Improved Immune Cell Expression with Circular RNA (oRNA™) *in vivo*

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Background

Ex vivo CAR-T therapies have demonstrated extraordinary promise in treating certain cancers but are challenged with preconditioning-associated toxicity, as well as complex, costly, and poorly scalable manufacturing processes tailored for each patient. Off-the-shelf protein-coding RNA therapeutics capable of *in situ* CAR expression offer an attractive alternative to *ex vivo* therapies, but they have been limited by effective delivery of RNA to immune cells and the inability to achieve meaningful protein expression from mRNA.

To overcome these barriers, we are developing two key proprietary technologies to achieve *in situ* CAR therapy: 1) protein-coding circular RNA (oRNA) and 2) effective delivery solutions based on proprietary lipid nanoparticles (LNPs).

Linear mRNA vs. oRNA

Therapeutic protein-coding RNAs have remarkable potential for many applications requiring protein synthesis, including vaccines, protein replacement, and cancer.

- **Linear mRNAs** consist of a 5′ cap, 3′ polyA tail, and coding region flanked by UTRs (untranslated regions); base modifications are often incorporated to reduce immunogenicity and increase protein expression.
- **oRNAs**, which do not require a cap or tail, are fully synthetic, in vitro-transcribed, unmodified, self-splicing circular RNAs which translate protein via an internal ribosome entry site (IRES).[1,2] (**Figure 1**)

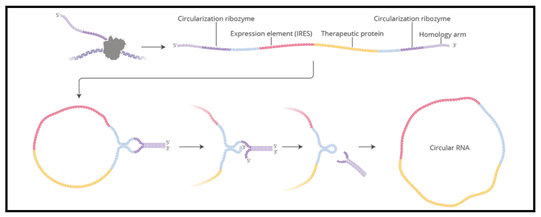


Figure 1. *Structural features and synthesis of oRNA; Orna's technology relies on a self-circularizing mechanism to co-transcriptionally create full-length circles robustly and efficiently.*

In this study, we investigate how oRNAs and mRNAs differ when formulated into lipid nanoparticles (LNPs) in terms of characterization and potency *in vivo* and *in vitro*.

Note: mRNA performance is impacted by its cap, tail length, presence of impurities, UTR sequences, etc. Unless otherwise noted, all mRNAs were commercially-available CleanCap® AG, 5moU-modified, and silica-column purified (Trilink Biotechnologies).

oRNA Readily Formulates into Lipid Nanoparticles

Four-component LNPs consisting of an ionizable lipid, phospholipid, cholesterol, and lipid-anchored PEG are among the most clinically-advanced RNA delivery vehicles, having recently received FDA approval for the delivery of siRNA and linear mRNA (Onpattro®, Spikevax® [mRNA-1273], Comirnaty® [BNT162b2]).[3] We first explored whether oRNA could be formulated similarly to conventional linear mRNA (**Figure 2**).

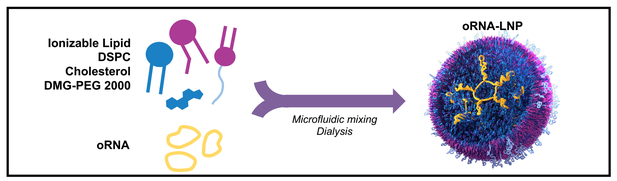
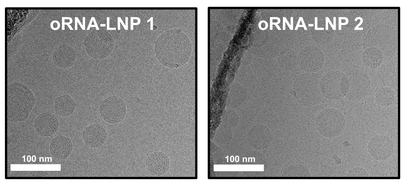


Figure 2. *Four lipids (ionizable lipid, phospholipid, cholesterol, and lipid-anchored PEG are combined with oRNA to make lipid nanoparticles.*

Representative oRNA-LNPs 1 and 2 **(Figure 3)** had favorable characterization results. Transmission electron cryo-microscopy (cryo-EM) imaging revealed the LNPs were generally unilamellar with few solvent-filled bleb compartments.[4]



Metric	Assay	oRNA-LNP 1	oRNA-LNP 2
Avg. Diameter	Dynamic Light Scattering	65 nm	68 nm
Polydispersity Index (PDI)	Dynamic Light Scattering	0.03	0.04
oRNA Encapsulation Efficiency	Ribogreen	96%	93%
Post-dialysis oRNA Concentration	Ribogreen	360 µg/mL	398 µg/mL

Figure 3. *Cryo-EM images and characterization information of two oRNA-LNPs (LNP-1 and LNP-2 use different ionizable lipid chemistries).*

oRNA-LNPs Have Improved Formulation Characteristics Compared to mRNA-LNPs

Generally, oRNA-LNPs and mRNA-LNPs have similarly low PDI (<0.1) and high encapsulation (>90%) across all tested ionizable lipids (A-H) and construct lengths (#1-4). Lipids A-H include both externally-published and proprietary ionizable lipids with diverse head and tail chemistries.

