Brief progress report for AURIS foundation – asked for a "succinct" summary and discuss how important the machine was

We are incredibly grateful to the AURIS Foundation for supporting our research this year. Below is a brief summary of our progress using the electroporation device, which has been purchased with the funds from the AURIS Foundation.

The principal goal of our current research project is the development of a novel technique applicable in the therapeutic treatment of age-related macular degeneration (AMD), the leading cause of blindness in elderly people in the Western world. It is estimated that AMD will affect nearly 200 million people by 2020 and 300 million by 2040 (1, 2). AMD is characterized by the degeneration of the central retina, or macula, the region with the highest concentration of photoreceptors and responsible for sharp central vision. AMD presents two forms, dry or atrophic and wet or neovascular. Wet or neovascular AMD (nvAMD), characterized by abnormal blood vessel growth, represents only 10-15% of all cases but it comprises the majority of cases with severe AMD-related vision loss (3, 4).

Current research indicates that nvAMD results from an imbalance in the levels of growth factors that normally regulate blood vessel growth and vascular permeability, specifically the balance between vascular endothelial growth factor (VEGF), which promotes vascularization, and pigment epithelium-derived factor (PEDF), which inhibits vessel growth (5-7). Currently the standard treatment for nvAMD is frequent anti-VEGF injections into the vitreous to reduce pathological blood vessel development in the retina. However, to be effective this course of care is highly unpleasant, expensive, and must be repeated indefinitely at monthly intervals (8). Further, response can vary significantly: in two large American studies, vision was maintained in over 90% of people, but only improved significantly in 25-40% of cases after treatment (5, 9-11) and over an extended period in many cases the diseases progresses, albeit at a slower rate. Thus, there is an unmet need for improvements of durability and efficacy of the therapy. One obvious step would include modifying both the drug delivery system and its targeting strategy.

In effort to create a more lasting nvAMD treatment, recent approaches have explored gene therapy to modify DNA responsible for pathological protein production, rather than combat later symptoms. Most experiments use a modulated virus (viral vectors) to introduce outside DNA into cells (a process called "transduction"). However, while viral vectors can provide efficient gene transfer, they often only offer a temporary solution because added DNA is not integrated into the genome; viral vectors that integrated the added DNA into the cells' genome are available but can cause severe side effects including cancer. Repeated treatment is often impossible due to the production of immunological reactions against the virus while the first treatment usually does not (12). Creation and production of viral vectors is also costly and time-consuming, reducing the potential for scaling up to industrial production.

In our lab we use electroporation in combination with the non-viral *Sleeping Beauty (SB)* Transposon System, which results in high transfection efficiency and safe, long-term transgene expression (13, 14).

Transposons offer a more lasting, safe, and inexpensive method of gene delivery than viral vectors (15). Transposon systems have two components: a DNA plasmid encoding the gene of interest and a second plasmid encoding the SB transposase enzyme, which "cuts" out the gene of interest from the plasmid and "pastes" it into the existing genome of the host target cells. The *Sleeping Beauty* transposon system has been shown to be a particularly promising option for clinical therapy, with strengths including convenience, biosafety, ability to transpose fairly long DNA sequences, and permanence, so only one successful treatment would be required to last for life (16). The Sleeping Beauty system was so named because it was repurposed for biotechnology following a "long evolutionary sleep" and was the first to be used effectively for gene therapy in vertebrates (15). Currently, there are 12 trials worldwide using this vector, with none in eyes and none *in vivo* (17).

We hypothesize that rebalancing VEGF and PEDF levels would restore proper retinal vascularization patterns by increasing PEDF secretion. Our team and partners have previously shown how the *hyperactive Sleeping Beauty* (*SB100X*) transposon system can be used for *ex vivo* electroporation to transfect retinal and iris pigment epithelial cells with PEDF (13, 14). In the *TargetAMD* project, cells were removed from iris tissue samples and transfected using electroporation and subsequently transplanted into the eye. The next step toward a practically useful method for application in humans would be *in vivo* treatment, to avoid any possible complication associated with and cell manipulation outside the body and cell transplantation.

