R E V I E W

By **Prof. Dimitrinka Yordanova Atanasova-Dimitrova, PhD**

Member of the scientific jury, approved by Order No. P-109-182/08.04.2025

of the Rector of the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna

of the dissertation work

of Radoslav Hristov Spasov

on the topic

"ROLE OF PAX6 TRANSCRIPTION FACTOR IN MOUSE CEREBELLAR DEVELOPMENT"

For awarding the educational and scientific degree "Doctor of Philosophy"

In the scientific specialty "Anatomy, Histology and Cytology"

In professional direction 7.1 Medicine,

An area of higher education 7. Health care and sports

Scientific supervisor of the PhD student: Prof. Dr. Anton Bozhidarov Tonchev, MD, PhD, DSc

This review has been prepared for the public defense before an academic jury of the doctoral dissertation submitted by doctoral student Radislav Hristov Spasov, MD for the acquisition of the educational and scientific degree "Doctor of Philosophy" in the scientific specialty "Anatomy, Histology, and Cytology". The submitted documents and materials fully comply with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Implementation, and the Rules for the Development of the Academic Staff at the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna. The procedure has been correctly observed.

I. Brief biographical data about the doctoral student

Radoslav Hristov Spasov was born on October 14, 1988 in Kyustendil, Bulgaria. He graduated from the Medical University "Prof. Dr. Paraskev Stoyanov" in Varna in 2014, where he also obtained a specialty in anatomy, histology and cytology in 2021. He has been an assistant professor at the same university's Department of Anatomy and Cell Biology since 2015.

Since 2018, he has been a PhD student in anatomy, histology and cytology. Spasov's scientific interests are related to the molecular mechanisms of neurogenesis, especially the role of transcription factors in developing the cerebellum in mice. He actively participates in research

projects related to cerebral ischemia, molecular neurogenetics and pharmacological studies on models of metabolic syndrome.

Spasov's scientific output includes several original articles published in renowned international and national journals, including *International Journal of Molecular Sciences*, *Neuropharmacology* and *Varna Medical Forum*. He is a co-author of over five scientific publications and has participated in numerous symposia with oral and poster presentations. Among his latest research is the current dissertation work, dedicated to the role of the transcription factor Pax6 in the development of the mouse cerebellum, developed under the supervision of Prof. Dr. Anton Tonchev, MD, PhD, DSc.

Currently, Radoslav Hristov, MD is a member of the Bulgarian Anatomical Society, the European Federation of Experimental Morphology (EFEM), the International Federation of Anatomists' Associations (IFAA) and actively participates in the development of scientific projects funded by National and European sources.

II. General impressions and relevance of the topic

The presented dissertation is a scientific study dedicated to a fundamental problem in neurodevelopment – the molecular mechanisms regulating cerebellum development in mammals, focusing on the transcription factor Pax6. The author demonstrates excellent anatomy, embryology and molecular neurobiology training, combining classical morphological methods with modern genetic approaches (such as conditional knockout with the Cre/lox system). The structure of the work is strictly logical and scientifically sound, with clearly defined goals, objectives, methodology and consistent interpretation of the results.

The dissertation topic is highly relevant, as the development of the cerebellum is directly related to both motor and cognitive functions. The cerebellum is a central structure in regulating fine motor skills, balance, and some aspects of learning and emotional regulation. The transcription factor Pax6 is of key importance for many processes in the nervous system, and its impaired expression is associated with severe congenital malformations and neuropathologies, including autism spectrum, medulloblastoma and anencephaly.

The use of *conditional knockout mice* for Pax6 allows one to study its function in isolation after birth, a problem that cannot be investigated with a general knockout due to perinatal lethality. Given the rapidly growing interest in regenerative neurobiology and the application of stem cells

and gene therapy in neurological clinical practice, this makes the study particularly valuable and timely.

An impressive aspect of the study is the monitoring of structural and quantitative changes in brain tissue by morphometry and immunofluorescence microscopy, which confirms the role of Pax6 not only as a transforming, but also as a supporting factor in the differentiation and migration of glutamatergic neurons. These results have potential applications in developing models of diseases associated with impaired neurogenesis.

III. Structure and clarity of the exposition

The dissertation on the topic "*Role of Pax6 transcription factor in mouse cerebellar development*" is written on 144 pages and is divided into sections as follows: *Table of Contents* – 4 pages, *Introduction* – 2 pages, *Literature review* – 21 pages, *Aim and objectives* – 1 page, *Material and methods* – 18 pages, *Results* – 74 pages, *Discussion* – 7 pages, *Conclusions* – 1 page, *Contributions of the dissertation* – 1 page, *Bibliography* – 9 pages, including 124 literary sources, all in latin. *Publications and reports related to the dissertation* – 1 page, *Abbreviations used* – 3 pages, *Acknowledgements* – 1 page. The work is illustrated with 71 figures (most combined photomicrographs), 33 diagrams and three tables.

