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Evaluation of the Doctoral Dissertation prepared by Matisa Alla, Msc, a PhD student at the Institute of Human Genetics Polish Academy of Sciences.

Title of the thesis:

"A Dominant Gain of Function NANOS1 Variant Disrupts WNT Signaling and Impairs Human Germ Cells Specification"

The dissertation presented for review was completed under the supervision of Prof. dr hab. Jadwiga Jaruzelska. The aim of the study was to investigate the double variant p.[Pro34Thr; Ser78del] of NANOS1 protein, which has been found in infertile man, and to find disrupted pathways critical for human germ cells specification. The PhD Candidate, Matisa Alla, hypothesized that this variant causes infertility by impairing the NANOS1 ribonucleoprotein (RNP) interactome. Four specific objectives of the project were clearly defined, all of which are closely aligned with the project's primary goal.

The thesis's structure is classical and it is written in proper language. However, although the dissertation was prepared very thoughtfully and with great attention to detail, the PhD candidate did not manage to avoid some minor editorial issues. Gene symbols are not written in italics, which is the standard and widely accepted practice. Also, the labels on some of the figures are almost impossible to read unless they are magnified several times. In addition, in some cases, the text describing results was not fully reflecting what was on the figure. This mostly concerns description of results of assessment of differentiation efficiency of cell lines after antibiotic-based selection of clones. For example, on the page 50 it is stated: "At 6 h pre-me, the pluripotency markers KLF4, OCT4, and MYC were higher than in stem cells, as expected, while on the contrary NANOG expression decreased, albeit not significantly (Fig. 7B)." However, according to figure 7B expression of *MYC* at 6 pre-me was lower than in stem cells and in the case of *OCT4* there was no

significant difference between stem cells and 6h pre-me. Moreover, descriptions of figures 2A and 2C are switched. The agarose gel is on Figure 2B not 2A, and RT-qPCR results are presented on Figure 2A not 2B.

The first task of the project was to generate the PCG differentiation model with temporally regulated expression of wild-type and variant NANOS1 proteins. This was successfully achieved by differentiating the W15 human embryonic stem cell (hESC) line, which is endowed with a NANOS3-tdTomato fluorescent marker, into mesendodermal precursors (pre-me). However, generating stable edited cell lines after cloning PiggyBac plasmids and inserting a 3x-FLAG tag appeared to be problematic. The first approach, which was based on antibiotic selection, proved ineffective, so a second approach based on fluorescence-activated cell sorting had to be employed. The second approach was successful and the PhD candidate's determination to obtain stable and reliable cell lines and her diligence in testing the effectiveness of the methods used should be recognized here.

I have two questions regarding this part. The first concerns the method used to estimate the expression of pluripotency and primitive streak markers. In the first approach, RT-qPCR was used; in the second, fluorescence was used as the basis for estimation. I would like the PhD candidate to explain the rationale behind this difference in method selection. Also, five pluripotency markers and three primitive streak markers were used in the first approach. However, the second approach did not include two pluripotency markers (KLF4 and MYC) and one primitive streak marker (MIXL). Was there a particular reason for this, or was it simply a matter of practicality and reducing the time required for the experiment?

The first objective was to prepare the cell lines for the main experiments. The second objective focused on searching for NANOS1-binding RNA targets using the enhanced crosslinking and immunoprecipitation (eCLIP) method. The experiments performed led the PhD candidate to conclusion that the p.[Pro34Thr; Ser78del] variant induces a functional shift in NANOS1, with the MUT-NANOS1 protein predominantly losing the intronic-binding specificity of the WT-NANOS1 protein. This resulted in a broader, less selective RNA-binding profile. However, the conclusions drawn from the comparison between WT-NANOS1 and MUT-NANOS1 were based on unnormalized data. Although normalization should have been performed at this stage, it was not carried out until the eCLIP and RNA-seq results were combined. The lack of normalization led to an incorrect conclusion about the dominant intronic-binding specificity of WT-NANOS1. As this could have happened during the research process, it should have been corrected when preparing the thesis.

Following the eCLIP experiment, RNA sequencing was carried out. Information about the sequencing technology used was not included in the methods description. I assume it was Illumina,

and I would like to ask whether using a different technology, such as long-read sequencing, could potentially affect the results of the analysis, and if so, how?

Following the initial analysis of the RNA-seq data, the results were combined with the eCLIP data. This was followed by a very detailed gene set enrichment analysis. This analysis revealed that the p.[Pro34Thr; Ser78del] variant exhibits a premature repressive gain-of-function effect, downregulating canonical Wnt signaling in pre-me cells. Several additional analyses identified WNT pathway components as targets of MUT-NANOS1. As part of the fourth objective, an in-depth exploration of these targets was undertaken to further validate their roles. Several methods, approaches and combinations were employed, including NANOS1 overexpression experiments. The results led to the final conclusion that MUT-NANOS1 disrupts the RNA interactome. This subsequently impairs PGC competence by downregulating canonical WNT signaling in pre-me cells. In PGCs, MUT-NANOS1 insufficiently represses WNT/TGF-β components, triggering aberrant WNT activation and a shift towards mesendoderm/mesoderm fate. This altogether provides an explanation for the absence of a germline in infertile male patients harboring the MUT-NANOS1 variant.

It should be emphasized here that the doctoral student approached her results very critically and analyzed them thoroughly, as exemplified by such detailed checking of the derived cell lines. She showed great insight and awareness of both the limitations of the methods used, which can affect cellular processes in unforeseen ways, and the possible certain randomness of some results. She made the limitations of her research very clear by pointing out the need for further experiments.

In summary, the doctoral dissertation presented is of a very high quality and provides some fascinating results. It makes a significant contribution to our understanding of germ cell development processes and provides a strong scientific basis for explaining the infertility of carriers of the p.[Pro34Thr; Ser78del] variant. The volume of work, the quality of the research and the number of tests and results obtained are truly impressive. Matisa Alla has done an excellent job. She has also demonstrated that she possesses the necessary knowledge and skills and is prepared to take on substantial research projects. Although she made some editorial errors, these do not affect the overall quality of the thesis in any way.

In my opinion, the dissertation meets the conditions set out in Art. 187 of the Act of 20 July 2018 on Higher Education and Science (Journal of Laws 2024, item 1571) and therefore recommend admitting Matisa Alla to the next stage of the proceedings. Considering the quality of the research and the significance of the results presented, I propose that the dissertation be awarded.