Conditional Manipulation of Gene Function in Human Cells with Optimized Inducible shRNA

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The difficulties involved in conditionally perturbing complex gene expression networks represent major challenges toward defining the mechanisms controlling human development, physiology, and disease. We developed an OPTimized inducible KnockDown (OPTiKD) platform that addresses the limitations of previous approaches by allowing streamlined, tightly-controlled, and potent loss-of-function experiments for both single and multiple genes. The method relies on single-step genetic engineering of the AAVS1 genomic safe harbor with an optimized tetracycline-responsive cassette driving one or more inducible short hairpin RNAs (shRNAs). OPTiKD provides homogeneous, dose-responsive, and reversible gene knockdown. When implemented in human pluripotent stem cells (hPSCs), the approach can be then applied to a broad range of hPSC-derived mature cell lineages that include neurons, cardiomyocytes, and hepatocytes. Generation of OPTiKD hPSCs in commonly used culture conditions is simple (plasmid based), rapid (two weeks), and highly efficient (>95%). Overall, this method facilitates the functional annotation of the human genome in health and disease. © 2018 by John Wiley & Sons, Inc.

Keywords: knockdown • shRNA • human pluripotent stem cells • gene editing

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INTRODUCTION

Human pluripotent stem cells (hPSCs) represent an invaluable model to both understand human development and generate a wide variety of cell types useful for disease modeling and drug screening (Avior, Sagi, & Benvenisty, 2016; Pourquié, Bruneau, Keller, & Smith, 2015; Trounson & DeWitt, 2016). However, functional analyses of complex developmental and disease mechanisms using hPSCs require efficient ways to probe gene function. Unfortunately, manipulation of gene expression in hPSCs is extremely complex, as most traditional methods such as transfection and transduction are poorly effective (Cao et al., 2010; Costa et al., 2007; Ellis, 2005; Herbst et al., 2012; Luo, Lü,

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Pan, & Long, 2016; Yao et al., 2004). Moreover, manipulation of gene expression is complicated by the need for maintaining such cells in a self-renewing undifferentiated state (thus complicating the study of genes regulating pluripotency), and by the difficulty in determining the stage-specific role of genes involved at multiple steps during lineage specification. Overall, stable loss- or gain-of-function experiments in hPSCs have only limited value, while conditional manipulation of gene expression is crucial to fully harness the power of hPSCs for functional genetics applications.

Inducible gene knockdown using short hairpin RNAs (shRNAs) is an established method to study gene function in many cell types (Boettcher & McManus, 2015; Lambeth & Smith, 2013). In this context, the tetracycline-derepressible (TET-OFF) system is the most widely used approach (Kappel, Matthess, Kaufmann, & Strebhardt, 2007; Lambeth & Smith, 2013). This relies on a modified RNA polymerase III (Pol III) promoter that is responsive to a tetracycline-sensitive Repressor protein (tetR). Following simple tetracycline treatment, the tetracycline-bound tetR undergoes a conformational change that prevents its binding to the Pol III promoter, which is therefore derepressed. As a result, the shRNA is induced and drives gene knockdown. However, application of the TET-OFF system in hPSCs has proven challenging for two main reasons: (1) tight control of shRNA expression is difficult to achieve; (2) induction of the shRNA rarely works in differentiated derivatives. Indeed, very high and homogenous expression of both the tetR and the inducible shRNA is required to obtain a potent yet controlled knockdown. However, standard methods rely either on lentiviruses or on randomly integrated plasmids (Laperle et al., 2015; Lian et al., 2012; Massé et al., 2011; Tsuneyoshi et al., 2012; Zafarana, Avery, Avery, Moore, & Andrews, 2009), which are plagued by heterogeneous expression and transgene silencing in hPSCs, in particular when such transgenes are located in regions where heterochromatin forms following cell fate choices (Herbst et al., 2012; Raya et al., 2009).

We recently developed an alternative approach to implement optimized inducible shRNA expression in hPSCs (Figure 5C.4.1A; Bertero et al., 2016). This method takes advantage of recent advances in gene editing based on programmable nucleases to induce controlled transgenesis into human genomic safe harbors (GSHs), regions in the human genome that are expressed in virtually any human cell type, are resistant to gene silencing, and can be genetically modified without negatively affecting cellular functionality (Gaj, Gersbach, & Barbas, 2013; Hockemeyer et al., 2009; Pawlowski et al., 2017; Sadelain, Papapetrou, & Bushman, 2012; Smith et al., 2008). We developed an all-in-one targeting approach to deliver a TET-OFF-inducible shRNA expression cassette to the AAVS1 GSH, and combined this method with an optimized tetR protein that virtually eliminates premature shRNA expression in the absence of tetracycline. This technology, which we named OPTiKD for OPTimized inducible KnockDown, allows streamlined generation of hPSCs in which expression of individual or multiple genes can be conditionally manipulated in a homogeneous, rapid, dose-responsive, and reversible fashion (Figure 5C.4.1B). Further, we demonstrated that this approach is fully functional even after differentiation of hPSCs into a panel of more than twelve hPSC-derived lineages, including cell types of major clinical interest such as neurons, cardiomyocytes, and hepatocytes (Bertero et al., 2016).

