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RNA Replicon Platform to Enable Long-lasting Transient Expression in Primary and Stem Cells

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ABSTRACT

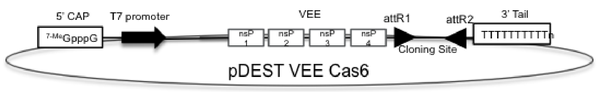
RNA replicons are an emerging platform for delivering complex genetic content into mammalian cells. Replicons are synthetic mRNA molecules that include viral nonstructural proteins (nsP1-4) from Alpha viruses such as Semliki Forest Virus and Venezuelan Equine Encephalitis Virus (VEE). Replicons can be transfected into cells and can self-amplify by virtue of a self-encoded RNA-dependent RNA polymerase. These molecules provide long-lasting, high-level gene expression from a few initial RNA molecules, making their use ideal for gene transfer applications needing sustained expression.

Current mRNA generation kits are suited for smaller (5kb or less) transcripts sizes and thus are not ideal for RNA replicons that are typically over 10 kb. In addition, the 5' end is less amenable to capping, necessitating enrichment and quantification of the capped functional replicon. Here we propose construction of a robust self-replicating vector platform with intrinsic structures that enable enrichment of functional mRNA. As a proof of principle we constructed a vector encoding self-replicating EmGFP and sustained GFP expression was verified in human dermal fibroblast and resting as well as activated T cells. The utility of this platform was further extended in other primary cell types and stem cells as well. Applications that might benefit from replicon use include RNA Chimeric Antigen Receptor – T cell immunotherapy, iPS cell reprograming strategies using multiple factors to modulate cell fate, and engineering via synthetic gene circuits.

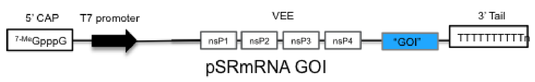
INTRODUCTION

The robust Replicon gene expression vector system with accompanying enrichment and QC solutions will provide a platform for generation and purification of self replicating mRNA (srRNA). This molecule provides prolonged expression in mammalian cells thus overcoming current constraints with synthetic mRNA that requires repeated transfections for sustained expression.

Step 1: Vector Construction



Step 2: Insertion of GFP



Step 3: mRNA Generation

Step 4: Testing in cells

The goal here is to **(A)** create a self-replicating RNA gene expression technology platform for diverse applications **(B)** test mRNA generated using this platform in different primary and stem cells and examine persistence of expression in T cells.

MATERIALS AND METHODS

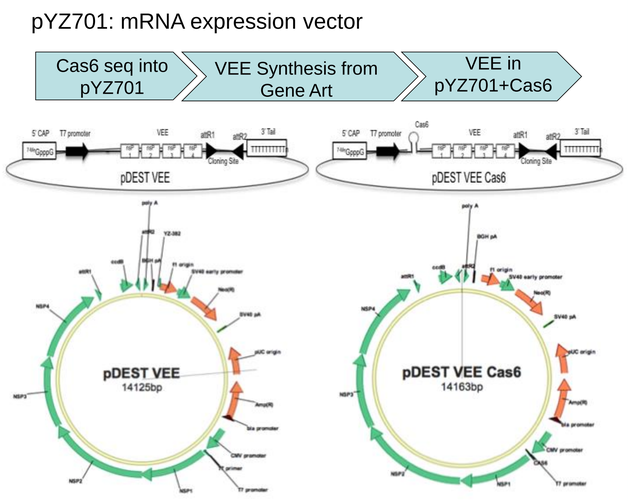
All materials are from Thermo Fisher Scientific unless specified otherwise.

Primary cell transfection:
Cells were sensitized with B18R containing Opti-MEM for at least 2 hrs before transfection. Approximately 100 thousand cells (at about 70-80% confluence) were transfected with 500ng SrRNA using Lipofectamine™ MessengerMAX™ . Media was changed 4 hr post transfection. Cells were maintained with B18R containing media for the rest of the experiment and fed every alternative day. Images were captured using EVOS™ XL Core Imaging System . Sr-GFP expression was analyzed using Attune™ NxT Flow Cytometer.

T cell culture and electroporation:
Purified CD3+ cells were cultured with Dynabeads™ Human T-Expander CD3/CD28 and 100U/mL recombinant human IL-2 CTS™ OpTMizer™ T Cell Expansion SFM for 12 days. Three million cells at a density of 20 million/mL in a 100 ul tip was electroporated with 3 ug of RNA in T buffer using Neon Electroporation System

