Targeted Delivery of siRNA to Proximal Tubule Cells in Kidney

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ABSTRACT

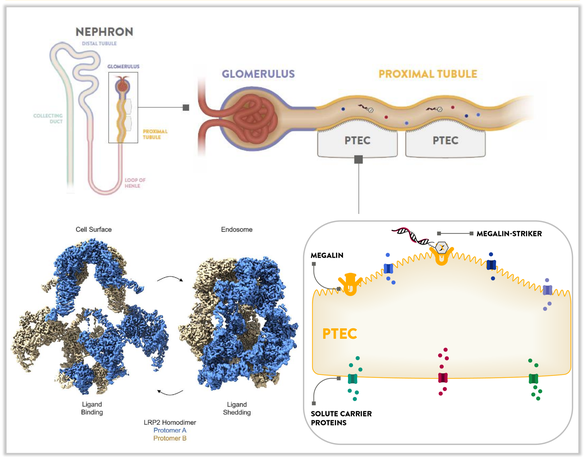
Small interfering RNA (siRNA) is a clinically validated therapeutic modality, which silences gene expression via RNA interference (RNAi). Although siRNAs are secreted through the kidney, siRNA mediated knock-down in the kidney is still limited, largely due to challenges with optimal delivery. Proximal tubule epithelial cells (PTECs) within the nephron are attractive targets for utilizing RNAi, where the primary mode of entry is likely endocytic uptake. Here we describe a targeted approach to deliver conjugated siRNAs exploiting the PTECs' internalizing receptors. Understanding the mechanisms of siRNA uptake in kidney PTECs is pivotal for developing efficient and safe delivery platforms targeting renal diseases.

In the present study, we investigated siRNA uptake in vitro and in vivo using conjugated siRNAs called STRIKERs . A well-differentiated, opossum kidney (OK) PTEC cell line was used as a model system to mimic proximal tubule biology both morphologically and functionally, for studying siRNA uptake. Fluorescently tagged siRNAs were employed to observe uptake and intracellular trafficking. Additionally, siRNAs conjugated with target ligands were administered in mice to assess exposure in vivo, measured by mass spectrometry and microscopy. We further measured siRNA mediated knock-down across multiple genes in mouse kidneys.

The siRNAs were taken up by OK wildtype cells in both time and concentration dependent manner, predominantly from the apical surface. We further studied the mechanism of conjugated siRNA uptake using specific inhibitors and cell specific knockouts of various PTEC endocytic receptors. Conjugation with selected ligands of megalin significantly enhanced siRNA uptake both in OK cells and in mouse kidneys. In addition, these conjugations led to significant gene knock-down across multiple targets.

The work suggests that PTECs employ megalin to facilitate siRNA uptake via endocytosis and can be used for gene silencing. Elucidating the mechanism(s) for siRNA uptake in the proximal tubule aids in the development of RNA-based therapeutics. Future work will focus on ligand conjugation optimization and chemical modification to improve kidney specific silencing activity.

BACKGROUND



- Kidney is a complex organ with dozens of cell types, making it hard for oligo mediated delivery.
- Megalin (LRP2) is an endocytic cell-surface receptor which is highly expressed on PTECs and recognizes a wide range of substrates. The rapid internalization, slow degradation and high recycling capacity makes it an ideal entry point for intracellular kidney-targeted delivery of a ligand-siRNA conjugate.
- Megalin-STRIKERs program aims to use the megalin receptor to specifically deliver siRNA therapeutics to the proximal tubule of the kidney to silence mRNA expression of specific solute carrier proteins (SLCs), thereby inhibiting the uptake of circulating solutes linked to systemic diseases.

(Megalin structures refer to PDB code 8EM4 and 8EM7, Beenken, et.al., Cell 2023, 186, 821–836)

Polarized OK cells recapitulate endocytic and morphological features of the proximal tubule epithelial cells

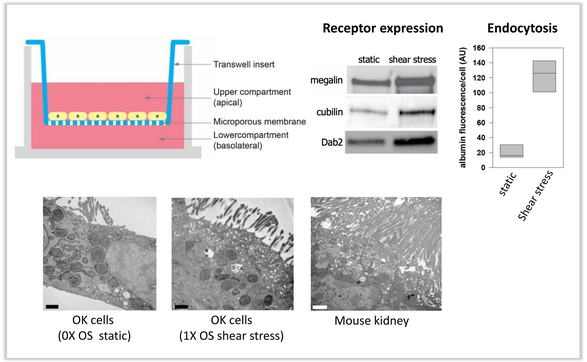


Figure 1. OK cells were grown on Transwell® permeable supports. After overnight incubation, the filters were transferred to an orbital platform shaker and rotated at 146rpm [1X orbital speed (OS)] for 72 hr. Apical regions of cells grown at 0X and 1X OS are shown in comparison to a similar section of PT in mouse kidney (by transmission electron microscopy). OK cells cultured under shear stress mimic features of native PTEC and megalin/cubilin-mediated endocytosis.

