Barcoding of small extracellular vesicles with guide RNA enables analysis of gene editing and uptake in tissue repair

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INTRODUCTION

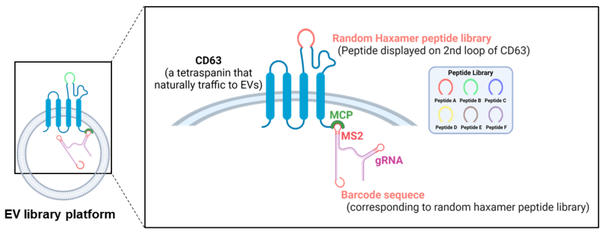
The therapeutic potential of extracellular vesicles (EVs) in promoting tissue repair has been established in animal models of impaired wound healing, however, there is an unmet need to engineer EVs to direct cell type-specific uptake and promote endosomal escape. Since EVs are efficient nanocarriers of small RNAs, we have focused on the engineering of EV payloads to deliver gene editing payloads in a CRISPR/Cas9 system based on modifications of select tetraspanins like CD63 that we have shown is highly expressed on EVs.

We have engineered CD63 to express an RNA binding peptide sequence (MCP) that facilitates loading of guide RNAs (gRNAs) that express the MCP-binding RNA sequence (MS2). We show that the loading of CD63 engineered EVs with gRNA is dependent on the interactions of MS2 with MCP. Furthermore, we show that with a gRNA designed to target lox sites, we can deliver gRNA-loaded EVs that induce a fluorescent reporter (tdTomato) that is under the control of an upstream lox-STOP codon-lox cassette. We provide *in vitro* cell culture imaging and *in vivo* imaging following systemic and topical treatments to demonstrate gRNA-specific activation in the presence of Cas9 of this reporter in skin and lung using topical treatments and in lung, liver and other organs following intravenous delivery.

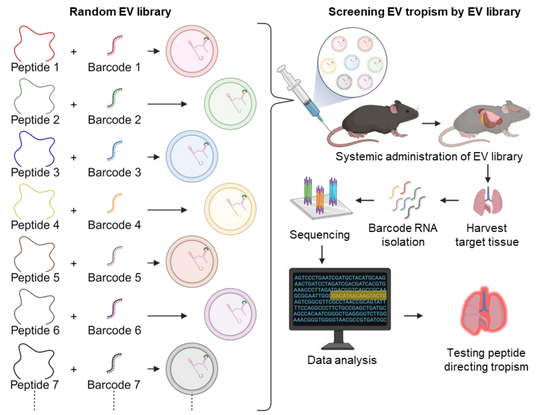
With these studies demonstrating the capacity for gRNA-loaded EVs to delivery gene editing payloads, we also provide proof-of-principle for how CD63 has been engineered for peptide display of a gRNA barcoded combinatorial library to identify novel mediators of EV uptake and endosomal escape. Together, this approach is designed to enhance the tropism and specificity of EV therapeutics in wound healing.

METHODS

Construction of bifunctional engineered EVs



- **Constructing a bifunctional EV capable of both targeting and payload delivery**
- **EVs displaying external random hexamer peptide libraries are paired with barcode sequences of internal gRNA cassettes**
- **Simultaneous target-specific delivery screening with gene editing capabilities**



- **Platform technology enabling systematic screening of target-specific drug delivery systems**
- **High accuracy by directly reading the base sequence paired with the peptide**

RESULTS

Engineered EVs charaterization

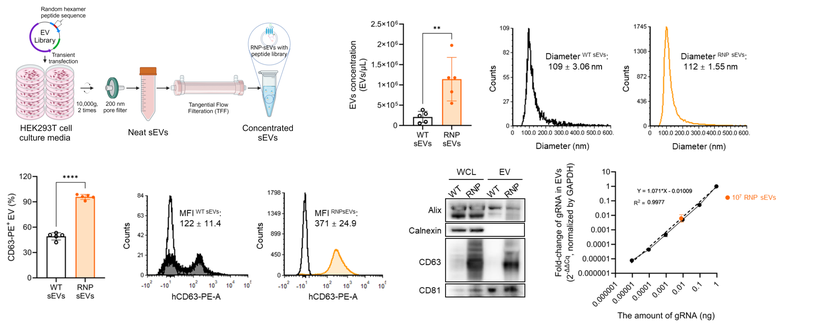


Figure 1. Characterization of random peptide displayed sEV library.

