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Review of Matisa Alla's doctoral dissertation entitled
„A Dominant Gain-of-Function NANOS1 Variant Disrupts WNT Signaling and Impairs Human Germ Cell Specification"

The PhD thesis by Ms. Matisa Alla was prepared at the Institute of Human Genetics, Polish Academy of Sciences in Poznan under the supervision of Prof. dr hab. Jadwiga Jaruzelska.

In general, the research topic concerns understanding the molecular basis of male infertility, and the supervisor's team has experience and excellent competence in this research. The problem of infertility is complex and socially significant. In this work, the PhD student investigated the influence of mutations in the NANOS1 p.[Pro34Thr; Ser78del] protein, identified in infertile male patients, at the early stage of development of human gametogenic cells (hPGCs). The team's previous research has shown that this variant impairs the interaction of NANOS1 with the DEAD-box helicase GEMIN3 and causes upregulation of some mRNAs encoding pro-apoptotic proteins. In addition, it influences proliferation, cell cycle progression, and apoptosis in the TCam-2 seminoma germ-cell-like model. The hypothesis developed on this basis assumes that these specific mutations cause infertility by impairing the NANOS1 RNP interactome during the specification and early differentiation of hPGCs. The specific objectives of the PhD thesis included: (1) Generation of a PGC differentiation model with temporally regulated expression of wild-type and variant NANOS1 protein, respectively. (2) A comprehensive global search for NANOS1-binding RNA targets in both wild-type and variant NANOS1 contexts using enhanced crosslinking and immunoprecipitation (eCLIP), including identification of specific NANOS1-RNA interaction

sites. (3) RNA sequencing (RNA-seq) analysis of the transcriptome in both cell lines, wild-type and variant, at different stages of hPGC specification to assess the altered RNA targets in a dynamic manner. (4) Validation assays of selected NANOS1-bound RNA targets differentially altered between wild-type and variant cell lines.

The choice of research topic and experimental model is well justified and supported by the team's previous results. Post-transcriptional gene regulation during germ cell development is little known, and the NANOS1 RNA interactome has not been explored in human PGCs. Therefore, the goals set by Ms. Matisa Alla are ambitious and innovative, and most of them have been fully achieved.

This very extensive doctoral thesis has a classical structure containing the following sections: an Abstract, Introduction (13 pages), Research aim and objectives, Materials and Methods (12 pages), Results (> 100 pages), Discussion (12 pages), Conclusions, Significance, Limitations, and References. I particularly appreciate the preparation of the Significance and Limitations chapters, which are not common in doctoral theses and demonstrate the ability to evaluate research findings critically. The thesis also includes a list of abbreviations and Supplementary tables and figures. The Introductory chapter covers all the important aspects discussed in the doctoral dissertation. It provides information about the development of the germ line and differences in germ cell specification between mice and humans. In the further part of the Introduction, the author briefly characterizes the models of human PGC-like cells and signaling pathways critical for their specification. Finally, the author presents information about NANOS proteins and their participation in male infertility. In summary, this chapter is an interesting and valuable introduction to the research topic and proves the PhD student’s excellent theoretical preparation for conducting research.

The next chapter, Materials and Methods contains information on culture conditions of human embryonic stem cells, detailed protocol of hESCs induction to precursor and hPGCLC stage, design and generation of plasmids used in the dissertation, insertion of a 3xFLAG tag using CRISPR-Cas9 system, protocols for cell sorting, immunofluorescence and imaging, karyotyping, protein and RNA analysis, etc. These descriptions are generally very detailed and well-written, which should allow for the reproduction of the experiments. The descriptions are accompanied by several tables and figures, which are helpful, especially in visualizing complex genetic constructs. The author indicated which experiments/analyses were performed in collaboration (e.g., bioinformatic analyses or karyotyping). It should be

noted that despite the vast number of experiments, most were performed independently by the PhD student.

Ms. Matisa Alla began her research by preparing cell models and developing a protocol for deriving PGC lines from hESCs. This required a series of optimizations and characterization of cells at different stages of differentiation. Using a cell line expressing the NANOS3-tdTomato reporter enabled the sorting of differentiated PGCs. Due to the lack of specific antibodies for NANOS1, it was necessary to edit the gene and generate cell lines expressing NANOS1-FLAG. Because the expression level of endogenous NANOS1 was low, cell lines with inducible expression of WT- and MUT-NANOS1 were established (the PiggyBac and Tet-ON systems were utilised). During this research stage, Ms. Matisa Alla had to solve several problems resulting from low editing efficiency, sensitivity of cells to antibiotic selection and sorting, and low differentiation potential. Finally, she developed a protocol based on FACS selection and 96-well cell seeding, which resulted in the generation of four stable (2 WT- and 2 MUT-NANOS1) cell lines capable of differentiating into PGCs. They were characterized in great detail regarding NANOS1 expression after dox induction, karyotype, expression of stem cell markers, etc. The author observed several cell line-specific effects.

