

Hi, my name is Shannon Boye, and I'm an assistant professor in the Department of Ophthalmology at the University of Florida. And the focus of my research program is developing a gene therapy for myosin VIIA Usher syndrome, or USH1b.

As you know, Usher syndrome can be divided into three main categories, depending on the degree of severity. Patients with type 1 Ushers are profoundly deaf from birth and tend to experience their first loss of vision before the age of 10. Mutations and multiple genes can account for type 1 Usher syndrome, including myosin VIIA. And actually myosin VIIA mutations account for the most frequent cause of Usher syndrome type 1.

The myosin VIIA gene makes a complex protein called myosin VIIA. What I'm showing you on this slide, is a picture of the different domains of the protein, and put simply on one end of this protein is what's called the motor domain. This motor literally allows this protein to walk along actin filaments in the cells of our body. The other end of the protein is the tail domain. And this tail domain can actually bind to other proteins in the cell, and take them along for a ride.

Myosin VIIA protein is known to be expressed in the hair cells of the inner ear, as well as the photoreceptor cells of the retina. You may be wondering why both hearing and vision are affected in USH1b. And the answer lies in the structural similarities between the hair cells in the inner ear, and the photoreceptor cells in the retina.

Both of these cell types contain a very specialized feature called the cilia. In the hair cells, it's called the kinocilium. And in the photo receptors, it's called the connecting cilium. And when myosin VIIA is absent from either of those two structures, those cells become unhealthy, ultimately can degenerate, and thus a loss of hearing or vision can result.

It's thought that Usher's patients experience a loss of hearing first, because myosin VIIA is important for the development of the hair cells of the inner ear. Whereas in photoreceptors, perhaps myosin VIIA isn't required for development, but rather for the maintenance of photoreceptors after they're already formed.

So what do the retinas of these patients look like? On this slide, I'm summarizing findings from my clinical colleague Dr. Samuel Jacobson's lab. In this study, he studied 33 different patients with USH1b. All of whom had severe bilateral hearing loss in early childhood. Visual acuity within the first two decades in these patients, on average, was about 20/63, but by the sixth decade had dropped to less than 20/200.

These patients first lost their rod-mediated vision. In other words, their ability to navigate under dim light conditions. That was followed by a loss in cone-mediated vision. We use our cones to see under daylight

conditions, to see each other's faces, to read a book, et cetera. But cone-mediated vision is lost after rod-mediated vision is lost in this disease. And the rate at which cone vision is lost in these patients can vary greatly. With some patients maintaining normal cone vision even into their fourth decade. After this time, all USH1b patients, examined in this study, had lost cone-mediated vision.

Importantly, one result of this study showed that the initial site of the disease in USH1b is the photoreceptor cells of the retina. There are other cell types in the retina, like the retinal pigment epithelium, that are affected. But these effects appear to be secondary, to the defect in the photoreceptor cells.

My goal is to develop a gene therapy strategy for USH1b. And when we develop a gene therapy strategy for any disease, one of the first steps is to find a good animal model. In other words, one that faithfully mimics the human condition, in which the disease can be characterized, and in which therapies can be tested.

One mouse model that's been a very famous model of USH1b, is called the shaker 1 mouse. It carries a mutation in myosin VIIA, and as a result expresses no myosin VIIA protein. This mouse exhibits vestibular defects from birth, circling behavior, and balance problems. Really interesting though is that this mouse does not have any retinal degeneration, and no robust ERG phenotype. What I mean by that, is that there is no obvious loss of retinal function in these mice.

And if you remember the patient characterization I just described, that this is quite different from the patient condition. Where there is a profound degeneration of photoreceptor cells, and a loss of both rod and cone function.

So as you can see the shaker 1 mouse is by no means a perfect model of USH1b. Nevertheless, in the late 1990s investigators set out to find any small way that the shaker 1 mice might look different from a normal mouse. So that those parameters could be used to test gene therapies. To tell us whether or not they were effective.

And one of the things that investigators noticed was that in the shaker 1 retinal pigment epithelial cells, or the RPE, there are these little pigmented granules, called melanosomes, and they failed to move down towards the photoreceptors that were right next door. Whereas in a normal mouse RPE, those pigmented melanosomes moved towards the photoreceptors and hugged their outer segments. So in normal mice they move, and in shaker 1 USH1b mice they don't move.

Another subtle phenotype that was observed in the shaker 1 mice, had to do with Rhodopsin trafficking. Rhodopsin is a very highly expressed protein in the eye. It's made in the photoreceptor inner segment and then it has to transport to the photoreceptor outer segment through this very narrow feature, called the connecting cilium, which we've discussed before.

