[MUSIC PLAYING]

KIMBERLY BROTHERS: So clarity of the cornea, which you all know is the transparent front part of the eye, is essential for vision. And an intact epithelium is a very important barrier that protects from microbes. The eye is exposed to them on a daily basis. However, our tear film washes them away. But if you get a breach or compromise in that barrier, and the bacteria can get inside, once they get into the stroma, they'll multiply out of control. You'll get a lot of neutrophil infiltration and bacterial toxins that are a major cause of damage.

So this is a Pseudomonas aeruginosa corneal ulcer. This is a picture from the Campbell lab. And you see a lot of those results of inflammation here, as well as the cornea no longer being transparent. So wound healing is very important for maintaining that protective epithelial barrier.

There are more than 100,000 people in the US that have suffered vision loss from corneal infections caused by microbial keratitis, or inflammation of the cornea. And the vision outcome is not great. Half of all patients have reduced vision, and a quarter of them become legally blind.

So you're going to be hearing more about that kind of stuff from Dr. Espendar. What I'm going to talk to you about is the project we had in the lab, where we believe bacteria inhibit corneal wound healing. So the bacteria, or what they secrete, could build up on your contact lens or in your contact lens case, and then be introduced to the ocular surface and influence the corneal cell behavior.

So how do we do this? We have a 96-well plate-based assay where we'll put this silicone stopper into the well. And it has this two-millimeter tip. And that creates a cell-free zone. We seed corneal cells in, and they will grow right up to that stopper. Then we stratify them so they're layered like a real cornea. Then we'll remove that stopper, add different treatment conditions to give them time to migrate, or not, and we'll do a fluorescent viability stain at the end to see if what we're adding is killing the cells.

So what we do is we take an overnight culture of bacteria, we normalize it so it's the same optical density, and then we'll filter it to remove the bacteria to see if just what the bacteria secretes can prevent these cells from moving in. These are our layered corneal cells. We can also just add live bacteria to this assay, as well.

So just to walk you through a particular experiment, this is what an endpoint looks like. If you just leave the stopper in and take it out at the end of the experiment and add the fluorescent viability stain, this black area is that cell-free zone. This could only capture half of it on the [INAUDIBLE] with this objective. But you also see this nice green fluorescence so you know the cells are viable.

If we take just what we grow the bacteria in, LB, you see the cells have completely filled in that cell-free zone, and they're nice and fluorescent and viable. So what we did is we took bacteria that are associated with contact lenses and microbial keratitis, and we tested them in this cell migration assay. And a lot of them heal. See? Nice viability, the cells all fill in the area.

But these two don't, Pseudomonas aeruginosa and serratia marcescens. You see they look identical to the control wound in this experiment. So there's no cells in this original barrier area. So we've tested a variety of different strains, and a lot of them I got from Reeg in the Campbell lab. A lot of keratitis strains. So here's my tribute to Reeg.

And what this table is showing you is what we did is we looked at how many inhibited cell migrations, so had this phenotype, how many healed. We calculated their percent inhibitory out of the total number that we tested. And then we also separately compared our keratitis isolates.

So what we found is three of them, Pseudomonas aeruginosa, serratia marcescens, and staph aureus, which are top ocular pathogens, all were quite capable of inhibiting corneal cell migration. So we took it a step further and did some timelapse imaging of our migration assays. And you're just looking, this is top-down. The circle that you see in the beginning here is going to be your original wound area. These are just bubbles. They go away.

But you see the cells migrate as a unit, together. And they close that gap in less than 24 hours. This is just if you add LB, which is what we grow the bacteria in. This is E. coli. Again, same thing. Closes that gap in less than 24 hours. And we did this by taking images every 15 minutes for 24 hours and then reconstructing them into a movie.

If we look at serratia marcescens, it looks very different. You see the cells no longer move as a unit. The layer gets quite patchy. Very thin. And that gap is never closed.

This one is one of my favorites. This is Pseudomonas aeruginosa. It tries to move forward. It goes back. And then the whole thing just snaps off completely. And this is in about two hours. And the whole cell sheet is contained like a rubber band. So we're exploring the migration dynamics and quantifying the cell movement. So that's some of the work we're doing now.

