REVIEW

by Prof. Albena Jordanova, PhD - Sofia University "St. Kliment Ohridski", Faculty of Medicine, Department "Chemistry and Biochemistry, Physiology and Pathophysiology", member of the scientific jury, appointed by order No. 132-OB/03.09.2024 of the Director of the Institute of Molecular biology "Acad. Rumen Tsanev"

Assoc. Prof. Anastas Gospodinov, PhD

of a PhD thesis for awarding the educational and scientific PhD degree, in the Field of higher education: 4. Natural sciences, mathematics and informatics; Professional field: 4.3. Biological sciences; Scientific specialty: Molecular biology

Author: *Aleksander Sergeev Atemin - PhD student in the Laboratory of Genomic Stability, Institute of Molecular Biology "Acad. Rumen Tsanev" – Bulgarian Academy of Sciences (BAS)*

Dissertation topic: *Studying the dynamics of processes in living cells through modern microscopic approaches*

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In the PhD thesis by Aleksander Sergeev Atemin - a full-time PhD student at the Laboratory of Genomic stability, Institute of Molecular biology "Acad. Rumen Tsanev" - BAS, the dynamics and kinetics of the process of entry of the SARS-CoV-2 virus into the host cells were studied in depth and visualized and analyzed, as well as the dynamics of the levels of various replication-related proteins (RIF1, ORC1, MCM6, Claspin, PCNA) during the cell cycle.

It is known that COVID-19 is a disease caused by a new strain of coronavirus, called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which hit the human population after December 2019. On March 11, 2020 the World Health Organization (WHO) declared it a pandemic and this viral attack turned the lives of all people on Earth upside down, causing the death of over 7 million people. Bulgaria was in 87th place in the world in terms of the number of infected people per 1 million

inhabitants (over 187 thousand) and in second place in the world (after Peru) in terms of the number of deaths per 1 million inhabitants (over five thousand) - a total of 37,965 deaths. On 4 May 2023, the WHO Director-General announced that COVID-19 was no longer a public health emergency of international concern and emphasized that despite increased immunity and fewer hospitalizations and deaths, there are still question marks surrounding how SARS-CoV-2 infects cells and evolves.

That is why the goal set in the dissertation work of Aleksander Atemin is relevant and not only fundamental, but also socially significant: to study the dynamics of the processes accompanying the entry of SARS-CoV-2 virus-like particles into host cells, and in addition - the dynamics of the levels and distribution in the cell of various proteins involved in DNA replication during the cell cycle are also investigated.

To realize the set goals, six main tasks are planned for implementation, and virus-like particles possessing all the structural proteins of SARS-CoV-2 and non-essential genetic information were used and tested. These particles are known to enter host cells by a known mechanism, but cannot reproduce and infect other cells. In order to follow the dynamics and kinetics of the process of entry of viral particles into the cell, various structural proteins of SARS-CoV-2 were labeled in the viral membrane and genome, and in addition, pH changes in the viral particle were determined. In addition, the dynamic behavior of various replication-associated proteins was also investigated by creating dual-labeled *HeLa Kyoto* cell lines that express EGFP-ORC1, MCM6, Claspin, RIF1, or PCNA-tagged PCNA together with mCherry-tagged PCNA. Through highly informative microscopic techniques, the expression and distribution of the investigated proteins in the analyzed cell lines was followed. What's more - after a comparative analysis of the captured microscopic images with a very high time resolution, the dynamics of the tested proteins throughout the entire cell cycle was established.

The dissertation is designed extremely carefully with some minor technical errors and logically leads the reader from the in-depth and detailed Literature Review, through the set Goals and Objectives and Materials and methods to the Results and Discussion section. The PhD thesis covers 128 pages, contains 24 figures, and numerous panels or dozens of photographs are presented on them. The bibliographic reference includes 247 up-to-date literary sources from renowned and indexed scientific publications, the majority of which are from recent years.

