SCIENTIFIC COUNCIL OF MEDICINE
UNIVERSITY OF BELGRADE

At the meeting of the Scientific Council of the Faculty of Medicine in Belgrade, held on 03.11.2014. No. 4600/11, the Evaluation Board for the assessment of the completed PhD thesis entitled:

"Expression of neural adhesion molecules (NCAM) in health and diseased renal tissues" ("Ekspresija neuralnih adhezionih molekula (NCAM) u zdravom i obolelom bubrežnom tkivu")

written by candidate Sanja Ćirović, a graduate of molecular biology and physiology, employed at the Institute of Pathology, Faculty of Medicine, University of Belgrade as an associate (expert advisor), was appointed. The scientific advisor of the thesis is Prof. Dr. Jasmina Markovic-Lipkovski.

The members of the appointed Evaluation Board are:

1. Prof. Dr. Claudia Müller, Professor of Medicine, University of Tübingen, Federal Republic of Germany
2. Prof. Dr. Jovan Vasiljević, Professor of Medicine, University of Belgrade,
3. Prof. Dr. Svetislav Tatić, Professor of Medicine, University of Belgrade,

Based on the analysis of the submitted PhD thesis, the Evaluation Board submits to the Scientific Council of Medical Faculty the following

REPORT

A) The contents of the thesis

PhD thesis of molecular biologist and physiologist Ćirović Sanja is written on 106 pages and is divided into the following sections: Introduction, Objectives, Materials and methods, Results, Discussion, Conclusions and References. The thesis contains a total of 38 images. Doctoral dissertation contains a Summary in Serbian and English, the Candidate's biography, information about the Board and one Appendix.

In the Introduction, the most common physiologic abnormalities of renal function are described, as well as the problems facing nephrologists and patients in the treatment of renal insufficiency and renal tumors. In the text neural cell adhesion molecule (NCAM), its gene and protein structure is described, which leads to the manifestation of different NCAM isoforms due to the presence of alternative splicing of NCAM mRNA; its role in mediating homophilic and heterophilic cell adhesion as well as the possibility of post-translational modification of NCAM molecules. Further, the role of NCAM during differentiation of the kidney is described; a brief review of the mesenchymal-epithelial transformation during the development of nephron, and the presence of NCAM as a marker of fetal renal progenitor stem cells is included. Also in the Introduction NCAM expression in kidney tumors and its role in tumor invasion is described. In short, there is an overview of the presence of NCAM in interstitial fibrosis and possible co-expression of NCAM and molecules responsible for the occurrence of interstitial fibrosis i.e. FGFR1, intergin α5β1 and αSMA.

The objectives of the work are clearly defined. They consist of the examination the expression of NCAM isoforms in fetal kidney tissue, health adult kidney, and adult kidney tissue with an initial interstitial fibrosis, renal cell carcinoma and renal cell cultures. Further immunomorphological characterization and correlation of NCAM+ cells with other well defined cell surface markers was done in other to more precisely defined NCAM role in interstitial cells and in renal progenitor cells. Also, new mesenchymal markers (antibodies W5C4C5 and W8B2), as well as antibody W1C3 (obtained in the laboratory of Prof. Dr. JH Bühring, University of Tübingen), as possible new clone for NCAM were tested on renal tissue and correlated with NCAM in order to better characterize NCAM+ cells.

In the Materials and Methods section it is noted that the experiments were done at the Center for Medical Research (ZMF), University of Tübingen and in the Laboratory of the Institute of Pathology, Faculty of Medicine, University of Belgrade. The experiments were conducted in accordance with ethical principles. Methodology of immunohistochemistry staining of fetal, fibrotic, tumor and normal renal tissue was precisely described. Different NCAM antibodies were used to detect expression of NCAM isoforms in tissue. Also markers such as CDH9, CD34 and W1C3 were analyzed by imunohistochemistry staining. Next, double immunofluorescent method was used to examine co-localization of NCAM with CD24, CD133, PSA-NCAM, Ki-67, FGFR1, α5β1 integrin, EpCAM, Six2 etc. For detection of NCAM expression in renal cell lines FACS analyses were done. Furthermore, Western blot and immunoprecipitation were used to detect presence of NCAM isoforms

at protein level. While, after mRNA isolation and reverse-transcription into cDNA, RT-PCR analyses were performed to determine presence of NCAM isoforms at nucleotide level. The numbers of NCAM+ FGFR1+ cells in kidney tumors, as well as the numbers of NCAM+ cells in the tissue with initial interstitial fibrosis were obtained by semi-quantitative method, while statistical data were processed using IBM SPSS software.

