UNIVERSITY OF WARSAW
FACULTY OF BIOLOGY
INSTUTUTE OF ZOOLOGY
DEPARTMENT OF CYTOLOGY
Prof. Maria Anna Ciemerych-Litwinienko
Head of the Department of Cytology
Vice dean of the Faculty of Biology

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Referee's report on PhD thesis entitled
"The role of microRNA-378a in skeletal muscle differentiation, angiogenesis and hind limb ishemia in mice"
by
Bart Krist

The doctoral dissertation by Bart Krist resulted from the project conducted under the supervision of prof. Jozef Dulak and dr. Urszula Florczyk-Soluch at the Department of Medical Biotechnology, Faculty od Biochemistry, Biophysics and Biotechnology, Jagiellonian University.

Bart Krist's PhD thesis presents the results of multiple, well planned experiments testing the role of miR-378a in the processes occurring during the skeletal muscle regeneration and also angiogenesis. The choice of the study object, i.e. miR-378a, based on the multiple lines of evidence showing that this particular microRNA could be involved in the skeletal muscle function. Available data on the miR-378a missed many crucial information on its precise function. Thus, the Author decided to test the hypothesis stating that miR-378a may be involved in the myogenic differentiation and generation of new blood vessels in ischemic skeletal muscles.

The novelty of the proposed study is unquestionable. However, the expectations that its results might serve as a ground for the development of new therapeutic approaches were not fully reached. The role of miR-378a in the processes studied appeared to be not as central as expected.

Bart Krist realized all of the aims of the study. As a result, his PhD thesis presents the description of numerous, well-planned, and logically designed experiments allowing the precise examination of the role of miR-378a in myogenic differentiation and neovascularization. To do so Bart took an advantage of multiple molecular and cell biology tools and experimental approaches, such as: in vitro culture of cells (e.g. myoblasts, endothelium) and tissue explants; in vitro models allowing to study formation of blood vessels; in vivo models of ischemic muscle injury, histological analyses of skeletal muscles, immunolocalization of selected antigens, extensive flow cytometry analyses, sqRT-PCR. Next, he successfully overexpressed miR-378a and also inhibited its functions in in vitro

Ilji Miecznikowa 1, room 216C, 02-096 Warszawa
tel. 22 55 42 216, fax: 22 55 42 202
e-mail: ciemerych@biol.uw.edu.pl

cultured cells and also analysed the phenotype of animals lacking miR-378a expression. These tools and materials allowed Bart Krist to perform widespread analyses of miR-378a function.

Dissertation starts with a very good introduction that gives the reader the appropriate background – introducing the pathological conditions affecting blood vessels and muscle function in that miR-378a was shown or predicted to play a role. Also the microRNAs are introduced and very nicely described. The beauty of the ***Introduction*** lies in its clarity and also brevity. The reader gets information sufficient to appreciate the experiments presented within the thesis – information which is just sufficient and not overwhelming. Plus, the ***Introduction*** is supplemented with very good schemes and one helpful table.

Materials and Methods section is well presented. It contains all info necessary to repeat the experiments and also good schematic diagrams of some of the procedures.

First part of the ***Results*** describes the analyses documenting the expression of miR-378a in various cell lines and tissues and confirming that this microRNA is characteristic for the skeletal muscle. Next, the search for the role of miR-378a in skeletal muscle is presented. The Author aimed to show that in the absence of this factor in vitro differentiation of myoblasts will be affected. It was not. Neither in the cells cultured in normoxia nor under the hypoxic conditions. Various in vitro and in vivo analyses, involving silencing or overexpression of miR-378a, proved that this particular microRNA is dispensable for the myogenesis, arguing with the published data. The body of the evidence collected by Bart Krist supporting this notion is really immense, but in his opinion still not sufficient to completely exclude the role miR-378a in muscle regeneration. This is nicely argued in ***Discussion***. Thus, additional experiments allowing to verify the role miR-378a were proposed by the Author.

Fortunately, many "does not" describing the involvement of miR-378a in studied processes did not prevent the Author from further investigations. As a result Bart discovered that myoblasts lacking miR-378a fail to properly stimulate endothelial cells to form new capillaries. Next, lack of this factor decreased the endothelial sprouting from aortal rings, however, the effect was not as spectacular as in case of myoblasts-endothelium interactions. Additional, very detailed analyses of skeletal muscles damaged as a result of experimental ischemia, proved that miR-378a plays a role in the quality of inflammatory response accompanying regeneration. In the absence of analysed microRNA the proportions of some types of immune cells infiltrating the muscle (e.g. granulocytes, macrophages M2) were different, as compared to control. Also some inflammatory markers (TNF-a) were downregulated in such muscles. Thus, Bart Krist provided the multiple evidence questioning the role of miR-378a in the myoblasts differentiation and skeletal muscle

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Ilji Miecznikowa 1, room 216C, 02-096 Warszawa
tel. 22 55 42 216, fax: 22 55 42 202
e-mail: ciemerych@biol.uw.edu.pl

regeneration. At the same time he showed that this microRNA might be important for the proper function of myoblasts as stimulators of the formation of new blood vessels. However, at least in muscles, which damage was induced by ischemia, miR-378a was dispensable for the regeneration. Summarizing, obtained results bring the new and important information on the role of miR-378a in skeletal muscles.

Facing such detailed analyses presented in such clear manner I feel helpless in the search for anything what would be worth to add to this study. Reading the ***Results*** I had some suggestions to Bart. Unfortunately, he included my ideas into the ***Discussion***. I also find nothing truly in the need for my criticism.

In the ***Discussion*** Bart Krist critically analysed the results of the study. Especially interesting is the part in which the Author analysed the possible causes of differences between results of experiments he conducted and those ones presented by other research groups. He also proposes additional experiments crucial to better understand the role of miR-378a in skeletal muscle. ***Discussion*** proves that Bart is mature scientist able to perfectly analyse results of his study and also to digest the huge amount of available data and present it in comprehensive but concise manner.

From the editorial point of view presented PhD thesis is an example of the dissertation almost perfect. It is well written, illustrated with good quality photos and graphs. It contains appropriate schemes making the text easy to absorb by the reader. "Almost" referrers to the mistake in the reference to one of the procedures (p. 46) and to one unfinished sentence (p. 76). Truly, this is nothing.

Summarizing, Bart Krist successfully completed the PhD project. He presented well documented results which characterized the miR-378a in skeletal muscles. He uncovered not known functions of this microRNA in myoblasts impacting at the capillary formation and also in the modulating the immune response in the regenerating muscle. He proved to be well trained scientist, able to independently formulate hypotheses and to test them. He also learned wide range of molecular and cell biology techniques. **To conclude, in my opinion the PhD Thesis by Bart Krist meets all the formal requirements of the Degree of Doctor of Philosophy in Biological Sciences in accordance with Polish law. Thus, I do support Bart Krist's application and ask the Council of the Faculty of Biochemistry, Biophysics and Biotechnology to grant him such degree.**

M. A. Ciemerych

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tel. 22 55 42 216, fax: 22 55 42 202
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