CHAPTER FOUR

A guide for drug inducible genome editing with HIT systems

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Abstract

Technologies toward precise control of biological events are desired for biomedical research and potential clinical applications. Our recently reported HIT systems based on CRISPR/Cas9 and TAL effectors can achieve temporal and dose dependent drug control for genome editing. Methods are presented for the application of these optimized HIT systems in human cells in this chapter.
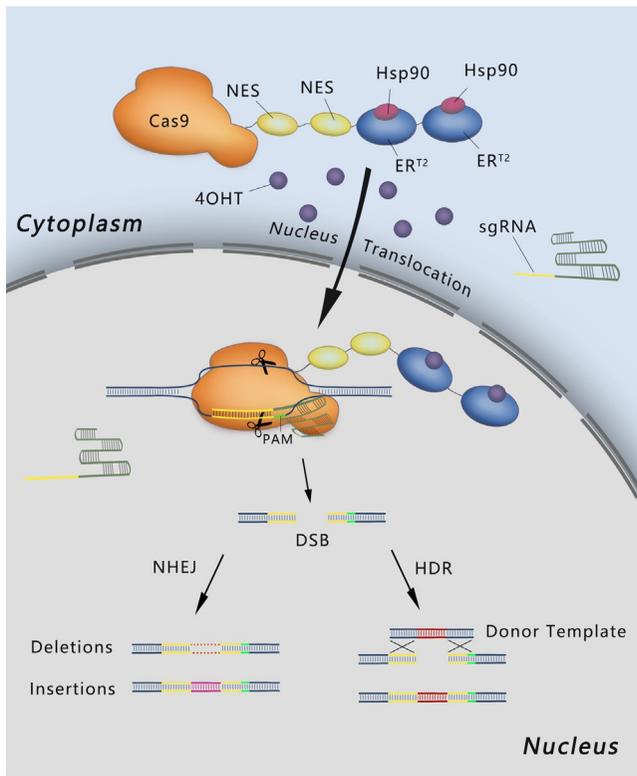
1. Introduction

Systematic interpretation of gene function requires the ability to manipulate genes in a robust and controlled inducible manner. Recent discoveries of TALEs (transcription activator–like effectors) and CRISPR

(clusters of regularly interspaced short palindromic repeats) have enabled targeted genetic modifications in cultured cells, as well as in whole organisms with high efficiency and specificity (Kim & Kim, 2014; Wei et al., 2013). Investigating gene functions under drug control may provide potential avenues toward safer gene therapies because it reduces undesired off-target events and allows genetic modification in greater precision. One way to achieve drug control is by utilizing estrogen receptor (ER), as exemplified by its successful coupling with Cre recombinase (Branda & Dymecki, 2004; Indra et al., 1999). Upon ligand binding, ER translocates from the cytoplasm to the nucleus. Consequently, fusion with an ER domain enables access of a protein to the nucleus, where genomic DNA resides in. ER^{T2}, an artificial ER mutant bearing three mutations G400V/M543A/L544A, shows high selectivity to the synthetic estrogen antagonist 4–OHT over endogenous ER ligand β–estradiol, reducing undesired perturbation from background activity (Feil, Wagner, Metzger, & Chambon, 1997). Inspired by the success of $CreER^{T2}$, we developed and optimized a series of chemical inducible devices based on the fusion of ER^{T2} to CRISPR and TALE systems, which we named hybrid inducible technologies (HIT). Herein, we describe the methods for drug inducible genome editing with our HIT systems.

