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GENE EDITING AND MODULATION TOOLS FOR LONG NON-CODING RNA APPLICATIONS

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ABSTRACT AND INTRODUCTION

Approximately 98% of the human genomic sequence is classified as non-coding RNA. Long non-coding RNAs (lncRNAs), are characterized as having a minimum of 200 nucleotides in length and they are referred to as master regulators of protein coding genes. Despite the growing understanding of their relevance in various diseases including cancer (1-3), the biological purpose of majority of lncRNAs remain unclear due to their low and cell specific expression levels, varied cellular localization, shared genomic sequences with coding transcripts, and lack of effective investigating technologies. Here we evaluated gene editing and modulation technologies that can be applied for studying lncRNA function and role (1) Transient CRISPR-Cas9 RNA format: Co-delivery of two synthetic gRNA with Cas9 mRNA into cells resulted in gene knockouts through target gene deletion; (2) Lentiviral based CRISPR system: we established a dual gRNA lentiviral system that allows expression of two gRNAs for deleting user defined genomic DNA sequences; (3) RNA interference: transfection of small interfering RNA (siRNA) enabled repression of targeted mRNA. Discussed here are advantages and limitations of each technology and lncRNA editing applications in cell models, including, but not limited to, stem cells.

1. Important functions of long non-coding RNA

Figure 1. LncRNA Function. LncRNA mediate numerous biological processes and are essential for regulating the protein coding genome.

2. Gene Editing and Modulation Tools for Long Non-coding RNA Applications

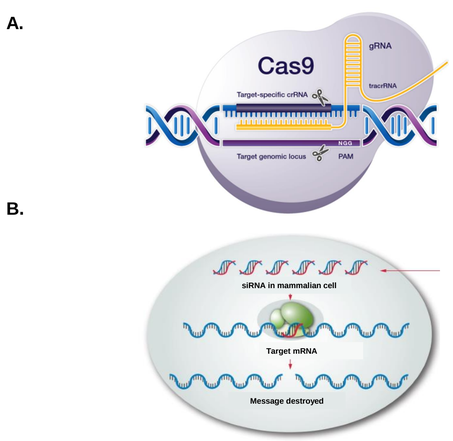

Figure 2: Schematics of gene editing and modulation tools. A. The CRISPR Cas9 system contains an RNA guided (gRNA) endonuclease (Cas9) that cleaves genomic DNA at a specified location resulting in a double stranded break. **B.** SiRNA are short double-stranded RNA approximately 21 nucleotides in length, with 3' overhangs at each end. SiRNA can be used to interfere with the translation of proteins by binding to and promoting the degradation of messenger RNA at specific sequences.

MATERIALS AND METHODS

iPSC cell culture and Transfection: Gibco Human Episomal iPSC Line (Cat. No.: A18945) were maintained in Gibco™ StemFlex™ medium (Cat. No.: A3349401) at 37 ° C with 5% CO2. iPSC's were transfected with Lipofectamine™ Stem Transfection Reagent (Cat. No.: STEM00001) according to manufacturer's instructions.
Genomic Cleavage Detection assay: iPSC's transfected with gRNA's were assayed for gene editing efficiency using the GeneArt™ Genomic Cleavage Detection Kit (Cat. No.: A24372) to assess the performance of gRNA designs
qRT-PCR assay: Cells were transfected with 25 nM Silencer™ Select siRNA against FEZF1-AS1 using Lipofectamine™ RNAiMAX Transfection reagent (Cat. No.:13778075). Cells were harvested 72 hours post-transfection for qPCR to measure the mRNA levels of FEZF1-AS1. qPCR was performed using TaqMan® Cell-to-Ct™ 1-Step Kit (Cat. No.:A25602) to measure gene expression.