In vitro gRNA delivery and gene editing by EVs

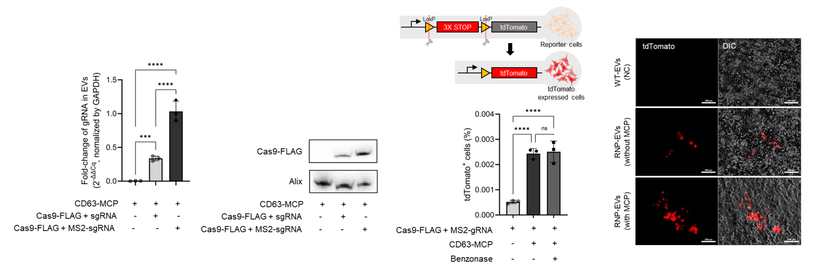


Figure 2. Testing of the delivery of gRNA by sEVs and their gene editing in recipient reporter cells

Systemic administration and intrinsic tropism of RNPL-EVs

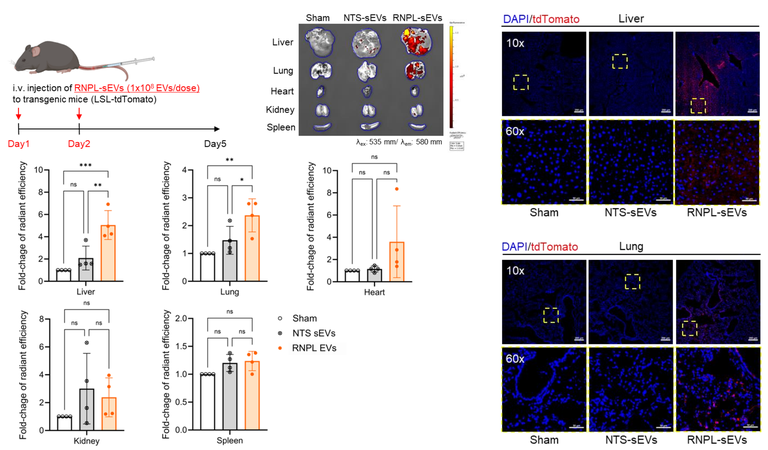
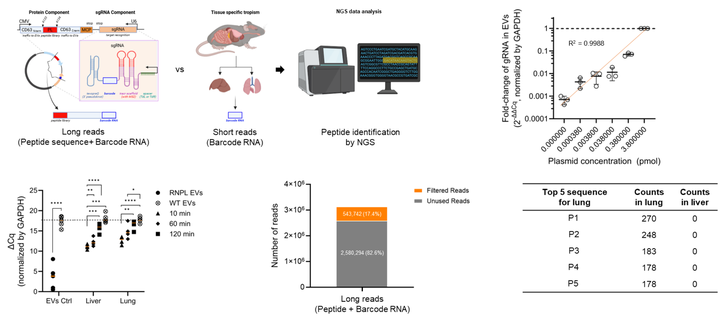


Figure 3. Systemic distribution of ribonucleoprotein with peptide library (RNPL) sEV and their tropism in mice.

Screening lung specific peptide displayed EVs by NGS



Top 5 sequence for lung	Counts in lung	Counts in liver
P1	270	0
P2	248	0
P3	183	0
P4	178	0
P5	178	0

Figure 4. Screening the lung specific peptide displayed sEVs by next generation sequencing (NGS)

Testing peptide displayed sEVs directing tropism

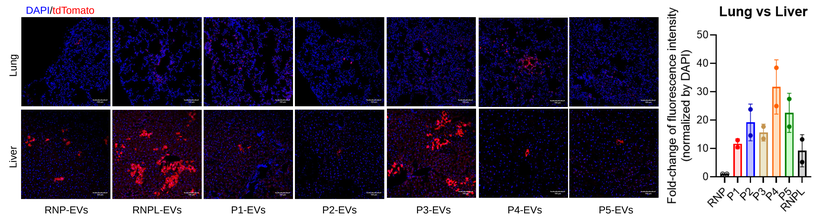


Figure 5. *In vivo* testing peptide displayed sEVs directing lung tropism vs. liver tropism

CONCLUSIONS

1. Engineered CD63 with MCP sort the gRNA by MS2 sequence into EV lumen.
2. Engineered EVs preserved and delivered gRNA and edited the gene in recipient cells.
3. Random peptide library-displayed EVs intrinsically showed significant tropism to liver and lung.
4. NGS allowed us to select individual barcode sequences paired with individual peptides and select five lung-specific peptides.
5. Among the five peptides, P4 displayed EV showed high lung-specific gene delivery, and the other four also showed higher affinity than random peptide displayed EV.

Our study provides a systematic screening technology for target-specific gene delivery and editing, and enables determination of EV tropism and functional drug delivery through bifunctional engineered EVs.

REFERENCES

1. Luke H. Rhym, et al., “Peptide-encoding mRNA barcode for the high-throughput in vivo screening of libraries of lipid nanoparticles for mRNA delivery” Nature Biomedical Engineering 7 (2023) 901-910.
2. Guochao Wu et al., “Adeno-associated virus-based gene therapy treats inflammatory kidney disease in mice” The Journal of Clinical Investigation 134(17) (2024)e174722.

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