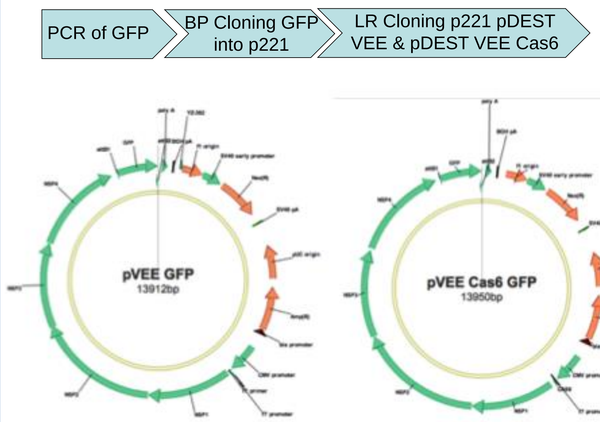
RESULTS

Figure 1. Vector Construction



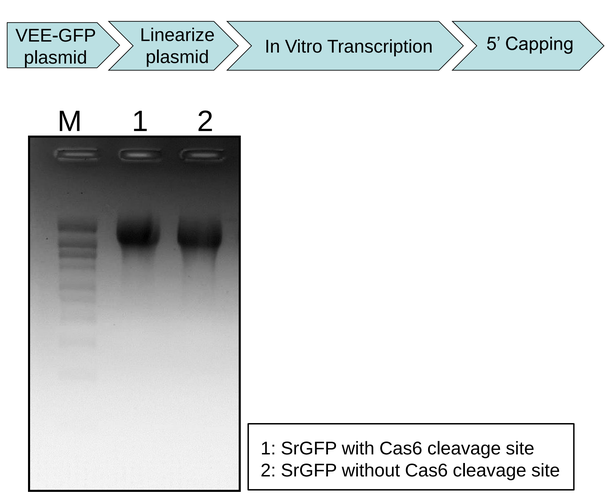
The mRNA vector pYZ721 is a T7 mRNA expression vector with polyT, which was used as the base vector for insertion of VEE and Cas6 cassettes. The 7.5kb region comprising nonstructural proteins 1-4 (ns1-4) or the VEE portion was produced at GeneArt. The Cas6 recognition sequence comprised of a 30 nucleotide with cleavage occurring between 22 and 23rd nucleotide was synthesized as an oligonucleotide. The three DNA fragments was cloned together to generate the pDEST VEE and pDEST VEE Cas6

Figure 2: Insertion of GFP using MultiSite GateWay Cloning



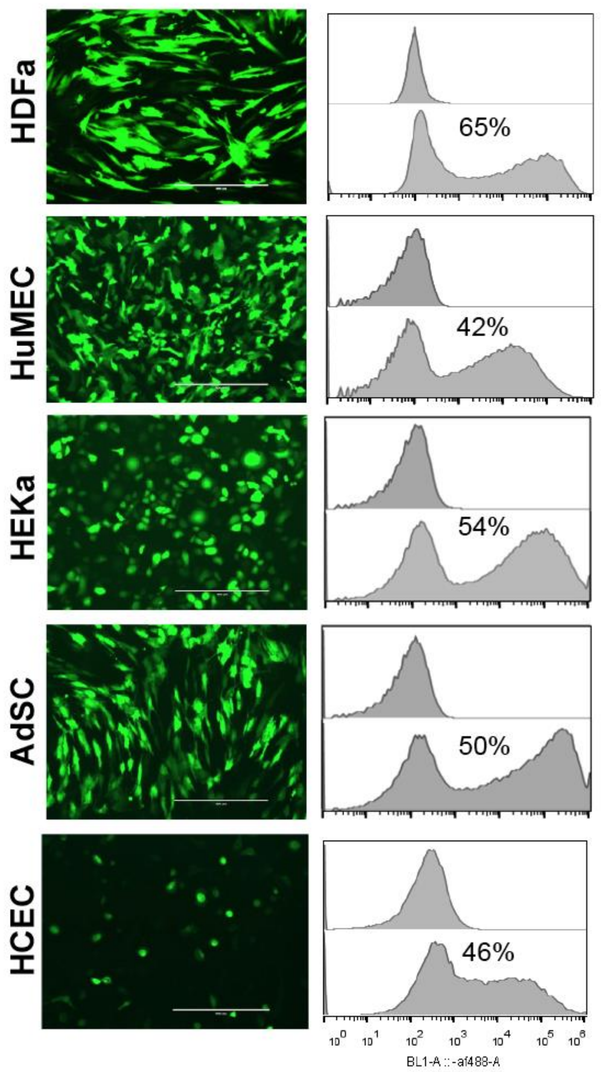
To test the functionality of the generated VEE vectors, GFP was cloned into the DEST vectors via MultiSite GateWay cloning and the resulting expression vectors were confirmed via restriction endonuclease digestion and sequencing.

Figure 3: mRNA Generation



mRNA was generated using optimized Thermo Fisher IVT kit and other reagents. Resulting mRNA was analyzed for length and integrity on 2% denaturing agarose gel.

Figure 4. SrRNA Delivery and expression in primary & stem cells



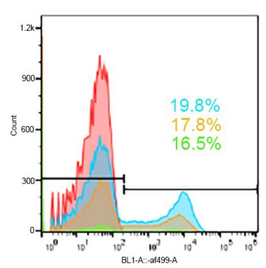
Sr-GFP RNA efficiently expresses in multiple cell types. Cells were transfected with Lipofectamine™ MessengerMAX™ Transfection Reagent and imaged 24 hours after transfection. Percentage of GFP expression was obtained by flow analysis of sister well with Attune™ NxT Flow Cytometer from Thermo Fisher Scientific 24 hours post transfection.