However, oRNA-LNPs were generally smaller than their mRNA-LNP counterparts **(Figure 4)**. Smaller LNPs may be desirable for the clinic, as they have fewer manufacturing risks during filtration steps and may be safer for applications requiring intravenous administration.

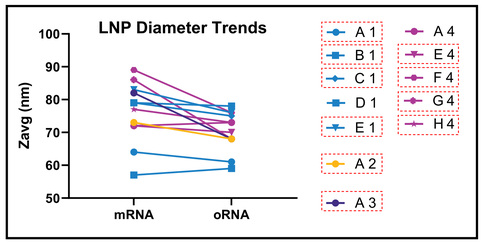


Figure 4. *LNP diameter (as measured by Dynamic Light Scattering, Zavg intensity) of mRNA-LNPs and oRNA-LNPs made with the same lipids or constructs. In most (but not all) cases, oRNA-LNPs are smaller than mRNA-LNPs (shown in dashed red box). Construct 4 is firefly luciferase, with corresponding in vivo expression data plotted in Fig. 4 and 5 below.*

oRNA-LNPs Express Higher in Spleen and Shift Biodistribution to Spleen Compared to mRNA-LNPs *in vivo*

Splenic expression of oRNA-LNPs and mRNA-LNPs were compared in mice using a firefly luciferase (fLuc) reporter **(Figure 5)**. Significantly higher spleen expression was observed with oRNA-LNPs compared to those formulated with industry-standard linear mRNA (Trilink Biotechnologies). The spleen, a reservoir of effector immune cell subtypes including T cells, is a particularly relevant tissue for *in situ* CAR therapies.

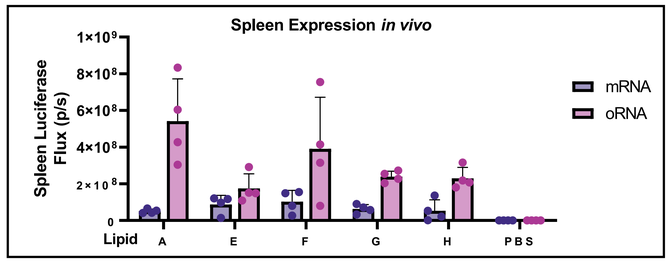


Figure 5. *Spleen luciferase flux of fLuc mRNA-LNPs and oRNA-LNPs 6 hr after i.v. dosing in C57BL/6 mice at 0.5 mg/kg as measured by IVIS bioluminescence imaging.*

Comparing *in vivo* biodistribution, oRNA-LNPs consistently demonstrate higher relative flux in the spleen compared to mRNA-LNPs, which express predominately only in the liver **(Figure 6)**. Different ionizable lipid chemistries result in unique levels of spleen biodistribution with oRNA-LNPs.

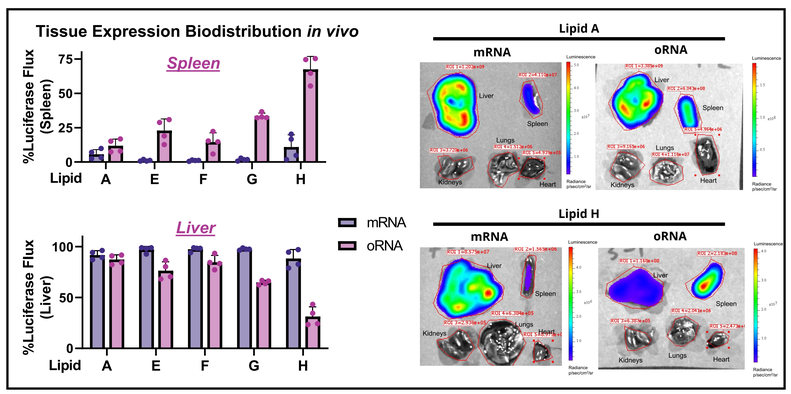
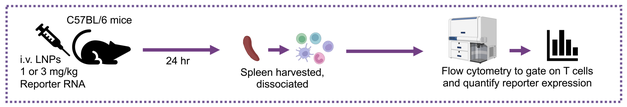


Figure 6. *Left: relative luciferase flux biodistribution of fLuc mRNA-LNPs and oRNA-LNPs 6 hr after i.v. dosing in C57BL/6 mice at 0.5 mg/kg as measured by IVIS bioluminescent imaging. Right: representative IVIS images of tissues ex vivo for two different ionizable lipids.*

oRNA-LNPs Have Higher Splenic T Cell Expression than mRNA-LNPs



At a cellular level, LNPs delivering oRNA result in superior mouse splenic T cell expression compared to those delivering linear mRNA (Trilink Biotechnologies) **(Figure 7)**. This result held across two different ionizable lipids and three different reporter proteins.

