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Name

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EVALUATING THE EFFECT OF EXTRACELLULAR Ca2+ ON CELL MIGRATION IN U2OS CELLS

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Student Major

ACS Certified Chemistry/Biochemistry major

Narrative Questions

1. **Describe how you came to choose your topic, specifically noting any pre-research that you did. What sources did you use in this pre-research? To what extent did you consult with librarians, faculty, or others? How did this pre-research lead you to your topic?**

   Have you ever wondered how wounds heal? I have. I noticed the wounds I got from falling off the bike started to form a scrape days after. Being curious enough, I rubbed the scrape just to discover a newly formed reddish area of skin underneath. While it was fun to think that was magic, I soon realized something else happened in the body that tells the skin cells to come together and heal the wound. Yet, what is that? My question was only answered when I joined Dr Martha Grossel's Molecular Cell Biology (BIO 309). She introduced me to one of the most inspiring talks ever, "The electrical blueprints that orchestrate life" by Dr Micheal Levin from Harvard University. I could never forget the Picasso Frog that he presented, going from a distorted facial structure to a normal frog face. "So it turns out that all cells, not just nerves, but all cells in your body communicate with each other using electrical signals" - he said. "Cell communication" were the two words that kept my interest up until this point. I was confused and curious at the same time since I previously thought that only nerve cells have the capability to communicate through signals. Although the topic was so interesting, it was not the main theme of my BIO 309 class. Therefore, I decided to sign up for an online course called "Introduction to Bioelectricity" taught by Dr Pedro Irazoqui, Dr Rebecca Bercich, and Dr Dan Pederson from Purdue University. There, they presented me with an experiment done by Zhao et al.'s group in 2002 in which they applied an electrical field with polarity opposite to the default wound healing direction. To my surprise, the reversal of the endogenous electrical polarity caused the cells to migrate away from the wound. From biology classes, I have been taught that contact inhibition release and population pressure are some of the most influential cues that can direct wound healing. However, the bioelectric signals proved to be the dominant cue that won over other cues and make the body do something opposite to its instinct (Zhao 2009, 674-682). I directly emailed Dr Pederson to ask more about that experiment and we talked about bioelectricity for an hour. Seeing my interest, he suggested I do my research project in bioelectricity and see it in real life. Therefore, when I was given a chance to conduct research in Dr Grossel's class, I told her about my intention, to which she suggested I either use ion addition or ion channel blockers. After reading a few articles about the toxicity of ion channel blockers, I decided to use ion addition as my experimental design. Yet, I did not know where to start. I sought help from the library and was advised by two awesome librarians, Andrew Lopez and Kathy Gehring, to use the powerful ConnColl library website, where I was able to find databases listed by field of interest. They helped me figure out about the memberships for these databases, which allowed me to find great

resources for my research. I ended up using ScienceDirect, Scopus, Google Scholar, Nature, and PubMed for my resources. Dr Deborah Eastman was kind enough to show me a trick where I can not only search for my topic but also use the keywords embedded in the topic. Dr Martha Grossel helped me with brainstorming ideas and forming my hypothesis. Thanks to all of these wonderful people, I was able to finish my research project and present it to the scientific community. References: Zhao, Min. 2009. "Electrical Fields in Wound healing—An Overriding Signal that Directs Cell Migration." Seminars in Cell & Developmental Biology 20 (6): 674-682. https://www.sciencedirect.com/science/article/pii/S1084952108001535. Levin, Michael. "The electrical blueprints that orchestrate life." Posted March 31, 2023. TED, 19:40. https://youtu.be/XheAMrS8Q1c

2. **Describe your process of finding information for your project. Note specifically the tools you used to undertake your research, as well as the specific search strategies you used within these tools. (Note: “Ebsco,” being an umbrella vendor, is not a specific enough response when identifying tools; listing the “library database” is also an unacceptably vague answer. Specific tools include JSTOR, America: History & Life, Web of Science, etc., along with OneSearch, the new library system.)**