RESULTS

3. Dual gRNA Lentiviral Based System

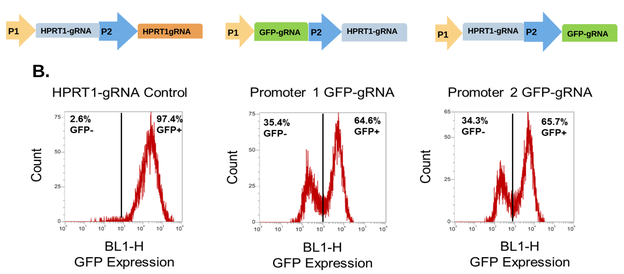

Figure 3: Dual gRNA vector construct. A. The dual gRNA lentiviral construct was developed to guarantee delivery of 2 gRNAs into the genome at once to increase editing efficiency for lncRNA knockout. The plasmid has two RNA pol III promoters, each expressing a different gRNA. **B.** Dual gRNA vectors were transfected into U2OS cells expressing GFP and Cas9 protein using Lipofectamine 3000 . The total GFP expression was measured by flow cytometry 5 days post transfection.

4. Validation of Gene Deletion Using Dual-gRNA Vectors

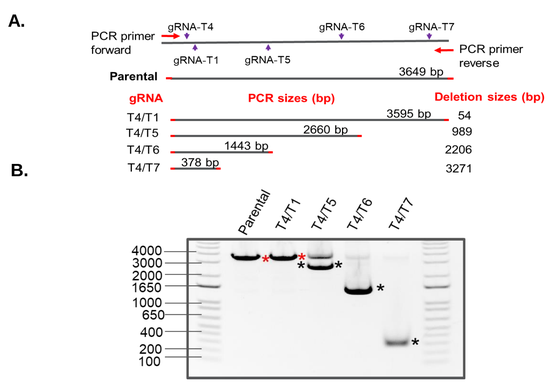

Figure 4: Dual gRNA mediated gene deletion. A. HPRT 1 gene map flanking gRNA targets. Dual gRNA target pairs and their predicted corresponding PCR base pair length. **B.** The lentivirus based dual-gRNA vectors were transduced into GripTite-293 cell line stably expressing Cas9 for validation of gene deletion. Stars in red indicate parental PCR band. Stars in black indicate the expected PCR bands if deletion happened.

5. Functional Validation of Dual-gRNA Vector through Viral Infection

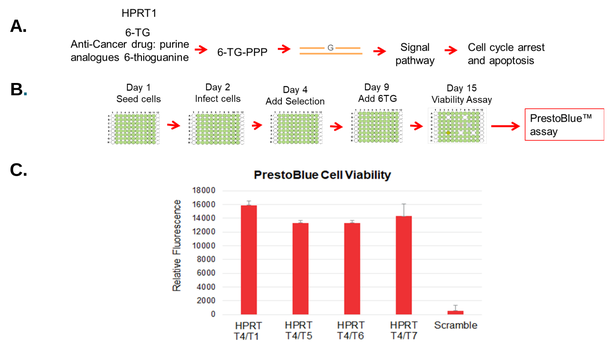

Figure 5: Functional validation of dual-gRNA vector. A. 6TG signal pathway in HPRT. **B.** Workflow of lentiviral gRNA particle infection followed by selection with Puromycin and 6TG treatments. **C.** HT1080 Cas9 stably expressing cells were infected with dual-gRNA lentiviruses. Cells that have gene deletion on HPRT1 are resistance to 6TG treatment. The cell viability was measured by PrestoBlue™ cell viability assay.

