To develop a lipid nanoparticle (LNP)-mRNA vector-based antiglaucoma gene editing therapy

PI: Prof. Wenjun Xiong Co-I: Prof. Zongli Zheng

Background of Research

Glaucoma

Glaucoma is a leading cause of irreversible blindness characterized by progressive damage of retinal ganglion cells (RGCs), affecting more than 65 million people worldwide (Quigley and Broman, 2006; Tham *et al.*, 2014). A global investigation revealed that around 3.6 million blindness cases in those aged 50 years and older were caused by glaucoma in 2020 (Bourne *et al.*, 2021). Noteworthily, primary open-angle glaucoma (POAG) is the most prevalent form of glaucoma, accounting for approximately 70% of all glaucoma cases (Tham *et al.*, 2014). The persistent elevation of intraocular pressure (IOP) caused by the resistance to drainage is the main symptom of POAG. High IOP leads to the damage to the optic nerve in retina and eventually the apoptosis of RGCs (Nickells, 2007). Reducing the inflow of aqueous humor from the ciliary body and increasing the outflow via the uveoscleral route or trabecular meshwork are currently the major approaches to relieve intraocular hypertension (Braunger, Fuchshofer and Tamm, 2015). Either in the east or the west countries, methods to decrease IOP are mainly surgery, laser treatment and medicinal treatment. Although surgery and laser treatment are the rapidest ways in lowering ocular hypertension, highly trained surgeons and intensive monitoring are required and extreme mental or physical pain for patients are inevitable.

Caveats of current IOP-lowering medications

Nowadays, the most common and non-invasive approach used in clinics for glaucoma is medicinal treatment, which includes prostaglandin analogues, carbonic anhydrase inhibitor, β-adrenergic blockers, α-adrenergic agonists, rho-kinase inhibitor as well as cholinergic or miotics. However, these IOP lowering medications are imperfect due to the low bioavailability, drug resistance and a series of side effects (Weinreb and Tee Khaw, 2004; Ganvaveet *al.*, 2015; Yadav, Rajpurohit and Sharma, 2019).

1) Low bioavailability

Since most anti-glaucoma drugs are in eye-drop forms, the absorption and bioavailability are heavily restrained by the pre-corneal contact time and the duration of drug release (Robinson and Mlynek, 1995). It was reported that only 1% to 7% of applied drop solution can reach the anterior segment and the majority of drugs are quickly drained (Ghate and Edelhauser, 2008). Because of the low absorption and bioavailability of anti-glaucoma eye drops, high frequency administrations are required in order to maintain a stable IOP. Poor medication persistence of glaucoma patients has also been attributed to inconvenient therapeutic regimens, with a reported therapy discontinuation rate of up to 65% (Zhou *et al.*, 2004; Quek *et al.*, 2011).

2) Lack of effectiveness

Glaucoma is a chronic progressive disease, and medications are used on a prolonged basis. This sometimes results in unexpected drug resistance and an increase in the dose of medication (Baudouin, 2007). It was reported that the efficacy of anti-glaucoma drugs, such as carbonic anhydrase inhibitor (brinzolamide) and β-adrenergic blocker (timolol), in reducing IOP decreases after 6-month continuous treatment (March *et al.*, 2000; Oddone *et al.*, 2015). The dissipation phenomena of the timolol, the most common used glaucoma medicine, is well known in the clinical management of glaucoma (Boger, 1983). The reduction of timolol's efficacy can be observed over a few days (short-term escape) and over months or years (long-term drift). Lack of effectiveness hampers the long-term use of timolol in glaucoma patients.

3) Severe systemic side effect

Systemic side effects are the biggest safety concern of anti-glaucoma eye drops. Sufficient amount of unabsorbed agent can get into the systemic circulation via nasopharyngeal mucosa and induce severe systemic effects, such as bronchospasm, heart block, central nervous system effects, bradycardia and so on (Everitt and Avorn, 1990). A comprehensive survey by Chen *et al.* (2020) investigated 1236 patients diagnosed with glaucoma from 2000 to 2010, and found that β-adrenergic blockers, one of the most frequently prescribed first-line topical therapies for the reduction of IOP, was tightly associated with acute respiratory failure and ischaemic stroke.

