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**Evaluation of PhD thesis "Identification of mechanisms remodeling mitochondria during adaptation of adipose tissue to changing ambient temperature" by Paweł Nowialis, prepared under the supervision of prof. Leslie P. Kozak at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn**

The dissertation concerns reversible adaptive changes in the adipose tissue of mice following restoration of the normal ambient temperature after a long-lasting treatment by cold. Deciphering of the basic mechanisms behind the "cold-to-thermoneutral" physiological mode may be important for understanding of the cellular and biochemical regulatory processes underlying nonshivering thermogenesis in adult mammals. The appearing of so called "brite cells" in white adipose tissue is known to be a crucial element allowing the use of triacylglycerol stores in the thermogenic mode, owing to the dispersal of the mitochondrial membrane potential by uncoupling protein 1. This mechanism is similar to that occurring in the brown adipose tissue of newborn or hibernating mammals although the origin and features of BAT and brite cells are substantially different. The hallmarks of thermogenic adipocytes are a highly active respiratory chain and increased content of uncoupling protein 1 (UCP1). These features may be due in part to an increased amount of mitochondria. Interestingly, restoration of normal thermal conditions causes the "cold phenotype" to disappear, which may easily be traced by a gradual decrease of the UCP1 content. Thus it is fully justified that mitochondria were the focus of this dissertation. One could stress here that while the phenomena recalled above are well known, at the descriptive level the mechanisms of the emergence of brite cells in response to cold and their disappearance on re-warming are still elusive. Both the differentiation of brite adipocytes from stem cells and alternatively, reprogramming (by induction of adaptive changes) of differentiated white adipocytes as the source of brite cells have staunch advocates as well as opponents. Also the mechanism of the disappearance of brite cells upon restoration of thermoneutral conditions has not been explained. Both processes are of high importance in the regulation of thermogenesis in adult mammals. The latter is the main subject of this thesis.

The dissertation is short (52 pages including list of references) and has a standard layout (Contents, List of abbreviations, List of Figures, List of tables, Abstracts, Introduction, Objectives, Materials and Methods, Results, Discussion, Conclusions, and References). The title reflects the presented results although mitochondrial remodeling is a very complex phenomenon, encompassing numerous mitochondrial properties, which needs a broader spectrum of techniques to be conclusively investigated. In fact, remodeling of mitochondria was not shown satisfactorily in this study.

The dissertation is written in English and I do not feel qualified to evaluate its linguistic correctness. The only part in Polish - the Abstract - is astonishingly poorly written as if it was translated word for word from the English Abstract by a computer program.

**Introduction**

In this section (14 pages and containing 6 figures) the Author clearly presents the issues addressed in the experimental part and acquaints the reader with the subject of the thesis. He also explains the rationale of this study. This part of the dissertation indicates his good grasp of the matter of his investigations. At the end of this section two goals of the dissertation are listed. I do not have major comments regarding this part of the dissertation. I only suggest that mitofusins form GTP but not ATP hydrolysing hetero-oligomers (page 13 bottom).

**Materials and methods**

In the experiments three strains of mice were used: recombinant AXB8/PgnJ, AKAP1$^{-/-}$ knockouts in the C57BL/6J background, and AKAP$^{+}$/$^{+}$ as a control. It would be advisable to explain the reason for such a choice. Particularly the usefulness of AXB8/PgnJ should be justified, because the experiments with AKAP$^{+}$/$^{+}$ mice are to some extent a repetition of those performed with the former. This issue was in fact explained in the Discussion, but giving this information early in the text would be helpful.

Before the experiments the animals were maintained at 23°C, then the temperature was decreased to 4 degree for 15 days and then increased to 29°C for the next 3 days. Why was the recovery (post-chilling) temperature higher by 6°C from that at the beginning of experiments? An additional variable was introduced by this experimental design. This is particularly strange in view of the recent paper concerning a similar issue, published by the same group [Gospodarska E. Nowialis P. Kozak LP. (2015) J. Biol. Chem. 290: 8243-8255]. In those experiments both the initial and the post-chilling temperatures were the same (29°C).

The spectrum of methods used in the experiments reported is rather limited, the major technique being Western blotting. Although, this method is routinely used in many biochemical and biological laboratories its description in a particular situation must contain all relevant information allowing critical evaluation of the obtained results, including the method of preparation of cellular lysates. This is key for reliable identification and semi-quantitative detection of specific proteins. Some proteins need cellular lysates to by boiled for a few minutes with the sample buffer while in the case of others (for instance, respiratory complexes) the lysates may be heated up to 45°C, but they should not be boiled. In this dissertation there is no information concerning this issue.

**Results**

Experimental data are described on 5 pages of text and 12 pages containing figures. Placing all the figures together with their descriptions at the end of the chapter rather than next to the relevant part of the text makes the evaluation of the data very difficult. I consider such a layout a major editorial error. It would be more reader-friendly to put each figure in an appropriate site within the text.

