientist in the 21st century is implementing new tools to generate robust quantitative data. Valeriy notably did this twice in the master's project, namely the 'EzColocalization' and 'Mitochondria Analyzer' plugins for the open-source Fiji image processing package. These tools were then effectively used to quantify membrane potential and the degree of mitochondrial network fusion in various cell lineages and conditions, to supplement beautiful images with solid quantitative data. In fact, Valeriy's efforts were so impressive, they have inspired me to implement the 'Mitochondrial Analyzer' tool in my group's research.

So, in conclusion, Valeriy has demonstrated that already as a master's student, he has a very well-developed skill set that allows him to acquire data, process and analyze them by sophisticated quantification and statistics, plus even implement new sophisticated tools that are appropriate for answering his research questions. Furthermore, his thesis demonstrates that he has the required scholarship skills needed for performing research. He appears to have read a wide range of relevant research on far ranging topics such as embryo development, cell signaling, mitochondrial biology, and image acquisition and analysis. However, this is an impression I have from reading between the lines of his often difficult to read thesis, which brings me to the next part of the evaluation.

2) Poor communication of scientific results

Unfortunately, the text of the thesis often fails to do justice to Valeriy's scientific achievements. The thesis was quite often needlessly difficult to read, regularly trying to densely pack information into run-on sentences. The flow of the text was disjointed, as I frequently felt like I had to connect the dots among various topics that Valeriy mentioned but did not connect himself as the writer. It should be understood that the writer bears the burden of connecting various themes into a synthesis, not just describe various themes in depth, place them adjacently, and assume the reader will see the author's train of thought. However, I admit this is a common problem of early-stage scientists like Valeriy, but it is important to stress that the burden of flow is on the authors of grants, articles and theses, where you have to convince others of your understanding of the subject.

I will now enumerate the biggest problems that I identified when reading the text:

- Run on sentences that densely pack information in an overly confusing way. I will cite three glaring example that typifies the text in the thesis:
    - "Adhesion to the oocyte plasma membrane, causes a critical activated release of Ca^{2+} into the ooplasm, leading to exocytosis of oocyte cortical granules whose contents result in changes in molecular structure of ZP, resulting in inability of slower spermatozoa to penetrate it; thus preventing polyspermic fertilization events (Figure 1; Cheeseman et al., 2016; Tokuhiro et Dean, 2018)." [Page 2, Introduction].
        - This can be broken down into several smaller sentences that are easier to understand.
        - Or better utilize the cited figure and just highlight the important steps or state a general fact connecting all the depicted steps.
        - Why the semicolon? I noticed Valeriy relies excessively on this punctuation. I strongly suggest he abandon using it and if it appears, take it as a sign that

you should make the clause a sentence. Same goes for the equally abused hyphen (see below).

- "A solid number of relevant software solutions and protocols using 3D IF stained mitochondrial protein images were trialed (primarily driven by the relatively smaller size of mitochondria in preimplantation embryos, compared to pancreatic β-cells or neuronal cells – a frequently utilized and exemplary model culture system for such mitochondrial analyses)."
  - What an exotic collection of punctuation, with a hyphen taking place of a semicolon?
  - Text like this cries out to be broken into smaller, easier to digest sentences.
  - I noticed Valeriy's habit of putting text in parentheses, which I would also encourage him to give up. Putting text like this implies the text is not really important. But then why mention it? Seems the cited text is important, but not explained why.
  - As a general rule of thumb, a paragraph has a topic and each sentence is a 1 idea unit that in conjunction with the rest, fleshes out the topic systematically.
- "The observed mitochondrial hyperpolarization of TE cells, contrasting with low membrane potential across ICM mitochondria, is often experimentally evaluated utilizing potentiometric chemical dyes (such as MitoTracker Red – a rosamine derivative chemical, containing chloromethyl moieties that can react with free sulfhydryls located across the inner mitochondrial membrane, tetramethylrhodamine methyl ester, Jc-1 [sic] or many other deltapsim or carbocyanine-sentitive reagents, which bind to the mitochondria inner membrane and allow measurements of a quantifiable degree of intensity of the membrane polarization potential; Buckman et al., 2001; Lima et al., 2021; Rovini et al., 2021; Van Blerkom et al., 2002)."
  - The sentence is perfectly clear before the parentheses. The stuff contained within are tangentially connected, but seem to be important overall and could have been fleshed out in its own couple of sentences and/or paragraph

