Sergio Lozano

Sandia National Laboratory Internship

Project Description

Introduction

Intercellular communication plays a critical role in coordinating the activities of cell populations. Communication between non-adjoined cells is often accomplished through chemical signaling. However, it was recently discovered that membraneous "nanotubes" can physically link together cells separated by distances of up to 150 μm, and that these nanotubes can serve as conduits for intercellular trafficking of chemicals, biomolecules, and even organelles. Moreover, in phagocytic cells (e.g., macrophages) nanotubes can trap and shuttle bacteria for ingestion, and retrovirus-infected cells are observed to generate nanotube connections that promote efficient cell-to-cell transmission of the retrovirus. Despite the critical role of nanotubes in these processes, little is known about how the structures form, exactly what is being transported, or what cell activities are coordinated through nanotube-mediated intercellular communication.

Others in our group recently discovered that nanotube-like structures can be formed by synthetic lipid vesicles [Liu et al., Langmuir 24:3686 '08; Hayden et al., J Am Chem Soc 131:8728 '09; Stachowiak et al., Proc Natl Acad Sci USA 107:7781 '10]. Our objective is to compare synthetic nanotubes to those formed by live cells, primarily focusing on their formation kinetics, their selectivity in transporting biomaterials, and their ability to establish and maintain a functional connection between lipid vesicles and cells. As a first step in this effort, we have developed a protocol for long-time course microscopy-enabled observation of nanotube formation by lipid vesicles and cells. We

have initiated characterization of nanotube formation kinetics, as well as co-culture experiments that promote nanotube-mediated functional connections between lipid vesicles and cells.

Materials and Methods

The mammalian cell lines used in these experiments were purchased from ATCC and cultured according to their recommendations:

- RAW264.7 and HEK293T: DMEM + 10% FBS + 1% pen/strep; passaged every two days at 1:5 dilution
- P388D.1: RPMI 1640 + 5% FBS + 1% pen/strep; passaged every two days at 1:5 dilution
- L929: EMEM + 10% horse serum; passaged every two days at 1:4 to 1:5 dilution

In preparation for microscopy, ~80-90% confluent cell cultures were diluted 1:10 in fresh medium and 250 µl transferred to a sterile 35 mm diameter glass-bottom petri dish (MatTek P35G-1.5-14C). The cells were incubated at 37° C with 5% CO_2 for 1-3 hrs (the faster-growing RAW264.7 and HEK293T were incubated for 1 hr, while the slower-growing L929 and P388D.1 were incubated for up to 3 hrs), to give the cells an opportunity to settle down to the bottom of the dish and become immobilized. The petri dish was then transferred to an environmentally-controlled chamber (Tokai Hit INU-ONI-F1) mounted on the stage of an inverted microscope (Olympus IX71), and the cells maintained at 37° C with 5% CO_2 overnight. Cells were observed at 600X magnification, through phase-contrast microscopy as well as fluorescence microscopy

(Bodipy: Ex 530nm, Em 550nm; GFP-6xHis: Ex 488nm, Em 510nm). Time-lapse images were captured using Micromanager, and image sequences were imported to ImageJ and compiled into movies.

Synthetic lipid vesicles were provided to us by Frank Zendejas. They consist of a mixture of DSIDA-Distearylglycero triethyleneglycyl iminodiacetic acid (10%) and DPhPC-1,2-diphytanoyl-sn-glycero-3-phosphocholine (89.7%). Vesicles were prepared using an electroformation technique [Angelova et al, Faraday Discuss 81:303-311 '86]. The lipids were tagged with BODIPY dye (0.3%), such that they fluoresce when exposed to light of 530nm. This specific mixture of lipids produces DSIDA-rich domains in the vesicle where we can expect to see nanotubes forming. The vesicle are diluted 1:1 with buffer, (20 mM MOPS + glucose solution + $CuCl_2$; osmolarity of 370 Osm). The copper segregates with the DSIDA-rich domains and can be visualized through binding of GFP-6xHis; this makes it easier for us to predict where nanotubes should be projecting from.

To induce nanotube formation we introduce GFP-6xHis to the mixture at 2M and incubate the slide at RT in the dark for 1 hr before observation. The binding of the fusion protein's 6xHis tag to the copper-coordinating DSIDA-rich lipid domain stimulates nanotube formation. The mixture of vesicles, buffer, and GFP-6xHis is placed into a chamber created with a slide and cover slip separated by double-sided tape.

My Role

I was responsible for many parts of our experiment: passaging cells, setting up slide chamber, monitoring vesicles and cells, taking photos during observations, analyzing these images, compiling images into a video.

Passaging cells was critical for our experiment, we worked with four different cell lines and each cell line had its own specific requirements for growth. In addition, it was important that we determine the proper dilution ratio so that we could easily observe them during experiments. Once cells were passaged we would wait a couple of days before using them or repassaging them, this prevented over/under growth of the cells.

The vesicles we worked with were very delicate and needed to be handled carefully to prevent any rupturing, so for observations we were taught to make a slide chamber out of slides, coverslips and double-sided tape. The slide chamber created a gap between the slide and coverslip which prevented the material from crushing the vesicles and causing them to burst. I was responsible for making these slide chambers for vesicle observations.

