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Review of the doctoral dissertation
Title: **Cell lineage tracing in zebrafish heart development**
Author: **mgr inż. Eugeniusz Tralle**

Dear Faculty of Medical Sciences,

The doctoral dissertation submitted for evaluation was prepared in the Laboratory of Zebrafish Developmental Genomics at the International Institute of Molecular and Cell Biology under the supervision of Dr hab. Cecilia Winata. The thesis document comprises a summary in Polish and English, a broad introduction to the topic, a detailed scientific report of 2 unpublished projects with 24 figures, and a list of publications.

The candidate used the zebrafish model organism to investigate a heterogeneity of cardiac cell lineages. The vertebrate heart consists of cells from the early-differentiating first heart field (FHF) and the later-differentiating second heart field (SHF). While in mammals, this subdivision of progenitors provides the basis for ventricle septation, the contribution of two cell lineages to the zebrafish heart, which has only one ventricle, remain incompletely understood. Thus, the subject of this dissertation is highly relevant for our understanding of the evolutionary basis for the commitment of distinct cell lineages in the vertebrate heart.

In the first chapter of the doctoral dissertation, the candidate has written an informative introduction with an interesting review of the literature. Firstly, convincing arguments are formulated why the zebrafish is a suitable model organism for studying organogenesis. Indeed, heart morphogenesis occurs within a few days post-fertilization, and the developmental process can be traced at the cellular level. Next, the concept of heart compartmentalization is explained and compared between fish and tetrapods, providing a broad evolutionary context of the project. The focus was given to the heterogeneity of cardiac progenitor cells and master transcription factors of the FHF and SHF. A short section explains a suitability of scRNAseq technology and CRISPR/Cas9-based barcoding for studying cell heterogeneity with the goal to refine the categorization of cell subtypes. Finally, two main questions of the doctoral work are formulated in

relation to the knowledge gaps: What is the mechanism regulating *isl1a* expression in the descendants of the SHF? How is the cardiogenic program regulated by the Nkx2-5 and Isl1 genes? To these aims, the candidate conducted experimental work that is reported in two subsequent chapters. In my opinion, the introductory part is very well structured, written in an interesting manner, and it clearly integrates the scientific objectives.

The first project (Chapter 2) concerns the identification and validation of heart-specific *isl1a* enhancers. It starts with the materials and methods, describing the conditions of zebrafish husbandry, computational prediction of regulatory domains of the *isl1a* gene, cloning of seven putative enhancers, and all the steps for generating of GFP-tagged transgenic reporter lines. The next section presents the results of this experimental approach. Statistical graphs convincingly display various parameters during transgenesis, including survival rates of injected and control embryos, and a percentage of GFP-expressing embryos. Among seven reporter lines, only one *Tg(I3-e1b:EGFP)* was able to drive GFP expression in the embryonic heart. Importantly, crossing this reporter with *Tg(myl7:mRFP),* which is a myocardial reporter, allowed a precise mapping the enhancer activity to the atrioventricular canal and the inflow tract to the atrium in the developing heart. This result is very interesting because these tissues are thought to partially originate from the SHF. To isolate *I3-e1b:EGFP* expressing cells for gene expression analysis, the FACS approach was applied. Several cardiac and non-cardiac marker genes were assessed by RT-qPCR. These results suggested that *Tg(I3-e1b:EGFP)* includes SHF descendant cells, although a contribution of other cell types cannot be evidently excluded. I would have a question about *I3-e1b:EGFP* expression in other embryonic tissues beyond the heart. Finally, bioinformatic analysis of the *I3* sequence revealed putative binding sites for cardiac transcription factors, such as MEF2C, which is an evolutionary conserved regulator of cardiomyocytes. In conclusion, the newly identified enhancer may contribute to monitoring progenitor cell heterogeneity of the zebrafish heart, providing a valuable tool for future studies. The chapter is closed with a critical discussion of the obtained data and interesting ideas for follow-up experiments.

The second project (Chapter 3) aims to understand the role of *nkx2.5/nkx2.7* through single-cell RNAseq analysis of cardiac progenitors. At the beginning, the candidate validated that *nkx2.5/nkx2.7* morpholino-mediated knockdown recapitulates the expected mutant phenotype. After performing a control pilot experiment, sorting of cardiac progenitors for RNAseq experiment was done using unmodified and morphant *Tg(nkx2.5:EGFP)* embryos. This experiment turnout out to be particularly challenging, as the number of recovered cells was below thresholds to establish significant conclusions. The obtained results, however, provide preliminary indications about the heterogeneity of cardiac progenitors. In parallel to these analyses, the candidate established the scGESTALT system for lineage tracing. This is a very recent method enabling cell monitoring through Cas9 editing of a synthetic barcode sequence incorporated in a transgene. In combination with the *myl7:EGFP* reporter, this system could provide a valuable tool to determine the fate of cardiac subpopulations. The chapter is closed with a detailed discussion and specific perspectives for future work.

The fourth chapter provides a comprehensive summary of two projects in the context of the current knowledge. This chapter is very interesting, indicating the complexity of cardiogenesis in vertebrates. The references include 131 citations, suggesting that the candidate has a solid knowledge of the relevant literature in the field of research.

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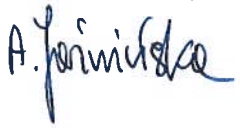
The last chapter provides a list of publications. The candidate is a co-first author of two original research articles that have been published in prestigious journals, namely *BMC Genomics* and *Nature Communications*. These scientific achievements are in accordance with the highest international standards for PhD candidates.

Taken together, the doctoral dissertation is very well written, and the structure of the work is appropriate. The experimental part includes many original ideas and top-notch recent techniques in molecular biology. This dissertation and the publications demonstrate that the candidate has a solid knowledge of the cardiac developmental biology. I am convinced that this work provides contributions to move forward the field of cardiogenesis in zebrafish. In conclusion, it is my pleasure to recommend this good dissertation written by Mr. Eugeniusz Tralle to be accepted for the degree of Doctor of Sciences at the Medical Faculty at the University of Warsaw.

Yours sincerely,



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