This unit describes all the steps involved in generating and validating OPTiKD hPSCs. The main method is subdivided into four basic protocols to be followed in chronological order (Figure 5C.4.2):

- Basic Protocol 1: Design of oligonucleotides for shRNA cloning
- Basic Protocol 2: Generation of inducible shRNA targeting vectors
- Basic Protocol 3: Generation of OPTiKD hPSCs
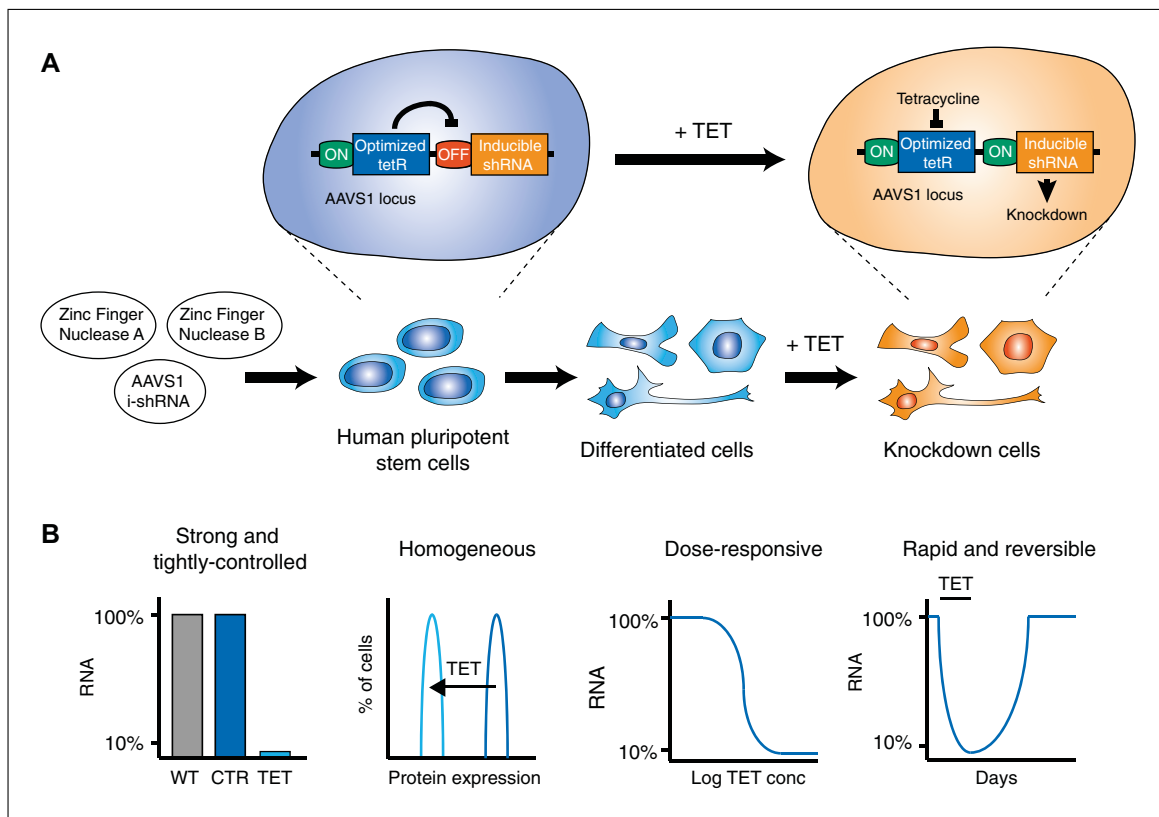- Basic Protocol 4: Validation of OPTiKD hPSCs

