SOAR Research Proposal - Summer 2018

Understanding the role of phosphorylation in tumor-suppressive function of Gap Junctions.

Faculty: Dr. Anastasia Thévenin, Assistant Professor of Biological Sciences
Student: Irene Bonetti, Biochemistry, Class of 2020
Project Duration: 10 weeks, May 21st through July 20th

Project Description:
Communication between neighboring cells is key to sustaining proper function of tissues, as well as of entire organs. Gap Junctions (GJs) are cellular structures that allow for direct communication between cells. GJs are made of proteins termed connexins (Cxs) that form pores between neighboring cells. These Gap Junction pores allow for small molecules and ions to move between adjacent cells. For example, Gap Junctions in the heart allow movement of calcium ions between cells. Because calcium ions are needed for muscle contraction, improperly functioning GJs cause heart arrhythmias. Thus, GJs are highly relevant to human health, as well as to understanding and treating human diseases, such as cancer, heart disease, deafness and many others.[1]

It was recently discovered that GJs in brain tumors can function as tumor suppressors - proteins that are able to counterbalance negative effects of tumor-causing proteins, or oncogenes. Specifically, GJs made of connexin 43 (Cx43) were demonstrated to recruit and inhibit a potent oncogene - Src.[2] The goal of our project is to characterize whether phosphorylation of Cx43 (a type of chemical modification) in or near the Src-binding region regulates Cx43-Src interaction and inhibition of Src oncogenic activity.

Connexin 43 Gap Junctions are heavily modified through phosphorylation of many different amino acids within the protein. Phosphorylation of Cx43 is known to regulate GJ function, from formation of the GJs, opening and closing of the GJs, as well as their removal from cell-cell contacts.[1] To study phosphorylation of Gap Junctions, our laboratory utilizes mutants of Cx43 (where individual amino acids that could be phosphorylated are mutated to amino acids that mimic phosphorylation). We can then use these phosphorylation mimics to study individual effects of phosphorylation on GJ function.[3] Thus, we aim to study Cx43 phosphomimetic mutants within the Src binding site to identify whether this interaction is regulated by Cx43 phosphorylation.

Previous students in the Thévenin Laboratory generated two Cx43 mutants of the Src-binding region. One mutant mimics phosphorylation at two serines (S) (S279E/S282E) by mutations to glutamic acids (E). The other mutant has serines mutated to alanines (A), thus preventing any phosphorylation from occurring (S279A/S282A). The specific goal of Irene's SOAR project is to express these mutants in mammalian cells and to study interaction between Cx43 mutants and Src by using co-immunoprecipitation techniques and western blotting analyses. Irene would use antibodies specific to Src (which have already been tested in our mammalian cell line) and precipitate Src from

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[1] Thévenin, A. F., Kowal, T. J., Fong, J. T., Kells, R. M., Fisher, C. G., and Falk, M. M. (2013). Proteins and Mechanisms Regulating Gap Junction Assembly, Internalization and Degradation. *Physiology 28: 93-116*.

[2] González-Sánchez, A., Jaraíz-Rodríguez, M., Domínguez-Prieto, M., Herrero-González, S., Medina, J. M., and Tabernero, A. (2016). Connexin43 recruits PTEN and Csk to inhibit c-Src activity in glioma cells and astrocytes. *Oncotarget* 7(31): 49819-49833.