So for the remainder of this talk, I'm going to focus on serratia marcescens because we have a lot of genetic tools available in the lab to figure out what is that factor that serratia is secreting that is inhibiting corneal cell migration. So we tested several serratia keratitis strains that we got from Reeg. And a lot of them-- this has been updated since the table. All but one of them inhibit corneal cell migration.

So they all have this very strong inhibitory phenotype. And it's so strong that we actually have to titrate the dose down or, as I will show you, this is what it looks like in the movie. This is even faster than Pseudomonas. The whole thing is gone. And most of it is just playing the dead cells on the bottom. So we'll play it again for you. So you just see the whole thing just come right off the dish.

So what we wanted to do next is see if this inhibitory phenotype is only specific to corneal cells, or if this phenotype is widespread across any type of cell line. So we did some cell migration assays using fibroblasts. And same thing. This is what your control wound looks like. Nice, viable cells.

The LB, the cells fill in the area completely. But with serratia marcescens, it doesn't. So we have this inhibitory phenotype in two different cell lines. And we know it's not just specific to corneal cells. So that led us to ask, well, can we do this experiment using actual tissue.

So this was the fun part of my post-doc, where I had to find a place to obtain a large amount of pig eyes. So I found a company in California where I can order them. They arrive in less than 24 hours of harvesting. They come in this box that says In a Pig's Eye. It's kind of creepy. And they come in two Ziploc bags. They're actually really quite disgusting.

And what we do is we take the cornea and we, when the whole eye is intact, will wound it with an Amoils scrubber, which you guys use in the clinic. It looks just like an electric toothbrush. Then we remove the cornea with some of the sclera still attached. Here is a freshly dissected one. And we will put it on an augorous collagen mold and add minimal media up to the sides so the cornea itself is exposed to the air.

Then we take our bacteria or their secretomes, exactly like we do in our in vitro assay, add them drop-wise onto the wounded cornea. We'll incubate for two days and give the tissue time to heal or not. And we'll stain with a solution called Richardson's solution. It'll stain any remaining wound area. And we'll compare our control wounds to the treated, and see if they healed or not.

So when we do this, just to walk you through this, this is what a control wound looks like. So the white part is the sclera here, like I'm showing you in the cartoon, the yellow is the cornea, and the blue is the stained wound. It's about 6.5 millimeter diameter. And then below is our in vitro phenotype, where you see the cell-free zone.

So, same thing. We took the LB, treated our wounded pig corneas. They heal, as well as the cells. They migrate in. The serratia does not heal also in our wounded pig corneas, just like in the in vitro experiments. So, very exciting. Same phenotype in different cell lines. And we can also do ex vivo with wounded pig corneas.

So that became the quest to identify what is WIF, or the serratia wound inhibitory factor. So we took many different steps where we fractionated the secretome. We did some proteomic analysis on different serratia mutant secretomes to compare to wild-type. We looked at the small molecule level by using metabolomics, where we took secretomes and secretome-treated corneal cells and sent them out for analysis. And then we also did some genetic analysis, where we looked at some serratia mutants and compared.

So by doing all of that, it became evident that our serratia wound inhibitory factor could be lipopolysaccharide, or LPS. So this is just a cartoon structure of LPS. It's got a lipid A bottom portion, followed by its core, and then its O antigen outer portion. So when I was testing these bacterial secretomes, we found many similar things to LPS. So the secretomes were resistant to hydrolytic enzymes. So is LPS.

We heat treated it at different temperatures, and found that whatever was inhibiting the cell migration, it was heat-stable at 90 degrees for 10 minutes. LPS is heat-stable. When I did different molecular weight cutoff filters, I found the inhibitory factor was between 10 and 30 kilodaltons. LPS is between 10 and 30 kilodaltons. It bound to HP20. LPS binds to HP20. And then lastly, it's freeze-thaw susceptible, and LPS is freeze-thaw susceptible.

So we did some experiments using some serratia LPS mutants. This one, WAAG, has a mutation in an LPS biosynthesis gene. So its LPS structure just has the lipid A portion and some of the inner core. And when we did our experiments, you see it's healing-- this one, the tissue hasn't completely healed. There's a tiny bit left. But you see the tissue is healing, and the corneal cells are migrating in to fill that gap. When we add back a functional copy of this gene, you see you go back to that inhibitory phenotype, where the wounded pig corneas aren't healing and the cells are not migrating.

