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Referee report on the doctoral thesis by MTech Manohara Mahadeva

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Title: *Membrane potential sets the tempo: Bioelectricity controls the rate of somitogenesis in chick embryos through mechanics*

The presented doctoral dissertation by MTech Manohara Mahadeva was conducted under the supervision of Dr. habil. Magdalena Kowacz in the Reproductive Pathology and Translational Medicine Team at the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, as part of the Sonata Bis 2020/38/E/NZ3/00039 project funded by the National Science Centre, Poland. The doctoral thesis primarily focused on the roles of bioelectricity and membrane potential in somitogenesis and tissue mechanics in chick embryos. The research explores the role of the membrane potential (V_m) in regulating somitogenesis, a critical embryonic developmental process in which somites form in a rhythmic, spatially coordinated manner. Using chick embryos as a model, the study shows that V_m changes as somites mature, with step-like hyperpolarization reflecting the onset of biochemical and structural changes.

Somitogenesis is a vital process in vertebrate embryonic development because it establishes the segmented body plan that underlies the axial skeleton, skeletal muscles, and dermis. By periodically forming somites along the anterior–posterior axis, this process ensures proper spatial alignment and proportional body growth. Somites develop into the sclerotome, myotome, and dermatome, which later differentiate into vertebrae and ribs, skeletal muscles, and the skin's dermis, respectively. Somitogenesis also regulates cell proliferation, migration, and differentiation in a highly controlled manner by integrating molecular signaling pathways, including Wnt, Notch, and FGF. Proper execution of this process is essential for normal organ formation and neural development, while disruptions can lead to congenital abnormalities, including scoliosis and other musculoskeletal defects. Therefore, somitogenesis is not only fundamental to vertebrate development but also serves as a valuable model for studying developmental regulation, the evolution of body segmentation, and potential regenerative medicine applications. Its influence extends beyond embryonic development, as insights into this process can inform many areas of biology and medicine. Understanding how somite formation is regulated may help develop strategies to prevent or treat congenital musculoskeletal disorders resulting from segmentation defects. Overall, advances in understanding somitogenesis have the potential to impact developmental biology, evolutionary studies, and clinical approaches to tissue repair and disease treatment. Thus, it is critical to understand the process and continually refine our understanding of the factors influencing somitogenesis, including the roles of bioelectricity, environmental influences, and cell and

tissue mechanics. In the last decade, new technologies have enabled single-cell or even molecular-scale research. Based on the obtained results, various detection systems have been developed that exploit electrical, optical, mechanical, chemical, and biological phenomena. They can be applied to monitor disease- or process-related alterations and to deliver information on specific mechanisms involved, thereby supporting their detection, diagnosis, and treatment. The research conducted by MTech Manohara Mahadeva explores fundamental biological processes and their implications for developmental biology, regenerative medicine, and pathological conditions. It is an essential component of mainstream research on identifying the mechanisms underlying somitogenesis and exploring potential strategies to delay disease progression. The title of the doctoral dissertation already fully reflects its content and indicates that the research topic lies at the boundary between biology and physics. Such an interdisciplinary approach has great potential to deliver results of great practical importance.

The doctoral dissertation is written in English. It is well organized, well documented, and well written, making the thesis enjoyable to read. It clearly presents the research subject, objectives, and means to achieve them. A helpful list of abbreviations and a list of related papers authored or co-authored by MTech Manohara Mahadeva are presented at the beginning of the dissertation, followed by a clear summary (abstract). The doctoral dissertation consists of two published articles and one submitted manuscript. The scientific works were published in journals listed in the Journal Citation Reports (JCR). The doctoral candidate is the first author of all these works, and her contributions are significant. The publications are thematically coherent and address the same research problem. In the summaries written in both Polish and English, the doctoral candidate presented the experimental results concisely and clearly.

The doctoral dissertation begins with an introductory chapter (Chapter 1) that provides background on the importance of studying somitogenesis and the current state of the art. Chapter 1 provides an introduction to somitogenesis, a rhythmic, tightly regulated developmental process essential to embryogenesis. It highlights the importance of understanding the mechanisms that synchronize somite segmentation and growth to maintain body proportionality during development. The chapter reviews existing models, such as the clock-and-wavefront model. It discusses the role of the membrane potential in regulating cellular processes, including migration, self-assembly, and proliferation, during somite formation. It also presents the research hypothesis that membrane potential may also regulate the rate of somitogenesis by altering the biomechanical properties of somite-forming cells. It outlines the study's objectives, exploring membrane potential modulation and investigating its effects on tissue mechanics and the rate of somitogenesis. The author of the presented doctoral thesis highlights the uncertainties and unresolved questions that remain. Such a problem description requires the candidate to possess substantial knowledge and insight into the research conducted to date. Indeed, the attached bibliographic list (over 260 positions) and references cited in previously published scientific papers attest to the extensive study of the subject and the Ph.D. student's commitment to the doctoral dissertation. Chapter 1 concludes with clearly defined thesis objectives. The objectives are clearly stated and well-defined. Each objective is

specific and avoids ambiguity, making the research's scope and purpose easy to understand. I would note that, for non-specialist readers, a separate chapter describing the methods used might offer additional clarity.