In summary, a therapeutic approach with better efficacy and safety profile is highly demanded.

Gene therapy for glaucoma treatment

Over the past decades, huge efforts were made to the development of recombinant adeno-associated virus (AAV) for in vivo gene delivery. Relied on its high efficiency and low immunogenicity, numerous clinical trials of gene therapies using AAV as a vector are in process for ophthalmic diseases (Gordon *et al.*, 2019). The very first FDA approved gene therapy was an AAV-RPE65 vector to treat Leber's congenital amaurosis (LCA), an inherited blindness disease (Maeder *et al.*, 2019). Meanwhile, there are many more ocular gene therapy trials being tested for various inherited and complex eye diseases.

However, there are unexpectedly fewer gene therapy trials targeting glaucoma despite the high prevalence of glaucoma and its significant impact on global health. This may be mainly due to several difficulties. Firstly, glaucoma is a multifactorial disease. The genetic factors contributing to glaucoma are still poorly understood, and thus it is not always straightforward which gene to target for glaucoma treatment. Secondly, fewer preclinical studies have been carried out to target key ocular structures, including trabecular meshwork and ciliary body, by AAV or other gene therapy vectors. Lastly, glaucoma treatment demands long-term efficacy without toxicity, which is a difficult hurdle to overcome.

An anti-glaucoma gene editing therapy using lipid-nanoparticle (LNP) mRNA vector

Here, we propose to develop a novel anti-glaucoma gene editing therapy with the following advantages: **1) scalable production, 2) low off-target effects, and 3) multiple gene targets**. With the excellent property to encapsulate messenger RNA (mRNA), lipid nanoparticles (LNP) are becoming another promising vector. The flexibility of LNPs in the fabrication process endows them with different chemical and physical properties, including size, shape, surface chemistry, charge, hydrophobicity, etc., allowing them to overcome biological barriers of cells and escape endosomes. Besides, the low immunogenicity, ability to deliver large fragments of transgenes, and good biodegradability make them ideal vectors. LNP surface can also be decorated with ligand proteins, giving it cell-specific delivery capabilities. LNP also has the advantage of industrialization, with a simple production process, low cost, and easy large-scale production. Compared to AAV, its ability to repeat administration and no packaging limits make LNP available for more comprehensive applications. Moreover, its transient expression lowers the risks for gene editing therapy as off-target edits will not accumulate over time. Therefore, the LNP-mRNA vector can make up for the deficiencies of AAV.

Preliminary data:

In the pilot study, we tested several LNPs encapsulating Cre-mRNA at first, as Cre is much more sensitive for reporting the infection phenotype *in vivo*. The optimized LNP production protocol is shown in Figure 1. Cre-LNPs were intravitreally delivered to the Cre reporter mouse lines, and three to five days post-injection, the eyeballs were harvested for subsequent cryosection and immunohistochemistry (IHC) (Figure 2). This time frame is sufficient for mRNA expression as indicated in the previous study (Patel et al., 2019). The large view of the whole retinal sections revealed patchy infection of Müller glia (MGs) by LNPs (indicated by white arrows), while the optic nerve head (ONH) and cells in the outflow facility exhibited strong fluorescent signals (Figure 2). Upon injection into the intravitreal chamber, LNPs have the chance to be uptaken by MGs through the inner limiting membrane (ILM), but largely, they might be eliminated in the vitreous humor via outflow facility including the ciliary body (CB) and trabecular meshwork (TM). This could explain why LNPs could only infect a few MGs while achieve strong expression in the outflow facility. The zoom-in views of the retinal sections showed predominant expression of Cre-mRNA in MGs (Figure 2). In the retinal outflow facility, strong Cre expression was observed in the CB, TM and iris (Figure 2). The CB structure comprises two main cell types: non-pigmented ciliary epithelium (NPCE) and pigmented ciliary epithelium (PCE). Figure 2 revealed that nealy all the NPCEs were labelled by tdTomato fluorescence, while PCEs showed a few signals as well. It has been reported that NPCEs in the CB contribute to the formation of vitreous humor (Farahbakhsh et al., 1987), while TM is responsible for its outflow (*Braunger et al., 2015*). Disruptions in NPCEs or TM can lead to disordered flow rates of vitreous humor, resulting in

abnormal intraocular pressure (IOP), which is one of the pathogenic factors in glaucoma (Alvarado et al., 1984). Considering the potent infection of LNPs in the outflow facility, particularly in NPCEs and TM, they hold promise as a highly effective delivery system for achieving gene therapy in glaucoma treatment.

