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Enrichment of cells with TALEN-induced mutations using surrogate reporters

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ABSTRACT

Targeted gene knockout using engineered nucleases such as transcription activator like-effector nucleases (TALENs) is a gold standard for investigating the functions of a gene of interest. Although most TALENs can cleave chromosomal DNA efficiently, the activities of designed TALENs are not always high enough to allow the efficient derivation of cells containing TALEN-driven mutations. Thus, simple methods to enrich cells containing TALEN-directed mutations would facilitate the use of TALENs. Here we describe the enrichment of such cells using surrogate episomal reporters coupled with flow cytometric sorting, magnetic separation, or hygromycin selection.

1. Introduction

Transcription activator like-effector (TALE) nucleases (TALENs) are promising tools for targeted genetic engineering [1–3]. TALENs are constructed of two modules: (i) a TALE, which serves as the sequence-recognition domain, and (ii) the catalytic domain of FokI nuclease, which cleaves DNA [4]. TALEs are proteins from *Xanthomonas*, a plant pathogenic bacterium, that activate transcription of endogenous plant genes [5]. A TALE comprises tandem arrays of 33- to 35-amino acid repeats, each of which recognizes a single base pair in the major groove of DNA [6,7]. The two amino acids at positions 12 and 13 of each repeat domain, the repeat variable diresidues (RVDs), determine base specificities for sequence recognition. For example, the bases guanine, adenine, cytosine, and thymidine are recognized by the RVD modules NN, NI, HD, and NG, respectively [4]. When two TALENs form a heterodimer through TALE-mediated sequence recognition, the catalytic domain make double strand breaks (DSBs) at the intervening sequence.

The gold standard for elucidating gene function is a comparison of the phenotypes of knockout cells or organisms with those of isogenic controls. Although knockout mice can be created via gene targeting, this conventional method requires substantial effort and time. In addition, it is difficult to achieve gene knockout in human and other higher eukaryotic cells without using programmable nucleases. The DSBs that these nuclease induce at specific genomic sites can be repaired through either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) [8]. HDR requires homologous templates such as donor DNA or single strand oligonucleotides, whereas NHEJ does not. Because NHEJ is an error-prone process, the repair of DSBs through NHEJ often results in small insertions or deletions (collectively called "indels"), which can lead to gene knockout.

TALENs, like zinc finger nucleases (ZFNs) and RNA-guided endonucleases (RGENs), represent programmable nucleases [8]. TALENs can be designed to target almost any given sequence, whereas the design densities for RGENs and ZFNs are limited [8]. Originally, TALENs only required a 5′ thymine at the end of the target sequence, a limitation that can be now overcome using TALENs with a modified N-terminal domain that recognizes all 5′ bases [9]. In addition, almost all designed TALENs are functional unless the target sites are methylated [8,10]. These features make TALENs an attractive tool for creating knockout cells.

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However, the efficiencies of designed TALENs are often low, hampering the generation of mutant cells created using these enzymes. The ability to enrich cells containing TALEN-induced mutations would greatly facilitate this process. Therefore, we previously developed three surrogate reporters that enable the efficient enrichment of cells containing programmable nuclease-induced mutations [11,12].

Several methods, including flow cytometric sorting, magnetic separation [13,14], and antibiotics selection, have been developed for isolating specific populations of cells, and we have used each for mutant cell enrichment; each isolation method has pros and cons [12]. Flow cytometric sorting has been widely used to isolate cells expressing or labeled with fluorescent factors. Although this technique is efficient, it has disadvantages; the availability of the expensive flow cytometry machines is limited and some cells are damaged due to exposure to strong lasers and hydrostatic pressure during the isolation process. As an alternative, magnetic separation enables the isolation of cells that express specific antigens. For our reporter system, we used a truncated H-2K^k that lacks a cytoplasmic domain as the distinguishing specific antigen to avoid any effects generated by the expression of the native antigen [15,16]. H-2K^k is a mouse MHC class I molecule expressed only in some rare mouse strains such as AKR/J or CBA/J, but not in human or most other murine cells [17,18]. Another method for isolating specific populations of cells is the use of resistance factors against antibiotics. Hygromycin B phosphotransferase, encoded by the hygromycin-resistance gene, phosphorylates hygromycin B, leading to its inactivation [19]. This gene has been used as a selection marker for transformed prokaryotes [20] and transgenic eukaryotes [21,22] in the presence of hygromycin B, which kills both prokaryotes and eukaryotes by inhibiting protein synthesis [21,23,24].

