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EVALUATION

of the Doctoral Dissertation prepared by Sara Mikac, MSc, Eng,

a PhD student at the Intercollegiate Faculty of Biotechnology

University of Gdańsk and Medical University of Gdańsk

Title of thesis:

"Identification of a novel, transcription-independent

role of Nrf2 in lung cells"

NRF2 is a transcription factor that controls the expression of hundreds of genes. NRF2/KEAP1 system has been recognized as a master orchestrator of cellular stress response and a critical defense mechanism. This field does not raise much controversy, and research data are relatively consistent. However, the reality is that some commonly accepted beliefs are oversimplification. Some experimentally important nuances may affect data interpretation and the awareness of such out-of-the-box mechanisms may broaden opportunities for modulation of the NRF2/KEAP1 system in translational research. Doctoral thesis by Ms. Sara Mikac is devoted to such overlooked issues. This is a very interesting choice of topic.

NRF2 is encoded by the NFE2L2 gene. NCBI reports that human NRF2 mRNA has 8 transcript variants coding for 6 different NRF2 isoforms, 505 to 605 amino acids long. According to the Ensembl database, there are 14 human NRF2 transcript variants, coding 12 NRF2 isoforms. However, not all transcript variants seem to really exist in cells. Two of them dominate in the level of expression over the others, both in basal and stress conditions. The identified ENST00000397062 and ENST00000397063 variants correspond to the NM_006164.5 and NM_001145412.3 sequences in the NCBI database. Interestingly, it is known that these variants use alternative first exons. Up to date, the shorter form of the protein has not been characterized. The research described in the doctoral dissertation of Ms. Sara Mikac started to fill this gap.

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Ms. Sara Mikac focused on the role of NRF2 in regulation of MHC-I expression and on the characterization of the shorter form of NRF2 in the lung cells. In the first part of the study, she investigated how activation of NRF2 affects MHC-I mRNA and protein levels. In the second part she analyzed the structure, expression and stability of the shorter form of NRF2 protein, named ΔN-NRF2. Her work, performed under supervision of Prof. Robin Fahraeus and Dr. Alicja Sznarkowska, is a direct continuation and logical follow-up of previous studies carried out and published or prepared for publication by the team.

In my opinion, the most interesting results obtained by the PhD Candidate are: i) Demonstration that NRF2 upregulates MHC-I protein level acting not as a transcription factor, but through the direct interaction and stabilization of MHC-I protein. ii) Characterization of ΔN-NRF2 as a protein lacking the first 16 amino acids, with impaired binding capacity to KEAP1, and thus not regulated by the KEAP1-dependent ubiquitination.

Implementation of the project required from the Candidate the application of siRNAs to inhibit the expression of genes of interest in cultured cells, as well as analysis of gene expression at mRNA and protein levels using qRT-PCR, western-blotting, immunocytochemistry and flow cytometry. Interactions between proteins were assessed using co-immunoprecipitations and proximity ligation assays. Detection of newly synthesized proteins was done using labelling with L-azidohomoalanine. The panel of methods is rather standard, but well-chosen to achieve the aims of the project. It is also a very good idea to re-use open science databases and the results of transcriptome analyzes to supplement the obtained results.

Importantly, help offered by the collaborators, Dr. Monikaben Padariya and Dr. Umesh Kalathiya is clearly indicated in the thesis. It also is worth emphasizing that the research was designed to allow for a mechanistic approach and experimental verification of the observed correlations.

Experiments were carried out by the Candidate using several types of primary cells isolated from the human lungs and on the wild type or modified human lung cell lines. Some modifications were done earlier (as clearly

indicated in the thesis), using the CRISPR/Cas9 technology. Others, mainly knockdown of genes using siRNA, were performed by the Candidate.

Dissertation by Sara Mikac consists of 117 pages, with 52 figures or supplemental figures and 7 tables or supplemental tables. The list of references contains 225 items. The thesis is not classically composed. I does consist of Abstract and Polish Streszczenie, Introduction, and Material and Methods, but then the Results are divided into two relatively independent parts: Part 1 describes studies on MHC-I regulation, whereas Part 2 deals with the characterization of ΔN-NRF2 protein. Both parts contain their own discussions.

I am not convinced of such a Dissertation structure as it leads to many repetitions in the text. A characteristic feature of this thesis, also inconsistent with the canon, is the inclusion of long paragraphs in the Results sections describing the justification of the research and the methodology of the experiments. Brief introductions are usually helpful, but in this case the proportions of the text are disturbed. However, this is a subjective editorial opinion, not a substantive comment.

The Dissertation ends with a joint Conclusion section, followed by Bibliography and Appendices. Whole text is supplemented by Contents, List of abbreviations, List of figures, List of tables, List of publications coauthored by the Candidate, Acknowledgments and Funding information. They are well prepared and facilitate reading of the thesis.

The Summary is short, condensed and correctly written. It would be more intriguing if it clearly indicated the gap in knowledge that the research is to fill. Nevertheless, the Summary properly introduces the topic, provides sufficient information on the methodology, and clearly summarizes the most important results. There is one mistake in the Polish "Streszczenie" – the constitutively active NRF2 in cancer cells promotes "chemooporność" (chemoresistance) and not "chemowrażliwość" (chemosensitivity).