1.1 Introduction to HIT-Cas9

The CRISPR/Cas (clustered, regularly interspaced, short palindromic repeats/CRISPR associated proteins) systems are adaptive immune defense machineries in bacteria and archaea (Bhaya, Davison, & Barrangou, 2011). Among them, type II CRISPR/Cas9 system has been widely used in site-directed genome editing in eukaryotic cells because of its ease of use and high efficiency (Mali, Esvelt, & Church, 2013; Mali, Yang, et al., 2013). With the lead of guide RNA (gRNA), Cas9 protein binds to its complementary DNA adjacent to a protospacer–adjacent motif (PAM), a species specific requirement for Cas9 recognition (Cong et al., 2013; Jinek et al., 2012; Mali, Esvelt, et al., 2013; Mali, Yang, et al., 2013). Upon binding, the nuclease activity of Cas9 protein introduces DNA double-strand breaks, which induces targeted gene editing from cellular DNA–damage response, either through error prone non-homologous end-joining (NHEJ) or precise homology directed repair (HDR) (Cong et al., 2013; Jinek et al., 2012; Mali, Esvelt, et al., 2013; Mali, Yang, et al., 2013).

To achieve drug inducible genome editing using CRISPR/Cas9 technology, we envisioned engrafting ER^{T2} to Cas9, a method to control the access of Cas9 protein to genome. After a series of optimization, we concluded at a HIT-Cas9 construct (Zhao, Zhao, et al., 2018) fusing two nuclear export signal (NES) peptides and two tandem ER^{T2} domains on the C-terminus of Cas9 (Fig. 1). The examination of both NHEJ and HDR events induced by C2N2E demonstrated that tight and efficient genome editing was accomplished in a 4-OHT inducible fashion across multiple human cell lines including a human embryonic stem cell (hESC) line H9, adipose derived human mesenchymal stem cells (MSCs), and a liver cancer cell line HepG2 (Zhao, Zhao, et al., 2018). In addition, HIT-Cas9 did not introduce significant off-target activity at several known sites. We also demonstrated that drug induction is titratable, selective and rapid, which grants further precision.

Fig. 1 Cartoon illuminating the mechanism of HIT-Cas9 system.

1.2 Introduction to HIT-TALEN

As transcriptional activators, TALEs found in plant pathogenic *Xanthomonas* can specifically bind and regulate genes during pathogenesis (Miller et al., 2011). TAL effectors are comprised of a central repeat domain mediating DNA recognition, nuclear localization signals (NLS), and an acidic transcriptional activation domain (Boch et al., 2009). Each repeat contains 33–35 amino acids specifying one target base due to its repeat variable diresidues (RVDs) in the middle (Boch et al., 2009; Moscou & Bogdanove, 2009). Fusion of a TAL effector DNA binding domain and a *Fok*I cleavage domain generates a TAL effector nuclease (TALEN), a powerful tool for targeted gene editing (Huang et al., 2011; Joung & Sander, 2013; Miller et al., 2011).

Similar to HIT-Cas9, Hybrid Inducible TALEN (HIT-TALEN) was generated based on fusion of ER^{T2} and TALENs (Fig. 2). TALEN constructs with two tandem ER^{T2} N-terminal fusions enabled chemical inducible genomic DNA cleavage despite background activity, whereas single fusion did not (Zhao, Zhang, et al., 2018). We further optimized the experimental protocol and observed background reduction while maintaining drug inducible effect by lowering the delivered amount of HIT-TALEN plasmid DNA.
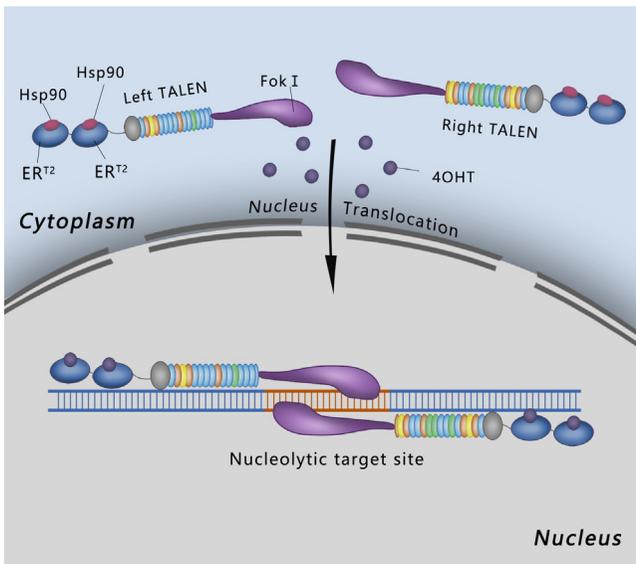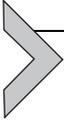

Fig. 2 Cartoon illuminating the mechanism of HIT-TALEN system.