Once we had prepared our vesicles and cells for observations, I was responsible for monitoring the experiment. Our experiments would last several hours and it was important that we continued to monitor it periodically to ensure the best results. Since the cells and vesicles were free to move around, it was important that we keep the microscope in proper focus to gather the best images, in addition, the cells needed a stable environment to survive and grow properly during observations so I would check on the cells condition throughout the experiment.

I was also responsible for capturing photos of the vesicles and cells during observations. Images were taken at different time intervals and were used to monitor the cells/vesicles activity and follow the formation of nanotubes. Once the images were taken I would use an imaging program to provide better contrast and look for nanotube formation. In addition, I would compile the images we collected and create a video for each experiment, this allowed us to visualize the dynamics ofcell and nanotube movement, growth, and morphology.

Accomplishments

During my internship I was able to achieve some project milestones that will help the team as they continue with future research. The first milestone we were able to achieve was to view nanotubes in both the mammalian cells and the lipid vesicles. Nanotubes had been viewed in the vesicles before, so we were more focused in finding a good vesicle sample that provided the optimal amount of vesicles and nanotubes for viewing. However, with the cells we were more focused with finding nanotubes between cells of the various cell lines we were using. This enabled us to meet the second project milestone, whichwas determining which of the cell lines should be used in our future research. Some of the cell lines did not grow properly under the conditions of the experiment. Others did not produce the type of nanotubes we were looking for; for example,we noted that some of the cell lines produced nanotubes that were too thick, or they were producing numerous thin and spiky nanotubes. The thicker nanotubes were difficult for us to observe properly and the spiky nanotube projections were too numerous to keep track of. However, other cell lines produced thin narrow nanotubes

that were perfect for our experiment. Based on this work we down-selected to two cell lines that we will continue to work with in future research.

Additionally, in the course of these experiments we discovered that our cell lines of interest can formnanotubes by two different processes. In process A the cells are at first joined together, they then separate leading to the nanotube formation. In process B one of the cells projects a nanotube that fuses with a neighboring cell. Our experiments suggested that process A was more common, but more quantitative work must be done before a firm conclusion can be made.

Finally, towards the end of my internship, we started to work on the third phase of the project. Our progress had been slowed by the lack of resources (vesicles) which were being made here at Sandia in another lab. In this phase of the project we were going to observe vesicles and cells together, the goal was to have a vesicle connect to a cell via a nanotube. Our initial work was to determine the proper quantity and density of cells to use with the vesicles; having too many cells will make it difficult to properly observe and keep track of nanotube formation so we must determine an ideal concentration. I carried out some preliminary experiments to frame out this line of research, and some future work will be needed to further optimize the conditions.

How Internship Experience Impacted My Academic/Career Planning Goals

Working at Sandia was the first time I worked ina research lab, and my first opportunity to conduct my own research. This experience has inspired me to pursue more research opportunities at the University of Texas. I had not planned on doing any other research because my course load is heavy, and my summers are usually busy,

but I now feel I would enjoy it and can learn a lot from other researchers. So, I intend to make changes to my academic schedule to allow for some opportunities to do research during the school year. In addition, until this internship I did not have a great understanding of the career paths and opportunities available to me through DHS, nor did I understand what a career as a researcher would entail. But, I have spent time discussing different career opportunities with my mentor, and feel I have a better understanding of what my life would be like as a researcher. I have found a great appreciation for the career, and will continue to seek more information to allow me to make the best decision for my career.

During my internship I was only able to make it to one of the lectures; it focused on the history of nuclear weapons. I found it very educational and learned a lot about the modern weapons age and the advancements that have been made since the first nuclear bomb was made. I also learned about the rigorous security measures that are in place to prevent espionage from other countries. In addition, Sandia did sponsor some intern events that were helpful for my personal development, the events helped me network with many other interns around the country that are involved in different fields of study.

Areas of Research That Need DHS Consideration

I think that the Department o Homeland Security should continue to support research with cellular communication. There are many different applications that are of importance to DHS and could help them accomplish its mission to protect against and respond to threats and hazards to the Nation. Further understanding of cellular communication and gaining the ability to manipulate messages are important goals that

could help the DHS respond quickly to biological warfare and other threats to the Nation. If a virus or bacteria is used as a weapon, it would be important for us to be able to achieve rapid communication between our cells, allowing the cells to form a network that spreads information about the pathogens. If we are able to manipulate what is transferred between cells during communication we could also have the cells rapidly transmit a medicine to help victims survive. Having control of rapid communication will give us the best opportunity to survive biological warfare.

Benefits

Working at the lab provided numerous benefits for my future including meeting and working with great people, acquiring new skills, attending educational and interesting lectures. Early in my internship I was taught how to use different equipment in the lab and as my internship continued I was introduced to more equipment. Learning how to use equipment in the lab will be very helpful in the future because I plan on doing more research and working in a lab. Throughout my internship I was working with different people around the lab which was a great opportunity to network and learn from them. I was also privileged to work with a great mentor, Steven Branda, he worked hard to make sure I had everything I needed to accomplish my goals. Despite his busy schedule he made himself available to me at all times, and assisted me when I was working on my poster presentation. After my poster presentation he offered great advice to improve my poster preparation and presentation skills; he also educated me on my career opportunities with Sandia.