The Introduction (consisting of 19 pages) is a well written broad overview, describing NRF2 as a transcription factor, and its role in maintaining of cellular homeostasis. The Author focuses on the regulation of NRF2 activity, with special emphasis on KEAP1 function but also with reference to KEAP1-

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independent pathways. A separate section is devoted to the dual role of NRF2 in cancers and in the immune response. The text is interesting, easy to follow and nicely introduces the readers to the Dissertation topic. It reflects a comprehensive knowledge of the Author, and very good understanding of the NRF2 regulation and function. The Introduction is illustrated with clear and well-prepared schemes. Particular subchapters create a cohesive and logical content. I only have one comment:

On page 15, the Author states that in response to oxidative stress, "Nrf2 is liberated from Keap1-mediated degradation, resulting in its translocation to the nucleus and activation of the transcription of its target genes". This statement should be more precise, as it directly concerns the topic of the research and can be misleading to the reader. Indeed, it is widely accepted that KEAP1 sequesters and represses NRF2 in the cytoplasm, thus impeding its translocation to the nucleus. In many publications NRF2 activation is described as translocation of molecules released from the KEAP1 complex. However, more detailed analysis indicates that, although NRF2 inducers cause conformational changes in KEAP1, NRF2 remains trapped due to the blockage of the complex in a close conformation. The ubiquitination machinery cannot dissociate but remains misaligned with NRF2 lysine residues, and the ubiquitination is not feasible. Thus, only *de-novo* synthesized NRF2 can enter the nucleus. In fact, the Author refers to this model in later parts of the Introduction, but in some places of the text (e.g. page 96 in the Discussion section) the description is not clear.

Materials and Methods used in the study are rather shortly described (on 9 pages), but generally the description is sufficient to understand the research strategies and technical approaches. Nevertheless, details of the procedures are not provided, and referring to the kit manufacturers' protocols is not always sufficient. This, however, is not a significant flaw.

The Results (25 pages for Part 1 and 22 pages for Part 2) are described in details and illustrated with well prepared graphs. Figure legends are understandable and detailed, except Fig. 13. Attempt to present the data in the way which underlies the logical connections between subsequent stages of work should be appreciated, although interpretation of experiments is not

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always straightforward. Importantly, the Author points out and warns the reader that the technical quality of some of the results was substandard.

Generally, the analyses are complementary and mutually supporting. It is a very good idea, for example, to compare the effect of silencing NRF2 expression using siRNA and the inhibition of its transcriptional activity after ML385 administration. Technical quality of most western blots is good, and quantitative analyses are reproducible, as illustrated by low standard deviations in experimental repetitions. Some conclusions, e.g. that the increase in the HLA-A RNA could represent a feedback loop aiming to increase HLA-A expression are too speculative, at least for the Results section and should be experimentally verified. A have a few comments to this part:

- In Figure 1B statistical significance is not indicated, while labels in the graphs presented in Figure 1C and D are not obvious: effects of siKEAP1 (1C) or siKEAP1 and siNRF2 (1D) appear to be significant, whereas the difference between control and scrRNA (1D) is rather marginal. The application of the t-test here is not optimal – ANOVA would be more correct (e.g. to compare control, siRNA and siNrf2/siKEAP1).

- What could be the reason for the complete absence of NRF2 band in some western blots of A549 KO (Fig. 10A) or its detectable levels in others (e.g. Fig. 11), whereas loading control is similar? Are there any technical differences (longer exposure time?) or it is just a biological variance?

- The conclusions from Figure 18 and Figure 19A are not convincing. How did the Author assess the colocalization of HLA-A or HLA-C with NRF2? Based on what criteria?

- Figure 28 would be more informative if standard errors were added. Overall, histograms showing the negative controls used in FACS analysis would be a better way of presenting the results.

- Figure 43: what could be the reason for the multiple bands detected with anti-Keap1 antibodies?

The Discussion is very short (6 pages), and divided into two independent parts placed after two parts of the Results. It has a rather narrow and technical scope, and mainly provides just an extended summary of the experiments that repeats the descriptions presented already in the Result

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section. In particular, the reference to the role of KEAP1 is missing in the Discussion. Is the availability of KEAP1 higher in cells with the dominant form of ΔN-Nrf2? If yes – what can the consequences for the cell?

Some statements in the Discussion are too strong. For example, the Author mention (page 69) that the presented results are the first example of regulatory role of NRF2 independent of its transcription factor activity. However, the effects of transcriptionally inactive NRF2 – e.g., on proteostasis or protein nitrosation – have been described, although they were based on another mechanism. Probably one of the reasons of such a limited form of this part of the Dissertation is the inclusion of elements typical for discussion in the Results sections.

The Discussions ends with final Conclusions, containing the Highlights followed by Future perspectives. This section summarizes the results again. Nevertheless, the proposed experiments with the use of overexpression of specific NRF2 isoforms, especially if they were performed on cells lacking the endogenous form of the protein and enriched with functional assays, are very interesting and worth implementing.

In editorial terms, the Dissertation is prepared correctly. It only contains a few typos or minor editorial errors. The thesis is written in English, in a communicative way. Nevertheless, the grammatical errors are quite numerous. However, they do not significantly affect the overall quality of the text.

To sum-up, research described by the PhD Candidate is a valuable study. It provides new and important information shedding new light on the seemingly well-known pathways of the cell's stress response. In my opinion, the Candidate, Ms. Sara Mikac, MSc, Eng, has achieved the aims of the planned research, and her Dissertation meets all criteria of doctoral thesis. Therefore, I recommend the Dissertation for acceptance.

Yours sincerely

Alicja Józkowicz

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