A protocol to generate SPH-OminiCMV-Ents mESCs

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Method Article

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Abstract

Detection of genetic information in the natural context is essential to understand the biological principles. However, the ability to sense the activity of endogenous genes is limited for conventional tools. Here, we developed a highly programmable sgRNA switch system (Ents) that enables detection of endogenous genes and lncRNAs, even with very low expression level. This protocol is related to the publication “Endogenous promoter-driven sgRNA for monitoring the expression of low-abundant genes and lncRNAs” in Nature Cell Biology.

Introduction

Manipulation of endogenous genetic information is crucial to understand the basic principles of biological systems. However, approaches to maniplulate endogenous information are limited, especially for the low-abundant genes and long non-coding RNAs (lncRNAs). Previous studies have shown that lncRNAs could play essential roles in various biological processes[1-4]. Nevertheless, functional characterization of a large population of lncRNAs remains challenging, mainly due to their low expression levels[5-7].

Here, we developed an endogenous transcription-gated switch (Ents) by driving sgRNAs expression under an endogenous promoter. When Ents is paired with the highly-sensitive reporter, we could detect the activity of endogenous protein coding genes and lncRNAs faithfully in living cells, including genes with very low expression levels (< 0.001, relative to *Gapdh*, qPCR analysis). This system bypasses the procedure of identifying efficient sensor-actuator pairs, and offers virtually an unlimited number of highly effective triggers. We showed that this system could be applied to monitor the dynamic changes of genes with different expression levels in mouse ESCs (mESCs), providing a powerful platform to detect the information of various genetic elements in the natural context. In this protocol, we described detailed procedures for generating Ents system in mESCs.

Reagents

General Reagents

EmbryoMax DMEM (Milipore)

Fetal Bovine Serum (Gibco)

Non-essential AA (NEAA) (Milipore)

Nucleosides (Milipore)

Penicillin-Streptomycin (Gibco)

L-Glutamine (Milipore)

2-Mercaptoethanol (Milipore)

PD0325901 (Selleck)

CHIR99021 (Selleck)

Mouse LIF (Milipore)

DPBS (Gibco)

0.05% Trypsin-EDTA (Thermo Fisher Scientific)

Lipofectamine3000 (Invitrogen)

OPTI-MEM (Reduced serum Medium) (Gibco)

Phanta Max Super-Fidelity DNA Polymerase (Vazyme)

6-wells plate (Corning)

12-wells plate (Corning)

24-wells plate (Corning)

96-wells plate (Corning)

Plasmids

Plasmid1: CAG-scFv-NLS-P65-HSF1-Flag-WPRE-PolyA-CAG-dCas9-HA-10xGCN4-Flag-WPRE-PolyA-PGK-PuroR-PolyA (SPH)

Plasmid2: 3 x TS-miniCMV-mCherry (OminiCMV-mCherry)

Plasmid3: tRNA-sgRNA- tRNA (one sgRNA or sgRNA Array) (sgRNA precursor)

Cells

Mouse Embryonic Stem Cells

Equipment

Cell culture incubator with 5% CO2, 37 °C

Clean bench

Microscope (Olympus)

Centrifuge (Eppendorf)

Water bath

Moflo XDP (Beckman Coulter)

MoFloAstrios EQ (Beckman Coulter)

C1000 Touch Thermal Cycler (Bio-Rad)

Olympus FV3000

Procedure

Generate SPH-OminiCMV cell line

Day 1

Seed mESCs on 6-well plate, using LIF-supplemented medium.

Day 2

Co-transfect 1 μg SPH (resistant to puromycin), 1 μg OminiCMV-mCherry and 0.6 μg PBase using Lipofectamine 3000.

Replace with 2 ml LIF-supplemented medium 12 hours after transfection.

Day 4

Digest mESCs with 0.05% trypsin for 4 minutes at 37 °C, inactivate trypsin and centrifuge at 1000 rpm for 3 min, then seed mESCs to new 6-well plates and treat with puromycin (InvivoGen, final concentration 1 μg/ml) for 4~5 days.

Day 8

Digest mESCs with 0.05% trypsin for 4 minutes at 37 °C, inactivate trypsin and centrifuge at 1000 rpm for 3 min, then seed mESCs to new 6-well plate.

Day 10

Digest mESCs with 0.05% trypsin and prepare for FACS into 96-well plates.

Day 14~15

Remove single colonies from 96-well plates to 24-well plates. Confirm SPH-OminiCMV positive colonies by transient transfection of sgRNAs and PCR analysis (SPH Primers: forward (5'-GCTTCCCTGAATCCGGGCTG -3') and reverse (5'- TGGCTCTGGCCCCTAGCTC-3'); OminCMV primers: forward (5'- GGAGGCCTATATAAGCAGAGC -3') and reverse (5'-ACAAAGGCATTAAAGCAGCGTA-3').

Generate sgRNA precursor cell line

Day 1

Seed SPH-OminiCMV mESCs on 12-well plates, using LIF-supplemented medium.

Day 2

Co-transfect 0.5 μg SaKKHCas9-GFP-sgRNA and 1 μg SpCas9 sgRNA precursor using Lipofectamine 3000 (Life Technologies).

As previously described, Homology-Mediated End Joining (HMEJ) or homology recombination (HR) strategy (800 bp homology arm) was used to insert the sgRNA precursor, as previously described[8].