In my freshman year, I was introduced to OneSearch and Refworks thanks to Professor Michael Chan. His workshop on finding the right references was very helpful and gave me more tools to find the sources for my paper. During his workshop, I showed us the trick to search in OneSearch, where you can type a chain of words, such as "technology + anime", which was relevant to my literature research back then. In my sophomore year, Dr Deborah Eastman showed me how to word my search in a better way. My research was on gene-editing tools; therefore, she suggested I find some synonyms and related words to find more papers since the titles can be written differently but have similar meanings. Instead of just typing "gene-editing tools", I opened my search to "CRISPR-Cas9", "RNA-guided CRISPR-Cas9", "EColi lacZ gene modifications", etc., which were the same thing. I also used the filter search on Scopus and OneSearch by selecting my research interests, which quickly gave me access to the resources I need. Then, when I joined Dr Martha Grossel's class, she gave me a very smart trick where I can start by finding research that I am interested in and then use the references in that paper to have more resources. She also shared with me one of her tips to find research articles when I have vague ideas about what I intend to write, which is using the review papers as the foundation. On PubMed, there is a filter search where I can specifically look for review papers and see if there is a full-text option. Using review papers as a start, I acquired more generalized information about bioelectricity and aspects that I can research on. The reference section in review papers is particularly long, yet they are broken down into subcategories; therefore, I can have a large pool of references related to my research interest. I ended up reading the review paper of Tyler (2017, 627), where they discussed the ability to move of cells under the influence of bioelectric signals. This was very close to my field of interest, and in the experiments mentioned by Tyler's group, the study of Lee et al. (2018, 1-16) "Elevated Extracellular Calcium Ions Promote Proliferation and Migration of Mesenchymal Stem Cells Via Increasing Osteopontin Expression" captured my attention and ended up becoming the inspiration of my research. However, I did not want to replicate his experiments. Instead, I researched a different type of cells, U2OS cells, to see if the results were comparable with one another. I used a fairly similar experimental design, which was a wound healing assay, but I altered the concentration of the calcium ion influx. In the end, my results were very similar to his, and they were also backed up by other researchers who conducted similar testing designs. Thanks to the wonderful help of the aforementioned people, I was able to prove my hypothesis

and contributed my research to the field of bioelectricity. References: Tyler, S. E. B. 2017. "Nature's Electric Potential: A Systematic Review of the Role of Bioelectricity in Wound Healing and Regenerative Processes in Animals, Humans, and Plants." Frontiers in Physiology 8: 627. doi:10.3389/fphys.2017.00627. Lee, Mi Nam, Hee-Su Hwang, Sin-Hye Oh, Amir Roshanzadeh, Jung-Woo Kim, Ju Han Song, Eung-Sam Kim, and Jeong-Tae Koh. 2018. "Elevated Extracellular Calcium Ions Promote Proliferation and Migration of Mesenchymal Stem Cells Via Increasing Osteopontin Expression." Experimental & Molecular Medicine 50 (11): 1-16. doi:10.1038/s12276-018- 0170-6.

3. **Describe your process of evaluating the resources you found. How did you make decisions about which resources you would use, and which you wouldn't? What kinds of questions did you ask yourself about resources in order to determine whether they were worthy of inclusion?**

After I started my literature review, as always, I struggled to decide what would be the most valuable resources for my research. Google Scholar and Scopus were very useful; however, they are big databases where millions of results can be found just after one click. I figured that I don't have that much time to read through all of the references; therefore, I used a strategy to cope with this. What scientific question do I seek to answer? - I asked myself. As I mentioned before, I was curious about bioelectric signals and their ability to provide a network of cells in the body for tissue development and other functions. Therefore, I started by reading a review paper to get an overview of the aspects included in the big umbrella of bioelectricity. After reading the review article, I was able to narrow down my research interest and start to form my hypothesis. Back to my initial curiosity about the wound healing process combined with my interest in bioelectricity, I questioned myself: How can I incorporate these two components in my research? In the review paper, I came across the study of Lee et al. (2018, 1-16) that discusses the phenomenon of increased cell motility due to exposure to calcium ion influx. I was then curious if the same phenomenon will happen in other types of cells than mesenchymal stem cells. Therefore, I chose U2OS cells as my testing subject. My hypothesis was to prove that cell migration is influenced by the influx of calcium ions in a concentration-dependent manner in U2OS cells. Since I based my research on Lee et. al's study, I utilized the references that he used in his research, read this small collection of resources, and evaluated which would fit my research the most. Lee's research focused on mesenchymal stem cells, which are also extracted from bones like U2OS cells (bone marrow cells). Therefore, his research was very valuable in terms of providing the cross-check and confirmation for my research. I also tried to look for research articles in Nature and PubMed that used either U2OS cells or mesenchymal stem cells with the ion addition method to add to my RefWorks folder of related articles. Then, I thought: Wouldn't it be better if my research results are comparable to a variety of testing subjects with similar experimental designs? Therefore, I started to look at other people's works that had similar ideas but tested on other testing subjects to provide a more logical hypothesis. I limited my search in ion addition so that it can be close to what I wanted. After finding very few articles, I decided to also search for research that can have different testing subjects and ions used but have the same testing design. I asked myself: If they use different methods and different testing designs, but they achieved similar results to those of Lee's group, can they also be valuable resources? By comparing and contrasting the results of more than 10 research groups in the field of wound healing powered by bioelectric signals, I was able to find the resources needed

