Ultrasound-mediated delivery of non-viral gene therapy vector expressing COL4a5 in X-Linked Alport Syndrome (XLAS) disease model mice and non-human primates

SONOTHERA™

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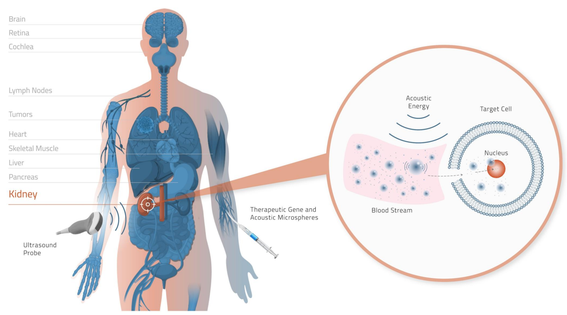
Introduction: XLAS is a hereditary disorder marked by progressive kidney dysfunction, hearing loss, and ocular abnormalities. The renal pathology results from structural defects in the glomerular basement membrane caused by mutations in the *COL4A5* gene. Lack of delivery technology allowing efficient delivery of a therapeutic transgene expressing full-length Col4a5 in podocytes prevents the development of genetic medicines for XLAS patients. Ultrasound-mediated gene delivery (UMGD) offers a non-invasive, non-viral and targeted approach for transgene delivery to renal cells, presenting a promising strategy for *COL4A5* gene replacement therapy in XLAS.

Methods: UMGD parameters were optimized to enable efficient transgene delivery to podocytes in both mice and non-human primates (NHPs). A series of non-viral gene therapy constructs encoding codon-optimized *COL4A5* sequences under the control of podocyte-specific promoters were engineered and screened *in vitro* in primary human cells to identify a podocyte-targeted expression cassette. The lead COL4A5 vector was delivered *in vivo* to the kidneys of an XLAS mouse model (G5X) and NHPs using non-viral UMGD. Safety of the gene delivery approach was assessed using established methods.

Results: To evaluate the safety and efficacy of UMGD for renal targeting, a reporter transgene was delivered to mice, resulting in kidney-specific and durable expression that was further enhanced with repeat dosing. An optimized podocyte-specific construct was developed which demonstrated robust full-length COL4A5 expression in primary human podocytes. The vector was successfully delivered to the kidneys of G5X mice using UMGD, resulting in strong transgene expression within the glomeruli. Further refinement of UMGD conditions enabled efficient delivery of the *COL4A5* transgene to NHP kidneys, with substantial delivery observed in podocytes. Safety and tolerability assessments using established endpoints confirmed a favorable safety profile, supporting continued advancement toward therapeutic application.

RIPPLE™ Delivery Platform

1. Ultrasound enhancing agents (a.k.a. microbubbles) co-infused intravenously with nucleic acid payload (e.g. DNA/RNA)
2. Ultrasound energy is externally applied to target organ
3. Expression of the therapeutic payload occurs in target cells



RIPPLE™ Platform is Titratable, Redosable, Durable, and Safe

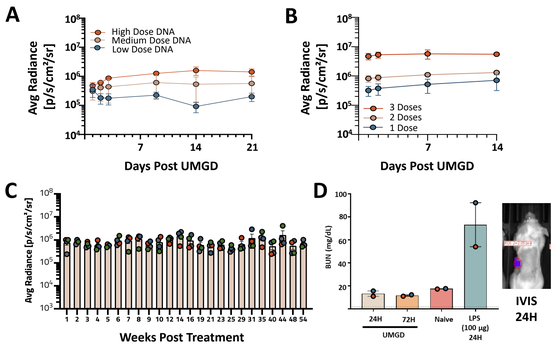


Figure 1. RIPPLE™ mediated delivery of DNA transgenic vector provides titratable, redosable, durable, and safe transgene expression. **(A)** IVIS measurement of luciferase transgene expression in the mouse kidneys treated with low, medium, and high DNA doses. **(B)** IVIS measurement of luciferase transgene expression in the mouse kidneys after single, double, and triple treatments. **(C)** IVIS measurement of luciferase transgene expression in treated mouse kidney for 54 weeks post treatment **(D)** Blood urea nitrogen (BUN) levels in 1- and 3-days post-treatment. LPS treatment was a positive control.

