Discovery of translation initiation elements enabled by a parallel arrayed screen of full-length viral UTRs in synthetic circular RNA

Kristen Ott, Trent Stevens, Yessica Wiryawan, Rahul Vungutur, Helen Flynn, Meaghan Richardson, Elena Minissale, Tatiana Fontelonga, Varun Shivashankar, Raya Shu, Jung Yang, Akshi Thakkar, Magnolia Chinn, Thomas Lee, Corey Ciullo, Sharmistha Kundu, Nelson Chau, Allen Horhota, Amy Becker, Shobu Odate, R. Alexander Wesselhoeft

Orna Therapeutics, 620 Memorial Drive 2nd Floor, Cambridge, MA, 02139

Background

Linear messenger RNA (mRNA) has emerged as a validated modality for vaccines and is being investigated for a spectrum of other therapeutic applications. We are developing a new class of synthetic, protein-coding circular RNA (oRNA™) that has advantages over mRNA including ease of production, high levels of protein expression, and enhanced stability. Lacking the cap structure and termini of mRNA, oRNAs require a sequence-based element, such as a viral internal ribosome entry site (IRES), to initiate translation. These IRES elements are often found in the 5' untranslated region (UTR) of viruses in the *Picornaviridae* and *Flaviviridae* families, and tens of thousands of unique UTRs containing putative IRESs are known to exist; yet only a handful of these sequences have been evaluated for activity.

The study of viral UTRs as translational initiation elements has several inherent challenges: 1) The length of putative IRES sequences (>500nt) is not amenable to oligo-based library screening, 2) expression must be tested in the context of oRNA, not plasmid DNA, 3) delivery method may change the result, and 4) differences in IRES translation may be expected between different cell types, cell states, and species, posing a barrier to therapeutic translatability.

To overcome these complications, we developed FoRCE™ (Formulated oRNA Cell-based Evaluation): a robust high-throughput platform that enables parallel arrayed synthesis, purification, LNP formulation, and cell-based screening of oRNAs. Using FoRCE™, we screen thousands of full-length viral UTRs for IRES activity in multiple primary human cell types and characterize hundreds of potent oRNA-active IRESs. These data define the "IRESome" in the context of translationally relevant oRNA, LNPs, and cell types and provide a strong foundation for the development of oRNA-based therapeutics.

oRNA is translated through a cap-independent mechanism

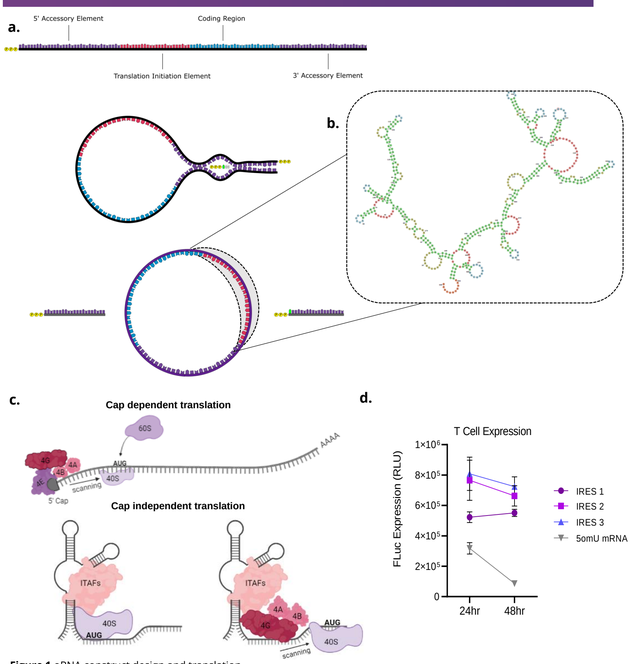


Figure 1 oRNA construct design and translation.
(a) Structural features and synthesis of oRNA; Orna's technology features a co-transcriptionally self-circularization mechanism that generates-covalently closed circular RNA[1,2]. (b) oRNA contains a translation initiation element such as a highly structured IRES that induces robust translation through a cap-independent mechanism. (c) Translation initiation of IRESs relies on several different IRES trans activating factors (ITAFs)[3] (d) Due to differing mechanisms of translation, oRNA translation can fine tuned more effectively than capped mRNA.