for my research and backed up my hypothesis. References: Lee, Mi Nam, Hee-Su Hwang, Sin-Hye Oh, Amir Roshanzadeh, Jung-Woo Kim, Ju Han Song, Eung-Sam Kim, and Jeong-Tae Koh. 2018. "Elevated Extracellular Calcium Ions Promote Proliferation and Migration of Mesenchymal Stem Cells Via Increasing Osteopontin Expression." Experimental & Molecular Medicine 50 (11): 1-16. doi:10.1038/s12276-018- 0170-6.

EVALUATING THE EFFECT OF EXTRACELLULAR Ca^{2+} ON CELL MIGRATION IN U2OS CELLS

By Binh Vo

I. INTRODUCTION

Cell migration is the process in which cells relocate themselves to reach the desired destination for a specific purpose in response to intracellular and extracellular cues (Pijuan et al. 2019; Te Boekhorst, Preziosi, and Friedl 2016, 491-526). It plays a significant role in ensuring proper cellular organization and protecting the well-being of cells, particularly for efficient immune response, tissue homeostasis, and wound healing (Trepat, Chen, and Jacobson 2012, 2369-2392). To effectively arrive at the right location for differentiation and proliferation (Zhao 2009, 674-682), cells must not only understand when to initiate migration but also move in which direction. The directional cue of cell migration for wound healing has long been proposed to be the applied electrical field since the intrinsic property of the electrical field is vectorial (Tai et al. 2009, 77-97). In fact, the endogenous electrical field was proven to be the dominant factor that provides guidance for cell motility over other well-accepted directional cues (Zhao 2009, 674-682). Zhao and his team conducted an experiment where the electrical fields with polarity opposite to the default wound healing direction were applied to the wound area. Experimental results showed that the reversal of electrical polarity caused the cells to migrate away from the wound area, leading to wound enlargement even though all the other cues including contact inhibition release and population pressure suggested the opposite route. Thus, they came to the conclusion that the cells follow the direction of the electrical gradients and ignore other directional cues in wound healing. However, how can bioelectric signals influence cell migration?

Since bioelectricity refers to the endogenous ionic current flows (McLaughlin and Levin 2018, 177-189), the answer might lie in ion fluxes, which were proven to be significant to the assembly for cell polarity and cell migration, as suggested by Campetelli, Bonazzi, and Minc (2012, 601-612). According to Huang et al. (2015, 2086-2097), an extracellular influx of Ca^{2+} was proven significant to cell polarization, which was necessary for cell motility (George and Bates 2022). Lee et al. (2018, 1-16) added a piece to the puzzle when suggesting a strong bond between

increasing extracellular Ca^{2+} and cell migration via the induction of osteopontin (OPN) expression from bone marrow mesenchymal cells triggered by extracellular Ca^{2+}. Our research was greatly based on the study of Lee et al. However, instead of utilizing mesenchymal stem cells, we employed U2OS cells, which were shown to have distinct morphological polarity in vectorial movement (Huang et al. 2015, 2086-2097). All these studies greatly inspired our research on examining the impact of extracellular Ca^{2+} on cell motility. Thus, we hypothesized that extracellular Ca^{2+} concentration enhances cell migration in a concentration-dependent manner. We utilized in-vitro scratch assay as a cost-effective and simple method to investigate cell migration since wound closure involves the movement of cells in order to reinstate structurally damaged tissues (Grada et al. 2017, e11-e16).

