Jana Balounova Ph.D.
Head of Immunology Unit
Phenotyping Module
Czech Centre for Phenogenomics
Institute of Molecular Genetics of the ASCR, v. v. i.
Prumyslova 595
CZ252 50 Vestec
Czech Republic
e-mail: jana.balounova@img.cas.cz | Phone: +420 325 873 219

Prague, January 20, 2021

Review of the PhD thesis: *Identification and characterisation of novel mechanisms regulating steady state and emergency granulopoiesis*

Proposed PhD thesis submitted by Petr Daněk summarizes the experimental data from four published studies. Petr Daněk is the first author of the paper published in *Blood* journal, co-author on the two papers published *Haematologica* and in the *Cell Death and differentiation* journals and shared first author of a video method article published in JoVE. Besides papers directly linked to the thesis, he coauthored two other articles.

In his thesis, Petr first comprehensibly summarizes current knowledge of steady state and emergency hematopoiesis with emphasis on granulopoiesis and transcription factors involved in regulation of myelopoiesis. He also provides an overview on Wnt signaling and its so far inconclusive roles in hematopoiesis.

The aim of the thesis is to characterize novel molecular mechanisms that might be involved in the regulation of neutrophil differentiation during physiological conditions and hematopoietic stress.

Results part of the thesis is split into three chapters, based on results shown in four published papers.

The projects discussed in the thesis are:

1. The role of the β-catenin-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. The role of the transcription factor *Cebpγ* in normal and stress-induced granulopoiesis

3. The mechanism of C/EBPα regulation of EVI2B expression, and characterization of the function of this transmembrane protein during granulocyte differentiation

Petr has clearly substantially contributed to all three projects.

In his first author publication he develops a new mouse model, that allows to specifically inhibit β-catenin-TCF/LEF mediated transcription by overexpressing dnTCF4 transgene. He clearly presents the benefits of this approach, which allows for investigating the role of canonical Wnt signaling. Using Vav-driven dnTCF4, Petr shows that β-catenin-TCF/LEF signaling promotes neutrophilic differentiation of HSPC via direct upregulation of G-CSFR in steady state and during emergency hematopoiesis in mice. To extend the data generated in the mouse model, he also shows that β-catenin-TCF/LEF signaling promotes differentiation of human HSPCs into neutrophils. Most of the experimental work including countless flow cytometry (FCM) analyses, mRNA expression profiling, ChIP-qPCR, numerous *in vitro* culture assays and *in vivo* functional assays, was done by Petr himself. Petr was not only designing these experiments, but also analyzing the data, preparing figures and writing the manuscript.

While addressing the role of the transcription factor Cebpγ in normal and stress-induced granulopoiesis, Petr was involved in generation and validation of hematopoietic-specific *Cebpγ* knock-out mouse model using FCM analyses of BM cell populations and colony culture assays. These and other experiments have revealed the absence of Cebpγ has negligible consequences on production of both immature and mature granulocytes during basal and several types of demand-adapted granulopoiesis as well as on HSC function, which is in contrast to data previously using the whole body *Cebpγ* KO.

Finally, Petr contributed to the third project, describing that *EVI2B* is a direct target of *C/EBPα* and revealing that EVI2B is essential for proper granulopoietic development *in vitro* and *in vivo*. Petr performed important *in vitro* experiments, showing that C/EBPα activation upregulates *EVI2B* expression in human cell lines transfected with different *C/EBPα* constructs. Using *EVI2B* KO mice and *EVI2B* deficient cell lines Petr showed that loss or downregulation od *EVI2B* is sufficient to block the differentiation of granulocytes *in vitro* and *in vivo*.

Structure of the thesis is logical, written in a very good English and is easy to follow.

I have the following questions:

1. You have shown that inhibition of β-catenin-TCF/LEF-mediated transcription is dispensable for the function of adult LT-HSC. However canonical Wnt signaling is employed during endothelial to hematopoietic cell transition in emerging HSCs and EMPs (Ruiz-Herguido et al., JEM, 2012, Frame et al., Stem Cells, 2015). Specifically, β-catenin activity is required in Cdh5 expressing cells before the stage of Vav1 expression (around E10–11) to generate HSCs and their progeny (Ruiz-Herguido et al., JEM, 2012).

1a. Have you used other drivers of dnTCF4 expression besides Vav-Cre (c-kit, CD31, Cdh5) to investigate the role of dnTCF4 in emergence of hematopoietic cells?

1b. Could you speculate what would happen when dnTCF4 would be expressed in hemogenic endothelium before emergence of HSCs or EMPs?

1c. You claim, that dnTCF4 is in your (Vav-Cre driven) model expressed from embryonic day 13.5, but I could not find the data neither in the manuscript nor in the thesis. Could you provide these data (expression data from E10.5-E13.5)?

2. ROS production, migration, and phagocytosis was not impaired in neutrophils of dnTCF4 animals as compared to wt, have you analyzed their ability to form NETs?

3. Referring to Figure 1C of the dnTCF4 paper, could you comment on the exceptionally high SD of the expression of β-catenin target genes (Axin2 and Nkd1) at 24 hours upon β-catenin pathway activation?

4. Referring to page 20 of Introduction "Neutrophils are predominantly characterized by their unique morphology (granular cytoplasm and ***rather small segmented nucleus)*** …" What is the size of neutrophil nucleus, is it really smaller than lymphocyte nucleus or it just looks smaller because the cell and cytoplasmic volume is bigger?

Taken together, I highly appreciate the quality of the submitted thesis. During his PhD studies Petr Daněk showed proficiency in the vast array of experimental methods including BM phenotyping using FCM, various *in vitro* cell culture assays, *in vivo* functional analyses of LT-HSCs, gene expression profiling, ChIP-qPCR and RNAseq analysis. Moreover, he has been awarded several grants and awards during his studies. In my opinion, the results of Petr's scientific projects clearly exceed the average level of PhD candidates. Petr's exceptional results and outstanding quality of his research papers prove that he is a mature scientist capable of independent work and therefore meets the requirements set by the Molecular and Cellular Biology, Genetics and Virology board of doctoral studies to be awarded the PhD degree.

Jana Balounova, Ph.D.

Průmyslová 595, 252 50 Vestec, Czech Republic
phone: +420 325 873 246
e-mail: ccp@phenogenomics.cz
web: www.phenogenomics.cz