The chapter is divided into two parts. In the first one changes of selected mitochondrial and other cellular parameters in inguinal white adipose tissue isolated from AXB8/PgnJ mice challenged by chilling and subsequent restoration of thermoneutrality were traced.

First, morphological changes of the adipose cells were shown. Then UCP1 transcript and protein levels were analyzed. Both UCP1-encoding mRNA and UCP1 protein were absent, that is, below the detection levels of the method used, in control mice kept under thermoneutral conditions and appeared at a substantial level after 15 days at 4°C.

Interestingly, they both increased further in the first 6 h of restoration of thermoneutrality, and then gradually declined. On the basis of the time-course of the reduction of UCP1 content in further

experiments the thermoneutrality period (i.e. maintenance of 29°C) was extended to 72 h. However, UCP1 protein level was still substantially higher than that found in the control mice kept under thermoneutral conditions.

To characterize accompanying changes of mitochondrial metabolism and other cellular parameters which could be helpful for uncovering the mechanisms responsible for the reversible induction of brown-like adipocytes (brite phenotype) the Author applied Western blotting for identification and semi-quantitative analysis of selected subunits of respiratory complexes and mitochondrial ATPase (parenthetically, complex V is not a component of the respiratory chain; see Fig. 11A), total and phosphorylated protein kinase A subunit RIIB (antibody against pS114, not pS112 see: Method chapter), and AKAP1 (A Kinase Anchoring Protein 1). Additionally, using the same method he evaluated relative amounts of proteins involved in autophagy and mitophagy (LC3II and LC3I, p62, Atg7, PINK1, phospho-PARKIN and PARKIN), apoptosis (caspase 3) and mitochondrial dynamics (pDRP1, DRP1, OPA1S and OPA1L). He also estimated *Atg7* transcript level using RT-PCR and mtDNA content using qPCR. Finally, he tried to detect reorganization of the mitochondrial network using electron microscopy.

These assays led to the conclusion that the restoration of thermoneutrality did not induce apoptosis in AXB8/PgnJ mice, but increased the tendency to mitochondrial fission. Unfortunately, high standard deviations of the data (probably because of high inter-individual variability or the presence of variable amounts non-fat cells in the tissue samples tested) did not allow the Author to convincingly confirm the participation of mitophagy in the involution of brite cells.

The second part of this chapter focuses on the putative role of AKAP1 in the white adipose tissue remodeling in the response to changing ambient temperature. This concept has emerged from the well documented data indicating that acclimation of adipocytes to low temperature is closely related to PKA activation in response to β-adrenergic stimulation. AKAP1 is a protein anchoring PKA to mitochondria and a mitochondria-stabilizing factor thus cells depleted of this protein could be debilitated in terms of AKAP1/PKA-dependent mitochondrial processes. To test this concept two strains of mice were used: $AKAP1^{-/-}$ knockouts and the AKAP1-positive control. Experimental protocols were basically the same as previously.

On the basis of the collected data it was concluded the AKAP1 knockout mice did not exhibit significant differences to their $AKAP1^{+/+}$ counterparts concerning mitophagy and mitochondrial fusion/fission process although substantial relative increase of OPA1S/OPA1L ratio in the former was observed. However this was interpreted (together with the increased caspase 3 activation) as a marker of enhanced apoptosis. This is in line with antiapoptotic function of AKAP1 protein. Thus it seems that AKAP1 is not involved in the re-adaptation of adipocytes to the restored thermoneutral conditions at least as concerns mitochondrial processes investigated. Despite this they did show one marked difference: the increase of the UCP1 protein level in cold-exposed mice was substantially more pronounced in the former (Fig. 16 B). Moreover it was constantly increasing despite restoration of the thermoneutral conditions. This observation seems to be inconsistent with much less evident effect of the exposition of AKAP1-depleted mice to low temperature on general morphology of the adipose tissue than observed in AKAP1-positive equivalents (Fig. 15).

Additionally, one should note, that the UCP1 expression patterns also differ between the AXB8/PgnJ and $AKAP^{+/+}$ mice exposed to cold and subsequently to normothermic conditions (see Fig. 10 B, UCP1, disappears quickly and Fig. 16 B, UCP1 content is constant or even grows steadily). The Author failed to comment this observation.

This part of dissertation provokes several critical comments and minor issues.