6. Functional Validation of gene knock out of LncRNA FEZF1-AS1 in iPSCs

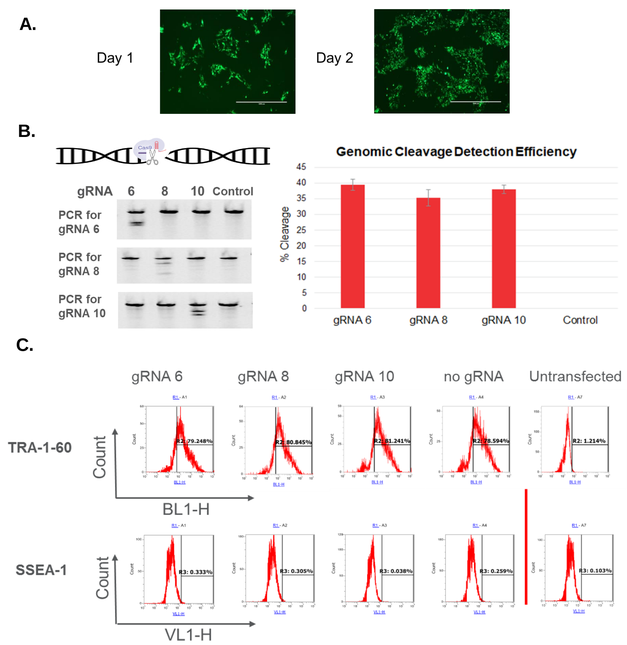

Figure 6: Single gRNA validation in IPSCs. A. Transfection optimization: iPSC were seeded at a density of 10,000 cells per well and transfected following day with 100 ng of GFP mRNA using Lipofectamine™ Stem Transfection reagent. Images were captured on EVOS™ cell imaging system at a 4X magnification 24 and 48 hours post transfection. **B.** iPSC's transfected with Cas9 mRNA and single gRNAs 6,8,10 targeting different loci within lncRNA FEZF1-AS1 were harvested 48 hours post transfection and assayed for gene editing efficiency using the GeneArt™ Genomic Cleavage Detection Kit to assess the performance of gRNA designs. The gel image band intensity directly correlates to target indel formation. **C.** Flow cytometry analysis of pluripotency markers TRA-1-60 (positive marker) and SSEA-1 (negative marker) expression in iPSCs that were transfected with single gRNA 6,8,10, after 48 hours of incubation.

7. Gene Deletion Through Co-Delivery of Two Synthetic gRNA with Cas9 mRNA into iPSCs

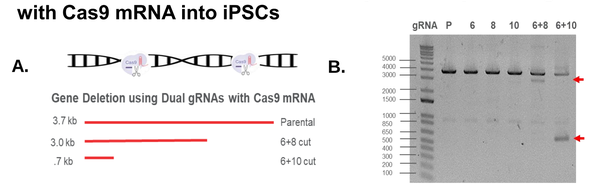

Figure 7: FEZF1-AS1 Gene deletion through CRISPR/Cas9 mRNA with dual gRNAs in iPSC. A. Diagram of expected base pair deletion fragment length using the gRNA pairs. B. Cas9 mRNA and two synthetic gRNAs were delivered into iPSCs using. Lipofectamine™ Stem Transfection Reagent gRNA for gene deletion. 72 hours post transfection, iPSCs were harvested for PCR verification. The red arrows indicate the PCR products resulting from the gene deletion

8. siRNA Mediated Knock Down of lncRNA FEZF1-AS1 in IPSCs

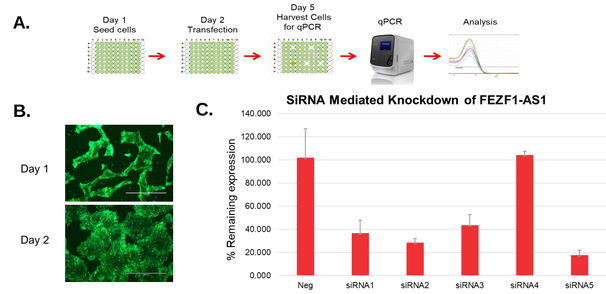

Figure 8: siRNA mediated modulation of FEZF1-AS1 in iPSC. A. Experiment setup workflow. **B.** GFP transfected iPSC as a transfection control. **C.** FEZF1-AS1 gene expression level as measured by qPCR 72 hours post negative control or 5 different FEZF1-AS1 specific Silencer™ Select siRNA transfection. GAPDH was used as internal control for each sample. The percent remaining expression was normalized to negative control siRNA transfected samples.

CONCLUSIONS

- siRNA efficiently mediated knockdown of lncRNA FEZF1-AS1
- LncRNA can be deleted via synthetic and lentiviral dual-gRNA systems
- Different methods can be selected based on lncRNA target sequence and downstream application.

Future Steps:
- Modulation of lncRNA using CRISPR activation and repression
- Functional validation of lncRNA after deletion and modulation

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TRADEMARKS/LICENSING

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