Endocytic uptake of megalin ligand-conjugated siRNA in OK cells is time and concentration-dependent and predominantly apical

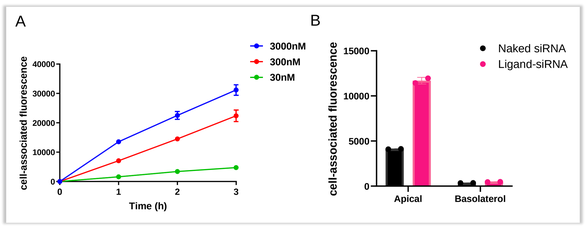
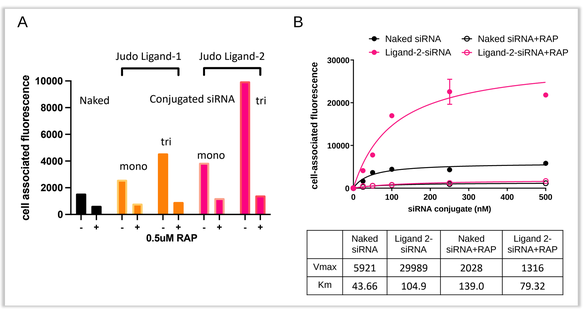


Figure 2. A. OK wildtype cells were treated apically with 30, 300, or 3000nM of conjugated siRNA for 1, 2, or 3hr. B. OK cells were treated apically or basolaterally for 3hr with 100nM unconjugated or conjugated siRNA. Endocytic uptake was assessed by spectrofluorimetry. Endocytosis of siRNA is in a time and concentration dependent manner and is predominantly from the apical surface.

Conjugation with selected megalin ligand increased uptake of siRNA in OK cells and the uptake was competed by RAP



	Naked siRNA	Ligand 2-siRNA	Naked siRNA+RAP	Ligand 2-siRNA+RAP
Vmax	5921	29989	2028	1316
Km	43.66	104.9	139.0	79.32

Figure 3. A. OK cells were treated with 3µM Cy5-labeled unconjugated siRNA or siRNAs conjugated to selected ligands of megalin +/- 0.5µM RAP for 1h. B. OK cells were treated with different concentrations of naked or conjugated siRNAs +/- 0.5µM RAP for 3h. Enhanced uptake of ligand conjugated siRNA was observed, and the uptake was competed by antagonist RAP (Lrpap1). Binding capacity (Vmax) of ligand-2 conjugated siRNA is ~5.1x that of naked siRNA.

The uptake of megalin ligand-conjugated siRNA in OK cells was abolished in *Lrp2* KO cells

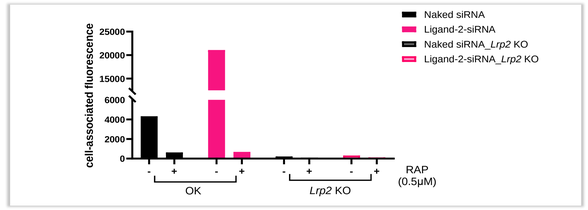


Figure 4. OK wildtype and *Lrp2* KO cells were treated apically with 300nM of Cy5-labeled naked or conjugated siRNA +/- 0.5µM RAP for 3h. Endocytic uptake was assessed by spectrofluorimetry. Uptake of naked and conjugated siRNA was abolished in *Lrp2* KO cells, and RAP profoundly inhibited uptake in both cell lines.

Conjugation with selected megalin ligand increased PT specific uptake of siRNA in mouse kidney

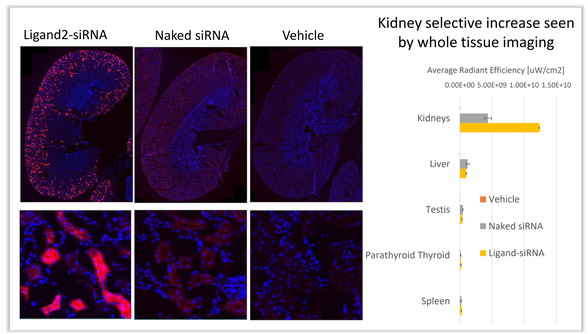


Figure 5. Mice were injected with Cy5.5-labeled siRNAs at a dose of 5 mg/kg and sacrificed 6 hours after administration. Fluorescence associated with different organs was quantified and shown in the graph; Tissues were sectioned for fluorescent imaging. Megalin ligand conjugation enhanced siRNA uptake in the kidney, specifically within PCTs. The enhanced uptake was specific to kidneys.