Replace with 1 ml LIF-supplemented medium 12 hours after transfection.

Day 4

Digest mESCs with 0.05% trypsin and prepare for FACS, and sort positive cells, then seed GFP positive cells into 12-well plates.

Day 8

Digest mESCs with 0.05% trypsin, then seed mESCs to new 12-well plates.

Day 18

Sort single cells into 96-well plates by FACS, successful insertion of the sgRNA precursor is determined by PCR.

Day 22

Remove single colonies from 96-well plates to 24-well plates. Confirm sgRNA precursor positive colonies by PCR analysis.

Day 27

Measure the fluorescent intensity of SPH-OminiCMV-Ents colonies by FACS, and take the fluorescence images under a confocal microscope (Olympus FV3000).

Troubleshooting

Please use SaKKHCas9 to insert the SpCas9 sgRNA precursor

Time Taken

Around one and a half months

Anticipated Results

We could faithfully detect the activity of endogenous genes and lncRNAs.

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Figures

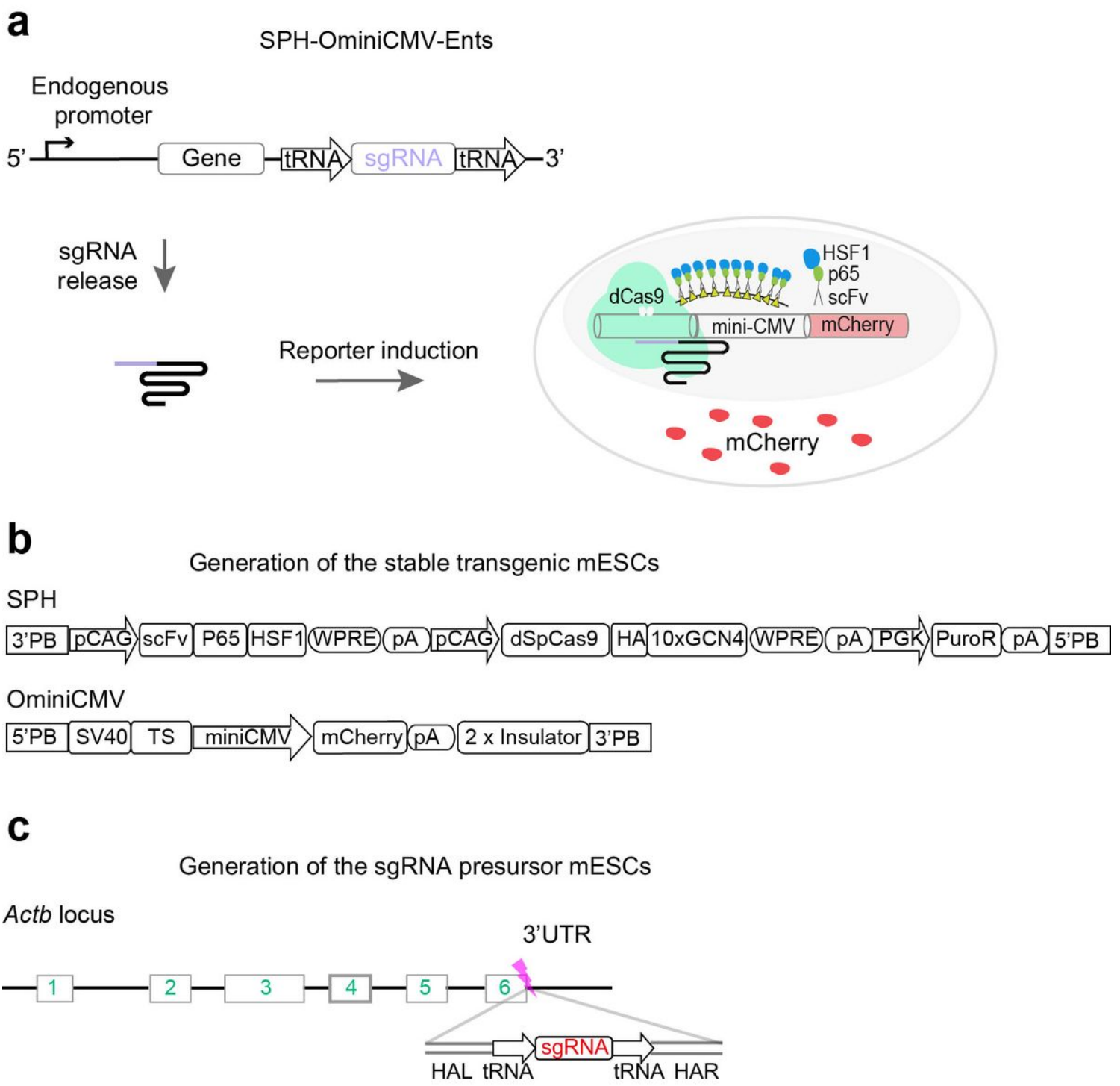

Figure 1

Design of the SPH-OminiCMV-Ents system. a, Schematic showing detection of endogenous gene expression using SPH-OminiCMV-Ents. The sgRNA is released by the endogenous processing mechanism, and then induce the expression downstream mCherry. b, Vectors to generate SPH-OminiCMV transgenic mESCs. c, Schematic showing insertion of an sgRNA precursor in the 3'UTR of Actb locus.

Supplementary Files

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