With the generous funding of the AURIS Foundation, we purchased the BTX Gemini X2 Twin Wave Electroporator and two sets of electrodes for *in vivo* gene therapy. These tools have played a central and essential role in our investigation. While other teams have previously used *in vivo* electroporation for transfection, they have done so in the eyes of significantly smaller model organisms or in non-ocular tissues. Thus, transitioning to porcine eyes has required significant experimentation. Porcine eyes can be easily obtained from the local slaughterhouse and are similar in size to human eyes. Eye size is an essential element for developing a protocol for human application because electroporation involves the induction of an electric field whose strength is dependent on distance. The BTX Gemini X2 is impressively versatile and provides experimenters with remarkable control over transfection settings. Most notably, the device can produce both square and exponential decay waves for different approaches. While we plan to use specialty electrodes for the delivery of transgenes *in vivo*, the instrument has options to electroporate using cuvettes with cells in suspension or high throughput plates. This single device makes it possible not only to target porcine RPE cells *in vivo*, but also to conduct future gene therapy experiments in other tissues as well as *in ovo* and *in utero*.

As a result of the flexibility of the BTX Gemini X2, we had many decisions to make regarding the electroporation protocol. Over the past few months, we have systematically tested >30 programs in triplicate, using porcine eyes provided by a local slaughterhouse. To design these programs, we began with a comprehensive literature review then selected nine variables to optimize: electrode (electrode type and position), DNA delivery (injection location, DNA concentration, and DNA volume), and pulse settings (voltage, number, length, and interval). With

respect to electrode settings, we have varied type and position. Using both noninvasive paddles and invasive needles (figure 1A), we experimented placing electrodes on either side of the eye ("opposite") or on either side of the injection site ("adjacent") (Figure 1).

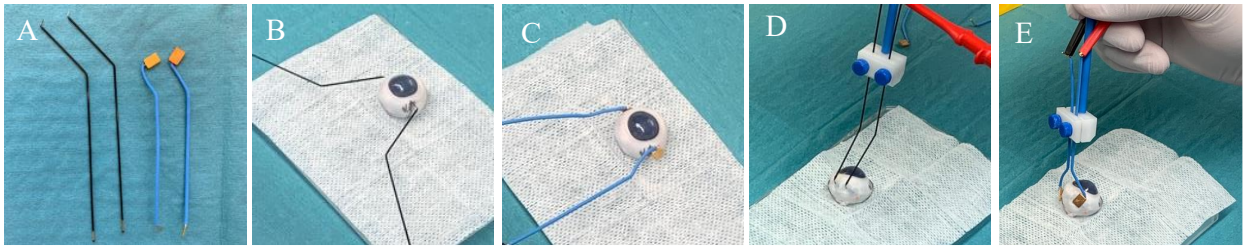

***Figure 1:* In vivo electroporation electrodes and positioning.** (A) Two different types of electrodes were evaluated: invasive "needle electrodes (left) and "paddle" electrodes (right), which cover a bigger area than the needle electrodes. Electrodes were positioned either opposite to each other, positive electrode always positioned directly at the injection site (B and C), or adjacent to the injection site (D and E).

To evaluate DNA delivery settings, we tried both intravitreal and subretinal injection locations and various DNA volumes, while keeping DNA concentration constant following previous optimization work. Finally, with respect to pulse, we tested a range of pulse numbers, voltages, lengths, and intervals between pulses. The whole procedure from injection of the plasmid DNA, electroporation, isolation and finally seeding of the cells for *in vitro* analysis is shown in Figure 2.