II. MATERIALS AND METHODS

Cell culture

U2OS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated GE – HyClone FBS, supplemented with 10,000 units/mL penicillin, and 10,000 µg/mL streptomycin (a kind gift of Professor Martha Grossel). The U2OS cells were split using trypsin on a biweekly basis (Kathy Barker 1998, 223) and grown to 80% confluence.

Wound assay

U2OS cell plates were plated on BioCoat collagen-treated 60-mm plates and ensured to have a confluence > 70%. After 48 hours, the plates were added with concentrations of $CaCl_2$ solution, ranging from 0 mM to 20 mM (Lee et al. 2018, 1-16), and incubated for 48 hours before wound scratching. Cells were washed, and wounds were scratched using a sterile 1000 µL blue pipet tip. Cells were then gently rinsed in PBS and fed with media. Images were taken under 50X magnification at 0, 6, and 24 hours after wound scraping (Slomiany et al. 2006, 635-646).

III. RESULTS

Low confluence in combination with $CaCl_2$ addition led to poor cell growth in U2OS cells

To determine the effect of $CaCl_2$ addition on the growth of U2OS cells, a pilot trial was conducted. The starting plates all had a confluence of approximately 40% and were incubated for 12 hours before being added with $CaCl_2$. After 5 days, the control plate observed a normal cell growth with a confluence of 90%, while the reverse was shown in plates treated with $CaCl_2$. The plates containing 4 mM, 10 mM, and 20 mM $CaCl_2$ saw traces of floating cells. Originally, the 4 mM $CaCl_2$ plate contained spherical cells, but it witnessed the emergence of spindle-shaped cells at 20% confluence after 5 days. Floating cells started to appear in the 10 mM $CaCl_2$ plate and increased their density while coexisting with dark spherical cells in the 20 mM $CaCl_2$ plate. Both plates indicated a confluence of 50%.

