Opponent´s Report on PhD Thesis of Petr Danek, MSc.

Title of doctoral thesis:	Identification and characterisation of novel mechanisms regulating steady state and emergency granulopoiesis
Title in Czech:	Identifikace a charakterizace nových mechanizmů regulujících bazální a pohotovostní granulopoezu
Supervisor:	Meritxell Alberich-Jorda, Ph.D.
Study program:	Molecular and Cell Biology, Genetics and Virology
Oponent:	Ing. Ota Fuchs, CSc., ÚHKT Praha

The submitted thesis reports the results, which were obtained within the research carried out in departments of Hemato-oncology, Cell and Developmental Biology, and Leukocyte Cell Signaling, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague. The aims of this doctoral thesis were: 1) to find the role of the β-catenin-T cell factor/lymphoid enhancer-binding factor (TCF/LEF) complex during neutrophilic differentiation in both steady-state and emergency granulopoiesis and its importance for the biology of hematopoietic stem and progenitor cells; 2) to elucidate the role of the transcription factor C/EBPγ on differentiation of neutrophils during steady-state and emergency granulopoiesis; 3) to investigate how C/EBPα regulates expression of *EVI2B,* and determine the function of this transmembrane protein during granulocyte differentiation. All these aims are important and their successful solution shows ways for future investigation.

Theoretical part (pages 19-44) covers all studied areas and is focused on mechanisms regulating the generation of granulocytes, their role in the innate immune system, and diseases associated with poor or excessive production of neutrophils (neutropenia, immunodeficiency or inflammatory and autoinflammatory diseases). Petr Danek, MSc. desribed the role of transcription factors in lineage commitment, the canonical Wnt signaling pathway and its role in hematopoiesis. The conflicting results were obtained in past about the function of the canonical Wnt signaling pathway in the hematopoietic system. To solve this problem, it was necessary to use more specific models. Danek et al. used a novel mouse model described by

Lucie Janackova et al. in the journal Genesis in 2016. The study is based on transgenic mouse strain enabling inducible expression of an N-terminally truncated variant of nuclear Wnt effector T cell factor 4 (TCF4). This variant acts as a dominant negative version wild-type TCF4 lacking β-catenin binding domain and prevents β-catenin-TCF/LEF-mediated transcription of activation of responsive genes (granulocyte colony-stimulating factor receptor /G-CSF/, a master regulator of steady state and emergency granulopoiesis). This strategy allowed to investigate β-catenin-TCF/LEF-mediated transcription in myeloid progenitors in both, steady-state and emergency granulopoiesis *in vivo*. Suppression of the β-catenin-TCF/LEF transcription results in reduced response to G-CSF stimulation. For induction of emergency granulopoiesis, mice were treated with lipopolysaccharide. The same results were obtained in human $CD34^+$ hematopoietic stem and progenitor cells by genetic (dominant negative form of TCF4) and pharmacological inhibition (cercosporin) of the Wnt pathway. Danek et al. described results of this study in the journal Blood with high impact factor 17.794 (Blood 2020; 136(22) 2574-2587). Their article was commented in the same number of this journal by Steffen Boettcher, University Hospital Zurich (Blood 2020; 136(22), 2487-2489). Therefore, the first aim was fulfilled and Danek et al. showed that the β-catenin-TCF/LEF complex directly regulates G-CSF receptor levels and consequently controls the differentiation of myeloid progenitors into granulocytes.

The second aim was studied with the help of a conditional tissue specific *Cebpg* knock-out mouse model. Excision of *Cebpg* was verified using RT-qPCR and western blotting. Bone marrow composition was determined in wild type and knock-out mice. Petr Danek was co-author of publication (Kardosova M et al. C/EBPγ is dispensable for steady-state and emergency granulopoiesis. Haematologica 2018; 103(8): e331-e335), where the results of these studies were described.

The third aim was solved in K562 cells because they do not express endogenous C/EBPα. Petr Danek was co-author and as a shared first author of two publications from these studies on expression of *EVI2B* and its function during granulocyte differentiation (Zjablovskaja P. et al. EVI2B is a C/EBPα target gene required for granulocytic differentiation and functionality of hematopoietic progenitors. Cell Death Differ 2017; 24(4): 705-716; Zjablovskaja P, Danek P et al. Proliferation and differentiation of murine myeloid precursor 32D/G-CSF-R cells, J Vis Exp 2018; 132: 57033). Authors introduced the wild-type and mutant C/EBPα proteins in the form of an estrogen receptor fusion protein. Treatment with β-estradiol induced expression of EVI2B only in full length p42 C/EBPα-ER expressing cells. Authors described *EVI2B* as a novel C/EBPα target gene. A subset of acute myeloid

leukemia (AML) patients characterized by mutated or silenced *CEBPA* has the downregulated *EVI2B* expression. Both C/EBPα and EVI2B are upregulated in murine 32D/G-CSF-R cells by G-CSF administration. Knock-down of *EVI2B* by two different shRNAs blocked granulocytic differentiation. Authors showed that a membrane glycoprotein EVI2B is important for proper differentiation of myeloid precursors to granulocytes.

Only few typing errors were found in this thesis: in the list of abbreviations-low-density lipoprotein receptor–related-protein 6 is abbreviated as LRP5; a membrane glycoprotein is described in italics *EVI2B* on the page 65-the second paragraph; M.Aderova in reference number 262.

I would like to address the author of this thesis with three questions listed below:

1) Cercosporin, a naturally occuring perylenequinone, was used as inhibitor of binding of TCF/LEF complex to β-catenin. However, cercosporin is also a potent selective inhibitor of protein kinase C (PKC) and was studied as photosensitizer in photodynamic therapy. Please, can you explain why this inhibitor was used?

2) There are differences in expression and function of LEF1 isoforms (the long isoform and N-terminally truncated isoform) in normal and leukemic hematopoiesis. Can you clear these differences?

3) What do you think? Will it be possible to regulate canonical Wnt signaling and granulopoiesis in order to treat neutropenia in congenital neutropenias and inherited bone marrow failure syndromes?

Petr Danek, MSc. is author or co-author of five impacted articles and this is very good outcome of his doctoral study. Since all these articles went through a thorough refereeing process, it warrants a high level of his research. I recommend the thesis of Petr Danek to defence and to a further procedure concerning the granting of the scientific degree PhD.

V Praze dne 17.1.2021

Ing. Ota Fuchs, CSc.