From the known IOP-associated genes, we selected four gene targets, which are ADRB2 (encodes β2-adrenergic receptor), CA2 (encodes carbonic anhydrase II) and ROCK1 and 2 (encodes two Rho kinases) (Table 1). When the expression of the selected genes is silenced, IOP lowering effects are expected given the known functions of the genes. Silencing the ADRB2 gene should mimic the effect of using beta blockers, such as Timolol Maleate, which works to decrease the production of intraocular fluid (Coakes, 1978). Silencing CA2 genes should mimic the effect of using Carbonic Anhydrase Inhibitors (CAIs), such as Brinzolamide, which also works to decrease the production of intraocular fluid (Bernard Becke, 1954). The mechanism of CAIs in lowering IOP is to inhibit the enzymatic activity of carbonic anhydrase to convert carbonic acid to water in the NPCE. Silencing ROCK genes should mimic the effects of using Rho Kinase Inhibitors (RKIs), such as Netarsudil, which works to increase drainage of intraocular fluid by improving outflow of the trabecular meshwork (Honjo, 2001; Wang, 2015). Combined medications have been offered to glaucoma patients who have little response to a single medication. For example, Rocklatan@ (Aerie Pharmaceuticals, Inc.) is the combination of RKI and beta blocker, while Cosopt@ (Akorn Inc.) is the combination of CAI and beta blocker. These combined medications provide evidence that targeting multiple pathways/genes in the aqueous humor production and outflow pathway can provide additive or synergistic treatment effects.

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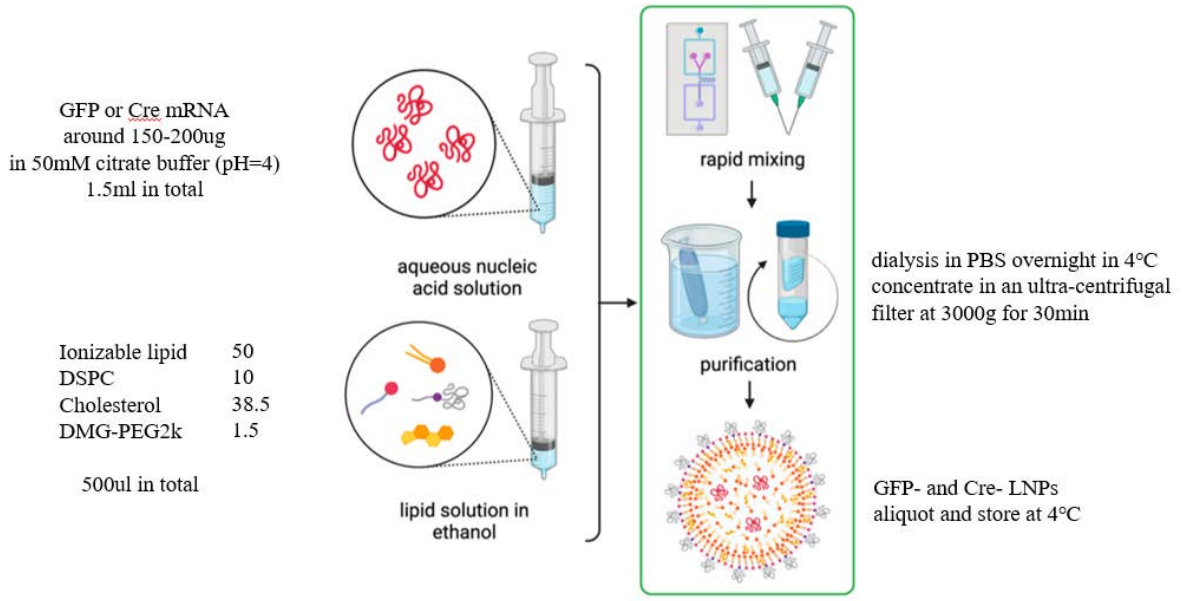

Figure 1. A schematic representation of LNP production flow. mRNA is diluted in the citrate buffer, and the four lipids in the ethanol using the indicated ratio. After mixing in the microfluidic chip, LNP was dialyzed in PBS and concentrated using the ultra-centrifugal filter.