The dissertation **introduction** is structured clearly and logically, covering essential aspects of the topic: the importance of the cerebellum, the role of neuronal populations, and the place of the transcription factor Pax6 in embryonic and postnatal development.

The doctoral student clearly defines the main scientific problem — the lack of knowledge about the postnatal role of Pax6 in cerebellar neurogenesis due to the lethality of total knockout. From here, the need for research using a conditional knockout model is logically argued. This lays the foundation for the scientific hypothesis and the experimental framework of the dissertation.

The **literature review** is arranged sequentially, starting with general information on embryonic development in mammals, then moving on to cerebellar development and ending with molecular markers and the specific role of Pax6. Such a structure facilitates the reader's orientation. The review demonstrates a good knowledge of embryonic neurology, including relevant genes such as Atoh1, Lmx1a, Tbr1, Tbr2, NeuroD1/2 and Pax6. Tracking the spatiotemporal expression of these markers in different layers of the cerebellum is particularly valuable. The use of contemporary and authoritative sources – including articles from the last 5–10 years, as well as classic works related to brain development – demonstrates good critical thinking in the selection of literature.

The review effectively prepares the reader for the scientific questions raised in the dissertation – it demonstrates why it is necessary to use a conditional knockout of Pax6 (rather than a global one) and the logic for choosing specific time points (E12.5–E17.5, P7–P21). The explanation of the role of the Rhombic lip, outer and inner granular layer, as well as transcriptional cascades (Ptf1a → Pax2 → Gsx1 → NeuroD2) directly explains the choice of markers used in the experimental part (BrdU, Tbr1, NeuroD2, etc.). The literature review has a high scientific value for building the methodological framework of the dissertation

Focused and specific goal: The main goal – to analyse morphological changes in the cerebellum upon reduced Pax6 expression – is formulated, scientifically justified and in full accordance with the dissertation topic. The formulation of the tasks is well-structured, logically consistent and corresponds to the scientific goal. Four distinguishable tasks are described, which follow logically: 1) generation of appropriate control and mutant samples; 2) histological processing of brain tissues at several time points (prenatal, postnatal and adult); 3) application of cellular and molecular markers; 4) quantitative morphometric and cellular analyses

The "**Materials and Methods**" section is well structured and described in detail. *Comprehensiveness*: The methodological part is exceptionally detailed and covers the entire experimental process, from the choice of animal model to the analysis of the images. This demonstrates excellent experimental culture and precision. *Selection of transgenic line*: The use of conditional knockout using Tel-Cre::Pax6fl/fl to avoid perinatal lethality is a scientifically adequate approach demonstrating an understanding of the model's limitations. *Technical precision*: Techniques such as immunohistochemistry, immunofluorescence, light and fluorescence microscopy are described in detail, including microscope settings, exposures, image formats used and analysis programs (e.g. Fiji ImageJ). *Listing of antibodies used*: Including tables with primary and secondary antibodies, including manufacturer and concentration, is an example of good practice in document management.

The **Results** section is structured, following a logical and experimental sequence. The main subsections include: 1) Normal expression of Pax6 in the developing cerebellum, establishing the baseline expression pattern; 2) Data from the Tel-Cre::Rosa26 reporter mouse line to confirm the spatiotemporal activity of the Cre system; 3) Studies on the Tel-Cre::Pax6$^{fl/fl}$ mutant line, which is the central part of the analysis, with rich quantitative processing. This section is further expanded into several subsections, covering: quantitative analyses of area, thickness and length of the layers

(granular, molecular, Purkinje), assessment of cell density, and statistical significance of deviations in the mutant phenotype. A particular approach was used: immunofluorescence, microscopy (Axio Imager Z2), analysis with Fiji ImageJ (for area, thickness, length, number of cells), validation with NeuN, Calbindin, Parvalbumin and other markers.

Results from the reporter line (Tel-Cre::Rosa26): Cre recombinase expression was demonstrated in the target cell populations (Pax6-positive), including granule cells. This validates the model's applicability for studying the transcription factor Pax6 at later stages of development (E12.5–P21).

Key findings from the mutant model (Tel-Cre::Pax6$^{fl/fl}$): The mutant cerebellum shows significantly reduced cortical area (up to 48% decrease) and granular layer (up to 32% decrease). Differences in thickness are less pronounced (11% decrease), and at some levels do not reach statistical significance. This gives rise to the interesting conclusion that area depends more strongly on the length than on the thickness of the layers. Measurements show a significant shortening of the length of the molecular layer (over 40% decrease) with preserved thickness – this is a key contributing element of the analysis. The reduced number of Purkinje cells and granular cells is statistically significant. This suggests impaired not only morphogenesis, but also neurogenesis.

