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Opponent's review

PhD thesis of **Aleksandar Mihajlović**, MSc.

Title: "The involvement of the Hippo signalling pathway in the first two cell-fate decisions of pre-implantation mouse embryo development"

Topicality of the research:
During his PhD Aleksandar Mihajlović focused his effort on understanding the molecular mechanisms regulating the first two differentiation events during mouse early embryonic development, which create distinct cell lineages, namely trophectoderm and inner cells mass during the first and epiblast and primitive endoderm during the second event. Mammalian early development is characterized by dramatic and rapid changes accompanying transition from totipotent blastomeres into highly specialized cell lineages. However, most of the regulatory mechanisms are still not completely understood. It is also very important and sensitive period of development, during which cells are prone to chromosome segregation errors. Aneuploidy and developmental defects are frequently arising. Therefore I believe that the subject of the thesis was well chosen and it is extremely important.

Introduction of the problem and relevant literature:
The introduction section summarizes comprehensively recent knowledge and hypotheses in the field and all relevant literature is discussed. My minor criticism here would be the length of this chapter, which I believe could be reduced to developmental events relevant to the thesis.

Methods and techniques used in the study:
To obtain his results Aleksandar used a large variety of techniques ranging from molecular biology approaches, such as qPCR, throughout mainstream cell biology techniques, such as immunodetection and confocal microscopy, up to advanced manipulation of cells, including microinjection and creation of embryonic chimeras. All techniques are described in sufficient details allowing reader assessing not only procedures and protocols but also numbers of cells in the study etc.

Main objectives:
Two main objectives are presented in the thesis, namely to address whether the manipulation of the activity of Hippo signalling pathway can influence the decision between epiblast and primitive endoderm and then to asses the importance of Rho-associated kinase 1/2 for preimplantation development and Hippo signalling.

Characterization of the results and achievements:
The results were published in two important scientific journals - Scientific Reports and Reproductive Biomedicine Online, unpublished results are presented in the thesis. In summary work of Aleksandar and his colleagues showed that the differentiation decision between trophectoderm, primitive endoderm and epiblast cells is flexible and controlled by exposure to trophoblast phenotype related differentiation factors rather than predefined path leading rigorously into TE cells commitment first followed by decision between primitive endoderm and epiblast fate. Results related to the role of Rho-associated kinases showed the

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overall importance of this regulation for establishing the apical-basolateral polarity and formation of tight junction, which are both essential for differentiation events in early embryo, according to the prevailing hypothesis. On molecular level Rock inhibition leads into disruption of spatial arrangement of Hippo signaling mediated by Angiomotin. The published and unpublished results presented in the thesis are highly relevant and important for our understanding of the factors controlling the origin of differentiated cell lineages during mammalian early development.

Questions for the candidate:

1. In his work Aleksandar used embryonic chimeras. Is there any role, for example during establishing of cell polarity, for the zona pellucida or can aggregated blastomeres of early embryos recapitulate similarly all events without its mechanical support?

2. Recently published paper by Korotkevich et al. demonstrated the role of cellular apical domain in TE differentiation, the independence on Cdh1 and regulation of spatial expression pattern of Angiomotin. The candidate should discus the link between his results and the data published recently.

3. Could candidate comment on a possible link between the sizes of the individual blastomeres, the absolute levels (not relative concentration) of protein factors important for differentiation and the cell fate?

Conclusion and recommendation:
Aleksandar Mihajlović demonstrated his scientific quality and critical and independent thinking; the thesis is well prepared and extremely informative. The experiments were well executed and the results are showing great skills in laboratory work and data analysis. Therefore I recommend proposed thesis for defense and candidate for receiving PhD degree.

In Brno, 24. 03. 2017.

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Warsaw, 24 February 2017

Examiner's report on PhD thesis entitled „The involvement of the Hippo signaling pathway in the first two cell-fate decisions of pre-implantation mouse embryo development" presented by Aleksandar Mihajlović

In the thesis submitted for examination the candidate presented and discussed the results of an extensive research project aimed at the elucidation of some of the mechanisms, which govern cell differentiation during preimplantation development of the mouse. During this period in the blastocyst two extraembryonic lineages: trophectoderm (TE) and primitive endoderm (PE) are separated from the pluripotent epiblast (EPI).

In the first part of the study candidate made an effort to clarify whether differences between the inner blastomeres of the embryo, which ultimately lead to the formation PE and EPI of the blastocyst, may be related to their origin from two rounds of differentiative divisions during which inner cells (ICM) are separated from the outer TE precursors. The second part of the work aimed at the clarification of the role of Rho-associated protein kinases 1 and 2 (Rock1/2) in the regulation of Hippo signaling pathway, which control TE versus ICM cell-fate decision.

Preimplantation mouse embryos are an excellent experimental system to study the self-organization process of development. However, despite great progress observed during last two decades, many aspects of mechanisms controlling the first differentiation events, which occur during preimplantation development of the mammalian embryo, are still a subject of a heating debate. Thus it has to be emphasized that the research which the candidate undertook related to a very important problem of mammalian developmental biology.