In wild type mice, the transport of Rhodopsin from the inner to the outer segment, is fast enough that if you were to look at a photoreceptor, you wouldn't usually see any Rhodopsin detectable moving actively through the connecting cilium. In the shaker 1 mouse however, when the investigators looked at the connecting cilium, they saw the presence of Rhodopsin. Even though it ultimately did get to the outer segment, its transport there seemed to be slowed. So again this was another possible outcome measure to be used in testing gene therapies for this disease.

So how does gene therapy work. This is a simple concept, but one that you should know before we go on with the talk. First what we do is we take a piece of DNA, and we put it inside a vector. You can think of the vector like a taxicab and the gene as the passenger inside. My job as a gene therapist is to tell that taxicab where to go and drop off its passenger.

So in our case, the piece of DNA is going to be myosin VIIA, and the taxicab we're going to use is actually a virus. And what we do to make this safe is we gut out all of the virus' native viral DNA, and replace it with a normal gene, a normal myosin VIIA gene. We then ask that virus to infect the cells of the retina, release that normal copy of myosin VIIA. Which then can go on to make a normal myosin VIIA protein and hopefully restore vision to those animal models or patients.

So the very first attempt to use gene therapy to correct those phenotypes I talked about in the shaker 1 mouse, was dictated by the size of the myosin VIIA gene. So the myosin VIIA gene is relatively large, about 7,000 base pairs. And at the time, the only taxicab or virus that was available that could accommodate that size of a gene, was called lentivirus.

And so investigators tested a lentivirus containing therapeutic myosin VIIA, for its ability to drive myosin VIIA protein expression in cells from the shaker 1 mouse, and showed that indeed it was possible, and the protein expressed exactly where it should express. They then went on to deliver the lentivirus containing myosin VIIA to the retinas of the shaker 1 mice, and they found that this led to robust expression of myosin VIIA in the retinal pigment epithelium of those mice. Notably however, expression of myosin VIIA in the photoreceptor cells of those shaker 1 mice was hardly detectable. And we'll come back to this concept in a moment.

But excitingly this lentivirus, containing myosin VIIA, was able to correct both that melanosome migration and opsin trafficking defect phenotype, that we discussed briefly before. And this was a very exciting proof of concept. That gene therapy could be used to deliver myosin VIIA to the retina. And possibly restore vision to these patients. And it actually formed the basis for ongoing clinical trials using lentivirus, containing myosin VIIA, to treat USH1b patients. This is a drug known as USHStat.

And in good news, this was a safe and well tolerated drug. Unfortunately though, this treatment with lentiviral myosin VIIA provided no evidence of biological activity. And again if you reflect back on what the investigators found when they tested lentivirus in the shaker 1 mouse, there was little to no expression of the therapeutic protein in retinal photoreceptors.

And if you'll remember the clinical characterization, photoreceptors are the initial site of this disease. So perhaps the failure to confer therapy had to do with the fact that lentivirus does not infect retinal photoreceptors very effectively. The jury's still out on that though. It's just a hypothesis at this point.

But that brought us to the concept that perhaps we should start thinking about using AAV, or adeno-associated virus, to deliver this large gene. AAV has really become the gold standard in retinal gene therapy. But as I mentioned before, it has a relatively small packaging capacity. It can only fit a piece of DNA that's about 5,000 base pairs long. And again myosin VIIA is bigger than that. It's about 7,000 base pairs.

The first vector platform that we tried was called heterogeneous or fragmented AAV. And to understand what this means I just have to go over a few simple concepts associated with AAV packaging. AAV is a single strand of DNA virus. So it has an exterior shell called the capsid. And inside that capsid is a single stranded piece of DNA. Now whenever a single strand of a piece of DNA gets into the body, it needs to form double stranded DNA.

So when we're packaging AAV, inside one capsid a plus strand of DNA can get sucked in, and then another capsid a minus strand of single strand of DNA can get sucked in. When those two strands of DNA find each other, after both of those vectors have been put inside a cell, they can find each other via a region of overlap, and then go on to form the full length myosin VIIA gene. And then make full length myosin VIIA protein, which will hopefully restore function to the cell. So again these are called heterogeneous or fragmented AAVs.

The diagram I'm showing you on the top left of this slide, is representative of the fragmented AAVs. And so what you're seeing are those single stranded pieces of DNA that are cut off in a heterogeneous fashion. And they're cut off at around 5,000 base pairs, because the AAV capsid can only hold 5,000 base pairs. So the DNA gets sucked in and then it gets cleaved around that natural carrying capacity of that virus system. The problem with this system is that you wind up packaging heterogeneous sized pieces of DNA, and we'll get into that in a moment.

