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Expert Opinion on Ph.D. Thesis

Ph.D. Candidate: **Pablo Bora, MSc.**

This doctoral thesis by Pablo Bora is focusing on the understudied aspects of early mammalian embryogenesis, specifically on the role of p38-MAPK pathway in differentiation of pluripotent stem cells into epiblast and primitive entoderm cell populations.
Ability to generate extra-embryonic cell types during pre-implantation development (trophoectoderm and primitive endoderm) is a necessary prerequisite for successful implantation and further development in utero. Therefore, understanding of molecular mechanisms behind blastocysts maturation is of great importance especially in the light of assisted reproduction when these fate decisions are undertaken in vitro and may affect the outcome of infertility treatment.

The aims of the thesis were defined as follows: a) refine temporal window of required p38-MAPK function during blastocyst maturation, with particular emphasis on primitive endoderm specification; b) identify and experimentally validate potential targets of p38-MAPK phosphorylation activity that is necessary for normal preimplantation development and lineage specification; and c) identify system-wide effects of p38-MAPK activity and define its role towards regulating blastocyst maturation and associated lineage specification.

In order to examine the role of p38-MAPK pathway in cell fate decisions, Mr. Bora employed a variety of techniques typically used in the field, including mouse embryo culture, microinjections, mRNA and transcriptomic analysis, western blot, immunofluorescence and proteomic analyses. The methods chosen were appropriate for the corresponding scientific questions.

As the whole, the dissertation thesis is meticulously elaborated, its language is sound and sophisticated, and its individual parts are well balanced. The core of the thesis comprises 3 first author papers in peer-reviewed journals. Concerning the quality of the research that makes the grounds of this thesis, I may conclude that it is exquisite. The strategies and findings fully conform to the original plan, and are all published in high visibility journals in the field. The significance for science is unquestionable.

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I have the following comments and questions:

1. In all three studies SB220025 was used to inhibit p38-MAPK signaling. This small molecule inhibitor is known to affect production of TNFalpha – cytokine involved in multiple signaling cascades. Could Mr. Bora explain why this particular inhibitor was chosen? What alternative approaches may be used: siRNA or other inhibitors?

2. Phenotype observed under conditions of p38-MAPK inhibition is marked by decreased total number of cells comprising mouse embryo as well as decreased blastocyst volume. This is in line with involvement of p38-MAPK pathway in cell survival and proliferation via multiple other pathways. Has Mr. Bora noticed any cell cycle disturbance during live-embryo imaging? Could the phenotype be rescued by extension of in vitro culture after wash out of p38-MAPK inhibitor?

3. In the study number 3. (Front Cell Dev Biol) Mr. Bora shows involvement of p38-MAPK pathway under conditions of amino acid depletion induced cell stress. Interestingly amino acid supplementation nearly completely restored the number of epiblast cells under the conditions of p38-MAPK pathway inhibition but still did not fully rescue primitive endoderm population. This peculiar finding supports the fact that p38-MAPK signaling is essential for primitive endoderm formation. Could Mr. Bora suggest the explanation for rescuing effect of amino acids for epiblast but not primitive endoderm cells? Is it possible that epiblast cells are more prone to amino acid depletion induced cell stress than extra-embryonic cell lineages?

4. In the study number 2. (Manuscript) Mr. Bora demonstrate original finding that p38-MAPK effect on mouse embryo development may be mediated through influencing of rRNA processing, specifically by orchestrating of DDX21 localization to the nucleolus. Mr. Bora shows that the localization of DDX21 changes upon transition from 16-cell stage to blastocyst stage and inhibition of DDX21 results in a similar phenotype to that observed after p38-MAPK inhibition. Mr. Bora also suggests that changes in DDX21 expression may be associated with zygotic genome activation. Is there any information on activity of p38-MAPK prior to zygotic genome activation? How would inhibition of p38-MAPK pathway during cleavage stage affect primitive endoderm formation?

In conclusion, based on my opinion detailed above, I strongly recommend this thesis to its oral defense and I suggest the candidate to be awarded by the title "Doctor of Philosophy".

Brno, June 24, 2021

Doc. MVDr. Aleš Hampl, CSc.