In this article, we provide protocols for enriching TALEN-directed mutant cells using surrogate reporters coupled with flow cytometry, magnetic separation, or hygromycin selection. The step-by-step protocols described here will greatly facilitate the generation of knockout cells using TALENs, especially when low activity TALENs are used.

2. Materials

2.1. Plasmids encoding a pair of TALENs

Plasmids encoding a pair of TALENs (e.g., Addgene, Cellectis Bioresearch, Life Technologies, Toolgen, Transposagen Biophamaceuticals)

2.2. Reporter plasmids

1. Reporter vectors (Fluorescent reporter, Magnetic reporter, Hygromycin reporter; previously described in Refs. [11,12,25]; *commercially available from Toolgen (184, Gasan digital 2-ro, Geumcheon-gu, Seoul, Korea, 153-783, South Korea; http://www.toolgen.com/)*)
2. Restriction enzymes (*Eco*RI, *Bam*HI (New England BioLabs, Inc.)) to prepare reporter plasmids for insertion and ligation of the target sequences
3. Oligonucleotides containing the TALEN target sequence (custom-ordered, e.g. Bioneer, South Korea)
4. Chemically competent cells (e.g. DH5α, Invitrogen)
5. Ligase (e.g. New England Biolabs)
6. LB broth
7. Kanamycin stock solution (100 mg/ml)
8. LB agar containing 100 μg/ml kanamycin

2.3. Cell culture

2.3.1. Cell culture medium for 293 cells

1. DMEM (Gibco/Invitrogen)
2. 10% fetal bovine serum (FBS, e.g. Gibco/Invitrogen)
3. 1% Penicillin/Streptomycin (e.g. Gibco/Invitrogen)

2.3.2. Cell culture reagents and plasticware

1. 0.25% trypsin solution (Gibco/Invitrogen)
2. Phosphate buffered saline (PBS) without magnesium and calcium (e.g. HyClone)
3. 35-mm culture dishes (e.g. BD biosciences)
4. 60-mm culture dishes (e.g. BD biosciences)

2.4. Transfection reagents

Transfection reagents (e.g. Lipofectamine (Invitrogen) or polyethylenimine (PEI) (Polyscience))

2.5. Enrichment of mutant cells

2.5.1. Flow cytometry

1. Flow cytometers (e.g. Aria (BD biosciences))
2. Flow cytometry buffer (2% FBS in PBS)

2.5.2. Magnetic separation

1. PBE: PBS supplemented with 0.5% bovine serum albumin and 5 mM EDTA (e.g. Miltenyi Biotech, Germany)
2. Anti-H-2K^k antibody conjugated with magnetic beads (MACSelect K^k microbeads (120-000-450); Miltenyi Biotech, Germany)
3. MACS buffer (e.g. Miltenyi Biotech, Germany)
4. Separation column (MACS MS column (130-042-201) or LS column (130-042-401); Miltenyi Biotech, Germany)
5. MACS separator (130-090-976; Miltenyi Biotech, Germany)
6. MACS Multistand (130-042-303; Miltenyi Biotech, Germany)

2.5.3. Hygromycin selection

1. Hygromycin B (e.g. Invitrogen)

2.6. Determination of TALEN-induced mutations

2.6.1. PCR amplification of the target region

1. Genomic DNA Extraction Kit (Promega, USA)
2. RNase A (e.g. Invitrogen)
3. High-fidelity DNA polymerase (e.g. *Pfu*, Promega or Phusion polymerase, New England Biolabs)
4. Thermocycler (Biorad)
5. Water bath

2.6.2. T7E1 (T7 endonuclease I) assay

1. T7E1 enzyme and NEB buffer 2 (NEB, USA)
2. 6× DNA loading buffer (e.g. Elpis biotech, South Korea)
3. Agarose for preparation of a 2% agarose gel (Sigma)
4. 100 bp DNA ladder (Bioneer, South Korea)

2.6.3. DNA sequencing

1. T-vector (e.g. pGEM-T Easy vector, Promega)
2. Miniprep kits (e.g. Bioneer, South Korea)