Nevertheless, this fragmented or heterogeneous vector system proved capable of expressing full length myosin VIIA in the mouse retina that was of the appropriate size. It was just as big as wild type myosin VIIA. Excitingly we were able to use this fragmented AAV to correct both the melanosome migration phenotype, as well as the opsin trafficking defect that we saw in the shaker 1 mice.

As I mentioned with the fragmented or heterogeneous AAV system, there are heterogeneous pieces of DNA of different sizes contained within each AAV capsid. And we thought to ourselves, this probably isn't the best idea if we're pushing forward to the clinic. Because if you approach the FDA and you say, I'd like to deliver a virus containing different sized pieces of DNA to this patient, the FDA might not think that that was the safest approach.

So what we decided to do was design dual AAV vector systems, where in each capsid contains a piece of DNA that was completely defined. In other words, we knew exactly how big it was, and what the sequence of that DNA was, that was contained in each capsid. And the first platform that we tried was something called the simple overlap platform.

In this platform, the first virus contains the front half of the myosin VIIA gene. The second vector contains the back half of the myosin VIIA gene. And shared between them is the small region of overlap. And again once those two capsids get inside the cell, those two pieces of DNA can find each other via that region of overlap, recombine to form the full length gene and then go on to make full length myosin VIIA.

Excitingly, we were able to show that the simple overlap dual AAV vectors were capable of restoring both the melanosome migration, and the opsin trafficking defect, that we saw in those shaker 1 mice. One thing noted in the study however, was that the rescue was spotty. Rescue was possible, but some cells exhibited rescue, and other cells did not.

And so at this point, we reasoned that we really needed to increase the efficiency of our dual vector systems. And so we did just that, by designing multiple other dual AAV vector platforms, that differed in their molecular design. And in the interest of time I won't go into that right now. But then we compared them for their ability to express myosin VIIA protein.

And what we found after we infected cells with all of these dual vector platforms, was that if you delivered the fronts and the back half vector together, in other words, the 5 and the 3 prime vector, that we were able to deliver or drive myosin VIIA expression that was of the appropriate size. Which you can see here for reference.

However, when we delivered only the front half vector, in several of these platforms we could see a truncated piece of protein that was produced. More frequently when it was just the front half vector, but some tended to linger even if the front and the back half vector were delivered together. And what we think is happening here, is that that front half AAV vector may be producing some kind of truncated myosin VIIA protein. The jury is still out on that. But that's an idea that we're pursuing right now.

We show that fragmented AAV, simple overlap AAV, and AP hybrid dual vectors were able to deliver myosin VIIA to the retinal pigment epithelium and photoreceptors of the mouse retina. However, you should note that in the

bottom panel in the AP hybrid dual vector platform, that there was profound thinning of the retina after treatment.

And we don't know yet why this is. It could be that we have over-expressed the therapeutic protein. It could be that we have expressed it in a cell type that it doesn't want to be in, and we'll come back to that in a moment. It also could be that that formation of that truncated protein product is having a toxic effect on the retina. And those are all ideas that we're exploring now.

The next question we asked was what did the sequence of the recombined gene look like. In other words, when the front half and the back half came together, did they come together properly, or were there any genetic insertions, mutations, deletions, et cetera, associated with that recombination event. And so we did a variety of experiments to prove that indeed the AAV mediated myosin VIIA sequence was 100% normal. And so this means a lot from a regulatory standpoint. That these dual AAV vectors may be actually safe for use in the clinic.

So what had been accomplished at this point so far. First, we showed that fragmented AAV vectors were capable of driving full length myosin VIIA protein in the shaker 1 retina. We also showed that these fragmented AAV vectors could correct the shaker 1 phenotypes.

We also showed that simple overlap dual AAV vectors with defined genetic payloads, could also correct the shaker 1 phenotype. Using a more efficient dual vector platform, the AP hybrid dual vector platform, we showed that we could drive even higher levels of myosin VIIA expression, both in vitro and in vivo. And importantly, we also showed that the sequence of the dual AAV mediated myosin VIIA transcript was 100% normal.

So we felt strongly that we had laid the groundwork, at this point, for development of an AAV based treatment for USH1b. However, there were some lingering questions. And that was why don't shaker 1 mice have a loss of retinal structure and function. And number two, will these truncated proteins that we're seeing have an impact.

So to our first question, why don't shaker 1 mice have a loss of retinal structure and function. Well interestingly, whether or not myosin VIIA is actually expressed in mouse photoreceptors, remains controversial. There are some studies suggesting that it is in mouse photoreceptors. There are other studies suggesting that it's not in mouse photoreceptors. However, there is no question that myosin VIIA is expressed in non-human primate or monkey, and human photoreceptors. There's no debate about that in the literature.