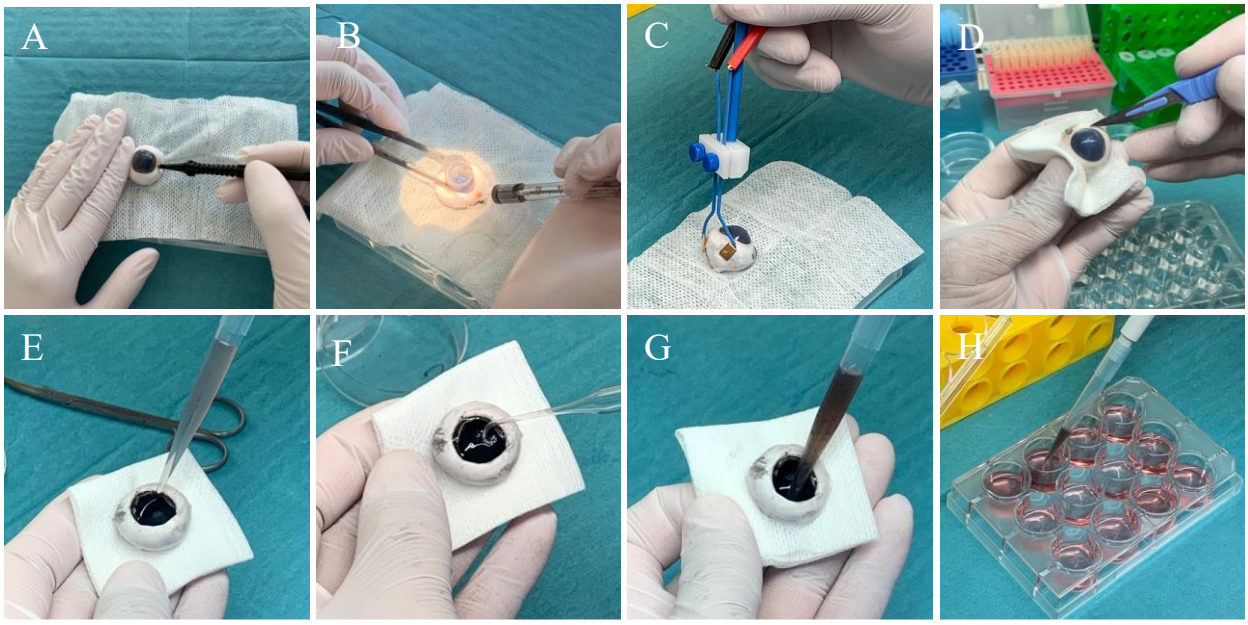

***Figure 2: In vivo* electroporation procedure of porcine donor eyes.** (A) The porcine eye was placed on a 12-well plate on a sterile gauze compress and a trocar was inserted to access the interior of the eye. (B) Using a lens and a surgical microscope, the plasmid DNA was injected subretinally through the trocar with a microinjection syringe. (C) *In vivo* electroporation was performed using either needle or paddle electrodes positioned opposite or adjacent to the injection site, as shown in figure 1. (D) After electroporation, the eye was cut approximately 3 mm posterior to the limbus and the anterior segment, the vitreous and the retina were removed. (E) Culture medium was added to the posterior segment and (F) RPE cells were mechanically removed using a bent Pasteur pipette. (G) Isolated RPE cells were gently resuspended and (H) plated in 12-well plates in culture medium supplemented with 10% FBS (Fetal Bovine Serum).

To enable visual assessment, using the BTX Gemini X2 we transfected the RPE cells *in vivo* with the Venus gene, which expresses the yellow fluorescent marker protein *Venus*. At this time, four programs of 30 have yielded fluorescent cells, indicating successful transfection (Fig 3). After observing the first transfected cells, we began the optimization process by making smaller adjustments to variables. Although these results are preliminary, the presence of bright yellow cells at 21 days post-transfection suggests stable integration of the *Venus* transgene.

