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Using Genome-Editing Tools to Develop a Novel In Situ Coincidence Reporter Assay for Screening ATAD3A Transcriptional Inhibitors

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Abstract

Transgene-based reporter gene assays have been used for discovery of inhibitors targeting vital gene transcription. In traditional assays, the reporter gene is commonly fused with a cloned promoter and integrated into a random genomic location. This has been widely applied but significantly dampened by disadvantages, including incomplete *cis*-acting elements, the influence of foreign epigenetic environments, and generation of false hits that disrupt the luciferase reporter activity. Therefore, there is a need to develop novel strategies for developing in situ reporter assays closely mimicking endogenous gene expression without disrupting its function. By employing the CRISPR-Cas9 system, we developed an effective in situ coincidence reporter system with a selection marker in the endogenous locus of ATAD3A, which provides a means of screening for transcription-targeted lead compounds with high confidence.

Keywords

ATAD3A; CRISPR-Cas9; Coincidence reporter; In situ; Transcriptional inhibition

1 Introduction

Transgene-based reporter gene assays have been widely used to identify transcriptional inhibitors targeting vital gene transcription on a large scale. In traditional transgene-based reporter gene assays, reporter genes, such as firefly and Renilla luciferases, are commonly fused with a cloned promoter and integrated into a random genomic location. Success has been largely hampered by the lack of complete *cis*-acting elements, the influence of foreign epigenetic environments, and the generation of false hits that disrupt the luciferase enzyme activity [1, 2]. To circumvent these disadvantages, several genomic editing tools including the adeno-associated virus (AAV) system [2], zinc finger nucleases (ZFNs) [3], transcription

activator-like effector-based nucleases (TALENs) [4], and clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) system [2, 5] have been developed to mimic the endogenous regulation of the target gene transcription more precisely.

Compared with other genome-editing systems, the ability of CRISPR-Cas9 to precisely and efficiently alter endogenous gene expression through targeted genome editing makes possible the wide use of this system as in situ reporter gene assays [6]. The insertion of a reporter gene into genomic deoxyribonucleic acid (DNA) can be achieved through homology-directed repair (HDR) in the CRISPR-Cas9 system. Given the fact that single reporter gene assay is frequently influenced by false hits in high throughput screening (HTS) [7], several strategies have been developed to circumvent this, including generating dual reporter genes with recombinase-mediated cassette exchange (RMCE) [2], and recruiting a coincidence reporter gene system [8]. pCI9.4, a promoterless version of FLuc-P2A-NLuc coincidence reporter plasmid containing a puromycin resistance gene [puromycin N-acetyltransferase, (pac)], has been constructed recently as an ideal template vector to develop in situ coincidence reporter gene assays [9]. The expression of firefly luciferase (FLuc) and nanoluciferase (NLuc) is simultaneously driven by the same target transcription regulatory elements, such as the promoter and enhancer regions. Moreover, insertion of coincidence reporter genes in the target gene locus through HDR can be efficiently enriched by puromycin selection.

ATPase family AAA domain containing 3A (ATAD3A) is a gene encoding a ubiquitously expressed mitochondrial membrane protein that contributes to mitochondrial dynamics, nucleoid organization, cell growth, and cholesterol metabolism [10, 11]. Interestingly, elevated ATAD3A gene expression is strongly associated with low survival of patients with breast, lung, and other types of cancer [10–12]. Knockdown of ATAD3A by lentivirus-mediated small harpin ribonucleic acid (shRNAs) significantly induces repression of tumor growth and metastasis [10], indicating that ATAD3A may be an attractive target for cancer treatment. Currently, directly targeting ATAD3A using small molecule inhibitors is challenging and not feasible.

Here, we offer an effective way to search for ATAD3A-targeted transcriptional inhibitors by developing a novel in situ coincidence reporter gene assay. Our novel reporter assays are powerful in identifying transcription-targeted lead compounds with high confidence, which should interest a range of cancer scientists and clinicians who seek to assess the feasibility of manipulating currently undruggable targets for therapeutic purposes.

2 Materials

1. Vectors: pCI9.4 and pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene; Watertown, MA, USA) (*see* Note 1).
2. 1 µg/µL sgATAD3A oligonucleotides.