2. Drug inducible genome editing by transient transfection

2.1 Transient transfection of HIT-systems into human cells

In the HIT–Cas9 system, effective sgRNA(s) targeting specific gene locus is required. Drug inducible genome editing can be achieved by co-transfecting C2N2E and sgRNA into target cells using transfection reagent. As for HIT–TALEN system, a pair of customized TALENs targeting specific gene sites is fused with two tandem ER^{T2} for controlled gene editing. We here described the transfection protocol for both HIT-systems using HEK293T cells as an example.

2.1.1 Equipment
• CO_2 incubator

2.1.2 Buffers and reagents
• Phosphate buffer saline (PBS)—137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4
• 0.25% Trypsin (Thermo Fisher)
• Dulbecco's Modified Eagle Medium (Thermo Fisher)
• Fetal Bovin Serum (FBS) (Thermo Fisher)
• GlutaMAX (Thermo Fisher)
• Penicillin-Streptomycin, $100\times$ (Thermo Fisher)
• Transfection reagent—Polyethylenimine (PEI), Linear, MW 25000, 1 mg/mL
• Opti-MEM (Thermo Fisher)
• 4-Hydroxytamoxifen (4-OHT)—Dissolve 10 mg of 4-OHT in 2.58 mL of DMSO to reach a stock concentration of 10 mM, then aliquot and store it at $-20\,°C$

2.1.3 Procedure
This protocol is used to transfect HIT–Cas9 or HIT–TALEN systems into HEK293T cells in a 24-well plate. All amounts and volumes are given on a per well basis. Readers can scale up or down according to cell number and plate type.

1. HEK293T cells are maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM GlutaMAX (Thermo Fisher), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C and with 5% CO_2 until the cultures are over 80% confluent.
2. The day before transfection, cells are washed by PBS and then dispersed using 0.25% trypsin. After counting the cell number, around $1.5–2 \times 10^5$ cells are pre-seeded per well in 500 μL of complete growth medium.
3. On the day of transfection, a total of 800 ng of HIT-Cas9 constructs (C2N2E and sgRNA at a molar ratio of 1:1.2) is used. As for HIT-TALEN system, 200 ng of each $2ER^{T2}$-TALEN in the pair are enough for low background activity under drug control. To achieve tight and effective genome editing using HIT-TALEN, readers can also decrease the amount of either one $2ER^{T2}$-TALEN construct under 100 ng while keeping 500 ng of another one. For each well, dilute both plasmids in 25 μL of Opti–MEM and incubate at room temperature for 5 min.
4. For each well, dilute 2.4 μL of PEI (1 mg/mL) in 25 μL of Opti–MEM and incubate at room temperature for 5 min.
5. Mix DNA and transfection reagent solutions gently and incubate 15–20 min at room temperature to form transfection complexes.
6. Transfer 50 μL of DNA-PEI complexes directly to each well and mix gently by rocking the plate back and forth.
7. Incubate the cells at 37 °C in a CO_2 incubator for 4–6 h, then replace the medium of each well with fresh complete growth medium supplemented with 500 nM 4-OHT.
8. Optional: Co-transfect a control plasmid expressing GFP or other fluorescent proteins with HIT-systems constructs to examine transfection efficiency, particularly for those cells with low transfection efficiency, such as MSCs, Hela and HepG2 cells.

2.1.4 Notes

1. The optimal cell confluence for transfection is approximately 60–70%. Higher or lower cell density may decrease the transfection efficiency.
2. All plasmids applied for the transfection must be endotoxin free.
3. The ratio of DNA (μg):PEI (μL) varies from the cells applied for the transfection. Basically, 1:3 ratio of DNA:PEI is suitable for most cell types such as HEK293T cells, Hela cells and HepG2 cells, while 1:1 ratio works better for mesenchymal stem cell (MSC) with lower cellular toxicity.
4. Transfection efficiency can be improved if the medium is changed prior to the incubation with DNA-PEI complexes.