Figure 5C.4.1 The optimized inducible knockdown (OPTiKD) system. (**A**) Schematic of the generation of OPTiKD human pluripotent stem cells (hPSCs) and of the resulting genetic circuit which allows tetracycline (TET)–dependent induction of gene knockdown both in hPSCs and hPSC-derived cells. i-shRNA: inducible short hairpin RNA. (**B**) Schematic of the properties of OPTiKD (refer to Figure 5C.4.7 for example of expected results). WT: wild-type; CTR: no tetracycline.
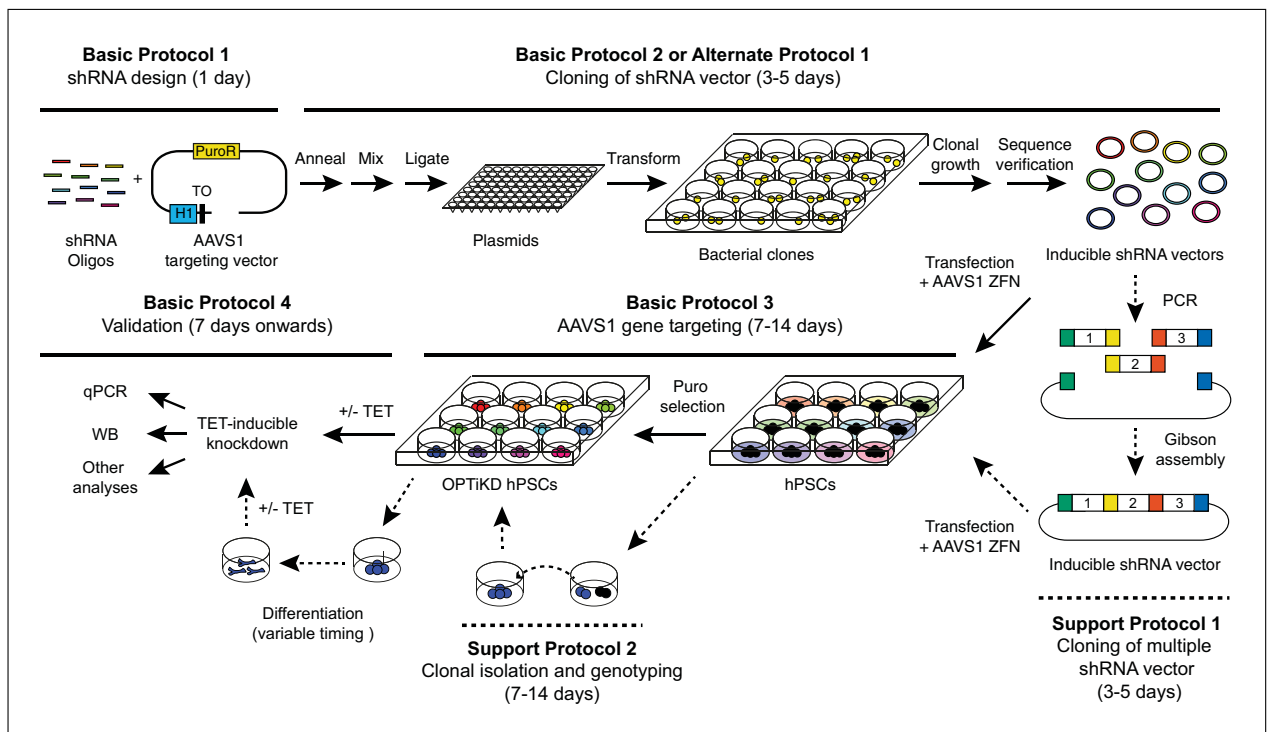

Figure 5C.4.2 Generation and characterization of OPTiKD hPSCs. Schematic overview of the procedure detailing how the various protocols relate to each other. Optional steps are indicated by dashed arrows. PuroR: puromycin resistance; H1: H1 Pol III promoter; TO: tet operator; ZFN: zinc-finger nucleases. Refer to the text for other common abbreviations.

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In addition to this, the Alternate Protocol provides a method for medium-throughput generation of several shRNA targeting vectors in parallel. Finally, two support protocols describe optional steps for the generation of OPTiKD targeting vectors expressing multiple inducible shRNAs (Support Protocol 1) and for the clonal isolation and genotyping of OPTiKD hPSCs (Support Protocol 2). The whole procedure can be completed in 3 to 9 weeks, depending on the experimental design and expertise of the investigator, and allows generation of OPTiKD hPSCs with >95% efficiency.

DESIGN OF OLIGONUCLEOTIDES FOR shRNA CLONING

The first step in generating OPTiKD hPSCs consists in obtaining oligonucleotides to be used to clone the shRNA sequence in the targeting vector. First, a suitable shRNA sequence must be identified. Second, oligonucleotides are designed to add appropriate 5′- and 3′-end overhangs to facilitate directional cloning.