In the Results section, all the results are presented clearly and in detail. The discussion is written clearly and transparently, with comparative examination of the results of the doctoral dissertation with the data in other studies (references).

The Conclusions summarize the most important findings that have emerged from the results of the work. The Bibliography contains a list of 119 references.

B) A brief description of the results achieved

This research using specific primers for RT-PCR showed presence of NCAM-120 /-140 /-180 isoforms in healthy and diseased kidney tissue. Also, it was observed that the expression of NCAM on renal cell line depends on the density of cells in culture, namely, at a density between 40-50% cells do not express NCAM, while presence of NCAM was detected only if the density in the culture was above 70%. Double-immunofluorescent staining which showed co-localizations of NCAM with CD24, a marker of adult renal progenitors and of fetal nephron precursor cells, but not co-expression with CD133, also a marker of adult stem cells, as well as co-expression of NCAM with a marker of epithelial cells (EpCAM, CD326), was used to more easily identify NCAM+ cells in the kidney that have recently passed the mesenchymal-epithelial transformation. It is interesting to point out that the NCAM molecules that were present in the cells of condensed mesenchym and its derivatives (pretubular aggregate, renal vesicles, "Comma" and "S-shaped body"), were posttranslational modified by polysialic acid (PSA). Results of co-expression of NCAM and these markers indicate that human fetal precursors may be divided into at least two populations: a) PSA-NCAM + CD24 + CD326-, i.e. nephron progenitor cells and b) PSA-NCAM- NCAM+ CD24-CD326- potential precursor cells of renal stroma. The results of this thesis also show aberrant expression of NCAM molecules in tumor tissue as well as in tissue with interstitial fibrosis of the kidney. In both those tissues it was observed that NCAM and tyrosine kinase FGFR1 receptor responsible for cell proliferation and aggressive behavior in some tumors of epithelial origin were co-expressed. Presence

of membranous and cytoplasmatic NCAM/FGFR1-complexes was detected in renal cell carcinoma (RCC), while in oncocytoma localization of NCAM/FGFR1 interaction was exclusively cytoplasmatic. Bearing in mind the fact that RCC is considered a malignant tumor and oncocytoma a benign, different localization FGFR1 and NCAM molecules suggests a multifunctional role of NCAM/FGFR1 interaction in kidney tumors. A significant result of this dissertation also represents detection of interstitial NCAM/integrin α5β1 and NCAM/FGFR1 positive cells in tissues with an initial interstitial fibrosis. The functional interaction between NCAM and the β1 subunit of integrin, as well as with FGFR1 could activate the intracellular signaling of the ERK /MAP kinase pathway and simultaneously modulate adhesion to extra-cellular matrix. This suggests that cell therapy with NCAM+/integrin α5β1+ cells could lead to a new strategy in the recovery of tissue in patients with initial interstitial fibrosis.

C) Comparative analysis of the doctoral thesis with the results from the literature

Relatively small numbers of investigations have examined the expression of NCAM isoforms in the renal tissue. Study on mouse embryos (Klein et al., 1988), for the first time showed the presence of three NCAM isoforms: NCAM-120, NCAM-140 and NCAM-180 in kidney. This NCAM isoforms were at that time described as a characteristic of adult neurons. They also detected that NCAM expression in kidney was rapidly lost after birth. The results presented in this thesis are in accordance with the research of Klein et al. Yet it is interesting to point out that the results of Western blot and immunoprecipitation, presented in this paper showed NCAM molecules larger than 180 kb, i.e. in the range of 140-250 kb, suggesting that in human fetal kidney tissue NCAM molecule is posttranslational modified by polysialic acid (PSA). The presence of PSA-NCAM has been demonstrated in the works of other authors, further indicating the specificity of PSA modification of NCAM molecules (Omer et al., 2013). The presence of NCAM in human renal progenitors was studied mainly in the cell cultures. A much smaller number of papers examined the role of NCAM+ cells in the renal progenitors *in vitro* in the fetal kidney tissue. The role of NCAM molecules in fetal progenitors of the human fetal kidney on cell lines has been extensively studied over the past year. Thus, using FACS analysis the existence of different populations of renal progenitor cells expressing the NCAM was postulated (Metsuyan et al., 2009). In this thesis, by double immunofluorescence PSA-NCAM+EpCAM+ progenitors, precursors of the nephron, and NCAM+EpCAM-, most likely progenitor cells of the stroma, which