Figure 5. SrRNA Expression in T cells

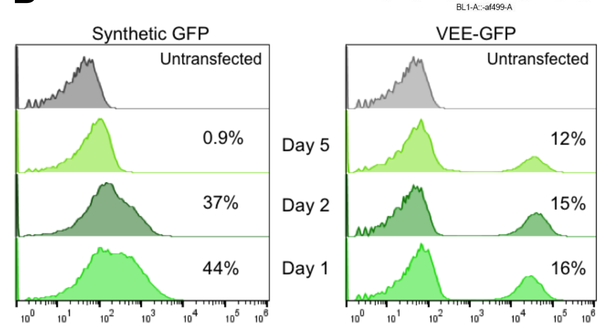
A

Neon Condition	Volts	ms	Pulse #
Condition 1	1650	10	3
Condition 2	2150	20	1
Condition 3*	2400	12	2

*Resulted in <5% viable cells at 24h

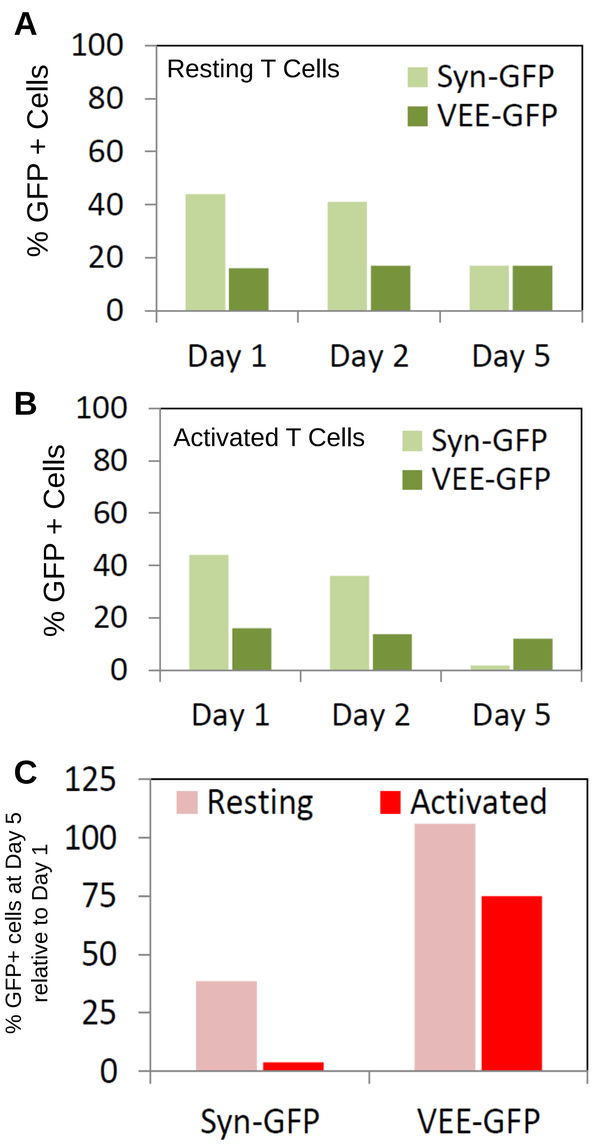


B



A. mRNA delivery into 3 million human Adult Purified CD3 Pan T Cells was optimal with Neon electroporation with 3 ug RNA in T buffer at 1650V, 10ms and 3p using Neon Electroporation System.
B. Cells were transfected with synthetic RNA or SrRNA and activated with CD3/CD28 beads. GFP expression was monitored at Day 1, 2 and 5 post transfection and activation

Figure 6. Persistence of SrRNA expression in T cells



T cells were transfected either with synthetic GFP (Syn-GFP) or Sr-GFP (VEE-GFP). GFP fluorescence was monitored both in **(A)** resting as well as **(B)** CD3/CD28 bead activated T cells. **(C)** Percent GFP positive cells at day 5 relative to day 1 following transfection for synthetic GFP and Sr-GFP in resting as well as activated T cells. Synthetic GFP signal drops significantly whereas Sr constructs exhibit persistent GFP expression with minimal decrease.

CONCLUSIONS

- We were able to create a self-replicating gene expression technology platform that exhibits persistence expression in a variety of primary cells as well as resting and activated T cells.
- Large Sr-GFP RNA has been efficiently transfected into a variety of primary cell lines with minimal cytotoxicity.
- This platform is suitable for gene delivery both by lipid-based transfection as well as electroporation.
- Strategic incorporation of Cas6 cleavage site near 5' of the construct eases quality control issues for large-scale manufacturing as uncapped mRNA induces cytotoxicity.
- This technology shows promise and has potential future applications in modulating cell fate (iPS cell reprogramming) and T cell immunotherapy (CAR-T cell generation).

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