- The flow of the text was often difficult to follow. Examples include:
  - For example, the Results is organized like this: 1) measurement of membrane potential in various lineages by MitoTracker Red (MTR); 2) determination of mitochondrial network status; 3) co-localization of MTR with a mitochondrial marker *in situ*; 4) levels of transcripts encoding respiratory chain complex subunits. It would have been easier to follow if the membrane potential chapters followed each other, then maybe discuss how this can be correlated with mRNA levels measured and then conclude with how these may be linked to the overall morphology of the network.
  - In Mitochondria chapter 1.3 of the introduction, mitochondrial membrane potential is mentioned tangentially in the context of 'mitochondria in oocytes and early embryos' (1.3.2) but would have nicely fit with prior discussion about the respiratory chain, which generates and consumes the membrane potential.
- One should robustly edit the text, meaning identifying extraneous topics that are not related to the thesis. For example, Valeriy discusses the sub-compartmental organization of mitochondria and protein import into those compartments. I am of course a fan this topic and am happy that Valeriy finds this interesting, but this is not really needed for understanding the thesis.

    - Also, you give the reviewer enough rope to hang you with: Porins allow molecules 4-5 kDa to diffuse through the outer membrane. The quoted 10 kDa is big enough to allow many small intermembrane space (IMS) proteins to leak back into the cytosol. Also, I do not agree with the statement that the IMS is the similar in content to the cytosol, namely for the reasons I state above.
    - The author should focus on the important topics pertaining to the thesis (e.g. mitochondrial dynamics, p38-MAPK signally pathway, preimplantation embryo development) and better connect these themes into a syntheses, allowing the author to clearly discuss their results.
    - Sometimes things are mentioned without any prior explanation, such as 'MYBBP1A' and 'POLRMT', which may be extraneous or important. It is impossible to tell from their brief mentions here, but most likely fall into the former category.
- There are also assorted typos (which I highlight in a printed copy of the thesis that I will provide to the candidate), wrong word choices (*e.g.* calling supplemental elements figures instead of tables), long passages of text without citations to support statements (in striking contrast to other parts that are replete with appropriate citations, some mistakes in citations (format, quoted years, etc, pointing to wrong figures (*e.g.* Sup. Fig. 6 cited instead of correct 5).

In conclusion, I found the text very difficult to read and understand, which is a real shame as I feel that the author really did put a lot of effort into conceiving it. Valeriy chose nice illustrative figures in the introduction, collated a lot of his data, cited a lot of articles. Sometimes he even made some nice attempts at synthesis like the following:

"However, despite the involvement of other members of the MAPK superfamily, such as ERK and JNK, in the regulation of DRP1-mediated mitochondrial fission (Cook et al., 2017; reviewed in Ren et al., 2020), little is generally known about how MAP kinases specifically regulate mitochondrial dynamics in preimplantation mouse embryos. However, some clues, in the context of the data presented here, can be found in human disease research, as p38-MAPK over expression in a post-subarachnoid haemorrhage (a type of stroke, caused by a pathological accumulation of blood into the subarachnoid space of the brain) model lead [sic] to depolarization of mitochondria and subsequent ROS leakage to cytoplasm (Huang et al., 2013; reviewed in Ki et al., 2013)." (Pages 55-56).

These brief bursts of sunshine show to me that if Valeriy puts his mind to it, and perhaps develops stronger self-editing skills, he can synthesize complex information and present it in a clear, logical way.