are correlated with the results Metsuyan et al., were detected. It also showed co-expression of NCAM and CD24 markers of renal progenitors, but also the expression of CD133, (Ivanova et al., 2010; Bussolati et al., 2005), as well as expression of stem cell markers TRA-160 (Fesenko et al., 2010) in fetal renal tissue. In contrast to adult renal progenitors which express CD24, and CD133 (Lindgern et al., 2011; Sagrinati et al., 2006), cells that were positive for CD24, CD133 and NCAM were not detected in fetal tissues. In this thesis it was shown presence of specific NCAM isoforms in kidney tumors, which correlates with the work of other authors (Gattenlöhner et al., 2009). Results of this thesis confirmed the important role of aberrant, re-expression of NCAM molecules in kidney tumors (Daniel et al., 2003; Cirovic et al., 2012), as well as the expression of FGFR1 (Tsimafeyeu et al., 2011). The functional interaction between NCAM/FGFR1 otherwise well studied in nerve cells (Francavilla et al., 2009; Kiselyov et al., 2005; Kiselyov et al., 2003) and ovarian tumors (Zecchini et al., 2011), in this dissertation, was detected for the first in kidney tumors. The specific NCAM isoforms (120, 140, 180) were detected by RT-PCR analysis in the adult kidney (Gattenlöhner et al., 2009). In this study increased expression of NCAM+ interstitial cells in the early stage of interstitial fibrosis (Marković-Lipkovski et al., 2009), as well as the heterogeneity of NCAM + interstitiaal cells that co-expressed FGFR1 (Kochoyan et al., 2008) or the integrin α5β1 were demonstrated (Schmid et al., 2009; Takagi et al., 2003).

D) The published papers resulting from the thesis

1. **Sanja Ćirović,** Jelena Vjestica, Claudia Mueller A, Svetislav Tatic, Jovan Vasiljevic, Sanja Milenkovic, Gerhard Mueller A, Jasmina Markovic-Lipkovski. NCAM and FGFR1 coexpression and colocalization in renal tumors. Int J Clin Exp Pathol 2014; 7 (4): 1402-1414.

E) Conclusion (explanation of scientific contribution)

The PhD thesis "Expression of neural adhesion molecules (NCAM) in healthy and diseased kidney tissue" by the PhD candidate Sanja Ćirović, a graduate molecular biologist and physiologist, is an original scientific contribution to the understanding of the role of NCAM molecules in fetal, adult fibrotic and adult tumor tissue. This research clarified the expression of specific isoforms of NCAM in fetal and adult kidney tissue, as well as presence of posttranslational modifications of the

NCAM molecule (PSA-NCAM) in fetal tissue during kidney development. Also, colocalization of NCAM with FGFR1, CD24, CD326 and integrin α5β1 and NCAM colocalization with a new mesenchymal marker W1C3. The results of this doctoral thesis may be useful in further research and identification of fetal and adult renal progenitors. Also, the results obtained in kidney tumors may help in better understanding of the role of NCAM/FGFR1 interaction (in signal transduction) in tumor cells.

The work on this PhD thesis is done according to the principles of scientific research. The objectives are clearly defined, scientific approach was original and carefully chosen, and a working methodology was contemporary. The results are clearly and systematically presented and discussed, and from them the appropriate conclusions are derived.

Based on all abovementioned, and in view of the current research work of the candidate, the Board proposes to the Scientific Council, Faculty of Medicine, University of Belgrade to accept the PhD thesis of the molecular biologist and physiologist Sanja Ćirović, and approve its public defense, in order to acquire the academic title of doctor (PhD) of medical sciences (area - molecular medicine).

Belgrade, 20.11.2014.

Members of the Board:

Prof. Dr. Claudia Müller

Prof. Dr. Jovan Vasiljević

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Prof. Dr. Svetislav Tatić

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Scientific Advisor:

Prof. Dr. Jasmina Markovic-Lipkovski

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