IRES translation efficiency varies by screening modality

Screening modality	Cellular mechanism	Translation efficiency in oRNA

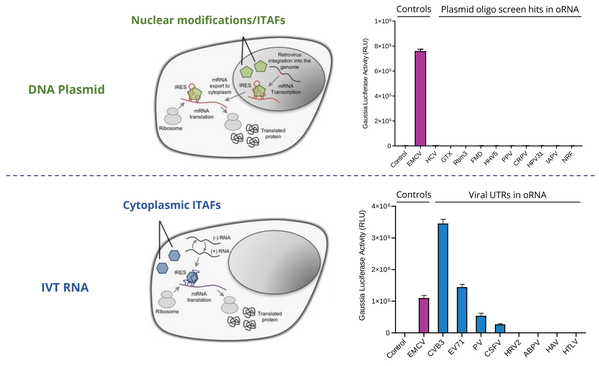
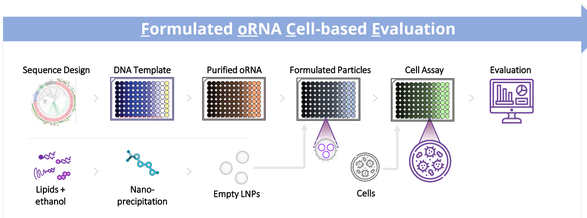


Figure 2 The importance of screening modality for oRNA translational elements.
DNA plasmid-based screening allows resulting RNA transcripts to access nuclear proteins before export to the cytosol while exogenously delivered oRNA does not enter the nucleus[4]. Preliminary screening showed that putative IRES candidates derived from plasmid-based screens[5] commonly failed to express protein when tested in oRNA [HEK293, top graph[1]]. Productive translation from viral UTRs in an oRNA context reveals a preference for full-length virally-derived IRESs [bottom graph, HEK293 expression[1]].

FoRCE™ high-throughput screening

Formulated oRNA Cell-based Evaluation



~3,000 IRES candidates were computationally identified

Novel oRNAs were synthesized and formulated into LNPs

IRESs were tested in primary human T cells, myotubes, and hepatocytes

Figure 3 Development of the Formulated oRNA Cell-based Evaluation (FoRCE™) high-throughput screening system
Approximately 3000 sequences were computationally identified from viral genomes in Genbank to screen in the context of oRNA. The completely automated process begins with a plate of DNA templates which are then linearized, *in vitro* transcribed, and purified. The plate-based formulation process enables high throughput encapsulation into LNPs. These formulated oRNAs can then be transfected into cell types of choice; here we utilize 3 primary human cells types: T Cells, myotubes, and hepatocytes.

FoRCE™ yields high-quality data

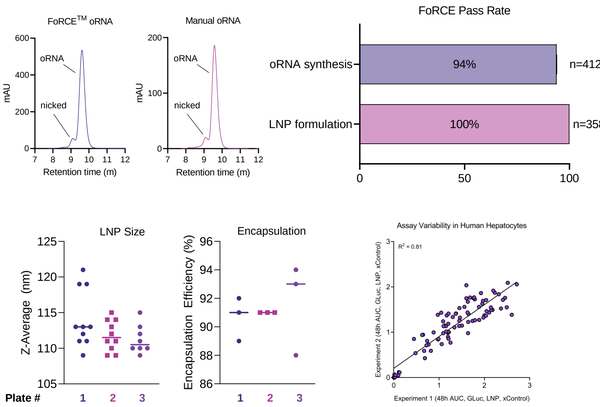


Figure 4 FoRCE™ generates high quality oRNA comparable to manual synthesis.
The fully automated FoRCE™ method consistently produces high quality oRNA with very few reaction failures. A plate-based formulation process then encapsulates the oRNA into lipid nanoparticles that are of consistent across plates. Finally, the oRNA-LNP complex is transfected into the cell type of choice. Expression readouts from FoRCE™ produced oRNA-LNPs show experimental consistency.