In the ***Literary review***, a thorough analysis of the considered problem was made, describing the modern ideas about the etymology of SARS-CoV-2, the genome, structural and non-structural proteins of the virus, as well as the importance of 9 additional factors with a specific function in the viral replication. The life cycle of SARS-CoV-2, the attachment of the virus to the surface of the host cell via the S-protein, the implementation of membrane fusion, the formation of the replication-transcription complex of the virus, synthesis of the viral RNA, self-assembly and release of the formed SARS-CoV-2 viruses are described in detail. The ***Literature review*** includes up-to-date information on current treatment strategies for COVID-19, vaccines used against COVID-19, and the dynamics and distribution of various replication-associated proteins in *HeLa Kyoto* cells. My only remark about the presented Literature review is the lack of sufficient illustrative figures to present the considered processes more informatively.

The ***Literature review*** logically leads to the research goals that Alexander Atemin has set for himself:

1. Research the dynamics of the processes accompanying the entry of SARS-CoV-2 virus-like particles into host cells.

2. Study of the dynamics of the levels and distribution in the cell of proteins involved in DNA replication during the cell cycle.

To achieve these goals, the following 6 main tasks are planned for implementation:

- Visualization of the entry and tracking of the movement of SARS-CoV-2 virus-like particles in the cell.
- Measurement of the velocity in 3D of the particles entering the cell.
- Measurement of pH change during entry of SARS-CoV-2 virus-like particles.
- Measurement of nucleocapsid release dynamics from SARS-CoV-2 virus-like particles upon entry into the cell.
- Measurement of changes in rate, pH as well as nucleocapsid release dynamics of SARS-CoV-2 virus-like particle variants.
- Determination of the levels and distribution of replication-associated proteins ORC1, MCM6, Claspin, RIF1, PCNA during the cell cycle at the single cell level.

The ***Materials and methods*** section includes information on the three cell lines used, VeroE6, U2OS, and A549, transfection steps with fluorescently labeled proteins, Andor Dragonfly spinning disk confocal system time-lapse microscopy of living cells, electron microscopy of MLE-12 cells, information on the synthesis of VLPs, inhibition of SARS-CoV-2 virus-like particles with recombinant Anti-SARS-CoV-2 Spike Glycoprotein S1 antibody, tracking and analysis of virus-like particle dynamics, and analysis of images obtained by microscopy for extended period of time and normalization of results when tracking all proteins during the cell cycle. Appropriate and up-to-date software programs and statistical methods were used in the analysis of the results.

The obtained ***Results*** and the subsequent ***Discussion*** are presented and analyzed in detail in 59 pages, with rich and detailed illustrative material in the form of real-time microscopic images, graphs, diagrams and schems. The results are presented in three main directions: dynamics of the internalization of virus-like particles, study of the dynamics of the levels of replication-related proteins during the cell cycle and development of a database for the purpose of visualization of the obtained results. In studying the internalization dynamics of SARS-COV-2 virus-like particles, original results are presented on the mode of entry of virus-like particles, the role of dynamin in the endocytosis of virus-like particles, the dynamics of acidification of virus-like particles, by labeling with pHluorin, the kinetics of virus-like particles lacking the Furin protease recognition site, the kinetics of virus-like particles containing all mutations characteristic of the Omicron variant of SARS-CoV-2, and the rate of nucleocapsid release from virus-like particles. In the study of the dynamics of the levels of replication-related proteins during the cell cycle, results were presented that indisputably prove the ability of the PhD student to plan and independently conduct molecular biological experiments: by creating double-labeled cell lines, they were studied protein levels during the cell cycle; measured the fluorescence of the protein PCNA (a key factor in DNA replication and repair) as an indicator of early, mid, and late S-phase, as well as the dynamic changes in other replication-related proteins during the cell cycle in single eukaryotes cells: Rif-1, Orc-1, Mcm-6 and Claspin. At the conclusion of the large-scale research conducted, a database was developed to visualize the results obtained. Access to the database https://covidynamics.imb.bas.bg/ is free and the obtained results for the internalization of the virus-like particles are available to scientists from all over the world, with detailed descriptions of the experimental conditions, the fluorescent markers