2.2 Harvest of transfected cells and preparation of genomic DNA

For cells with high transfection efficiency such as HEK293T, genomic DNA can be prepared directly from transfected cells by treating with lysis buffer. Otherwise, fluorescence-activated cell sorting (FACS) is recommended for the enrichment of the transfected cells.

2.2.1 Equipment
- Centrifuge (Eppendorf)
- Digital Dry Bath Incubator

2.2.2 Buffers and reagents
- Phosphate buffer saline (PBS)—137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4
- 0.25% Trypsin (Thermo Fisher)
- PCR Lysis Buffer—10 mM Tris-Cl (pH 8.0), 2 mM EDTA (pH 8.0), 2.5% (vol/vol) Tween-20, 2.5% (vol/vol) Triton-X 100, 100 μg/mL of Proteinase K

2.2.3 Procedure
1. After 48 h induction of 4-OHT, cells are washed by PBS and then dispersed using 0.25% trypsin.
2. Stop digestion by adding complete growth medium and collect cells by centrifugation at 1000 rpm for 5 min.
3. The pellets are washed with PBS and centrifuged again at 1000 rpm for 5 min.
4. For cells with relative low transfection efficiency, it is recommended to co-transfect a plasmid expressing fluorescent proteins such as GFP or RFP, and thus the transfected cells can be enriched by sorting fluorescent cells using flow cytometry. Collect all selected cells by centrifuging at 1000 rpm for 5 min.
5. Remove the supernatant and add the lysis buffer at a concentration of no less than 1000 cells/μL to resuspend the pellets followed by a lysis procedure: 50 °C for 60 min, 95 °C for 15 min. Store the cell lysate at −20 °C until use. Typically, 1 μL of prepared lysate is used in a 25 μL PCR reaction system.

2.2.4 Notes

1. Cell confluence after 48 h of incubation with 4-OHT should be at least over 80% for HEK293T cells and proximately 100% for MSCs, Hela and HepG2 cells.
2. Lysis buffer can be directly added to the cells without trypsination if fluorescence-activated cell sorting process is not required. Usually, 100 μL of lysis buffer is enough for one well in a 24-well plate.
3. Homogenize cell lysate and PCR reaction by pipetting up and down.
4. Dry bath incubator can be replaced by a PCR instrument to perform lysis procedure or add corn oil to avoid the evaporation if a small sample volume (below 20 μL) is applied.

2.3 Examination of genome editing under drug control

In this section, we describe the protocol to examine genome editing of an EMX1 site by HIT-Cas9 in HEK293T cells. Two paralleled methods, Surveyor assay and TIDE assay, are used to evaluate the editing efficiency.

2.3.1 Equipment

- PCR system (Thermo Fisher)
- Vertical & Horizontal electrophoresis System
- Digital Dry Bath Incubator
- Gel imaging system (BioRad)

2.3.2 Buffer and reagents

- AccuPrime Taq DNA Polymerase System (Thermo Fisher)
- 1 × TAE—40 mM Tris base, 20 mM acetic acid and 1 mM EDTA
- 1% Agarose gel—Dissolve 1 g agarose in 100 mL distilled water
- DNA dye—10 mg/mL Ethidium Bromide (EB)
- 6 × Loading buffer (NEB)
- SURVEYOR Mutation Detection Kit (Idtdan Transgenomics)
- 10 × TBE—0.9 M Tris base, 0.9 M boric acid, 20 mM EDTA (pH 8.0)
- 20% Ammonium persulfate (APS)—Dissolve 2 g of APS in 10 mL distilled water, then aliquot and store it at −20 °C until use
- 3.5% Polyacrylamide gel (stacking gel)—850 μL of 30% Acrylamide-Bisacrylamide (29:1), 750 μL of 10 × TBE, 25 μL of 20% Ammonium persulfate (APS), 5 μL of N,N,N',N'-tetramethylethylenediamine (TEMED), 5.9 mL of distilled water
- 15% Polyacry-lamide gel (separating gel)—9 mL of 30% Acrylamide-Bisacrylamide (29:1), 1.8 mL of 10 × TBE, 75 μL of 20% Ammonium persulfate (APS), 7.5 μL of N,N,N',N'-tetramethylethylenediamine (TEMED), 7.2 mL of distilled water