ACADEMIC QUESTIONS (addressed directly to Valeriy; restricted to presented data):

1) It is unfortunate that you did not have time to try to measure the mitochondrial networks at single cell resolution. Admittedly, the program was made for imaging single cells in culture, not multicellular animals. How would you go about analysing single cells of the embryo? Were you able to at least look at branching in the ICM and TE separately, given these are easily identifiable tissues? Given your results on the membrane potential in the various cell lineages of the embryo, what would your expectation be for their mitochondrial network properties? How will this property change upon p38-MAPKi, if at all?

2) Some technical questions about MTR. How was the 500 nM working concentration of the MTR determined? What would happen if you use less or more MTR concentrations? How does MTR cross the zona pellucida, since it is stated in the Materials and Methods that this is removed after MTR treatment?

3) Related to question 2, I always wonder whether the lower levels of MTR signal in the ICM is versus the TE is due to the longer, more impeded path of MTR to the ICM. Is there a complementary way to verify that the ICM cells truly have a lower membrane potential compared to TE cells? Is there anything in your data that suggests that my concern is not an issue in the pre-implantation mouse embryos?

4) Thanks to the results in Figure 18 A, you claim that the membrane potential in the inner cells is increased in the ICM, with high statistical significance. Yet, when I look at the population of values in the scatter plot, the control and p38-MAPKi appear more similar than the stated P-value suggests. The CTCF values on the y-axis appear to be plotted on a linear scale. Is there a way to make these differences more apparent visually?

5) It is quite interesting result that the polar TE cells have a lower membrane potential than the mural ones (Figure 17). Perhaps this was discussed in the discussion, but I did not get it, but what why would the mural TE cells have a higher membrane potential than the polar ones? Any why do you think the polar TE membrane potential is affected by p38-MAPKi whereas the mural one remains unaffected.

6) The result that the chosen transcripts encoding various respiratory chain complexes from the whole E4.5 embryo decreased across the board by p38-MAPKi. How is this linked to the effects on seemingly disparate effects on membrane potential in the ICM and TE? Did you measure the total membrane potential of all the cells in the E4.5 embryo? If yes, does this correlate with your results? Also, these transcript show different degrees of downregulation, including the complex assayed II subunit that you mention you discarded for technical reasons. Do they suggest which of the assayed complexes contributes most to the assayed membrane potential? Would a downregulation of complex V affect membrane potential in the same way as downregulation of one or all of the electron chain complexes? What about complex II?

7) Two questions about Figure 24. I do not understand what heatmap matrix is showing. Please explain. I notice 3-5 separate sub-populations in the scatter plot from AIF and MTR co-localization upon p38-MAPKi. What do these represent? The PrE, Epi and non-committed cells? If yes, then why are these not apparent in the control? Why are discrete subpopulations not seen in TE cells in either condition?

8) Figure 22 shows AIF localizations in the ICM. These signals are considerably more blobby in A than the skeletonized images found in B and C. How was the this signal skeletonized given the source images? Is it possible the signals in A are more saturated than the ones used for skeletonizations in B and C? What is the difference between the B and C images? Does DAPI really stain the nucleolus (or 'nucleous') as claimed in the legend?

CONCLUSION:

Overall, I am very impressed by the content and scope of Valeriy's thesis. As mentioned before, I think that Valeriy has demonstrated skills that make him a gifted early-stage researcher. He is

able to acquire, analyze and interpret complex data and seems to be able to place them in a broader context. The weakness of the thesis is in how he communicates the data. However, I hope that the feedback provided in this report will help him toward more clearly and simply conveying his ideas and thoughts. Without a doubt, he has fulfilled the thesis requirement for obtaining the Magister title in my opinion. I still am wavering between a 1 and 2 for a final grade. I reserve this final decision until the defence, seeing how he presents his project in a presentation format. However, I am sure that he will convince me that he deserves the highest grade in this forum. I will look for an understandable presentation where the hypotheses being tested and data are presented clearly and simply.

doc. Hassan Hashimi, Ph.D.

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