A protocol to build microRNA-inducible CRISPR-Cas9 platform

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Method Article

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Abstract

microRNAs \(miRNAs) are small noncoding RNAs that play important regulatory roles in plants, animals and viruses. However, measuring miRNA activity in vivo remains a big challenge. In this protocol, using a miRNA-mediated sgRNA releasing strategy and dCas9-VPR to drive a transgene RFP expression, we create a miRNA sensor that can faithfully measure miRNA activity at cellular levels. When sgRNAs are designed to target endogenous locus, we show this system can be adapted to achieve cell type specific activation of endogenous genes. Furthermore, when dCas9 is fused with a transcriptional repressor or a base editor, we show this system can be used to repress the expression of endogenous genes or mutate specific DNA bases of chromosome upon induction by cell type-specific miRNAs. This step-by-step protocol is related to the publication “A microRNA-inducible CRISPR-Cas9 platform serves as microRNA sensors and cell type specific genome regulation tools” in Nature Cell Biology.

Introduction

miRNAs are 21-24 nucleotide small RNAs regulating gene expression majorly at post-transcriptional level[1, 2]. The partial complementarity between a miRNA and its target mRNA leads to the destabilization and/or translational repression of the target mRNA, while the full or near full complementarity between a miRNA and its target mRNA leads to the cleavage of the target mRNA at specific positions[3-5]. miRNAs play essential roles in a variety of physiologic and pathologic processes in plants and animals[6-8]. Many miRNAs are only expressed in specific tissues, cell types, and developmental or disease stages[9, 10]. As a result, miRNA profiles have been successfully used to characterize the developmental lineage and differentiation status of human tumors, and in many cases are more accurate and informative than mRNA profiles[11]. A reporter for miRNA activity will be extremely useful for tracking differentiation status of stem cells and disease progression. However, due to the inhibitory nature of miRNAs on gene expression, a reporter that is activated by miRNAs has not been made possible. Furthermore, expansion the application of miRNAs rather than just as inhibitory tools remains to be challenging. The type II CRISPR system has recently been repurposed to a programmable gene editing system in plants, animals and microbes[12, 13]. Guided by a programmable chimeric CRISPR single guide RNA \(sgRNA), a Cas9 nuclease is recruited and induces a double-stranded break \(DSB) at complementary genomic sequences. Furthermore, the nuclease-deficient Cas9 \(dCas9) retains the ability to target genomic DNA without cleaving it[14]. When fused with different effector proteins, dCas9 system has been engineered as a platform to regulate transcription[15, 16], modify epigenetic status[17, 18] and mutate DNA bases at specific genomic loci[19, 20]. Traditionally, U6 or H1 promoters are used to driven sgRNAs expression while sgRNAs transcribed by Pol II RNA polymerase are inactive[21], probably due to the 5' Cap and 3' polyA tail structures. Here, using a miRNA-mediated sgRNA releasing strategy from the inactive pre-sgRNA, we created a CRISPR-Cas9 platform that can be turned on by specific endogenous or exogenous miRNA/siRNAs. We show that this system can be adapted as miRNA sensors and cell type-specific genome regulation tools. As illustrated in Figure 1a, the key component for our miRNA-mediated sgRNA production strategy is a sgRNA precursor \(pre-sgRNA), in which a sgRNA sequence is flanked by two miRNA complementary binding sites. Throughout this protocol, pre-sgRNAs are transcribed by RNA pol II under the control of CAGGS promoter, therefore can not be processed to functional sgRNAs due to the 5' Cap and 3' polyA tail structures. In the presence of cognate miRNAs, functional sgRNAs should then be released by AGO2-mediated cleavage reaction. Combined with different Cas9/dCas9-effector proteins, this platform may be adapted for genome editing, epigenetic modifications and regulating gene expression upon induction by specific miRNAs. As shown in Figure 1b, when combined with dCas9-VPR[22], in which three transcription activators including VP64, P65 and RTA are fused to the C terminus of dCas9, and a fluorescent protein reporter, this platform can be designed as miRNA sensors. Furthermore, when the sgRNAs are designed to target the transcription start site of endogenous genes, this platform can be used to activate endogenous genes in a miRNA induction manner \(Figure 1c) When dCas9 is fused with a transcriptional repressor or a base editor, we show this system can be used to repress the expression of endogenous genes or mutate specific DNA bases of chromosome depending on the expression of cell type-specific miRNAs \(Figure 1d, e). This protocol below contains a more detailed description for the construct and application of this microRNA-inducible CRISPR-Cas9 platform.