2.3.3 Procedure

1. Prepare a 25 μL of PCR reaction mix in a 0.2 mL PCR tube to amplify DNA fragment containing edited gene sequences using AccuPrime Tag DNA Polymerase. The mixture includes 500 nM of each primer, 1 × AccuPrime™ PCR Buffer II, 1 μL of genomic DNA from Section 2.2, 1 μL of AccuPrime™ Taq DNA Polymerase.
2. Place PCR tube in the PCR system and perform the following program: pre-denaturalized at 94 °C for 4 min, then 35 cycles of 94 °C for 20 s, 59 °C for 20 s and 68 °C for 35 s, finally 5 min at 68 °C. The annealing temperature varies depending on the primers designed for the amplification.
3. Pipette 5 μL of PCR products for agarose gel electrophoresis to determine successful amplification.
4. Keep 5 μL of PCR products for SURVEYOR examination and leave the rest of PCR products for Sanger sequencing.
5. SURVEYOR examination:
   - Mix 5 μL of PCR products with 4.5 μL of 1 × AccuPrime™ PCR Buffer II in a 0.2 mL PCR tube and perform the following program: denatured by heating to 95 °C and slowly reannealed from 95 °C to 85 °C by −2 °C/s followed by −0.1 °C/s from 85 °C to 25 °C for rehybridization using PCR system.
   - Add 0.5 μL of Transgenomic's SURVEYOR Nuclease into the reannealed products. Mix it gently and incubate at 42 °C for 20–30 min.
   - Add 1 × loading buffer to stop the reaction and apply all products for polyacrylamide gel (PAGE) electrophoresis. The gel is run usually at 100 V for 2 h and then stained by EB to visualize in Gel imaging system.
6. The sequencing results can be analyzed using TIDE web tool (https://tide.nki.nl/), while the results from PAGE electrophoresis can be analyzed by imageJ software.

2.3.4 Notes

1. To reduce non-specific cleavage by SURVEYOR nuclease, the PCR primers must be highly specific to produce a single robust DNA band. PCR primers with high T_m values often produce optimal results according to our experiences.
2. Pre-run the PAGE gel for half an hour at 80 V for clearer gel image.
3. Duration to incubate the mixture of reannealed products and SURVEYOR Nuclease at 42 °C is recommended not to be over 40 min to avoid non-specific cleavage.
4. Design additional primers flanking the cleavage site at a distance of 150 bp for Sanger sequencing and TIDE analysis.
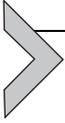
3. Drug inducible genome editing by lentiviral transduction

In certain circumstances such as scaled applications, it is desired to generate monoclonal stable cell lines expressing C2N2E and sgRNA(s) or a pair of $2ER^{T2}$ fused TALEN via lentiviral transduction. All plasmids introduced in this chapter are constructed using lentiviral expression vectors, which contain antibiotic resistant genes for enriching successfully infected cells. This protocol is an example to perform drug inducible editing of EMX1 site in human embryonic stem cell line H9.

3.1 Lentiviral packaging and transduction

We use three plasmid system to transfect 293T cells for lentivirus production. The packaged viruses need to be further concentrated by Amicon Ultra-15 centrifugal filter device before the infection of cells.

