Review of the PhD thesis written by Bart Krist and entitled: 'The role of microRNA-378a in skeletal muscle differentiation, angiogenesis and hind limb ischemia in mice'.

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The thesis was written based on research performed to investigate the role of the microRNA miR-378a in muscle differentiation and limb ischemia, in order to address the specific aims and research questions listed in the thesis on page 38. The research was performed in the research groups of Prof Jozef Dulak and Dr. Urszula Florczyk-Soluch at the department of medical Biotechnology (Jagiellonian University, Krakow, Poland) and addressed the study of muscle differentiation, vascular biology/angiogenesis and inflammation biology. The main findings of the presented work were (i) that miRNA-378a is overexpressed in myoblast cells, (ii) that overexpression of miRNA-378a in myeloblast cells does not influence the differentiation of these cells, (iii) that regulation of miRNA-378a does not influence the skeletal muscle cell differentiation or regeneration after hind limb ischaemia, (iv) that myoblast cells with suppressed expression of miRNA-378a have a decreased angiogenic potential in cultured endothelial cells and ex-vivo models, (v) that miRNA-378a has an anti-inflammatory role in ischaemic muscle.

Personal opinion
The PhD project was aimed at a very important subject being the biology of ischemic diseases. In-depth understanding of the intricate molecular, biochemical, molecular and cellular organization of this pathology is a prerequisite for development of therapeutic strategies for this disease. The work was approached in a straightforward way and dissected the problem to different levels of investigation. This approach was logical and effective, started with molecular/cellular work and progressed into in vivo models in mice. The candidate followed a strategic and sequential way trying to answer the most important research questions. The work was performed meticulously and the thesis that derived from it is therefore very well readable and extremely well put together in order to understand the flow of the work performed.
In many cases the outcome of the research indicated an absence of any role for miR-378a in the used assays. A good feature of the research performed was that the
excellent way of addressing the research questions allowed these conclusions. A drawback of such a situation is that results are more difficult to publish. I assume that this is the reason that no research papers on this work were published yet at this moment. This is, however, in no way a reason to deprecate this work. On the contrary, excluding a role for a miRNA can be as important as being able to assign one to it. Fortunately, there are enough interesting data resulting from this research to expect a number of sound research papers being published in the near future.

The thesis is well put together in terms of general structure. However, the text itself suffers a bit from grammatical and typographical issues, which is probably due to insufficient proofreading by colleagues. To give one example, a piece of text seems to be missing at page 76, line 7.

In general, I am impressed by the amount of work that was done and I certainly feel that the PhD thesis is of sufficient quality for proceeding to an official defense of the PhD thesis.

Comments to the work of the thesis

Relationhip miRNA-378a and PGC1b.
An issue that comes up a few times in the thesis is that miRNA-378a is embedded in the first intron of the gene ppargc1b (PGC1b). This gene is explained to be regulating metabolism of mitochondria and fatty acid and glucose metabolism. These processes seem to be extremely fundamental. Also, it has been described that the gene and its embedded miRNA are co-regulated. A general question that arises is about the relationship between a miRNA and the gene the miRNA is embedded in. A relationship is suggested, but is this a commonality among miRNAs and what is the situation for miRNA-378a and PGC1b?

In figure 28 (A and B) it is shown that both miRNA-378a and miRNA-378b are regulated negatively during response to induction of hind limb muscle ischemia. Both miRNAs are similarly and negatively regulated by day 3 and 7. The downregulation of both miRNAs gets restored to the level of the normal levels of untreated tissue over a period of 21 days. In this situation the expression of PGC1b is differently regulated, i.e. normal levels are reached again already after 7 days. It is possible to make a model of how this can be the case? Or is the relationship between the expression of the gene and the embedded miRNA not so exact?

Cell line(s).
The initial in vitro work makes abundant use of the myoblast cell line C2C12. Although this cell line is key to the muscle (and ischaemia) related work of this thesis, these
results would have been good to see repeated in more muscle-like cell lines. Why was only one cell line used? Confirmation in more muscle like cells would have contributed to the convincingness of the current work.