1. The bands representing LC3II and LC3I proteins shown in the Fig. 12 C and 17 C are marked incorrectly (the marking is reversed relative to that in the Abcam catalogue and published by other authors [Mizushima N. Yoshimori T. (2007) Autophagy 3:6, 542-545; Klionsky DJ. Abdelmohsen K. Abe A. et al., (2016) Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition), Autophagy, 12:1, 1-222, DOI: 10.1080/15548627.2015.1100356.]. It could be a simple editorial mistake in figure preparation or it could indicate erroneous calculation of relative abundancies of the two proteins. If the latter is true, then any interpretation of the data offered by the Author is fundamentally flawed, since where he claims an increased LC3II – to – LC3I ratio a decreased one is in fact present. I was unable to discriminate unequivocally between these two possibilities by inspecting the prints of the blot shown.
2. Changes of the PDRP1/DRP1 and OPA1S/OPA1L ratios indeed suggest a shift toward mitochondrial fission, but without further analysis of other proteins involved in the mitochondrial fission/fusion processes and, critically, visualization of the mitochondrial network (and co-localization DRP1 with the mitochondrial membrane) within cells a definite conclusion seems premature. (Similarly, the confirmation of the mitochondrial localization of the mitophagy-related proteins would also be desirable)
3. Fig. 14. is difficult to interpret. Intracellular structures are not enough clearly visible to be convincingly identified. Identification of mitochondria is plausible if both membranes can be discerned. The magnification should be much higher to allow observing both membranes and intramitochondrial structure. Visualization of the mitochondrial network with the use of electron microscopy requires a series of slices through the whole cell. How the mitochondrial area could be calculated on the basis of the TEM image shown here? I can only guess that Fig. 14 B is shown to compare mitochondrial size or compare combined surfaces of putative mitochondria visible on this cross section.
4. Figures showing Western blots are very small and therefore hardly readable.
5. Molecular masses of detected proteins are not provided.
6. Some blots are trimmed so much that protein bands of interest are partially removed (Fig. 12 K and 17 C).
7. Plots showing Western blot data for respiratory complexes are very small and illegible. It is difficult to discriminate between colors assigned to particular complexes. There is absolutely no reason to attempt saving space in a thesis of such a small volume.
8. Symbols indicating lack of statistical significance seem to be needless.
9. Figure legends must not straddle pages (e.g. Figs. 11 and 12) as it greatly complicates figure analysis.
10. Fig. 16. Descriptions A and B are interchanged.

**Discussion**

This chapter is well written and I want to appreciate careful and critical interpretation of the data. However, as are other parts of the dissertation, it is very concise and strictly focused on the presented results. This is not a major criticism although the Author could have used this part of dissertation to

demonstrate his expertise. The turnover of brite cells is an important regulatory part of thermogenesis and has been investigated for number of years, thus it would be appropriate if its various aspects and hypotheses were critically discussed in this part of the thesis.

Short but informative conclusions are the last part of the dissertation.

In keeping with the main text, also the list of references is modest and includes only 76 articles, 61 of them are not older than 10 years. Such a proportion indicates topicality of the subject matter.

**Conclusions**

Despite the numerous aforementioned imperfections and criticisms , I do appreciate the novelty of the presented study and I consider the results obtained Mr. Nowialis interesting and contributing to better understanding of the mechanisms allowing the white adipose tissue to participate reversibly in nonshivering thermogenesis at low ambient temperatures and to restore the white adipose tissue phenotype under thermoneutral conditions. Although to some extent preliminary, the findings presented in this dissertation are inspiring and provide a good basis for further, more advanced studies.

To sum I conclude that the PhD thesis presented by Mr. Paweł Nowialis meets the criteria of article 13 of the Act of 14th March 2003 on the Law on Academic Degrees an Title and Degrees and Title in the Arts (Official Journal of Laws of 2003 of the Republic of Poland No. 65, item 595, with amendments).

Therefore I recommend to the Faculty of Medicine with the Division of Dentistry and Division of Medical Education in English at the Medical University of Bialystok the admission of Mr. Paweł Nowialis to the next steps of the PhD conferment procedure.

**Wnioski końcowe**

Mimo wszystkich krytycznych uwag wymienionych w recenzji, w rozprawie doktorskiej mgr. Pawła Nowialisa dostrzegam i doceniam nowatorskie podejście i uważam, że przedstawione wyniki badań są interesujące i przyczyniają się do lepszego zrozumienia mechanizmów pozwalających białej tkance tłuszczowej uczestniczyć w sposób odwracalny w termogenezie indukowanej obniżeniem temperatury otoczenia. Wyniki te są w dużej mierze wstępne, lecz z pewnością dają dobrą podstawę i stanowią inspirację do dalszych badań.

Podsumowując uważam, że rozprawa przygotowana przez mgr. Pawła Nowialisa spełnia warunki stawiane rozprawom doktorskim zgodnie z wymogami określonymi w artykule 13 ustawy z dnia 14 marca 2003 r. o Stopniach i Tytule Naukowym oraz o Stopniach i Tytule w Zakresie Sztuki (Dz. U. z 2003 r., Nr 65, poz 595 z późniejszymi zmianami) i wnoszę do wysokiej Rady Wydziału Lekarskiego z Oddziałem Stomatologii i Oddziałem Nauczania w Języku Angielskim Uniwersytetu Medycznego w Białymstoku o dopuszczenie mgr. Pawła Nowialisa do dalszych etapów przewodu doktorskiego.