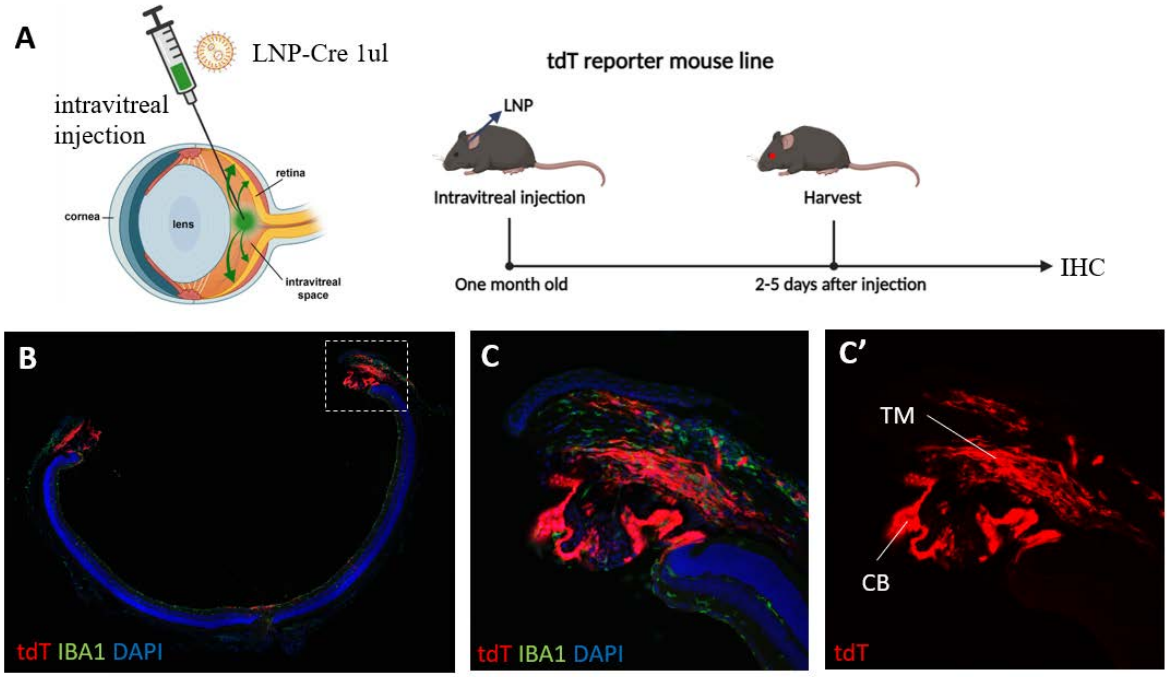

Figure 2. Targeting the outflow facility of mouse eyes by intravitreally delivered LNP-Cre vectors. (A) A schematic representation of the intravitreal injection. Adult mice were anesthetized for the operation. A thin wedged glass capillary was custom-made and loaded with LNP. About 1ul of LNP solution was slowly pushed into the posterior chamber. Lower right: demonstration of the ciliary body and trabecular meshwork infected by LNP vectors flowing from posterior chamber to anterior chamber. (B-C) A mouse eye section showing CB and TM infection. Mice were sacrificed at 5 days post LNP infection. Tdt signal was evident

in the NPCE and PCE, vessels of the ciliary body, and trabecular meshwork. PCE, pigmented ciliary epithelium; NPCE, nonpigmented ciliary epithelium; CB, ciliary body; TM, trabecular meshwork.

Gene	**Sequence (5'-3')**	**Region**
ADRB2	GTCCTGCACACTCAGCTCGT	50-100 bp downstream of TSS
CA2	GTGGTGGGACATGGTCACGC	50-100 bp downstream of TSS
ROCK1	GCAGGGTGGAGACTCCCTCG	50-100 bp downstream of TSS
ROCK2	GCCGGCTCATGCCGCTTCGC	50-100 bp downstream of TSS

Table 1. List of gRNAs designed to target the four glaucoma-associated genes.