Relative expression in myoblast cell line versus endothelial cells.
Early results show the overexpression of miRNA-378a in myoblast cells and the relative low expression in endothelial cells. The suggestion is posed (page 57, last sentence) that miRNA-378 has a minor role in endothelial cells because of the relatively low expression in these cells. The question how relative the expression is. The endothelial expression might still not be low in absolute terms. This might be important for its role in angiogenesis and cancer (see later).
This issue is especially important since effects in endothelial cells are quite convincing. In fact, the already low expression gives convincing results when further suppressed. This is observed in both in sprouting assays (matrigel and beads assay) as well as aortic ring assay.

Effects on endothelial cells and angiogenesis.
Micro- vs. macro vascular endothelial cells. It is concluded that there is a positive effect of miRNA-378a on angiogenesis. This effect is seen in in vitro assays (2 different ones) and in aortic ring assays. The fact that the result can not be seen in choroid tissues is explained by the fact that there must be a difference in micro- versus macro vascular endothelial cells. Why did the candidate not test microvascular HMEC cells in the in vitro assays to confirm this more solidly?
An alternative explanation is the non-robustness of the choroid assay, as the difficulty of preparing comparable tissue preparations from the eye tissue may play a role here. Did the candidate consider this to be the case? A whole page of discussion was spend on the issue of macro- vs. microvascular endothelial cells, but this alternative view was not discussed. But it may be quite important for the interpretation of the data, as a lack of effects in microvascular endothelial cells would devaluate the work for translation into e.g. cancer related investigation.

Silencing of miRNA-378a.
It is unclear what is meant with outward expression of the miRNA (page 62, line 9). Also, how can a gene be regulated by a miRNA at the protein level, but not at the mRNA level (page 62, bottom line)?

Effects vs trends.
At several locations in the thesis conclusions are drawn based on non-significant data or trends. E.g. in cases where the p value reaches almost the 0.05 level. This is
understandable, because in such cases it can be argued that a few more measurements will make the difference (e.g. the aortic sprouting, Figure 28 page 73). However, in some cases large differences are considered unimportant, while at 300% difference was observed. E.g. this is the case for the qPCR data on regulation of the target gene IGFR1 (Fig 17 page 63). It would have been expected to add discussion on these issues.
A similar situation is the case for the effect of miRNA-378a in reperfusion in response to hind limb ischemia (Figure 30A). Here a miRNA-378a mediated effect was denied to be the case. But in fact, a change in blood flow of 0.18 in a gross 0.45 increase of blood flow, going from control (day 0) levels to day 7 after induction of hind limb muscle ischemia. It is calculated like this a clear 40% increase in the blood flow. It would be nice if the candidate can elaborate on this alternative view on the data.

**Effect of miRNA-378a on inflammatory features.**
One interesting observation of the research presented in this PhD thesis is the effect on inflammatory cells. While blood levels of leukocyte subsets didn't differ, the number of immune cells in the ischaemic tissues was decreased after administration of AAV-miRNA-378a, mainly seen at the level of the macrophages. Why was foxP3 staining not used for detection of regulatory T cells? The mere definition of CD25 positivity selects the group of activated T cells in general. Activated CD4 cells do not necessarily have to be regulatory T lymphocytes.

Was it expected to observe a decrease in the number of leukocytes? And why?

**General issues to be discussed.**
In many experiments described in the thesis negative results were obtained. It can be discussed whether strong effects are expected when regulating the expression of a single miRNA. So the question might come up how many miRNAs have been described for which results have been convincingly strong in similar assays and models. In this regard it might have been beneficial to take along other miRNAs as control conditions, instead of only scrambled versions of the miRNA. Alternatively, other miRNAs may be known to have strong or convincing effects in muscle cell differentiation, to which the miRNA-378a effects could have been compared.

Having observed a pro-angiogenic and an anti-inflammatory profile of the effect of miRNA-378a, how does this relate to a possible therapeutic approach for ischaemic disease? If a therapeutic approach based on intervention at the level of miRNA-378a can be developed, how would the application be developed for treatment of cancer?
In general, delivery of miRNAs, or antagonirs, is a big subject of discussion for the development of therapeutics. How does the candidate think this issue would be solved?

Recommendation
I have certainly enjoyed reading the PhD thesis of Bart Krist. A big scientific effort was done to address the specific research questions posed. The work was well performed and described well in the thesis. I am convinced of the fact that the work is of sufficient quality for obtaining the PhD degree. It is for this reason that, based on my evaluation, I recommend the thesis for further procedures to the Council of the Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University in Krakow, and proceed with the process of providing Bart Krist with the degree of PhD.

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