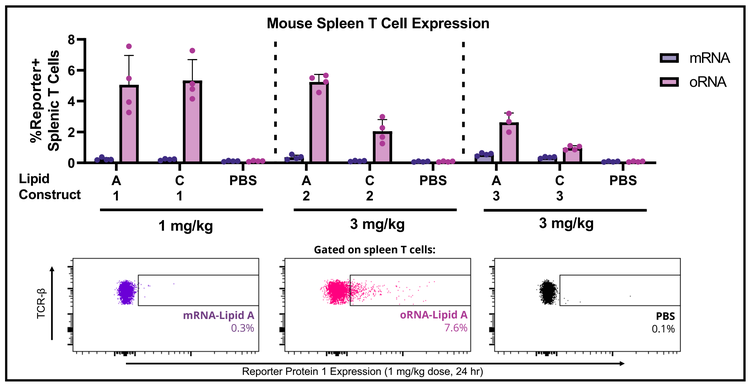


Figure 7. *Top: percent of splenic T cells expressing one of three different reporter proteins 24 hr after i.v.-dosing in C57BL/6 mice at 1 or 3 mg/kg. T cells were defined as CD45+, Live, TCR-β+, B220/CD11b/NKp46-. Bottom: representative flow cytometry scatter plots showing Reporter Protein 1 positive T cells for Lipid A mRNA-LNPs and oRNA-LNPs. mRNA for Reporter Protein 1 used PsU/5mC modifications (which we found to have much higher expression than 5moU for this construct).*

oRNA-LNPs Transfect Human T Cells *in vitro*

oRNA-LNPs display dose-dependent T cell transfection across human T cells *in vitro* **(Figure 8)**, demonstrating the species-to-species translatability of oRNA-LNPs across mouse and human.

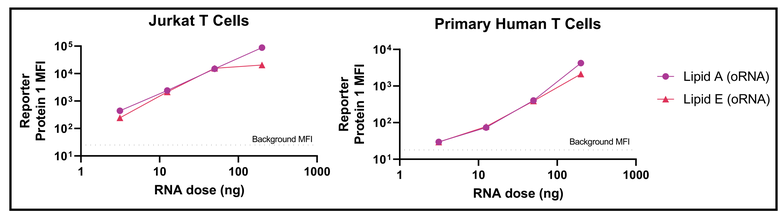


Figure 8. *Mean fluorescent intensity (MFI) of Reporter Protein 1 following transfection of Jurkat cells (left) or activated primary human T cells (right) in vitro with mRNA-LNPs or oRNA-LNPs coding for Reporter Protein 1.*

Conclusions

oRNAs are an exciting new therapeutic modality with the potential to treat numerous diseases but require LNPs for efficient delivery into target cells *in vivo*. In this study, we compared oRNA-LNPs to mRNA-LNPs primarily in terms of characterization and *in vivo* expression. The mRNA used in this study was Trilink CleanCap® AG, 5moU- or PsU/5mC-modified, and silica-column purified.

In this study, we found oRNA-LNPs:

- formulate into LNPs with favorable morphology, sizing, polydispersity, and encapsulation
- are generally smaller than mRNA-LNP counterparts
- express higher and distribute more to the spleen when administered i.v. to mice (compared to mRNA-LNPs)
- have significantly higher expression in mouse splenic T cells *in vivo* (compared to mRNA-LNPs)
- transfect human T cells *in vitro*

Applications like CAR therapy will require durable, high expression of oRNA in effector immune cell subsets (including T cells). **Together, these data suggest that our novel oRNA and LNP technologies favorably combine to maximize immune cell expression *in vivo* and support our plans to bring *in situ* CAR therapies to the clinic.**

References:
1. Wesselhoeft et al. "Engineering circular RNA for potent and stable translation in eukaryotic cells." *Nat. Comm.* **9**, 2629 (2018)
2. Wesselhoeft et al. "RNA circularization diminishes immunogenicity and can extend translation duration *in vivo*." *Mol. Cell.* **74**, 508 (2019)
3. Hou et al. "Lipid nanoparticles for mRNA delivery." *Nat. Rev. Mat.* **6**, 1078 (2021)
4. Brader et al. "Encapsulation state of messenger RNA inside lipid nanoparticles." *Biophys. J.*, **120**, 2766 (2021)
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