[3] Anastasia F. Thévenin, Rachel Margraf, Rachael Andrews, Charles Fisher and Matthias M. Falk. "Phosphorylation regulates connexin/ZO-1 binding and release, an important step in gap junction turnover" *Mol. Biol. Cell.* 28 (25): 3595-3608, 2017.

cells expressing Cx43 mutants. She will then test for presence of both Src and Cx43 in her precipitates. Presence of any Cx43 protein will tell us that we are observing a biochemical interaction between Cx43 and Src in these cells. Preliminary results from a current Honors student in similar experiments (but using a smaller portion of Cx43 mutants purified from bacteria) and Src from mammalian cells seem to indicate that Src-Cx43 interaction is phosphorylation dependent. However, because we were using a fragment of Cx43 protein from a non-mammalian source, low yields of Cx43-Src complexes made data analyses very difficult. *Irene's goal is to study these Cx43-Src interactions directly in cells while testing how this interaction affects functions of Src, as well as of Cx43 Gap Junctions.*

Roles and Responsibilities:

I, Anastasia Thévenin, will serve as Irene's direct mentor through the duration of the project. I will closely work with Irene in training her in the cell biology techniques this project requires, helping her plan, set up, and carry out her initial experiments, while further guiding her as she explores primary literature. In addition, I will gather all supplies and chemicals that Irene needs to carry out her proposed work.

Irene will keep a laboratory notebook (as is common in our discipline) and will report to me on a daily basis to go over the collected results, as well as to plan out the next set of experiments. Irene will prepare for her scheduled SOAR presentation. In addition, she will be asked to compile all her data into a research poster prior to completion of her SOAR project time, so she can present her work at the Pennsylvania Academy of Sciences 2019 Annual Meeting, Lehigh Valley Society of Cellular and Molecular Biology Symposium (TBD – April 2019) and at the Moravian College Undergraduate Student Scholarship and Creative Arts Day.

Project Timetable:

Because Irene is an international student, the timetable for the project had to be revised and modified based on the travel needs of both Irene and Dr. Thévenin. We will both need to travel to Europe to visit family in late July. We would like for the SOAR to start on Monday May 21st and end Friday July 20th for a total duration of 10 weeks.

WEEK 1 & 2: Learning mammalian cell culture, transfection of Cx43 mutants into mammalian cells and detection of active Src in cells by western blotting analyses.
WEEKS 3-6: Immunoprecipitation experiments between Src and Cx43 mutants (and wild type). Western blot analyses of immunoprecipitated samples.
WEEKS 7 & 8: Dye transfer assays in mammalian cells to determine if Cx43 mutants make functional Gap junctions upon Src activation. In these experiments, we will measure ability of our mammalian cells to transfer a fluorescent dye through Cx43 mutant vs. Cx43 wild type Gap Junctions. Fluorescence microscopy imaging will be carried out in the Falk Lab at Lehigh University Dept. of Biological Sciences (where Dr. Thévenin did her postdoctoral work in 2012-2014). Dr. Thévenin continues to collaborate with her former postdoctoral advisor, Matthias Falk.
WEEK 9: Repeats of successful experiments to have higher sample numbers and ability to carry out statistical analyses of any observed differences in Cx43 mutant interactions with Src.
WEEK 10: Remaining data analyses and poster preparation.
Experimental down time during the duration of the SOAR project: Irene will continue to research the topic through extensive reading of the primary literature to help her plan out and propose experimental set up and troubleshoot any issues that might arise. Irene will also work on preparing figures as results are obtained, to put together her SOAR presentation and research poster.

Student Engagement
Irene is currently a sophomore biochemistry major, who was enrolled in my Genetics class in the Fall 2017. Irene expressed her interest in pursuing summer research with me early last fall. She has spent the last couple of months actively reading some of the literature and meeting with me to discuss details on a few occasions. Her enthusiasm and curiosity about this project have been wonderfully refreshing to see. Her intention after graduating from Moravian College is to attend medical school, and this project could be a great insight into how Gap Junctions at the molecular and cellular levels are involved in cancer. Analytical and quantitative skills that Irene will gain from this research experience will help prepare her for an intellectually and analytically demanding medical training after Moravian College. Irene is highly interested in continuing work in my laboratory beyond SOAR, into independent study her junior year and eventual Honors project her senior year. Even though Irene has not had a biochemistry course, I have had the opportunity to evaluate her molecular biology skills during genetics lab. She is thorough and consistent in her bench skills and possesses a solid background in molecular/cellular aspects of living cells.