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Review of a Ph.D. thesis
by Pablo Bora, University of South Bohemia in České Budějovice, Faculty of Science, entitled "The role of p38-MAPKs in mouse preimplantation embryonic development: Regulating translation towards blastocyst maturation and lineage specification"

The thesis addresses an important topic of regulation of preimplantation mammalian development and early lineage specification. During first days of development, mammalian embryos undergo rapid changes, leading to specification of embryonic and extraembryonic lineages, namely: epiblast, primitive endoderm and trophectoderm. Proper differentiation of these lineages is indispensable for the continuation of development in utero, therefore identifying the factors and mechanisms governing this process is of vital importance.

The thesis investigates in great detail the role of p38 MAPKs (mitogen-activated protein kinases) in preimplantation mouse development, a factor which has been previously identified by the group of doc. Alexander Bruce as having a specific effect on differentiation of primitive endoderm lineage.

The thesis comprises a series of three publications, of which the candidate is the first author. Two of them (Chapter I: "p38-MAPK-mediated translation regulation during early

blastocyst development is required for primitive endoderm differentiation in mice" P. Bora et al., and Chapter II "DDX21 is a p38-MAPK sensitive nucleolar protein necessary for mouse preimplantation embryo development and cell-fate specification" P. Bora et al.) have been shared with the general public as pre-prints on bioRxiv repository website, and have been also accepted for publication by IF journals following peer-review process. Third publication (Chapter III) "p38-mitogen activated kinases mediate a developmental regulatory response to amino acid depletion and associated oxidative stress in mouse blastocyst embryos" P. Bora et al., 2019, have been already published in such a journal (Frontiers in Cell and Developmental Biology). This is a really commendable outcome, given the time consuming reality of embryological studies, and undoubtedly speaks of candidate's hard work and commitment. The thesis contains also clearly outlined aims, very comprehensive (perhaps even too detailed) introduction, and a comprehensive summary of results and discussion. Both the publications and all the auxiliary material are well written, and scientific literature is properly cited, referencing both essential classical and very recent publications (from last 3 years), confirming author's knowledge of the subject.

The author employed a vast range of techniques in order to uncover p38 MAPKs role in early development, including small molecule inhibition of specific pathways, RNA silencing following siRNA clonal microinjection, immunofluorescence analysis, time-lapse analysis of in vitro development, but also such large-scale essays such as mRNA sequencing and liquid chromatography–mass spectrometry (LC-MS) analyses of the embryonic proteome, rRNA and polysome profiling and indeed many others. This approach allowed him to uncover the details of p38 MAPKs function, including novel regulatory role on translational level. Importantly, he revealed two novel factors in blastocyst development and specification, DDX21 and MYBBP1A. His work also reveals important role of amino acid supplementation of mouse embryo in vitro culture. This has not only mechanistic, but very important practical implications, both for our understanding of health implications in the light of 'Developmental Origin of Health and Disease' hypothesis, and also for everyday experimentation and proper experiment design in embryological laboratory.

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Questions to the author:

regarding Chapter I:

To my understanding, embryonic diapause represents a state of arrested blastocyst development when epiblast and primitive endoderm lineages are already well sorted. According to transcriptomic analysis (Boroviak et al., 2015) diapaused murine epiblast profile is distinct from pattern of overlapping expression in early mouse blastocyst. Could you please provide evidence that "unspecified ICM" in p38MAPKs inhibited blastocysts indeed may be indicative of diapause state, or give an alternative explanation of the state these embryos are arrested in?

regarding Chapter II:

Presented here clonal loss-of-function analyses of *Ddx21* revealed cell-autonomous and non-cell-autonomous role of DDX21 in blastocyst development, with specific bias towards primitive endoderm (PrE) reduction. Could you also speculate on the possible phenotype of *Ddx21 KO/* silencing in the whole embryo, and on the relation between reduced volume of blastocyst cavity and reduced contribution PrE.

regarding Chapter III:

In your thesis you observed that amino acid supplementation affects the development of p38-MAPKs inhibited embryos. In a more recent paper (Frum and Ralston, 2020), the authors noted the influence of BSA supplementation on FGF4 treated mouse embryos. Could you please comment on the possible relation of these observations?

In summary, the work presented in this thesis is original, of high scientific quality and addresses an important topic in the field of developmental biology. As such, it represents a valuable contribution to our knowledge of developmental mechanisms. Therefore, I recommend the thesis for successful defense.

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