The results section demonstrates *high detail and technical competence* in quantitative analyses. *Strong logical connection* between aim, methodology and results. *This is a new model for the analysis of conditional* Pax6 expression, with potential for future studies on cell fate. Insight that *morphometric changes are due not only to cell loss but also to geometric changes* (reduced layer length).

The "**Results**" section in Radoslav Spasov's dissertation is an example of a well-organized, in-depth, and scientifically argued study that meets the criteria for a dissertation in anatomy and neurobiology.

The "**Discussion**" section is the culminating part of the dissertation work, in which the doctoral student integrates the main experimental results in the context of modern knowledge about the development of the cerebellum in mice and the role of the transcription factor Pax6. In this sense, the analysis is adequately structured, substantively justified and largely meets the requirements for critical scientific interpretation. The discussion follows the logic of the presented results – each discussed aspect has a direct empirical basis. The relationship between morphometric analyses and

the change in the surface/area of the cerebellum, immunomarkers and the state of granulosa cells, disorders in the clustering of Purkinje cells, and Pax6-dependent signals is particularly well traced.

The PhD student has reasonably identified Pax6 as a key regulator of the normal formation of the cortical structure of the cerebellum. The observations presented on the model (Tel-Cre::Pax6$^{fl/fl}$) are associated with specific phenotypic manifestations such as: 1) deformation and reduction of the cerebellar foliation; 2) aberrations in the location of granulosa and Purkinje cells; 3) impaired laminar arrangement. These findings are compared with existing data from *in vitro* and embryonic models, adding depth to the analysis and demonstrating thorough field knowledge.

Radoslav Spasov convincingly argues that the present study is **the first** to demonstrate a postnatal cerebellar phenotype upon conditionally reduced expression of Pax6 in vivo. This original scientific contribution expands the understanding of Pax6's postnatal functions beyond its embryonic role.

The study expands our knowledge of the role of Pax6 after birth, showing that it is required not only for embryonic brain formation, but also for: the correct postnatal organisation of the cerebellum layers; the proliferation, migration and positioning of neurons. A viable model has been created for the first time in which Pax6 expression is specifically reduced in the cerebellum before birth, but without compromising animal survival. This overcomes the limitations of global knockout models in which perinatal death occurs. Specific morphological and cellular abnormalities have been documented, such as reduced area, granulose layer thinning, ectopic cells, and loss of specific neuronal populations, all observed at different age stages. This highlights the role of Pax6 in the dynamics of neurodevelopment.

The model and methodology used in the dissertation provide a new toolkit for future research on molecular mechanisms of neurodegeneration, brain plasticity and recovery, and studies of human diseases associated with Pax6 (e.g., aniridia, atypical forms of autism, and cerebellar hypoplasias). The combination of quantitative (morphometry, statistics) and qualitative (histology, immunofluorescence) methods leads to reliable conclusions and increases the scientific value of the data.

The conclusions and contributions demonstrate that the dissertation: successfully fills a gap in knowledge regarding the postnatal function of Pax6; offers a new model and new data with strong

applicability in neuroscience; contributes not only to fundamental biology, but also to potential biomedical applications.

The doctoral candidate demonstrates real scientific activity through participation in an international forum and publications in scientific journals. Although the main results are published in a national publication, it is recommended to publish at least one leading article in an international peer-reviewed journal to increase the visibility of the contribution.

The *doctoral thesis abstract* of Radoslav Hristov Spasov is structurally complete, scientifically adequate and convincingly presents the main results, methods and contributions of the dissertation work. The presentation of the original model and its results is argued. The linguistic culture and stylistic consistency contribute to high scientific credibility.

IV. Conclusion

The presented dissertation work of Radoslav Hristov Spasov, MD, results from in-depth experimental work, scientific commitment and an original research approach. By developing a viable model with conditional loss of Pax6 function, the doctoral student successfully overcame a long-standing limitation in the development of neuroanatomical research, namely the impossibility of studying postnatal effects of global knockout of this key transcription factor. The results presented in the dissertation not only complement the existing knowledge, but also expand it with specific morphological, quantitative and cellular data on the development of the cerebellum. Of particular note is the establishment of the interdependence between glutamatergic and GABAergic neurons and the possible role of Pax6 in maintaining neuronal architecture and migratory processes. The content of the dissertation is scientifically sound, clearly presented, and reflects a highly experimental culture. The layout, language quality and published results further testify to maturity and professionalism in scientific work.

I confidently state that Radoslav Spasov, MD, possesses the qualities of an independent researcher. I also express a positive opinion about the developed dissertation work. As a member of the scientific jury for the procedure, I give my **positive vote** for awarding the educational and scientific degree "Doctor of Philosophy" to Radoslav Hristov Spasov, MD.

Prepared the

Prof. Dimitrina Atanasova Dimitrova, PhD

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Stara Zagora