Chapter 1 is followed by Chapter 2, which focuses on the dependence of a cell's membrane potential on extracellular voltage (V_z) using *Chara globularis* as the experimental model. The results were published in Mahadeva et al. *Biophysical Chemistry* 307 (2024) 107199, in which phD candidate is the first author. The key findings indicate the presence of extracellular voltage and its coupling to the membrane potential. The study demonstrated that the internal voltage and extracellular voltage can be independently modulated, as reflected in the overall membrane potential. Alongside the effects of external factors such as CO_2 and KCl, the evaluation was conducted. External CO_2 increases the negativity of extracellular voltage and expands the extracellular voltage zone (ZEV), while also depolarizing the cell. Similarly, KCl induces depolarization and enlarges the ZEV. The study confirms that the changes in extracellular voltage are not due to pH alterations but are caused by charge separation at the cell membrane/wall interface. The ZEV forms near regions of enhanced ion exchange, such as spines and wounded cells, suggesting that its formation is linked to ion exchange across the cell wall and membrane. Altogether, these results show the existence of a significant extracellular voltage zone near *Chara globularis* cells, explore its behavior under external stimuli such as CO_2 and KCl, and demonstrate its coupling with the cell's membrane potential. This research provides new insights into the generation and regulation of membrane potential beyond traditional ion-channel mechanisms. The study highlights the importance of considering extracellular voltage as part of membrane potential, as it influences cell physiology, ion exchange, and the local electrochemical environment. Consequently, the ZEV may act as a protective barrier against pathogens by excluding colloids and bacteria. While the study provides valuable insights into the combined effect of external and internal CO_2 on membrane potential, some aspects could be further explored or clarified to strengthen the findings, e.g.:

- The study mentions the additive effect of external and internal CO_2 on *membrane* potential, but it does not delve deeply into the underlying mechanisms. For example, how does external CO_2 interact with the cell membrane or extracellular voltage to cause depolarization? Similarly, how does internal CO_2 lead to hyperpolarization?
- The study suggests a coupling effect between the external and membrane environments. Still, it does not explicitly address how the combined effects of external and internal CO_2 influence extracellular voltage.
- The study mentions that the combined effect of external and internal CO_2 is additive, but it does not provide a detailed quantitative analysis or model to predict the exact relationship between the two factors.
- The study could discuss the broader implications of the combined CO_2 effect on cellular physiology, including its relevance to developmental processes, environmental stress responses, and pathological conditions such as tumorigenesis.

Moreover, Chapter 2 presents results that serve as a foundation for Chapters 3 and 4, establishing the critical relationship between membrane potential and extracellular voltage and demonstrating how external stimuli such as CO_2 and KCl can modulate cellular behavior. In Chapter 2, the experimental framework involving microelectrodes and controlled environmental conditions provides a robust methodology for exploring bioelectric phenomena, which is directly applied in Chapters 3 and 4. Chapter 3 builds on this model by investigating how membrane potential influences the periodicity of somite formation and growth in chick embryos, revealing that it synchronously regulates cell migration, self-assembly, and proliferation. Similarly, Chapter 4 extends the principles established in Chapter 2 to examine how membrane potential affects somite deformability, demonstrating that induced changes in membrane potential directly alter the mechanical properties of somite-forming cells. The experimental techniques, analytical approaches, and conceptual framework introduced in Chapter 2 are integral to the investigations in Chapters 3 and 4, thereby highlighting the interconnectedness of bioelectricity, biomechanics, and developmental biology. Therefore, given the significance of Chapter 2 to the overall PhD thesis objective, a summary of the extent to which algal cells can model somitogenesis (pros and cons) should be included.

Chapter 3 examines the role of the membrane potential in regulating somitogenesis in chick embryos directly. The study demonstrates that this potential can simultaneously control the periodicity of somite formation (cell migration and self-assembly) and somite growth (cell proliferation), ensuring consistent body patterning during early embryogenesis. It contains the results published in Mahava et al. *Developmental Biology* 517 (2025) 317, in the paper, in which the PhD candidate is the first author. The key findings showed that somites progressively hyperpolarize as they mature, with step-like changes in membrane potential observed between specific somite groups. These changes correlate with the onset of biochemical and structural transitions during development. Increased CO_2 and KCl levels depolarized somite-forming cells, whereas reduced levels hyperpolarized them. Depolarization accelerated somite segmentation and growth rates, whereas hyperpolarization slowed them. Overall, Chapter 3 establishes membrane potential as a critical factor in synchronizing somite formation and growth, offering a new perspective on the regulatory mechanisms of embryonic development. Some aspects are not fully considered or explored in the study presented in Chapter 3. For example, the study suggests that membrane potential may regulate gene expression through pathways such as β-catenin/Wnt signaling; however, the exact molecular mechanisms remain speculative. Another example, the study does not explore other bioelectric signals, such as endogenous electric fields or ion fluxes, which may also play a role in somitogenesis and embryonic development. Although no biomechanical data were presented in this Chapter or in the associated paper, it is postulated that depolarization softens cells, promoting faster segmentation and growth. In contrast, hyperpolarization increases rigidity, slowing these processes. The study highlights the role of membrane potential but does not fully explore how it interacts with other factors, such as mechanical forces or ECM composition, leaving this postulate speculative.