Contributions to the Discipline and Opportunities to Share Work:
The work proposed here will provide us with better understanding of how complex phosphorylation events at Gap Junctions regulate their ability to function as tumor suppressors. Because studying phosphorylation of Gap Junctions in cells has been inherently difficult, utilizing phosphomimetic mutants of Gap Junctions at our sites of interest simplifies analyses and data interpretation. Recently published work by A. Thévenin establishes approaches that Irene is proposing to use this summer.[4] Irene's results will provide highly desired molecular detail of how very specific phosphorylation events on Cx43 Gap Junctions recruit and inhibit Src – an oncogene present in many cancers. In addition, work we plan to conduct this summer will help guide our design of potential Src inhibitors, using relevant portions of Cx43 Gap Junctions as a recruitment scaffold. Irene's results, in combination with results from previous students and future (2018-2019 academic year) students will become an integral part of a future peer-reviewed publication and will be included as a part of a research grant proposal to NIH (R15 Area Grant) this fall and in other funding mechanisms in the coming months/years. As mentioned in the "Roles and Responsibilities" section, Irene will also have various opportunities to present her results at meetings and symposia during the 2018-2019 academic year: Pennsylvania Academy of Sciences Meeting, Lehigh Valley Society of Cellular and Molecular Biology Symposium and Moravian College Undergraduate Student Scholarship and Creative Arts Day.

Proposed Expenses (beyond stipends):
$255 - Src monoclonal antibody – Cell Signaling Technologies (Cat. # 2110). This antibody will be used in immunoprecipitations of Src from mammalian cells and in western blotting detection of Src.
$169 – Alpha-tubulin antibody – Thermofisher Scientific (Cat. # 62204). This antibody will be used as a loading control in our immunoprecipitation experiments.
$23 – Thermofisher shipping costs.
$25 – Cell Signaling shipping costs (approximate).

$472.00 – total amount requested

[4] Anastasia F. Thévenin, Rachel Margraf, Rachael Andrews, Charles Fisher and Matthias M. Falk. "Phosphorylation regulates connexin/ZO-1 binding and release, an important step in gap junction turnover" *Mol. Biol. Cell.* 28 (25): 3595-3608, 2017

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Student: Irene Bonetti
Major: Biochemistry
Expected graduation: May 2020.
On campus housing requested: yes

As a biochemistry-premed major with a specific interest in medicine, I was immediately fascinated by the proposed project and by Gap Junctions, in general. SOAR offers a terrific opportunity for me to explore this research field as an undergraduate student, especially giving me more time during the summer than during the academic semester. The project also allows me to apply what I learned in Genetics and Anatomy courses I took in the past year, to a concrete biochemical problem that is so relevant to human health and disease.

This SOAR project will grant me the opportunity to test my abilities in a laboratory environment and to acquire knowledge beneficial for medical school, such as solid analytical and critical thinking skills, while offering concrete experiences in a way that a normal lab-based course cannot. I am thrilled to work with mammalian cell cultures and understand the role of phosphorylated gap junctions in oncogene inhibition. Moreover, I will have the possibility to learn and apply new techniques that will allow the research to progress. I will have the chance to have a deeper understanding of molecular, cellular and cancer biology, all of which are crucial to understanding and treating human disease. Even though this project aims to understand the molecular detail of interaction between a tumor suppressor and an oncogene, it is hard to forget the human side of the terrible consequences that cancer has on people's lives. I am sure that taking part in this project will help make me a more competitive medical school applicant, as my chances of getting into medical school are lower than an average student, given the fact that I am an international student.

I always wanted to be a doctor; it has always been my ultimate goal. Therefore, this is the perfect opportunity to learn more about an important medical topic and link it to the class theory I have been learning so far. To conclude, I will be honored to participate in this SOAR project with Dr. Thévenin, and to be part of a research that has the potential to make a step forward, toward a possible cancer treatment.