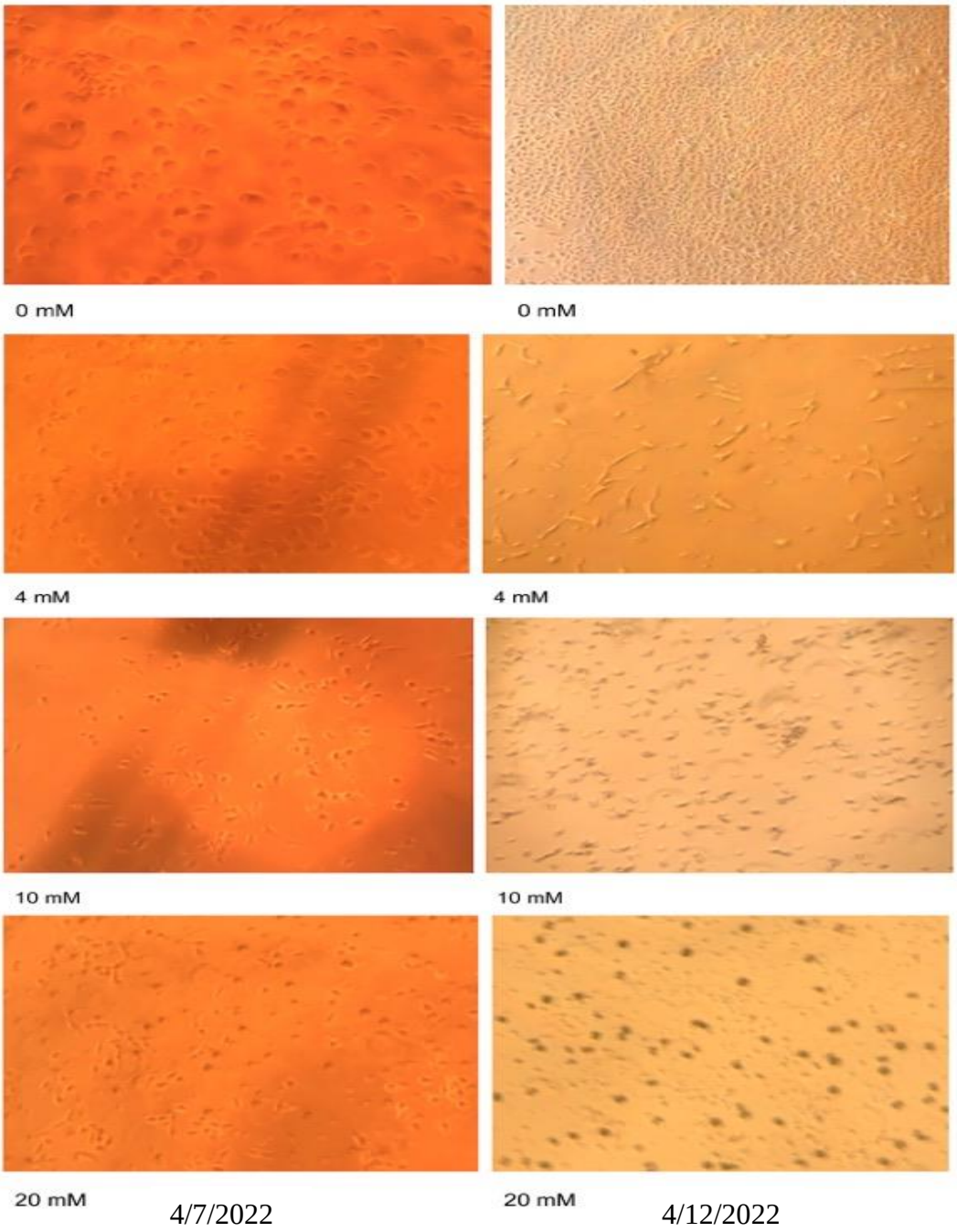

Figure 1. U2OS cells were grown on BioCoat collagen-treated plates and treated with increasing concentrations of Ca^{2+} from 0 mM to 20 mM for 5 days. Cells were checked for confluence under the microscope at 50X magnification.

Increasing extracellular Ca^{2+} ions promoted cell migration in human bone osteosarcoma U2OS cells in wound healing assay

To investigate the effect of extracellular calcium ions on cell migration in U2OS cells, a wound healing assay was implemented. Since the previous trial showed the negative impact of low confluence on cell growth, in this trial, the starting plates all had over 80% confluence before being treated with $CaCl_2$. After being added with $CaCl_2$ for 48 hours, all cell plates reached over 90% confluence, sufficient for wound healing assay. After 6 hours of wound scraping, there was a 20% minimization in the width of the wound in the control plate. The wound in the 4 mM $CaCl_2$ plate was reduced by 40%, while that in the 10 mM $CaCl_2$ plate closed by 50%. 20 mM $CaCl_2$ plate witnessed the most remarkable wound healing progress when its wound was reduced in width by 75% after 6 hours. After 24 hours, the control plate still observed no wound closure. However, full wound closure was seen in all plates added with $CaCl_2$. No traces of abnormal cells were observed in this trial.