So then we did an LPS depletion assay, where we took Polymyxin B-Agarose, which will bind LPS. So we added that in our secretomes, and then tested our post-treated sample. And you see it's healing. It's no longer inhibiting, whereas our Agarose B control is inhibiting, as well as our serratia wild-type. So further evidence that it's LPS. So lastly, I purified LPS from wild-type serratia, the LPS mutant, and E. coli. And what we found is only the wild-type-purified LPS inhibits wound healing in vitro and ex vivo. The LPS mutant doesn't, and the E. coli doesn't.

So just in summary for this part, what I've been talking to you up to this point, is the data in the *Scientific Reports* paper. So what I've shown you is LPS is necessary and sufficient to inhibit corneal wound healing. So bacteria from contact lenses or infections could affect the wound healing process. And this work allowed me to get an NEI F32 grant. So I was very excited about that.

So shifting gears to the host side, what genes are altered in the corneal cells when they're exposed to bacteria? So when I was on the training grant, I used some of my supply money to do some transcriptomic analysis with the department P30 molecular module. And what we found is we had increases in inflammatory genes, as well as autophagy genes when our corneal cells were treated with bacteria.

So what is autophagy? So autophagy is what happens when energy is low, conditions of cellular stress, or cellular organelles are damaged, autophagy will get activated and try to digest those damaged organelles, as well as, if there's a bacterial infection, autophagy will be activated to try to eliminate those bacterial pathogens. It will try to digest it.

So autophagy is very important in the eye. There's been a lot of work looking at autophagy with neurodegenerative diseases. And for my work, it's involved in corneal infections. So what we wanted to do is we wanted to develop an autophagy reporter cell line using the corneal cells. And what they have is it's the active form of LC3 that's tethered to GFP. So it will fluoresce green when autophagy is active.

So using the molecular module with Dr. Kitchington and Shang Wa, we developed an HCLE, which is the corneal cell reporter line, using lentivirus. So they're always making this LC3 protein. And we had to verify it worked using different inducers and inhibitors of autophagy.

So this is what it looks like if you just do mock treatment conditions. You see some basal levels of autophagy. So if autophagy is active, if you use a chemical autophagy inducer, you're going to see lots of bright green dots that they call puncta. And that's letting you know that autophagy is active in these corneal cells. And this came out really nice. We have to look on a very high magnification to be able to see them, but you see lots of beautiful bright green dots. So we were super excited. This is just recent work that we're doing in the lab.

So I took the keratitis strains from different bacteria, again associated with contact lenses and microbial keratitis, and tested them in this reporter cell line. And you see some of them you're not really seeing much. They do have some, like, basal green fluorescence background. But when you have active autophagy, you're going to see lots of bright green dots, like in this staph aureus strain, this serratia one. This one is pretty bright, the proteas and the moraxella.

So our autophagy genes from our transcriptomic analysis are induced when bacteria inhibit wound healing. These same bacteria activate autophagy. So our hypothesis was activation of autophagy inhibits wound healing. So we're looking at the other side of the coin here, the eye. I forgot to mention that.

So when we take this cell line and use the chemical inducer in our stratified corneal cells, unfortunately, it heals. It doesn't matter what dose. So activation of autophagy does not inhibit wound healing. So it's the complete opposite of what my data is showing me with the secretomes.

And to take it a step further, we use a broad inhibitor of autophagy, and look. As you increase the dose, you inhibit corneal wound healing. This is unfortunate. And then when we use a specific inhibitor of autophagy, again you see it inhibits wound healing. And at this point, we were able to purchase an objective that lets us image the entire wound on the confocal. So this is what it'll look like, if you can see that entire wound.

And again, the LB heals. And then as we add increasing concentrations of this autophagy inhibitor, it's not healing. So it's the complete opposite result of what my transcriptomic analysis is telling me. But I believe there's a link. I just have to figure out what that is.

So in summary, a variety of ocular pathogens inhibit corneal cell migration and wound healing. The serratia LPS inhibits corneal wound healing, both in vitro and ex vivo. So this is demonstrating the importance of LPS on wound healing and cell migration. And our data suggest that wound healing is autophagy-dependent just with what we see at the gene expression level, but we don't know how it's working since our chemical inducer is telling us the opposite.

So I've had a lot of people help me with this work. So I just want to thank them, as well as my sources of funding. And I'd be happy to take any questions.