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MASTER THESIS REVIEW

Student Name:	Pavlina Černá, Bc.
Thesis title:	"mTOR-regulated translation during first the wave of inner cell generation in mouse preimplantation embryos".
Supervisor:	doc. Alexander W. Bruce, Ph.D.
Co-supervisor:	Lenka Gahurová, Ph.D.
Reviewer:	dr habil. Zofia E. Madeja, Ph.D.
Reviewer's Affiliation:	Department of Genetics and Animal Breeding, Faculty of Veterinary Medicine and Animal Sciences Poznan University of Life Sciences, Poznan, Poland

General comment:

The presented Master Thesis is of high scientific value, fulfils all the requirements and meets the goals of the M.Sc. dissertation. Over all the introduction is well written, and it provides sufficient information (and figures) to understand the objectives of work. However, the Abstract is too wordy, the theoretical introduction is too long, the aims of work cannot be clearly elucidated form the text – it is not clear which part of the abstract refers to the "background knowledge", and which arises directly from this work, the results are somewhat lost within the text, and conclusion is not being clearly stated. In my opinion the final sentence is too broad, considering the amount of data (results) generated.

Material and Methods section has been meticulously prepared, and may in the future serve as a valuable "manual" for other undergraduate students, what in my opinion is also an important achievement of any M.Sc. dissertation. Similarly, the Results are described in detail and illustrated by numerous figures and graphs. The experimental setup included several study groups, this information (the number of embryos, experiment replicates, etc.) was not included in the body of text, only an information was given that "*all experiments have an extra suppl. table….*" – this creates unnecessary confusion and makes this crucial piece of information not straight forward. Also looking at the tables – for example Suppl. Tab. 1 - I am not quite sure how to understand it - in total used 151 embryos was used (72 + 79), but there were 5 biological replicates? I am not sure whether I understand it correctly - in total 5 experiments were done, and for each about 30 embryos were used? The scale of the experiment is important to understand the results (i.e. the

SD values).

The Discussion addresses the results in detail, but unfortunately it lacks the general conclusion – a few bullet points or "take home message". This is particularly important considering the amount of data (inhibitor/activator and microinjection experiments) presented in the results. Overall the Discussion is a strong part of the thesis, and it confronts the obtained results with the recent advances in the field (literature) and places this study within the greater picture of the ongoing project carried out by the group where the M.Sc. thesis was completed.

Detailed comments:

Introduction:

- pg 1: "…. *successive rounds of mitosis produce progressively smaller cells, referred to in the preimplantation embryo as blastomeres, and arise from so-called cleavage divisions in which resulting daughter cells do not grow during interphase*." In principle this statement is not wrong, but to be precise, it is not so much about "cell growth", as it is about lack of cytoplasm synthesis – oocyte's cytoplasm divides w/o increasing volume, which is accompanied by an abolishment of the growth period between G1 and G2 phase of the cell cycle. As the volume increases, cell's surface area increases.
- pg 2: *"….the combining of embryonic blastomeres of distinct genetic origin (to form chimaeras)….."* the statement "*distinct genetic origin*" - in my mind, may cause confusion. In genetic terms "distinct origin" rather implies phylogenetic distance (interspecies chimera). But here, the context (birth of viable pups) points to one species chimera.
- pg 3: "*An application of this strict definition of totipotency shows that isolated blastomeres from 4-8 cell stage embryos are not capable of supporting full development on their own*". It should be made explicit, that this staging refers only to the mouse embryo, in other species, where embryonic genome activation occurs much later in development than in the mouse, and where the preimplantation period is much longer, it is possible (although, not very effective) do derive viable offspring from a single blastomere of 4 and sometimes even 8 cell-stage embryo (sheep, goat).
- pg 3: "*nevertheless they are still considered pluripotent as they are able to contribute to all three blastocyst lineages when combined with other cells as chimeric embryos and form developmentally viable blastocysts*" . Does it refer to ICM, PrE and TE, or ICM, EPI and PrE? As definition of pluripotency clearly separates the ability to form both the embryonic and the extraembryonic lineages (totipotency - zygote) from the ability to solely contribute to the embryonic lineages (pluripotency – ICM, EPI).
- pg 3: "*Indeed, research has proven this pluripotent character persists until the early stages of blastocyst formation at the 32-cell stage*". Similar comment as above – by definition totipotency means the ability to contribute to all of the embryonic and extraembryonic