¹pCI 9.4 is a version of the FLuc-P2A-NLuc coincidence reporter plasmid but does not contain a promoter (instead it contains a 5' multiple cloning site for insertion of a promoter or response element of interest (<https://www.addgene.org/74230/>). pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang [6] and pCI9.4 was a gift from James Inglese [9].

3. FastDigest BpiI restriction endonuclease (ThermoFisher Scientific, Waltham, MA, USA) (*see* Note 2).
4. KpnI, HindIII, SalI, and PciI restriction endonucleases (New England Biolabs; Ipswich, MA, USA) (*see* Note 2).
5. QIAquick polymerase chain reaction (PCR) Purification Kit (Qiagen, Hilden, Germany) (*see* Note 3).
6. Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA).
7. PureLink™ Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA) (*see* Note 3).
8. Quick Ligation Kit (New England Biolabs, Ipswich, MA, USA) (*see* Note 3).
9. Super optimal broth with catabolite repression (SOC) medium, lysogeny broth (LB) agar plates containing 100 µg/mL ampicillin.
10. QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) (*see* Note 3).
11. NEB® Stable Competent *Escherichia coli* (New England Biolabs, Ipswich, MA, USA) (*see* Note 4).
12. Lipofectamine™ 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA).
13. HN12 cells (*see* Note 5).
14. Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin.
15. Puromycin (Sigma-Aldrich, St. Louis, MO, USA).
16. GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) (*see* Note 3).
17. 96-well solid white tissue culture-treated assay plates (Corning, Corning, NY, USA).
18. Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) (*see* Note 3).
19. GloMax® 20/20 Luminometer (Promega, Madison, WI, USA) (*see* Note 6).
20. SpectraMax® L Microplate Reader (Molecular Devices; San Jose, CA, USA) (*see* Note 6).

²-This protocol has been optimized for use with these restriction endonucleases. The same enzymes from other suppliers will work but optimization may be required.

³-This protocol has been optimized for use with these kits. Other kits may be used although optimization for specific use may be required.

⁴-This protocol has been optimized for use with these *E. coli* cells. Other *E. coli* cells may be used although optimization for specific use will be required.

⁵-HN12 cells are part of the OPC-22 oral and pharyngeal cancer cell line panel. These cells are available from multiple suppliers such as ThermoFisher Scientific, Promocell, and Accegen Biotechnology.

⁶-This protocol has been optimized for use with this equipment. Other similar devices may be used, although optimization for specific use will be required.

21. Actinomycin D (Sigma-Aldrich, St. Louis, MO, USA).

3 Methods

3.1 Generation of CRISPR-Cas9 Plasmid Targeting ATAD3A

1. Design target sequence of sgRNA for ATAD3A which locates in the upstream and next to transcriptional start site (TSS) of ATAD3A using online tool (<https://zlab.bio/guide-design-resources>) (see Note 7).
2. Add 1 μ L each oligonucleotide to 16 μ L double distilled water (DDW).
3. Heat at 95 °C 5 min on a heating block.
4. Add 2 μ L 1 M NaCl, replace on heating block and decrease temperature gradually to room temperature.
5. Dilute the annealed oligonucleotides 1:10 by adding 1 μ L each oligonucleotide to 9 μ L of DDW.
6. Digest pX459V2 plasmid with BpiI.
7. Run agarose gel and purify the linearized plasmid DNA using PureLink™ Quick Gel Extraction Kit.
8. Ligate the 10 ng annealed sgATAD3A oligos and 50 ng linearized pX459V2 using the Quick Ligation kit.
9. Transform the plasmid into a competent *E. coli* strain.
10. Pick three colonies into 5 mL LB with 100 μ g/mL ampicillin, incubate the culture and isolate the plasmid DNA from cultures by using the QIAprep spin miniprep kit.
11. Verify the sequence of each colony using the U6-forward primer.
12. Designate correct insertion of sgATAD3A into pX459V2 as pX459V2-sgATAD3A.

3.2 Preparation of Coincidence Reporter Gene Construct with Homology Sequences Targeting ATAD3A (Fig. 1)

1. Design primers for amplify the homology left arm and right arm of ATAD3A for the HDR template.
2. Amplify the homology left arm of ATAD3A (961 bp) with HN12 genomic DNA using the Q5 High-Fidelity DNA polymerase.
3. Run the products on a 1% agarose gel and purify the ATAD3A-left arm using PureLink™ Quick Gel Extraction Kit.

