REPORT ON THE DOCTORAL THESIS

of M.Sc. Erkut Ilaslan entitled „Distinct roles of NANOS paralogues in post-transcriptional mRNA regulation in human male germ cells"

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The presented dissertation is a thematically coherent series of three scientific publications – two experimental papers, and one illustrative review – which were published between 2020 and 2022 in peer-reviewed scientific journals from the Philadelphia list (ISI). The total Impact Factor (IF) of the publications included in the cycle is: when publications were published: 16,688, 5-year journals Impact Factor: 19,884 and 420 MNiSW points. In two of the publications, the PhD student is the first author, in the third publication, he is the second author. It should also be emphasized here that the PhD student's total scientific output is actually richer, as he is the co-author of six additional articles, the IF of which is: when publications were published: 29.657; 5-year journals Impact Factor: 38,176 and 800 MNiSW points. The evaluated thesis has been prepared within the following National Science Centre projects: „A study of a potential role of NANOS proteins in orchestrating 3'UTR-mediated translational regulation of functionally related mRNAs in human germ cells: A quest for the mechanism of the mammalian posttranscriptional regulons" (OPUS 2014/15/B/NZ1/03384) – Principal Investigator: dr hab. Kamila Kusz-Zamelczyk, and „NANOS1 RNP-interactome: structure and dynamics during specification/early stages of human germ cell development - significance for human reproduction" (OPUS 2019/35/B/NZ1/01665) – Principal Investigator: prof. Jadwiga Jaruzelska.

Unfortunately, the dissertation does not indicate whether the PhD student showed additional scientific or research activity, such as participation and/or presentation in national or international scientific conferences. Usually, such an addition conveys the information that the doctoral student is not only a specialist in their field of research but also has broader scientific and research interests.

The presented doctoral thesis consists of 120 pages. After the acknowledgements, the dissertation displays the table of contents introducing the 6 chapters: abstract, introduction, hypothesis and specific objectives, model of the study, results and discussion, and literature.

In the introductory chapter, the PhD student extensively describes what mammalian germ cells are, how they are formed, and the researcher indicates the differences between the human and mouse germ cell specifications. In addition, he describes the origin of germ cell tumor from abnormal primordial germ cell. In one of the subchapters, the doctoral student indicates three main groups of RNA-binding protein involved in post-transcriptional regulation during animal germ cell development. In a separate subchapter, the role of NANOS protein in primordial germ cell specification has been described, as well as the migration and its role in infertility. The introduction is enriched with exact figures, allowing for the clear understanding of the processes related to the researched issue. The doctoral student precisely indicates from what source the used figures are cited, using the appropriate citation pattern. Such an extensive, but at the same time accurate description introduces the reader to the issues directly related to the conducted research. The exemplarily detailed introduction displays the doctoral student's expertise in the field of research.

In the further part of the thesis, the PhD student presents the research hypothesis and specifies the main goals of the research. Then, the use of the selected model of the study during the implementation of research tasks is thoroughly described.

The "Result and discussion" chapter consists of a brief introduction and the most important results and conclusions regarding each of the three publications mentioned. Moreover, the doctoral student indicates the author's contributions, which show a significant participation of the PhD student in the conducted research. The author's contributions indicated in the dissertation correspond to those present in the published articles. Here, too, the PhD student placed copies of the publications included in the cycle. Due to the fact that the presented doctoral dissertation is a thematically coherent series of three papers, which are collective works, the PhD student has included relevant statements of all co-authors of the published scientific articles. It should be noted, however, that the statements themselves are much less legible than the other parts of the dissertation. The statements' scans should be prepared in a more aesthetically-accurate manner.

It should also be said that the layout of the doctoral thesis is transparent and raises no objections. The language of the presented dissertation is English. The doctoral student took care not only of the concerning the factual content and graphic aspects of the dissertation, but also (in evaluator's opinion) of the language, and the grammatical form. Another fact worth highlighting is that the discussion on the results, which is presented in the thesis, compared to

the already available reports in this area, clearly confirms the doctoral student's extensive knowledge in the field of research and shows the great involvement of the doctoral student in the implementation of the experiments. All of the points herein display an appropriate and well-mastered laboratory skills of the PhD student - skills in the field of independent use of cell and molecular biology methods (cell line cultivation and transfection, RNA-Seq, RT-qPCR experiment, western blot), and the performance of bioinformatics analyses.

As mentioned earlier, the dissertation is based on a series of three scientific papers that have already been published in international journals (all three in the International Journal of Molecular Sciences), so hereby I am convinced that their substantive correctness and interpretation of the results obtained have been verified by independent reviewers during the peer review process. Therefore, as a reviewer, I will only briefly analyze the content of individual publications in terms of their thematic consistency and innovativeness.