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lineages. Such capability is restricted to zygotes, and plausibly persists in embryonic cells after the very first cleavage divisions (more so in non-rodents, which activate their genome later, and have longer preimplantation development), pluripotency means restriction only to the embryonic lineages. However, at the same time, embryonic cells retain high level of plasticity. Thus, stating, that pluripotent character persists until the early blastocyst stage, implies, that at the blastocyst stage (expanded, hatched) pluripotent cells are no longer present, which is obviously not the case, as both the ICM and the EPI are considered pluripotent. To be precise in terms of pluripotency - mouse ICM is considered naïve and EPI primed.

Materials and Methods:

A small comment to confocal microscopy – what was the magnifying power of the objective used – was it the same for all of the images collected?

- pg 37: fluorescence quantification – I find this approach problematic if to be used for quantification of the amount of protein, even if the same confocal settings are going to be used, so many variables exist from one experiment to the other. It may be slightly different antibody binding affinity, the level of permeabilisation, the time for which embryos were "waiting" to be AB labelled (sometimes it will be done on the day of fixation, and sometimes on a later date), position of the embryo on the slide, and many others. Such changes for obvious reasons cannot be eliminated, and are normal for this type of studies, yet drawing conclusions "on the protein level" based on this approach is prone to create a bias. The only way to normalize the results would be to use a fluorescent reporter gene – such approach is an accepted tool to measure the changes in the protein levels in living cells. Alternatively (although it is difficult to be done, but not impossible on embryos) protein level may be established by Western Blot.

Results:

- pg 39: "*At this stage, only embryos comprising exactly 16-cell were analysed to distinguish and quantify three distinct types of blastomere….*" what about the embryos that were less than 16 cell stage? was there any delay in development observed in relation to the control group? This could also be a valuable information related to the impact of the inhibitors applied.
- Pg 55: Figure 22: "*….note cytoplasmic and nuclear HA-4EBP1 protein expression/localization….*" The signal is very weak signal - blends into background – not sure, whether it is not auto-fluorescence.
- Pg 70: "*…..the expression level in the entire embryo as a whole was quantified…*". Similar comment as made for pg. 37 (quantification of fluorescence), and also the use of the term "expression level" is not entirely precise, as the process of gene expression refers also to

transcription. Translation (especially during the preimplantation stage of development) may be happening at a different time point, than at which the protein is detected – sometimes even a few cleavage divisions earlier. Moreover, not all transcripts may be utilised at the same time. Also the whole process of gene expression from mRNA to protein may undergo spatio-temporal regulation in the early embryo. Therefore, bearing in mind future publication, it should be made clear when we talk about gene expression (transcription, translation) and protein localisation.

Discussion:

- pg 77: "*The last aim of the project was to test our hypothesis that mTOR does indeed play a specific role in translation regulation, that is important for spatial allocation of blastomeres by the 16-cell stage*" …..not sure of the link between translational regulation and cell number? factors responsible for cleavage/mitosis were not targeted?
- Pg 79: a paragraph starting with: " *In connection to…. We also measured the expression levels of endogenous p4EBP1….(…)….as increased p4EBP1 would be needed to drive translation of mTOR sensitive TOP-motif containing mRNA transcripts, required to ensure germane inner cell generation*". Since mRNA expression analysis (transcript) by real-time PCR (or other molecular method) was not performed it cannot be stated that the expression levels mere measured! To ensure that the whole protein "gene expression process" is studied, protein quantification (by WB) should be linked with transcript level evaluation.

References: The literature is well chosen (relevant).

Final evaluation: Over all, the thesis is of high scientific merit, and deserves a grade A (excellent).

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