Conjugation with selected megalin ligand increased siRNA exposure, specifically in kidneys

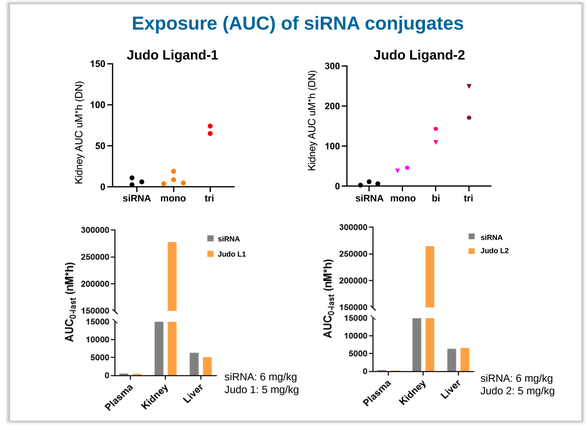


Figure 6. Ligand conjugated siRNA families of Judo Ligand-1 and Judo Ligand-2 were tested in mice for tissue and plasma exposure. Mice were administered with a single dose of naked or conjugated siRNA. Judo L1 and L2 were tested in multiple valencies attached to siRNA. siRNA exposure was measured by LC/MS. Both ligands conjugated to siRNA led to increase in kidney exposure over naked/unconjugated siRNA. The ligand mediated increase was specific to kidney.

Megalin STRIKERs mediated knock-down of different target genes in mouse kidneys

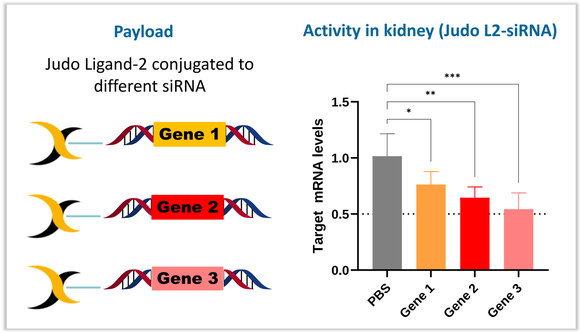


Figure 7. Judo Ligand-2 family ligands were conjugated to siRNA targeting three different gene targets. Mice (n=4 per group) were injected subcutaneously and necropsied at Day 7. A knockdown of approximately 30~50% was achieved in the kidneys across different mRNA targets. The STRIKE platform is modular, allowing for the same ligand linker to be used across different siRNA.

~70% durable target gene knockdown was attained in mouse kidneys with megalin-STRIKERs

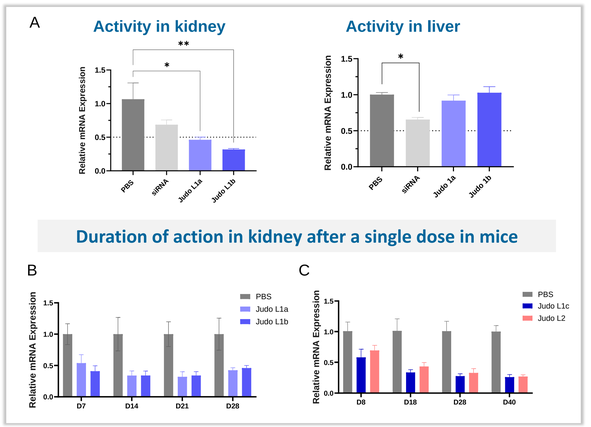


Figure 8. A. Mice were administered a subcutaneous dose of two variants of Judo Ligand-1 (Judo L1a and L1b). Significant knockdown of target gene was observed in the kidney at day 7. These ligand-conjugated siRNAs were ineffective in liver, consistent with lack of megalin expression in liver.

Duration of action was monitored in mice after a single dose of unconjugated or conjugated siRNAs with different variants of Ligand-1 and Ligand-2 for 28 (B) or 40 days (C). siRNAs conjugated with ligands of both family show sustained knockdown of target gene, lasting at least 40 days.

SUMMARY

- The JUDO STRIKE (Selectively Targeting RNA Into KidnEy) platform represents a significant advancement in targeted siRNA delivery for renal applications. By leveraging ligand-siRNA conjugates mediated by megalin, we achieved remarkable increases in siRNA exposure in a well-established proximal tubule cell line and in mouse kidney, as well as gene knock-down across multiple target genes specifically in the PTECs.
- These results highlight the utility of our innovative approach in optimizing gene silencing for therapeutic interventions in systemic and renal diseases. Future research will focus on refining ligand conjugation and chemical modifications to further enhance nephron cell specific silencing efficacy.

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