1.A: shRNA design

Identifying a good shRNA is the most critical parameter when generating OPTiKD hPSCs (see Commentary). Validated shRNA sequences can be obtained from various sources such as previous reports in the scientific literature, public shRNA databases, or commercial suppliers. Alternatively, shRNAs can be designed in house by using available algorithms (reviewed in Fakhr, Zare, & Teimoori-Toolabi, 2016). For genes that have no reported validated shRNA, we advise investigators to take advantage of the RNAi Consortium shRNA library (also known as the TRC library or the Genetic Perturbation Platform: *https://portals.broadinstitute.org/gpp/public/;* Moffat et al., 2006), which contains pre-designed shRNAs for all human genes, >50% of which have been experimentally validated. Such validation data is available from Sigma-Aldrich, one of the commercial distributors of the TRC library (*https://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/individual-genes.html*).

This basic protocol assumes that an shRNA from the TRC library is being used. When using alternative sources for an shRNA, ignore steps 1, 2, and 3 of this protocol and proceed directly to step 4. The OPTiKD method has been successfully used with a variety of shRNA designs (Bertero et al., 2016). When designing the shRNA in house, make sure to follow appropriate design rules according to the most recent literature on the topic (reviewed in Fakhr et al., 2016). Further, it is crucial to avoid shRNAs containing a poly(T) tract longer than four nucleotides, as this could lead to premature shRNA termination (TRC library shRNA are pre-screened to avoid this problem). We generated an electronic worksheet file that can be used to perform all the oligonucleotide design steps (steps 4 to 6) in an automatic fashion; we recommend using such tool once familiar with the design principles described here. The worksheet can be downloaded from *https://docs.google.com/spreadsheets/d/18jzVw-5kHzw8xAXkvvFYZizL-y5Hapb1r4Fp6zTXk4U/edit?usp=sharing*.

1.B: Design of oligonucleotides for cloning the shRNA into the targeting vector

The cloning procedure will be described in more detail in Basic Protocol 2. Briefly, two single-stranded oligonucleotides are annealed to form a double-stranded DNA sequence containing the shRNA sequence followed by a Pol III terminator (consisting of a stretch of four or more thymidines), as well as 5′- and 3′-end "sticky" overhangs that are complementary to those generated in the targeting vector following restriction digestion with *Bgl*II and *Sal*I (Figure 5C.4.3E and Figure 5C.4.4C). This design facilitates directional cloning of the shRNA into the targeting vector. Furthermore, the oligonucleotides are designed to destroy the *Bgl*II restriction enzyme site in the final plasmid in order to allow screening of correct recombinant clones by diagnostic restriction digestion (Figure 5C.4.4C). As transcription from the H1 Pol III promoter is more
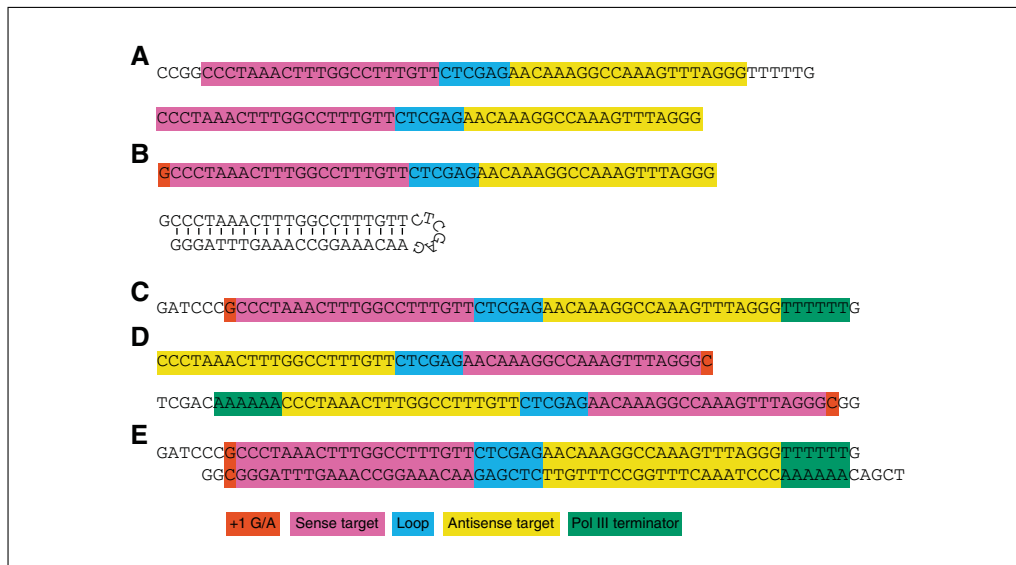
Figure 5C.4.3 Design of shRNAs for OPTiKD. Example of the design procedure described in Basic Protocol 1. The relevant sequence elements are color-coded. +1 G/A: guanine/adenosine found on the first translated nucleotide following cloning into the H1 promoter. (**A**) Sequence from the TRC shRNA database as obtained from the Sigma-Aldrich Web site (top) and after trimming of extra nucleotides (shRNA proper, bottom). (**B**) shRNA modified to start with a guanine to ensure maximal activity of the H1 promoter. The predicted hairpin secondary structure is shown on the bottom. (**C**) Top oligonucleotide for shRNA cloning. (**D**) Reverse complement of the shRNA in panel B (top), and bottom oligonucleotide for shRNA cloning (bottom). (**E**) Predicted double-stranded DNA obtained after annealing of the top and bottom oligonucleotides from panels C and D.