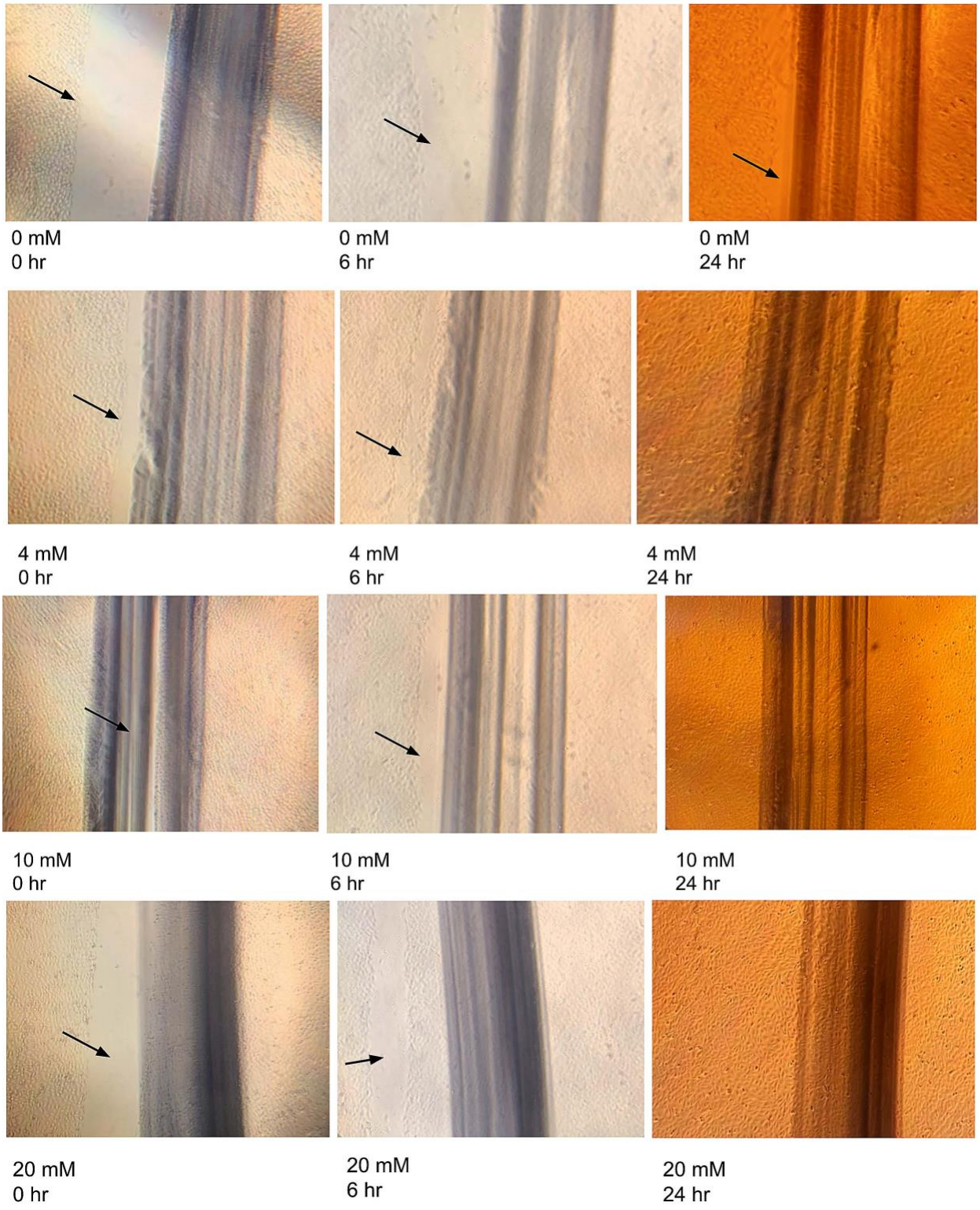

Figure 2. Images of wound healing assay of U2OS cells were taken at 50X magnification at 0, 6, and 24 hours after the wound scraping was performed. U2OS cells at 90% confluence were grown

on 60-mm BioCoat collagen-treated plates and added with elevated concentrations of Ca^{2+} from 0 mM to 20 mM.

IV. CONCLUSIONS

Cell migration serves as a significant procedure in several biological processes, including tissue formation, cancer progression, and immune defense (Pijuan et al. 2019). The determinant factor behind cell migration has long been proposed to be bioelectricity: the flux of mobile charged ions within the living cells (Tyler 2017, 627) that can influence cell motility (Campetelli, Bonazzi, and Minc 2012, 601-612). In this study, it is hypothesized that increasing extracellular Ca^{2+} exhibits a positive effect on cell migration, led by bioelectricity, in human bone osteosarcoma U2OS cells.

From the pilot trial, it was suggested that cells with low confluence cannot withstand the addition of $CaCl_2$. Therefore, even though the control plate had a 40% confluence as the other plates, the fact that it was not added with $CaCl_2$ explained the normal cell growth and 90% confluence after 5 days of incubation. The other plates with a similar confluence, after being exposed to $CaCl_2$, exhibited cell abnormality. After 5 days of incubation, as more $CaCl_2$ solution was added to the plates, more cell degeneration was observed in the form of floating cells and dark spherical cells, which were suspected to be caused by poor cell adhesion during cell harvesting protocol. Digital image of the pilot trial also showed that the confluence of cells treated with $CaCl_2$ remained low (< 50%) after the 5-day incubation in addition to abnormal cell development. Therefore, it was not possible to proceed to the next stage of the experiment, the wound healing assay.