Chapter 4 explores how membrane potential is linked to the mechanical properties of somite-forming cells during early embryonic development. The study demonstrates that membrane potential changes as somites mature, with progressive hyperpolarization and increased rigidity along the anterior-posterior axis of chick embryos. These results constitute the third manuscript submitted by the PhD candidate, who is the first author. In this Chapter, biomechanical data are presented, thus supporting the declaration of Chapter 3. The key findings of this part of the PhD thesis showed that abrupt changes in membrane potential between specific somite stages reflect the onset of biochemical and structural changes during development. Changes in somite deformability (aspiration length) are linearly dependent on induced changes in membrane potential, regardless of somite position or applied pressure. Somites exhibit viscoelastic properties, with younger somites being softer and less viscoelastic compared to older, stiffer somites. And finally, induced mechanics-related changes may be mediated by cytoskeletal alterations, particularly actin filament polymerization, which is sensitive to intracellular pH. Depolarization increases intracellular pH, promoting actin depolymerization and cell softening, while hyperpolarization decreases intracellular pH, stabilizing the cytoskeleton and increasing cell rigidity. My remarks here concern the conclusion that the mechanics of somites depend on actin filaments because actin is a key component of the cytoskeleton, which plays a central role in determining cell mechanical properties. The aspiration length of a few tens of microns was considered here to be influenced by actin filaments, which seems to be true if the aspiration length is smaller than $L = 0.2R_p$ (where R_p is the radius of the micropipette). For a used micropipette with an inner diameter (ID) of ~60 μm, L = 12 μm. Above this value, I would consider the effects from microtubules. Although biologists often use cell stiffness as a synonym for cell rigidity, the PhD thesis should include a clear definition of both. Stiffness is a measure of how much a system resists deformation when a force (or pressure) is applied. It is often defined as the slope of stress vs. elongation in the small-deformation regime, and it is not an intrinsic material constant because it depends on geometry (i.e., entire mechanical configuration). Young's modulus, as calculated in the PhD thesis, describes the material's elasticity and is an intrinsic material property.

The last part of the thesis, containing Chapters 5-7, is a discussion and summary that includes a list of open questions still to be resolved. The discussed chapters present membrane potential as a key regulator of somitogenesis, integrating bioelectric, mechanical, and biochemical processes during embryonic development, emphasizing the coordination of cellular processes like migration, self-assembly, and proliferation to produce somites of precise size and timing. The implications and future perspectives (Chapter 7) discuss in addition challenging traditional views of membrane potential generation and suggest its influence on cellular physiology, signaling, and pathogen defense. The study proposes links among membrane potential, cell mechanics, and signaling pathways. However, the study’s reliance on indirect evidence for key mechanisms, such as cytoskeletal reorganization and intracellular pH variations, weakens its conclusions. The study could benefit from a more critical evaluation of its limitations and alternative mechanisms, as well as robust experimental evidence to support its claims. Future research should address these gaps to strengthen the proposed model and its

applicability to other biological systems. Apart from the questions/remarks already asked, I have a general question: why is statistical significance, although mentioned in the PhD thesis, not presented there?

The noted inaccuracies and my comments do not affect the substantive value of the work, but they encourage a broader discussion. Manohara Mahadeva's doctoral thesis presents an innovative and original approach. The results are original and scientifically novel, and, most importantly, they open new research opportunities. They are valuable not only for elucidating the mechanisms of somitogenesis but also for offering a distinct perspective on tumorigenesis, which could affect the ongoing research. Moreover, the PhD thesis demonstrates that developing new biophysical methodologies is a promising approach to addressing multiple challenges. Understanding these processes at the molecular level is essential for addressing unresolved questions and for developing rational approaches to physiological processes and pharmacological treatments to combat these diseases.

Taking into account all aspects discussed in the review, I conclude that the dissertation fulfills all requirements for doctoral theses as stipulated by the Act on Academic Degrees and Title (Article 187 of the Act of 20 July 2018 – Law on Higher Education and Science, Journal of Laws of 2018, item 1571, as amended, in Polish: *art. 187 ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce, Dz.U. z 2018r., poz. 1571 z późn. zm.*) and as well as the applicable academic standards, and I assess it positively. Therefore, I recommend that MTech Manohara Mahadeva be admitted to the next stage of the doctoral dissertation defence procedure.

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