In the next step, Ms. Matisa Alla analysed the interactome of WT- and MUT-NANOS1 proteins using the eCLIP assay conducted at the premesendoderm (pre-me) stage of cell differentiation. The validation of the eCLIP protocol preceded this experiment. Figure 29C presents signals from RNA bound to the WT NANOS1 following RNAse I digestion. Is the specific pattern (2 bands) the result of incomplete digestion? Was this verified experimentally? **Were the RNAse I-treatment conditions changed in the subsequent experiment?** The signal from RNA in Fig. 30B is different. Western blot analysis of immunoprecipitated NANOS1-FLAG demonstrated differences between WT and MUT cells. The level of immunoprecipitated NANOS1 was higher in the case of the MUT variant (in input too), and one of the two WT replicates exhibited a slightly fainter RNA smear (Fig. 30). **Was this later taken into account in the analysis of the results?**

The eCLIP experiment revealed that WT- and MUT-NANOS1 proteins show preferences for binding different RNA regions. WT-NANOS1 binding was predominantly localized to intronic regions, whereas MUT-NANOS1 binds also to 3′UTR and CDS. After subsequent normalization of the eCLIP data, this preference became irrelevant, and both

protein variants favored CDS and 3'UTR. I want to note that the detailed description of NANOS1 binding preferences for different mRNA regions before and after normalization was misleading (especially if these results led to different conclusions and were separated in the dissertation by several chapters). Perhaps it would be sufficient to present the results after normalization. **I kindly ask the doctoral candidate to comment on this matter during the defense**. Detailed analysis of binding sites revealed that MUT-NANOS1 binds to a significantly greater number of genes, which may indicate a loss of binding specificity due to the mutation. Gene expression analysis following MUT-NANOS1 overexpression showed a significant downregulation of several genes critical for pluripotency and germ cell specification. In contrast to WT-NANOS1, genes associated with somatic development were upregulated in MUT-NANOS1. Combined eCLIP and RNA-seq data showed that in the pre-me differentiation stage, WNT-pathway genes are selectively bound and repressed by MUT-NANOS1, impairing PGC competence. In PGCs, MUT-NANOS1 insufficiently represses WNT/TGF-β components, triggering aberrant WNT activation and shifting toward mesendoderm/mesoderm fate. In addition, MUT-NANOS1 binds to and suppresses OCT4 and NANOG during the pre-meiotic stage and sustains their reduced expression in PGCs. These transcription factors are essential for establishing and maintaining pluripotency during early embryogenesis. Similarly, MUT-NANOS1 also binds factors responsible for differentiation, including MYC, AXIN2, and β-catenin.

To sum up, Ms. Matisa Alla demonstrated that a double mutation in the NANOS1 gene p.[Pro34Thr; Ser78del] causes a change in the interactome of this protein, disrupting gene expression and signaling pathways during early human germ cell specification and development. These complex changes in the post-transcriptional regulation of gene expression likely cause the reduced number of PGCs in vitro and the absence of a germline in infertile male patients harboring the MUT-NANOS1 variant. During the defense, **please comment on the likely mechanism of target mRNA repression by MUT-NANOS1**. The MUT-NANOS1 mutations encompass the NIM domain, which is responsible for recruiting the CNOT deadenylation complex. Is it known how these mutations affect the recruitment of this complex?

Although the work was written with great care, I found minor shortcomings listed below:

- Page 27 (*refer to Błąd! Nie można odnaleźć źródła odwołania*).
- Page 27 "*The key components of the transposon plasmid are shown in S3*" (should be Fig. S3)
- Page 41 - shortcuts – "*incubation duration*" incubation in what?

- Fig 2A and B – The descriptions in the figure legends are reversed
- it is a pity that the WB signals in Fig. 16 were not quantified; the same comment for Fig. 5
- page 90 - imprecise wording, "*Western blot analysis of immunoprecipitated WT-NANOS1 cell line*" (immunoprecipitated protein, not cell line)
- page 113 – "*Meanwhile, MUT-NANOS1 p.[(Pro34Thr; delSer78)] variant exerts a more pronounced impact, potentially disrupting pathways essential for PGC specification (Fig. 41), patients (Kusz Zamelczyk et al., 2013).*"

Summary

The doctoral dissertation of Ms. Matisa Alla is a complete work, presenting theoretical knowledge in the discipline and an original solution to a scientific problem. The results of this PhD project contribute significantly to understanding the regulation of gene expression in the early stages of human germ cell development in the context of infertility. The research goals were ambitious and required outstanding commitment and enormous work. The selected research model, experimental controls, and methods used were appropriate. The PhD candidate thoroughly characterized the generated cell lines, used two replicates of the eCLIP experiment for each line, validated the RNA sequencing results using RT-qPCR (on two independently generated MUT-NANOS1 cell lines), and performed rescue experiments. The scope of methods used in the work and the number of results are impressive. The research results were discussed in a very mature manner, taking into account the study's significance and limitations. Without a doubt, the dissertation demonstrates the doctoral student's ability to conduct scientific work independently.

In conclusion, I hereby declare that the dissertation being the subject of the review fulfills the conditions laid down in Article 187 Act of July 20, 2018, The Law on Higher Education and Science (Journal of Laws 2024, item 1571, as amended), and I request the Scientific Council of the Institute of Human Genetics PAS in Poznan to admit Ms. Matisa Alla to the further stages of the proceedings for the awarding of the doctoral degree in the field of medical and health sciences, in the discipline of medical sciences. Considering the exceptionally high level of the work and the novelty and significance of the results presented in the dissertation, I propose to award it a distinction.

Marta Olejniczak 2025.07.10 13:50:40+02'00'

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