of the cells and the virus-like particles, as well as real-time video recordings with and without available particle tracking. A database was also developed on the role of different proteins involved in DNA repair: visualization of the dynamics and their accumulation at the sites of DNA damage. The discussion part in the dissertation is relatively short, but summarizes the main results obtained during the development of the SPARTACUSS software for tracking SARS-COV-2 virus-like particles, the creation of the free databases https://covidynamics.imb.bas.bg/ and "DNArepairK: An interactive database for exploring the impact of anticancer drugs onto the dynamics of DNA repair proteins", a study of the dynamics of the entry of SARS-CoV-2 virus-like particles into the target cells, as well as a study of the dynamics of proteins, involved in DNA replication. My personal opinion is that the two sections Results and Discussion could have been combined, but even so presented do not in any way cast doubt on the highly informative results obtained.

The results obtained during the in-depth research conducted in the dissertation work are formulated in 8 main conclusions and 5 important contributions of scientific-applied importance. The results have been published in 3 scientific articles with a remarkable total impact factor of 13,457 in the period 2021-2023 and thus the legal requirements for acquiring the scientific and educational PhD degree have been fulfilled. In two of the publications, the PhD student is the first author, which is proof of his essential role, his underlined interest and commitment to the researched issues. The obtained results have been reported at 11 International and National Scientific Conferences with reports and posters, and the fact that in all the participations the PhD student is the first author is impressive. The presented abstract of Aleksander Atemin's dissertation work is perfectly designed, fully corresponds to the content of the PhD thesis and provides complete information about the conducted experiments, the obtained results, discussion and analysis of the research.

In conclusion, I would like to express my positive impression of the fact that the role of the team of the Genomic Stability Laboratory at IMB-BAS in carrying out the conducted research, as well as the contribution and presence of the unforgettable and loved by all of us Assoc. Prof. Marina Nedelcheva-Veleva.

I have the following questions for the PhD student:

1. How can you explain the increased susceptibility and faster internalization of virus-like particles in kidney VeroE6 cells compared to lung A549?

2. How can the results obtained by you and your team be applied in clinical practice to create therapeutic approaches in the treatment of viral infections similar to SARS-COV-2?

3. What are your future plans and in what direction will you continue your research?

CONCLUSION

The presented PhD thesis is the result of a precisely conducted study, the results of which are of significant theoretical and applied significance. The obtained innovative results reveal details of the way of entry of SARS-CoV-2 virus-like particles into the affected cells, the role of microtubules and dynamin in this process, the decrease of pH after the penetration of the particles, etc., as well as the changes in the levels and distribution of replication-related proteins RIF1, ORC1, MCM6, Claspin, PCNA, which proves the fine regulation of their expression and localization during the cell cycle.

The PhD student Aleksander Atemin has in-depth theoretical knowledge and professional skills in the scientific specialty Molecular biology, demonstrating qualities and skills for independent conduct of scientific research. He is able to apply a number of modern techniques and methods: cell culture, transfection of cell lines with different proteins, inhibition with different reagents, microscopy of living objects for an extended period of time, development of software solutions and databases, tracking and analysis of objects obtained after microscopic observation, which undoubtedly makes him a young and promising scientist, capable of independently analyzing the obtained results and comparing them with literary data in the field of research conducted.

Based on the above, I can confidently state that the large-scale dissertation thesis on the topic *"Studying the dynamics of processes in living cells using modern microscopic approaches"* represents an original scientific study. It meets all the conditions of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Rules for its Application and the Rules of the IMB "Acad. Rumen Tsanev" - BAS.

All this gives me the reason to fully convinced to recommend that the PhD student Aleksander Sergeev Atemin be awarded the educational and scientific PhD degree in the field of Higher education: 4. Natural sciences, mathematics and informatics; Professional field: 4.3. Biological sciences; Scientific specialty: Molecular biology.

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