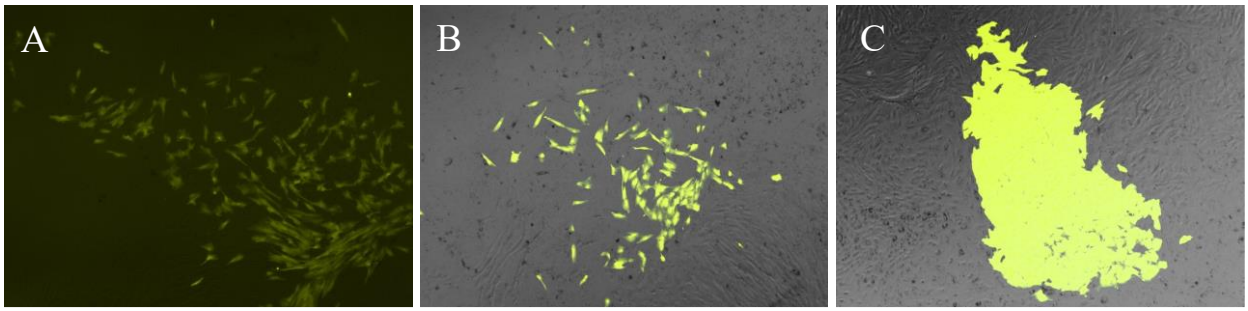

Figure 3: **Preliminary results of *in vivo* electroporation.** Electroporation was carried out using a plasmid encoding the *Venus* gene to allow visualization of transgene expression by fluorescence microscopy. (A) Fluorescence image of cells and (B-C) fluorescence overlay images of retinal pigment epithelial cells using different experimental programs. Clusters of transfected cells are visible in yellow, surrounded by non-transfected, non-fluorescent RPE cells.

Going forward, we continue to optimize these programs to increase transfection efficiency. We are currently planning additional experiments with the plasmid encoding the *Venus* gene but using an RPE-specific promotor to increase safety and efficiency. Once we have developed a successful program, we will transfect RPE cells in whole donor eyes with the *PEDF* gene followed by biochemical and histological analysis to qualitatively and quantitatively assess the transfection. Following the successful development of a protocol for the transfection of donor porcine eyes with the *PEDF* gene, we plan a safety and efficacy study by transferring the protocol from *ex vivo* porcine eyes to live rabbits, which have eyes of similar size.

References:

1. Eye Diseases Prevalence Research Group. Causes and prevalence of visual impairment in adults in the united states. Arch Ophthalmol. 2004:122(4):477-485.
2. Wong WL, Su X, Li X, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. Lancet Glob Health. 2014;2(2): e106–e116.
3. Ambati J, Ambati BK, Yoo SH, et al. Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. Surv. Ophthalmol. 2003; 48 (3):257-293.
4. Ferris, FL, Fine SL, and Hyman L. Age-related macular degeneration and blindness due to Neovascular maculopathy. Arch Ophthalmol. 1984;102 (11): 1640-2.
5. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med. 2006 5;355(14):1419-31.
6. Tolentino MJ, McLeod DS, Taomoto M, et al. Pathologic features of vascular endothelial growth factor-induced retinopathy in the nonhuman primate. Am J Ophthalmol. 2002; 133: 373–85.
7. Bhutto IA, McLeod DS, Hasegawa T, et al. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. Exp Eye Res. 2016; 82(1):99-110.
8. Amoaku WM, Chakravarthy U, Gale R, et al. Defining response to anti-VEGF therapies in neovascular AMD. Eye. 2015; 29(6):721-31.
9. Thumann G. Nonviral gene therapy for age-related macular degeneration. Expert Rev Ophthalmol. 2011; 6(1):81-93.
10. Brown DM, Kaiser PK, Michels M, et al. Ranibizumap versus verteporfin for neovascular age-related macular degeneration. N Engl J Med. 2006; 355(14):1432-44.
11. Brown DM, Michels M, Kaiser PK, et al. Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration : Two-year results of the ANCHOR study. Ophthalmology. 2009; 116(1):57-65.e5.
12. Izsvak Z and Ivics Z. Sleeping Beauty Transposition: Biology and Applications for Molecular Therapy. Mol Ther. 2004; 9(2):147-56.
13. Johnen S, Izvak Z, Stocker M, et al. Sleeping Beauty Transposon-Mediated Transfection of Retinal and Iris Pigment Epithelial Cells. IOVS. 2012; 53(8):4787-4796.
14. Thumann G, Harmening N, Prat-Souteyrand C, et al. Engineering of PEDF-expressing primary pigment epithelial cells by the SB transposon system delivered by pFAR4 plasmids. Mol Ther Nucleic Acids. 2017; 6:302-314.
15. Kebriaei P, Izsvak Z, Narayanavari SA, et al. Gene therapy with the Sleeping Beauty transposon system. Cell. 2017; 33(33):852-870.
16. Hudecek M, Izsvak Z, Johnen S, et al. Going non-viral: the Sleeping Beauty transposon system breaks through on the clinical side. Crit Rev Biochem Mol Biol. 2017; 52(4):355-380.
17. Gene Therapy Clinical Trials Worldwide. The Journal of Gene Medicine, December 2018. (http://www.abedia.com/wiley/)