3.1.1 Equipment
- Biosafety cabinet (Thermo Fisher)
- CO_2 incubator (Thermo Fisher)
- Refrigerated centrifuge with horizontal rotor (Eppendorf)

3.1.2 Buffer and reagents
- Buffer and reagents used for transfection assay (see Section 2.1.2)
- Essential 8 Medium (Thermo Fisher)
- 0.5 M EDTA (Thermo Fisher)
- Polybrene (hexadimethrine bromide, Sigma)—Dissolve 10 mg of polybrene in 1 mL of ultrapure water, sterilize by passage through a 0.2 μm filter and store at −20 °C until use.

3.1.3 Procedure
HIT–Cas9 device includes two constructs, C2N2E and sgRNA, containing different antibiotic resistant genes (zeocin and puromycin, respectively). HIT–TALEN system contains a pair of $2ER^{T2}$ fused TALEN targeting specific gene loci. The antibiotic resistant genes within the two constructs are different (zeocin and neomycin), too. All plasmids are constructed using lentiviral transfer plasmid. Co-transfect each transfer plasmid and packaging and envelope plasmids into the HEK293T cells for virus production.

1. The procedure for transfection is the same as what described in Section 2.1.3. The molar ratio of three plasmids (transfer plasmid:packaging plasmid:envelope plasmid) is 8:6:3 in our system.
2. Transfection assay is performed using HEK293T cells pre-seeded in a 10 cm cell culture dish. The cell culture supernatant is collected twice, 48 and 72 h after transfection. The total amount of supernatant is 15 mL:10 mL of medium from the first replacement 6 h post transfection and 5 mL from the second collection of supernatant.
3. Collect all the cell culture supernatant into a 50 mL sterile centrifuge tube and centrifuge at 2000 rpm in a refrigerated centrifuge for 5 min at 4 °C to pellet the cellular debris.
4. Filter the cell supernatant through a 0.45 μm filter to remove any remaining cell debris and large particles.
5. Transfer the filtrate into the Amicon Ultra-15 centrifugal filter device and then centrifuge at 4000 rpm for 20–30 min at 4 °C. The volume of concentrated cell supernatant is normally about 1 mL after the centrifugation.
6. Dispense the concentrated virus into 200 μL aliquots in 1.5 mL microcentrifuge tube and store at −80 °C until use.
7. Before the day of transduction, H9 cells by 80% confluence are passaged from one well of six-well plate to a matrigel pre-coated six-well plate at a dilution of 1:10 using 0.5 M EDTA. The cell confluence should be around 30–40% on the day of transduction.
8. Mix 1.8 mL of Essential 8 Medium, 2 μL of polybrene and 200 μL of concentrated viral solution in a 15 mL microcentrifuge tube. Change the medium with the mixture and incubate at 37 °C in a CO_2 incubator for 6–8 h followed by the replacement of fresh E8 medium. Change the medium every day and add antibiotic into the E8 medium after 2 days of transduction before screening monoclones.

3.1.4 Notes
1. The initial density of the HEK293T cells is critical for successful viral packaging. The cell confluence on the day of transfection should be between 60% and 80%.
2. Concentration of viral supernatant is necessary because the large size of the lentiviral constructs used in HIT-Cas9 and HIT-TALEN systems, which results in low titer.
3. It is not recommended that lentiviral supernatant is subjected to multiple freeze-thaw cycles. Do not use the viral supernatants if it has been stored at −80 °C over 6 months.

3.2 Generation of monoclonal stable cell line with HIT-systems

This protocol can be used to generate stable cell lines expressing all necessary constructs of HIT-systems. Optimal drug inducible genome editing can be achieved by carefully identifying monoclonal H9 cell line upon antibiotic selection.