Reagents

General Reagents Lipofectamine3000 \(Invitrogen) DharmaFECT 1 Transfection Reagent \(Dhamacon) OPTI-MEM \(Reduced serum Medium) \(Invitrogen) 24-wells plate \(Corning) 48-wells plate \(Corning) Oligonucleotides \(Life technology) FastPfu DNA polymerase \(Transgen) EcoRI-HF \(NEB) BamHI-HF \(NEB) ApaI \(NEB) T4 DNA ligase \(Thermo) HiPure Gel Pure DNA Mini Kit \(Magen) Trans-T1 Phage Resist Chemically Competent Cell \(Transgen) AxyPrep Plasmid Miniprep Kit \(Axygen) Blasticidin \(Gibco) Hygromycin \(Rocho) Zeocin\(Gibco) mRNA Universal SYBR qPCR Master Mix \(Vazyme) Universal DNA Purification Kit \(TIANGEN Biotech) **Plasmids** Plasmid1: pB-CAGGs-dCas9-VPR/KRAB/BE3-Hygromycin \(CRISPR/Cas9 effectors) Plasmid2: PB-CAGGs-miRT-gRNA-miRT-zeocin \(Pre-sgRNA) Plasmid3: PB-TRE3G-RFP-Bsd \(DNA Targets) **Cells** 293T cells Hela cells Mouse Embryonic Stem Cells Irradiated Mouse Embryonic Fibroblasts

Procedure

1. Pre-sgRNA plasmids cloning **I) Digesting pre-sgRNA plasmid with EcoRI-HF and BamHI-HF with the following reaction:** 500 ng PB-CAGGs-zeocin plasmid 1 μl CutSmart buffer 0.5 μl EcoRI-HF 0.5 μl BamHI-HF Add H_2O up to 10 μl. Incubate 2 hours at 37° C. After the digest, gel purify the linearized plasmid. **II) Clone pre-sgRNA** Pre-sgRNA contains a sgRNA sequence flanked by two miRNA complementary binding sites. Example pre-sgRNA sequence: 294T-sgRNA\(TRE)-294T: \(ACACACAAAAGGGAAGCACTTT)tacgttctctatcactgataGTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGC(ACACACAAAAGGGAAGCACTTT) Bases in \() indicate the miRNA binding sites which are reverse and complementary with mature miRNA sequence \(miR-294-3p). Nucleotides in the middle outside of \() indicate the sgRNA\(F+E) sequence[23], while the first 20 nucleotides of sgRNA \(lower case) are crRNA sequences which target to TRE promoter. We recommend using the “Guide Design Resources”: https://zlab.bio/guide-design-resources) to facilitate target selection. a) Order the following oligos: Oligo1 \(Forward) for the example pre-sgRNA sequence:

AGAATTCACACACAAAAGGGAAGCACTTTTACGTTCTCTATCACTGA Oligo2 \(Reverse) for the example pre-sgRNA sequence: AGGATCCAAAGTGCTTCCCTTTTGTGTGTGCACCGACTCGGTGCCAC b) Carry out PCR reactions on a thermal cycler as follows: 95° C for 2 minutes; 35 cycles of 95° C for 30s, 60° C for 30s, 72° C for 30s; 72° C for 5 minutes; 4° C forever. c) Run raw PCR product on 1.5% agarose gel electrophoresis, separate 164 bp amplicon and purify with HiPure Gel Pure DNA Mini Kit \(Magen). d) Digest purified PCR product with BamHI-HF and EcoRI-HF via the following reaction at 37° C for 2 hours: 26 μl Purified PCR product 3 μl Cutsmart buffer 0.5 μl EcoRI-HF 0.5 μl BamHI-HF total 30 μl. e) After digestion, purify with HiPure Gel Pure DNA Mini Kit \(Magen). f) Ligate pre-sgRNAs into the vector with reactions as follows: 1 μl Linearized PB-CAGGS-zeocin vector 7 μl Pre-sgRNAs 1 μl T4 DNA ligation buffer 1 μl T4 DNA ligase g) Incubate at room temperature for 1 hour. h) Transform into Trans-T1 Phage Resist Chemically Compotent Cell \(Transgen) according to the manufacturer's protocol. i) Use forward primer inside CAGGS promoter and reverse oligonucleotides as a reverse primer for PCR screening. Choice colonies which have a single band around == ~== 250 bp. Confirm the sequence of pre-sgRNA by Sanger Sequencing. j) Extract plasmids using AxyPrep Plasmid Miniprep Kit \(Axygen). Now plasmids are ready to use. **2. Generate miR-294-sensing-CRISPR-on mouse embryonic stem cells.** Day1 Plate 50,000 mouse embryonic stem cells in 24-well plates, using standard growth medium. Day2 Replace with 500 μl of growth medium. Prepare A mix with 200 ng dCas9-VPR, 200 ng 294T-sgRNA-294T, 100 ng TRE3G-RFP, 100 ng pBase and 1 μl P3000 Reagent in 50 μl OPTI-MEM. Add 1.25 μl Lipo3000 in 50 μl OPTI-MEM to get B mix. Vortex each mix well. Combine A and B mix, vortex thoroughly and incubate 5 minutes at room temperature. Add the solution to the cells carefully. Day3 Replace with 500 μl growth medium. Day4-7 Cells are treated with 10 μg/ml Blasticidin S \(Gibco), 150 μg/ml Hygromycin \(Roche), 100 μg/ml Zeocin \(Invitrogen). Day8 Plate 500 cells on feeder for colony picking. **3. MICR-ON and MICR-i** **For endogenous gene activation** Day1 Plate 50,000 HEK293T cells per well in poly-D-lysine-coated 48-well plate. Day2 18 hours later replace with 500 μl of growth medium. Mix 20 nM final concentration of miR-122 or NC in 25 μl OPTI-MEM \(amounts and volumes for a single well). Mix 0.5 μl DharmaFect 1 Transfection Reagent in another 25 μl of OPTI-MEM. Vortex to mix two solutions separately, incubate 5 minutes at room temperature. Combine the two solutions, mix gently and incubate 20 minutes at room temperature. Add the solution to the cells carefully. After 6 hours, replace medium with standard growth medium. Prepare A mix with 125 ng dCas9-VPR plus 125 ng pre-sgRNA plasmids or empty control plasmids and 0.5 μl P3000 Reagent in 25 μl OPTI-MEM. Add 0. 6 μl Lipo3000 in 25 μl OPTI-MEM to get B mix. Vortex each mix well. Combine A and B mix, vortex and incubate 5 minutes at room temperature. Add the solution to the cells carefully. Day4 48 hours after transfection, cells were harvested with Trizol for extracting total RNA for mRNA qRT-PCR \(mRNA Universal SYBR qPCR Master Mix, Vazyme). **For endogenous gene repression, use dCas9-KRAB instead of dCas9-VPR.** **4. MICR-BE** Day1 Plate 50,000 Hela cells per well in 24-well plate. Day2 18 hours later replace with 500 μl of growth medium.Mix 50 nM final conctration of miR-294 or NC in 50 μl OPTI-MEM \(amounts and volumes for a single well). Mix 1 μl DharmaFect 1 Transfection Reagent in another 50 μl of OPTI-MEM. Vortex to mix two solutions separately, incubate 5 minutes at room temperature. Combine the two solutions, mix gently and incubate 20 minutes at room temperature. Add the solution to the cells carefully. After 6 hours replace medium with standard growth medium. Prepare A mix with 250 ng dCas9-Apobec1-UGI \(BE3) plus 250 ng pre-sgRNA plasmids or empty control plasmids and 1 μl P3000 Reagent in 50 μl OPTI-MEM. Add 1.25 μl Lipo3000 in 50 μl OPTI-MEM to get B mix. Vortex each mix well. Combine A and B mix, vortex and incubate 5 minutes at room temperature. Add the solution to the cells carefully. Day4 48 hours after transfection, cells are treated with 500 μg/ml Hygromycin \(Roche), 100 μg/ml Zeocin \(Invitrogen). Day6 After 2 days, cells are harvested for extracting genomic DNA. Genomic regions are amplified with PCR with primers designed by "Primer-BLAST \(NCBI)":https://www.ncbi.nlm.nih.gov/tools/primer-blast/. Carry out PCR amplification as follows: 95° C for 10 minutes; 35 cycles of 95° C for 30s, 60° C for 30s, 72° C for 30s; 72° C for 5 minutes; 4° C forever. PCR products are purified using spin columns in Universal DNA Purification Kit \(TIANGEN Biotech). Anneal and digest purified PCR product with ApaI. Assemble reaction as follows: 200 ng PCR product 2 μl 10X NEBuffer 3.1 Add H_2O up to 19 μl. Heat up the reaction to 95° C, using thermocycler for 5 minutes and let it cool down to room temperature. Add 1 μl ApaI to the reaction, incubate 25° C for 3 hours. Run digested product on 2% agarose gel under 120V for 30 minutes. The intensity of PCR amplicon and cleaved bands is quantified using ImageJ. For each lane, the fraction of cleaved products is calculated by the following formula: Fc = \(b+c)/\(a+b+c), where a is the intensity of the undigested PCR product and b and c are intensities of each cleavage products. Sequence editing efficiency = $1-Fc^{1/2}$.