Applying FoRCE™ to define the IRESome

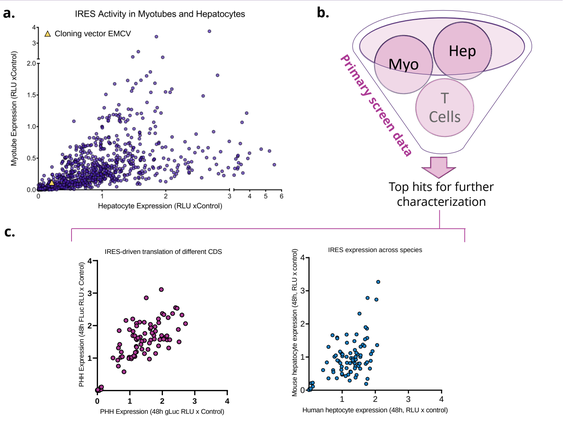


Figure 5 Characterizing the IRESome through parallel arrayed screening.
(a) Expression data from ~3000 novel viral UTRs was gathered and normalized to the expression level of our current best IRES. IRESs displayed a wide range of expression, both within the same cell type and across cell types. (b) Top hits from the primary screen were selected for further characterization (c) Viral UTR elements efficiently drive translation of both secreted and intracellular proteins and expression levels are consistent across human and mouse primary hepatocytes *in vitro*.

Highly expressed IRESs cluster based on sequence and structure

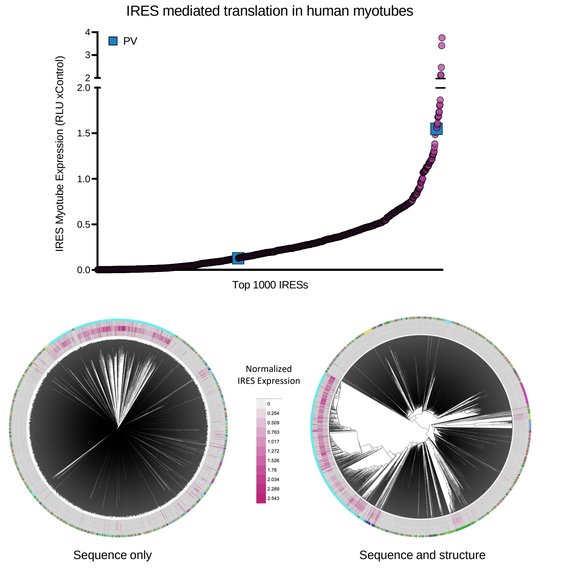


Figure 6 IRES structure analysis improves function prediction compared to sequence or phylogeny analysis.
IRESs derived from closely related viruses (ex: Polioviruses indicated by blue squares) sometimes show high variability in levels of expression. To determine the best method to predict IRES efficacy, IRESs were clustered by either total sequence similarity (left) or sequence guided structural similarity (right). The outer ring indicates genus, the three heat map layers correspond to normalized expression in different cell types, and the innermost tree indicates degree of similarity.

Conclusion

We applied FoRCE to almost 3,000 unique oRNAs containing UTRs extracted from viral genomes and discovered hundreds of IRESs that drive translation from synthetic oRNA in primary human T cells, hepatocytes, and myotubes. Many of these IRESs exhibited far greater activity than those commonly used by the broader scientific community such as the EMCV IRES. Sequence and structure analyses revealed a prioritization of structural conservation that was related to IRES function. This evaluation of viral IRES activity in oRNA, the most comprehensive to date, represents a major advance in our ability to control and maximize cap-independent translation. Continued exploration of both natural and synthetic translation initiation elements will further actualize the therapeutic potential of oRNA .

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. Mauger et al. "The genetic code as expressed through relationships between mRNA structure and protein function." *FEBS letters* **8**, 587 (2013)
4. Gritsenko et al. "Sequence features of viral and human Internal Ribosome Entry Sites predictive of their activity." *PLoS Comput. Biol.* **9**, 3 (2017)
5. Weingarten-Gabbay et al. "Systematic discovery of cap-independent translation sequences in human and viral genomes." *Science* **351**, 6270 (2016)