In cleaving preimplantation mouse embryo two (in some embryos three) rounds of differentiative divisions occur which give populations of outer and inner blastomeres. Subsequently, outer cells form a TE and inner ICM cells are precursors of EPI and PE. It was previously suggested that the origin of inner cells from the first round of differentiative division biases them to form EPI precursors and from the second toward the PE. Candidate decided to test the hypothesis that the time of the exposition of the parental cell to the positional clues, which induce outer cells into formation of TE, biases their daughter inner cells into the contribution toward PE or EPI lineages. Since outer position induced TE differentiation relays on the activity of Tead4 transcription factor, which is regulated by Hippo pathway, candidate made a decision to examine the differentiation of inner cells derived from blastomeres in which *Tead4* expression was down-regulated by RNAi, thus preventing their induction into TE. Inhibitory dsRNA was introduced into one blastomere of 2- and 4-cell embryos. Chimaeric embryos composed of 8-cell stage embryo and a single 1/8 blastomere to which dsRNA was injected were also created. Subsequently the localization of cells derived from blastomeres, in which Tead4 was inhibited, was determined at the stage of mature blastocyst. It was determined that invariably cells in which TE differentiation was inhibited, exhibited significantly biased contribution toward populating the EPI over the PE

lineage. Accordingly, candidate managed to demonstrate that interference into first stage of cell differentiation in the embryo (TE vs. ICM) influences the second differentiation process (EPI vs. PE). Consequently candidate proposed that positional clues which inhibit Hippo signaling in outer cells of the embryo and thus induce these cells to differentiate into TE, concomitantly prime the inner cells, which arise from differentiative divisions at the 8- and 16-cell stages, to differentiate into PE. So heterogeneity of the ICM cells may result from the length of the exposition of parental blastomeres of inner cell's to such clues, since TE-priming of inner cells derived from the 5^{th} cleavage division (second differentiative division of outer blastomeres of 16-cell embryos), is longer than inner cells created during 4^{th} division.

Next, in a series of meticulously designed experiments candidate demonstrated that the reduced ability of TE-inhibited inner cells to populate PE lineage is associated with decreased expression of PE markers and in consequence reduced capacity to respond to PE differentiative ques. Thus candidate provided evidence that two cell-fate decisions, which occur in preimplantation embryo, are indeed interlinked. On the basis of the results presented in his thesis candidate proposed an integrated ("time-inside time-outside") cell-fate decision model explaining the formation of EPI and PE lines in mouse blastocyst.

The second part of the research was aimed at the elucidation of the role of Rho-associated protein kinases 1 and 2 (Rock1/2) in the regulation of Hippo signaling pathway, which is involved in the regulation of TE-precursor cell line formation. To this goal candidate analyzed the phenotype of mouse embryos developing in vitro in the presence of inhibitors of Rock1/2. He observed that activity of Rock1/2 is indispensable for the proper blastomere polarization, formation of tight junction and proper F-actin distribution, which are required for the development of the blastocyst. In further experiments candidate managed to show, that activity of Rock1/2 regulates Hippo signaling pathway, and Rock1/2 inhibition results in displaced Hippo activation in outer cells of the embryo. Amot protein seems to be the element of the Hippo pathway which is affected by the inhibition of Rock1/2, and its mis-localization is responsible for the observed phenotype. Thus candidate has shown that Rho-associated protein kinases 1 and 2 may participate in the regulation of the first cell-fate decision in preimplantation mouse embryo.

In summary, in the experiments presented in the thesis submitted for review (which only very briefly were summarized here), candidate provided a very comprehensive research of the molecular basis of two waves of cell differentiation, which occur during mammalian preimplantation development, and which lead to the formation of two extraembryonic lineages: TE and PE, as well as EPI pluripotent precursor of the embryo proper. It has to be emphasized that evaluated study brings a valuable set of data, which are very important for the better understanding of the mechanisms which control the early mammalian development. Without any doubts the thesis as a whole constitutes a substantive original contribution to the knowledge in the area of developmental biology. Significant part of the thesis has already been published in respected international scientific journals (*Scientific Reports* and *Reproductive Biomedicine Online*).

It is important to draw attention to the fact that during this study the candidate demonstrated excellent experimental skills. He was able to apply an appropriate set of cell, developmental and molecular biology techniques. Difficult experiments, which are described in his thesis, were well planned and properly executed, and accompanied by appropriate set of controls. The presentation and interpretation of results in the examined thesis is very

good. General literary quality and the way of presentation of the thesis are of a high standard. The candidate showed exceptional familiarity and understanding of the relevant, vast literature of the subject. The only criticism is in regard to the terminology related to the mouse embryo, since author (many times thorough the thesis) is calling the cavity of the blastocyst as "blastocoel". However it is well accepted among mammalian embryologist that blastocyst cavity is not a homolog of the blastocoel of embryos of the animals belonging to other taxonomic groups. The examiner suggests candidate to research this subject since acknowledging this fact is important for the understanding the significance of preimplantation period of mammalian development, which is unique for this group. The good lecture on this topic is: *O'Farrell et al., 2004. Embryonic cleavage cycles: How is a mouse like a fly. Curr. Biol. 14, R35.*

Despite above mentioned critical comment, the opinion of this examiner is that research thesis submitted by Mr. Mihajlović constitutes a solid scientific contribution to the field of mammalian reproductive biology. On this basis I recommend that the thesis be defended and candidate be awarded the degree.

Marek Maleszewski