Timing

Pre-sgRNA plasmids cloning: 3 days Generation miR-294-sensing-CRISPR-on mouse embryonic stem cells: 14 days MICR-ON and MICR-i: 4-5 days MICR-BE: 6 days

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Figures

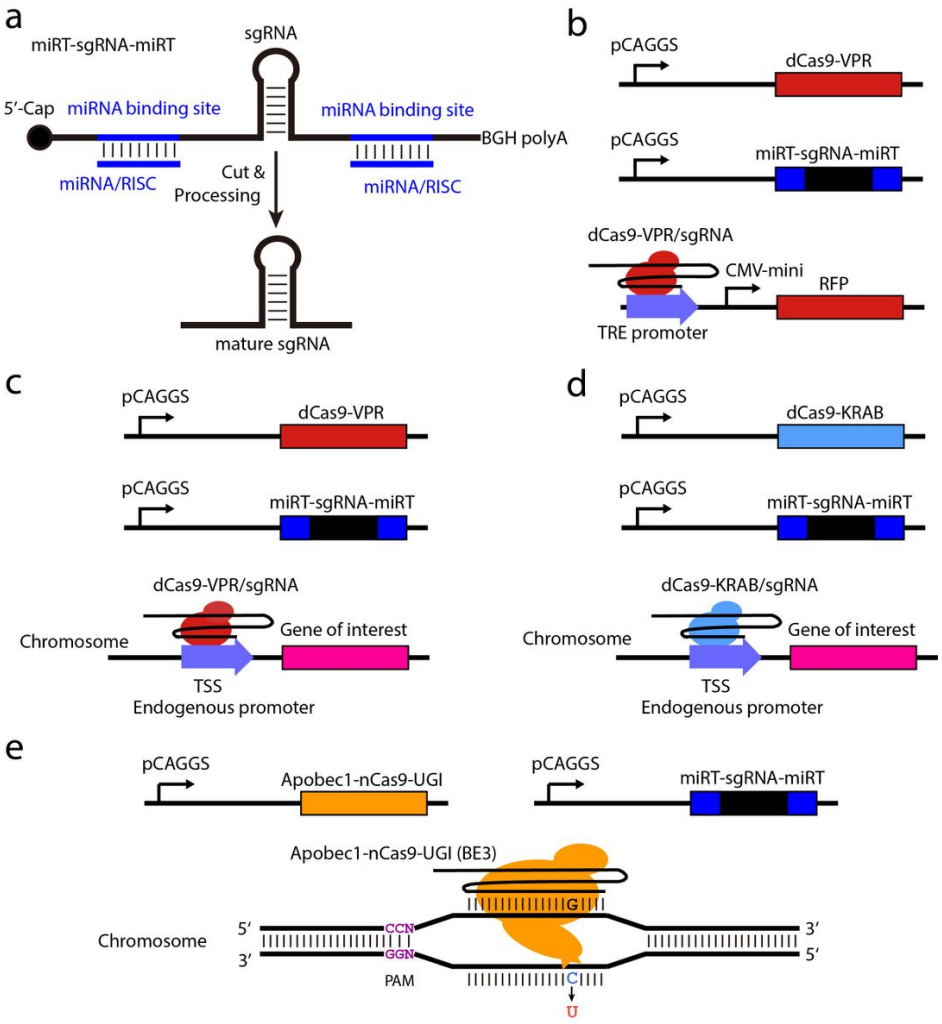

Figure 1

Design of MICR platform. (a) Design of pre-sgRNA. Schematic representation of the pre-sgRNA (miRT-sgRNA-miRT). Two flanking miRNA binding sites are drawn. (b) Design of MICR-ON system to activate RFP expression upon induction by miRNAs. The dCas9-VPR and pre-sgRNA were driven by a CAGGS promoter. The TRE promoter contains seven repeats of a sgRNA binding site with GGG PAM sequences. (c) Schematic representation of MICR-ON to activate endogenous gene expression. sgRNAs are designed to target the transcription start site of endogenous genes. (d) Schematic representation of MICR-i to repress endogenous gene expression. sgRNAs are designed to target the transcription start site of endogenous genes. (e) Schematic representation of MICR-BE. sgRNAs are designed to target endogenous DNA locus.