3.2.1 Equipment
- Biosafety cabinet
- CO_2 incubator

3.2.2 Buffer and reagents
- Essential 8 Medium (Thermo Fisher)
- Zeocin (10 mg/mL, Invivogen)
- Puromycin (10 mg/mL, Invivogen)
- Rock inhibitor (Y-27632, 10 mM, Selleck)
- TryplE Express (Thermo Fisher)

3.2.3 Procedure
1. H9 ESCs are maintained in E8 medium supplemented with antibiotic and 10 μM rock inhibitor at 37 °C and with 5% CO_2 after 48 h of transduction. The screening concentration used for zeocin is 2.5 μg/mL when infected with C2N2E viral particles, while that for puromycin is 1 μg/mL after sgRNA viral infection. The concentration applied for neomycin is 100 μg/mL when establishing the HIT-TALEN stable cell line.
2. Observe the infected H9 cells every day until the cells in the untransduced well are dead completely. Prepare cell stocks at liquid nitrogen once the polyclonal populations of resistant cells are expanded sufficiently.
3. Isolate individual cells from the polyclonal resistant cells by incubating with Tryple-E for 2–3 min to form the homogenized cell solution. Quantitate the cell concentration by a hemocytometer and transfer cells into a 10 cm cell culture dish precoated with matrigel at a concentration of 50 cells/mL. Cells can also be passaged to matrigel coated 96-well plate at a concentration of 10 cells/mL for monoclonal selection. Single-cell sorting by flow cytometry is another method to generate monoclonal cell line.
4. Maintain the cells in E8 medium supplemented with antibiotic and rock inhibitor in a CO_2 incubator for 7–14 days. Change the conditional E8 medium every 2 days within the first 7 days of culture and scan the plate for cell growth after 7 days.

5. Remove rock inhibitor from E8 medium once the cells appear as colonies in the well.
6. Transfer the monoclonal lines to 12-well plate by incubating with $0.5\,M$ EDTA for 5 min at $37\,°C$ when the cells have expanded to be confluent. Freeze replicated cell stocks in liquid nitrogen before screening the transgene expression and its function in genome editing.

3.2.4 Notes
1. It is recommended to generate and screen monoclonal H9 cell lines in two steps: first expressing C2N2E construct at a level for efficient and tight drug inducible performance, and second with sgRNA viral delivery.
2. The antibiotic selection should last at least 2 weeks for zeocin and neomycin or 1 week for puromycin until the control cells completely die. Use the same concentration of antibiotic for the following monoclonal selection.
3. Rock inhibitor is required when screening the stable cell lines by antibiotic selection, particularly for the isolation and growth of a monoclonal cell population.

3.3 Examination of drug inducible genome editing for monoclonal H9 cell lines

The methods to examine drug inducible genome editing are the same as what described in Section 2.3. It is noted that the concentration of 4-OHT applied for H9 cells is lower than that used for HEK293T cells due to the high sensitivity of H9 cells to drug induced cytotoxicity.

In addition, it is important to verify the expression of Cas9 or TALE protein prior to examination of genome editing efficiency for monoclonal cell lines. This should be executed for those monoclonal H9 cells survived after antibiotic selection. Both quantitative real-time PCR and western blot can be used to evaluate transgene expression.

3.3.1 Equipment
• See Section 2.3.

3.3.2 Buffer and reagents
• See Section 2.3.

3.3.3 Procedure

1. Maintain monoclonal H9 cell lines survived after antibiotic selection in E8 medium. Plate each clone in duplicate at 24-well plate and treat the cells with or without 250 nM of 4-OHT.
2. Extract genomic DNA from each well after 3 days of 4-OHT induction and perform PCR amplification as described in Section 2.3.
3. Examine the genome editing events by Sanger sequencing and SUR-VEYOR assay as described in Section 2.3 and compare the efficiency between 4-OHT treated and untreated samples. Select monoclones displaying high editing efficiency and low background activity for the future experiments.
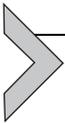
4. Summary and conclusions

We have designed and vigorously optimized drug inducible systems for genome editing based on CRISPR/Cas9 and TAL effectors via transient transfection in 293T cells. These systems displayed high efficiency and low background activity, and open new opportunities for precise chemical control of dynamic biological processes. Here we discussed their application by lipid particle based transient transfection and lentiviral infection. Other scenarios exist, such as in vivo delivery, and the protocols will need customized optimization to achieve optimal performance in a drug inducible manner.

Acknowledgments

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Further reading

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