RIPPLE™ Efficiently Delivers DNA to Major Cell Types of Mouse Kidney

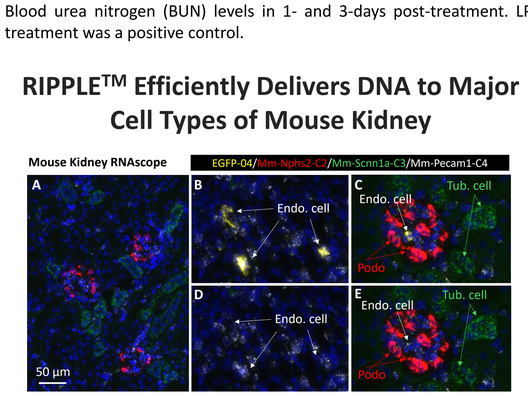


Figure 2. Mice were RIPPLE™ treated with a DNA vector expressing EGFP under control of the CAG promoter, and kidney from naïve and treated animals was stained using RNAscope with probes detecting transgenic DNA expression in podocytes (Nphs2+), endothelial cells (Pecam1+), and tubule epithelial cells (Scnn1a+) of mouse kidney was detected **(A)** Cell marker staining for naïve tissue. **(B–E)** RNAscope images of treated kidney, including EGFP and cell marker staining (B, C), or cell marker staining only (D, E). **(F)** HALO analysis of cell types detected in naïve mouse kidney. **(G–H)** EGFP+ biodistribution in kidney.

RIPPLE™ Efficiently Delivers DNA Transgene to Alport Disease Model Mouse Kidney

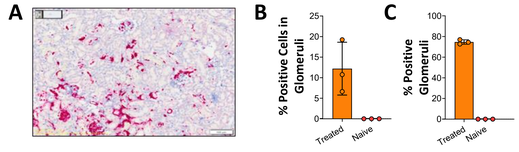


Figure 3. Transgenic DNA is efficiently delivered to the Alport disease model (G5X) kidney. **(A)** RNAscope detection of the transgenic DNA/RNA in the treated G5X mouse kidney. **(B)** HALO quantification of the % of cells with transgenic DNA/RNA in glomeruli. **(C)** HALO quantification of the number of glomeruli with transgenic DNA/RNA.

RIPPLE™ Efficiently Delivers Transgenic DNA to Major Cell Types of NHP Kidney

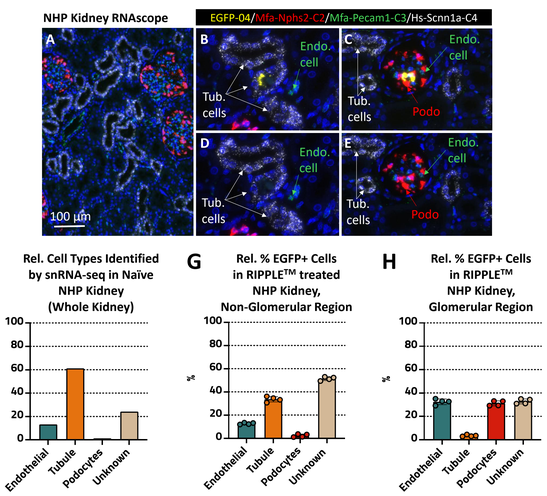


Figure 4. NHPs were RIPPLE™ treated with a DNA vector expressing EGFP under control of the CAG promoter, and kidney from naïve and treated animals was stained using RNAscope with probes detecting transgenic DNA/RNA, podocytes (NPHS2+), endothelial cells (PECAM1+), and tubule epithelial cells (SCNN1A+) of NHP kidney. **(A)** Cell marker staining for naïve tissue. **(B–E)** RNAscope images of treated kidney, including EGFP and cell marker staining (B,C), or cell marker staining only (D,E). **(F)** HALO analysis of cell types detected in naïve NHP kidney. **(G–H)** EGFP+ biodistribution in kidney.