In the second trial, the starting plates all had over 80% confluence and were incubated for 48 hours prior to $CaCl_2$ treatment. Since the concentration of $CaCl_2$ used in this experiment was the same as that in the pilot trial, higher cell confluence in the starting plates was believed to be one of the determinant factors for cell growth. As a result, all plates observed over 90% confluence after $CaCl_2$ was added for 48 hours. There were also no traces of abnormal cells observed in the plates, suggesting a better cell harvesting procedure. In the wound healing assay, all plates treated with $CaCl_2$ demonstrated faster and more efficient wound healing progress in comparison to the control plate. After 6 hours of wound scraping, the wound in $CaCl_2$-treated plates closed by more than 40%, while that in the control plate was only reduced by a fifth. After 24 hours, full wound closure

was seen in all $CaCl_2$-treated plates, while a small wound was still visible in the control plate, suggesting that $CaCl_2$ promotes cell migration in wound healing assay. Experimental results also showed that as the concentration of Ca^{2+} rose, the faster the wound closure. It can be seen from the wound minimization percentage after 6 hours of wound scratching. In the 4 mM $CaCl_2$ plate, the wound was closed by 40%, while that in the 10 mM $CaCl_2$ plate was reduced by one-half. 20 mM $CaCl_2$ plate demonstrated the fastest rate of wound closing since the wound healing progress reached 75% after 6 hours. These results served as evidence for our hypothesis that elevated extracellular Ca^{2+} ions enhance cell migration in U2OS cells.

Even though our conclusion on wound healing rate matched up with that of Lee et al. (2018, 1-16) and other lab groups who also used U2OS cells up to 10 mM $CaCl_2$, we had conflicting opinions about the effect of Ca^{2+} on cell migration in 20 mM $CaCl_2$. According to their data, the impact of calcium ions on cell motility at the concentration of 20 mM $CaCl_2$ decreased in comparison with that at other concentrations tested, while our data showed the reverse. This can be explained by the difference in assay used to assess cell migration (wound healing assay vs Transwell cell migration assay) and assessment tools. Our lack of use of cell counting method using a hemocytometer made our data slightly less reliable in terms of accuracy. It should be addressed that we only compared the size of the wound before and after wound scratching without quantifying the number of cells on each plate, as done in Admirabilis Kalolella's group. The fact that the marker lines covered some parts of the wound and the images of wound areas were inconsistent may also contribute to this difference in the outcome. Thus, if this experiment is repeated, we will consider adding the cell counting method to better validate our results. Overall, despite minimal errors, the data obtained supported our hypothesis, and our research contributed to the understanding of the effect of Ca^{2+} on cell motility. Although our research was greatly based on that of Lee et al. (2018, 1-16), we managed to add our understanding of the effect of bioelectric signals on cell migration using a different cell line and assessment tool. Our data added a piece to the puzzle of the relationship between bioelectricity and cell migration by observing how much faster and more efficient the wound healing progress was under the influence of the influx of extracellular calcium ions.

The next step for this research is to explore the mechanism behind the effect of calcium ions on cell migration. Although Lee et al. (2018, 1-16) proposed that the induction of OPN expression stimulated by extracellular Ca^{2+} was the reason, since OPN expression is found to be limited in the bone of human adults (Chen et al. 1993, 113-123; Lund, Giachelli, and Scatena 2009, 311-322), it is unknown if human bone osteosarcoma U2OS cells, a cancer cell line (Mohseny et al. 2011, 1195-1205), also expresses OPN. A study by Saidak et al. (2009, 2072-2080) on the effect of extracellular Ca^{2+} on cell motility in breast cancer cells via the activation of calcium sensing receptor (CaSR) captured our attention. Since U2OS is a cancer cell line, it is possible that it expresses CaSR. Thus, we suggest that extracellular Ca^{2+} concentration enhances cell migration via either the induction of OPN expression or the activation of CaSR. This is the aspect that differed our study from Saidak et al. and Lee et al. In future research, we hope to conduct western blots and Real Time Quantitative PCR (Saidak et al. 2009, 2072-2080) in order to confirm which protein, OPN or CaSR, is expressed in U2OS cells and serves as a gateway for calcium ions to affect cell migration. From there, we could explore the mechanism behind the effect of Ca^{2+} on cell migration in human bone osteosarcoma U2OS cells.

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