⁷CRISPR/Cas tools include software and bioinformatics to aid in the design of guide RNAs (gRNAs) for use with the CRISPR/Cas system.

4. Simultaneously digest purified 1 µg ATAD3A-left arm products and 1 µg pCI9.4 in separate microcentrifuge tubes using KpnI and HindIII DNA restriction enzymes.
5. Purify linearized pCI9.4 and ATAD3A-left arm using the QIAquick PCR Purification Kit.
6. Ligate the purified ATAD3A-left arm fragment and linearized pCI9.4 using Quick Ligation Kit using a 3:1 (fragment:plasmid) ratio (*see* Note 8).
7. Transform 2 µL ligation reaction into NEB® Stable Competent *E. coli* according to manufacturer's protocol.
8. Pick five single colonies and grow each overnight in 5 mL LB containing 100 µg/mL ampicillin.
9. Isolate DNA from 4 mL bacterial culture using QIAprep Spin Miniprep kKit.
10. Digest plasmids with KpnI and HindIII DNA restriction enzymes as above.
11. Run on an agarose gel to identify colonies that produce plasmids with the ATAD3A-left arm insertion (*see* Note 9).
12. Amplify the homologous right arm of ATAD3A (960 bp) from HN12 genomic DNA using the Q5 High-Fidelity DNA polymerase.
13. Run 1% agarose gel and purify ATAD3A-right arm using PureLink™ Quick Gel Extraction Kit.
14. Simultaneously digest 1 µg ATAD3A-right arm product and 1 µg pCI9.4-left arm ATAD3A in separate 1.5 mL microcentrifuge tubes using SalI and PciI DNA restriction enzymes.
15. Repeat **steps 3–11**, using SalI and PciI to identify colonies that produce plasmids with the ATAD3A-right arm insertion.
16. Designate as pCI9.4-ATAD3A (*see* Note 10).

3.3 Generate HN12-ATAD3A-FLuc-NLuc Cells (See Note 11)

1. Seed the HN12 cells in a 6-well tissue culture plate overnight at 37 °C under 5% CO₂.
2. Transfect sequence-verified pX459V2-sgATAD3A and pCI9.4-ATAD3A at a 1:1 ratio into HN12 cells.
3. After 24 h following transfection, seed cells into three 100 mm dishes and three 96-well plates using limiting dilution to obtain monoclonal cells.

⁸Using a higher molar ratio of the intended insert increases the chances of a successful ligation. This should be determined by optimization studies using different insert: plasmid ratios.

⁹This should be verified by DNA sequencing.

¹⁰This provides the template for HDR.

¹¹Ensure that all procedures for use of human cell lines have been approved by the appropriate agency.

4. Incubate cells with 1.0 µg/mL puromycin and select puromycin-resistant cells over 2–3 weeks.
5. Select single drug resistant clones and subculture these for expansion.
6. Determine the FLuc and NLuc expression in each clone using Nano-Glo® Dual-Luciferase® Reporter Assay System.
7. Verify correct insertion in the ATAD3A gene by PCR using standard procedures.
8. Prepare genomic DNA using GeneJET Genomic DNA Purification Kit and verify insertion of coincidence report genes with puromycin resistance gene into the locus of ATAD3A with the left and right arm-verified primers by PCR (*see* Note 12).
9. Confirm transcriptional repression of the coincidence reporter genes induced by potential ATAD3A transcriptional inhibitors (*see* Note 13).

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¹²The PCR product for the correct insertion of left side is 1346 bp and that for the right side is 1401 bp.

¹³Plate 1×10^4 cells into a 96-well white solid-bottom plate overnight and treat cells with the potential ATAD3A transcriptional inhibitors (e.g. 1 µM actinomycin D), followed by detection of the luminescent signal for each luminescent reporter within 16 h.

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Fig. 1.

The in situ coincidence reporter gene assay in searching for the transcriptional inhibitors of ATAD3A. (a) Schematic of pCI9.4-ATAD3A. The coincidence reporter genes and pac gene are adjacent to homology arms of ATAD3A. *LA* homology left arm, *RA* homology right arm, *pac* puromycin resistance gene, *FLuc* firefly luciferase, *NLuc* nanoluciferase. (b) Schematic figure showing insertion of a cassette into in the native ATAD3A locus on Chromosome 1. The cassette contains coincidence reporter genes (FLuc-P2A-NLuc) and the pac gene. *TSS* transcriptional start site