Podocyte-Specific Col4a5 Therapeutic Candidate Engineering

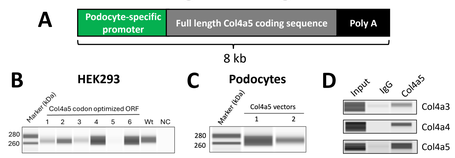
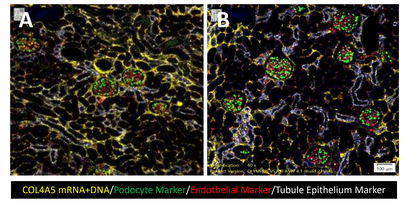


Figure 5. (A) Schematic representation of DNA vector expressing full length human Col4a5 under the control of a human podocyte-specific promoter. Codon-optimized Col4a5 ORFs were expressed in **(B)** HEK293 and **(C)** human primary podocytes. Transfected cell lysates were harvested and analyzed by capillary WB. **(D)** Co-IP assay was performed to test Collagen IV trimer formation. Cells were co-transfected with Col4a3, Col4a4, and codon-optimized Col4a5. Col4a5 and interacting proteins in lysates were precipitated, and protein samples were subjected to capillary WB analysis.

Efficient Transduction of Glomerular Cells and Nuclei in NHP Kidney



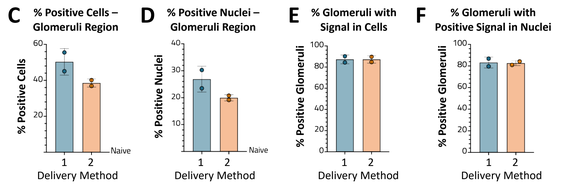


Figure 6. RIPPLE™ was applied to NHP kidneys to deliver transgene expressing human COL4A5. **(A,B)** RNAscope detection of transgenic COL4A5 DNA/RNA along with indicated cell-specific markers in treated NHP kidney. **(C, D)** Quantification of COL4A5 positive cells and nuclei in the glomeruli region across two different delivery variations. **(E, F)** Quantification of glomeruli cells or nuclei with COL4A5 signal across two different delivery variations.

Transgenic COL4A5 Protein is Expressed in NHP Glomeruli

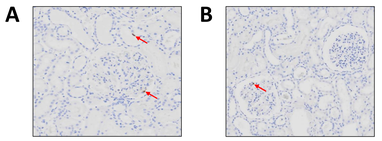


Figure 7. RIPPLE™ was applied to NHP kidneys to deliver transgene expressing human COL4A5. **(A, B)** COL4A5 protein was detected via IHC across 2 different delivery variations.

Favorable Safety and Tolerability Profile Following Delivery to NHP Kidney

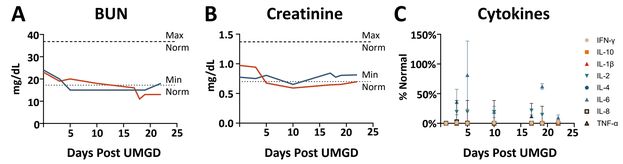


Figure 8. Renal functional response to RIPPLE™ treatment shows the tolerability of treatment. NHP **(A)** blood urea nitrogen levels, **(B)** creatinine levels, **(C)** average inflammatory cytokine expression after administration of RIPPLE™.

Conclusion:

- RIPPLE™ provides robust, durable, titratable, and redosable transgene delivery to kidney of small and large preclinical models including Alport disease mouse model kidneys.
- Podocyte-specific expression vector provides robust expression of full-length human COL4A5 in podocytes *in vitro* and in NHP kidney.