The research hypothesis and the purpose of the research were properly specified and included in a separate chapter of the dissertation. The first aim of this thesis has been to identify mRNAs under potential regulation of NANOS proteins by utilizing RNA-Sequencing (RNA-Seq). The second aim of this dissertation is the functional characterization of the pool of mRNAs under regulation of each NANOS paralogue by bioinformatic approaches, and validation of the biological implications of these regulations by functional experiments.

On the basis of the obtained results, the PhD student correctly formulated conclusions corresponding to the assumed objectives of the work. In the first presented study, the PhD student showed that an increased confluency of TCam-2 viable cells and an increased rate of cell proliferation compared to the controls are associated with overexpression of the wild-type NANOS1. In contrast, overexpression of specific – infertility-associated mutant NANOS1 leads to a reduction in total cell number, and proliferation rate compared to control, completely reversing the phenotypic effect of NANOS1. Additionally, overexpression of both; wild-type and mutant NANOS1, has been shown to lead to an increase in the proportion of TCam-2 cells in the G0/G1 phase, while reducing the S-phase cell proportion. Interestingly, as mentioned by author, it is partially confusing, thus the inhibition of the cell cycle by NANOS1, does not explain it's pro-proliferative role.

Further phenotypic analysis of TCam-2 cells showed that overexpression of wild-type NANOS1 resulted in a reduction in the number of apoptotic cells compared to the controls. In contrast, overexpression of mutant NANOS1 increased the number of apoptotic cells compared

to the control group. These results suggest that the mutations identified in infertile patients change the function of NANOS1 from anti-apoptotic to pro-apoptotic. In the next step of the study, wild-type NANOS1 was overexpressed in the TCam-2 cell line, and RNA-Seq analysis was performed to identify potentially apoptosis-related mRNAs regulated by NANOS1. In the performed research, ten pro-apoptotic genes that were downregulated upon overexpression of the wild-type NANOS1 (*GADD45A, GADD45B, GADD45G, RHOB, BCL10, STK17A, TP53BP2, RIPK1, SIAH1, JUN*) have been identified. Out of these ten genes, the downregulation of seven was confirmed by using RT-qPCR (*GADD45A, GADD45B, GADD45G, RHOB, BCL10, STK17A, TP53BP2*). Moreover, when mutated NANOS1 was overexpressed in TCam-2 cell line, it was unable to downregulate four of these proapoptotic genes, namely *GADD45A, GADD45B, GADD45G*, and *RHOB*, indicating that the infertility-linked NANOS1 mutation disrupts NANOS1's ability to repress four of these DNA damage-related proapoptotic genes.

In conclusion, the obtained results indicate that *NANOS1* overexpression in TCam-2 cell line is associated with increased proliferation, changes in cell cycle, and an inhibition of apoptosis. In effect, a mutation in NANOS1 could be responsible for switching of NANOS1 activity from an anti-apoptotic to a pro-apoptotic state.

Furthermore, in the next step of his research, the doctoral student tried to characterize functions of NANOS1 and NANOS3 in the TCam-2 cell line in order to gain insight into their functions during human germ cell development. Additionally, in the presented dissertation, NANOS1 and NANOS3 effects on the cell cycle have been indicated. Moreover, the NANOS3-PUM1-FOX1 axis in regulating mRNAs involved in G2/M phase and propose dysregulation of this axis as a factor contributing to testis cancers.

In the third part of the dissertation, the review article, the PhD student presents the latest reports indicating the role of mammalian NANOS RNA-binding proteins, and the mechanisms of their overexpression in cancer.

Having read the content of the scientific articles presented, which are the basis for the doctoral dissertation, I state that they were well-thought through, properly planned, and the research was properly selected and conducted.

As an evaluator, I declare that the doctoral dissertation of Mr. M.Sc. Erkut Ilaslan entitled: "Distinct roles of NANOS paralogues in post-transcriptional mRNA regulation in human male

germ cells" is an innovative, valuable work and an original contribution to the development of modern medicine related to human reproduction, using modern diagnostic methods based primarily on molecular biology.

Final conclusions

Summarizing, I unequivocally state that the doctoral dissertation meets the statutory requirements, and hereby I apply to allow Mr. M.Sc. Erkut Ilaslan to enter further stages of the doctoral program. It meets the requirements established in „Rozporządzenie Ministra Nauki i Szkolnictwa Wyższego z dnia 19 stycznia 2018 r. w sprawie szczegółowego trybu i warunków przeprowadzenia czynności w przewodzie doktorskim (Dz.U.2018 poz 261)."

Decision: positive evaluation.

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Questions:

a) **Referring to the PhD student's research activity, I would like to inquire if the student took part in any national or international scientific conferences.**

b) **What kind of difficulties were faced by the student during the research process?**

c) **What did the student base his choice on while deciding on the timeframes for the experiments/measurements?**

d) **Is it possible to conduct similar research on a feminine model?**