And we think this discrepancy may have something to do with the structural differences seen in mouse versus primate photoreceptors. And I've taken this image from the Peti lab, who has very nicely shown that in primate photoreceptors, which you can see here on the right-- This is in the cat retina.

These are high resolution, electron micrograph images of a primate photoreceptor. Showing this neat little structure that you can see in the cartoon in orange. And then within the EM itself, the structure that forms a collar around the base of the inner segment, and the bottom of the outer segment. It essentially forms a collar at that junction. And those are called calycal processes.

And if you look at where myosin VIIA is expressed in the primate photoreceptors, you'll notice that it is localized right to those calycal processes. Now interestingly, on the top left, you'll see that mouse photoreceptors lack calycal processes. So it may be that because mouse photoreceptors don't have calycal processes, that indeed they don't express myosin VIIA. And this may be the reason why the shaker 1 mouse does not exhibit degeneration of its retina, or a loss of retinal function.

Recently in my lab, we showed that myosin VIIA is expressed quite differently in the non-human primate, versus the mouse retina. When you look at the non-human primate retina, you'll see that the vast majority of myosin VIIA expression is present in the neural retina. Which is an area of the eye where the photoreceptors are present. Whereas little to no expression could be detected in the retinal pigment epithelium. The exact opposite is true in the mouse retina, where the majority of myosin VIIA expression is found in the retinal pigment epithelium. Whereas little to no expression is found in the neural retina.

So again this may explain, why the only very robust phenotype we could see in the mouse was that melanosomes migration phenotype, because myosin VIIA is indeed in the mouse photoreceptors. Whereas again, in the human disease, in the primate retina, photoreceptors are the initial site of insult. And you can see why, here in this Western blot on the left, because myosin VIIA expression is very high in the neural retina of primate. over.

On to our second question, and that was what is the impact of these dual AAV vectors, or the front half vector alone, or the back half vector alone, at different doses following injection in wild type mice or normal mice. And what we found was, put simply, the more dual vectors that you delivered, the greater the loss of retinal function in these mice. There was a dose at which no loss of retinal function was observed, and this may be the safe dose.

Similarly, the more front half only vector you gave to these mice, the greater the loss of retinal function. And again there was a dose at which no loss of retinal function that was observed. The back half vector alone had no impact on retinal function whatsoever. But this tells us something about the dual vectors, and maybe more importantly about the front half vector alone. And so my lab currently is exploring ways to re-engineer this dual vector system, so as to prevent formation of a potentially toxic front half truncation product.

In summary, up to this point, it's been shown that a lentiviral based gene therapy is proving ineffective for correcting the retinal phenotype in USH1b patients. And whether this is due to the failure of lentivirus to efficiently

transduce photoreceptors, is up for debate, but it's possible. In USH1b patients we know that photoreceptors are the primary site of disease. So that's the cell type we need to target, if our gene therapy is going to be effective.

Myosin VIIA is differentially expressed in the mouse versus primate retina. And that could be due to the fact that mice lack calycal processes. We know that AAV is the gold standard for delivering genes to photoreceptors, and so we went on to show that fragmented AAV and dual AAV myosin VIIA vectors are capable of correcting melanosome migration in the shaker 1 mice.

Importantly, we showed that the dual vector mediated myosin VIIA sequence is 100% normal. And we also showed that there was a loss of retinal function in a dose dependent fashion. In other words, the more dual vector or the more front half factor alone that you added, the greater the loss of retinal function. And so some re-engineering, or simply a dose reduction, may be in order.

So in terms of where we're at now, and also future directions, it is our strong belief that for a gene therapy for USH1b to be successful, vectors must be designed to recapitulate the expression pattern of myosin VIIA in primate retina. So recall that we found stark differences between where myosin VIIA was expressed in mouse versus monkey. And in order for these vectors to be really tested, we need to test them in a species where we can express the protein where it should be in the humans. It might not be informative to be testing these vectors in the mouse retinas.

So we've begun to do just that in collaboration with our colleagues at the University of Alabama, at Birmingham. And depending on the results of those studies, say we see no toxicity whatsoever, because now we're working in a species that is amenable to myosin VIIA expression in photoreceptors, we may be able to push this therapy directly forward into IND enabling studies. However, if we still see issues with toxicity, we have plans to further evaluate methods to get rid of that production of that truncated front half protein product.

And I'm very happy to be the recipient, very recently, of an FFB Gund-Harrington Scholar Award, to pursue this work. Because I think that we have a lot of good ideas, and we're headed in the right direction. And I think that a dual AAV vector and mediated gene therapy for USH1b is a worthy pursuit, and it's something that's certainly on the horizon. Thank you so much for your time.