efficient and precise when a guanine ("G") or an adenosine ("A") base is found on the +1 site (Ma et al., 2014; Ranganathan, Wahlin, Maruotti, & Zack, 2014), the shRNA is modified to satisfy this criteria if needed.

Materials

SnapGene software (GSL Biotech LLC)

1. Obtain a shRNA target sequence for the gene of interest from the TRC library (*https://portals.broadinstitute.org/gpp/public/gene/search*).

    Search the gene of interest by its official gene symbol: a list of all available shRNAs will appear and can be visualized online or downloaded as a comma-separated (CSV) file. In this list, identify the 20- to 21-bp-long target sequence (sense shRNA target site).

2. Check that the 21-bp sense shRNA target site maps to the desired transcript by using BLAST (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*).

    A 100% match is to be expected. The shRNAs from the TRC library have been designed to target all annotated isoforms of a given gene, but as annotations are ever evolving it is possible that the isoform of interest might not be targeted by the selected shRNA. If so go back to step 1 and select a new shRNA.

3. Design the shRNA sequence by joining the following segments in 5′ to 3′ orientation:

    a. The sense shRNA target site identified in step 1.
    b. The 6-bp "loop" sequence 5′-CTCGAG-3′.
    c. The reverse complement of the sense shRNA target site identified in step 1 (antisense shRNA target site).

    The resulting shRNA should be 46 to 48 bp long (Figure 5C.4.3A).

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Note that the shRNAs listed on the Sigma-Aldrich Web site will most likely have initial "CCGG" and final "TTTTTG/TTTTG" sequences, which must be ignored as these are not part of the shRNA proper. When the shRNA sequence is transcribed by Pol III, it will form a hairpin structure with the complementary sense and antisense target sites forming a stem through Watson-Crick base pair interactions, and an extruding 6-bp loop (Figure 5C.4.3B).

4. Check to see if the sense shRNA target site starts with a guanine ("G") or an adenosine ("A"): if not, insert a "G" at the beginning of the sense shRNA target site (Figure 5C.4.3B).

   Note that a complementary final "C" should not be added to the antisense shRNA target site, as the extra "G" hanging on the 5-end of the shRNA will not create any issue and it is better to preserve the structure of the 20- to 21-bp-long hairpin.

5. Design the "top" oligonucleotide by:

   a. Taking the shRNA sequence from step 4.
   b. Adding 5′-GATCCC-3′ to the 5′-end.
   c. Adding 5′-TTTTTTG-3′ to the 3′-end (Figure 5C.4.3C).

6. Design the "bottom" oligonucleotide by:

   a. Obtaining the reverse complement of the shRNA sequence from step 4.
   b. Adding 5′-TCGACAAAAAA-3′ to the 5′-end.
   c. Adding 5′-GG-3′ to the 3′ end (Figure 5C.4.3D).

   The additions from this and the previous step contribute to creating the appropriate overhangs for subsequent directional cloning and the Pol III terminator sequence.

7. *Optional:* Use SnapGene software for *in silico* simulation of the subsequent cloning step by:

   a. Annealing the oligonucleotides.
   b. Digesting pAAV-Puro_siKD with *Bgl*II and *Sal*I (for the sequence see Materials in Basic Protocol 2).
   c. Ligating the two fragments.

   Save the resulting plasmid map for later use.

   We strongly recommend this step to verify that the oligonucleotides are predicted to anneal perfectly and form a structure with the overhangs required for the subsequent cloning step (Figure 5C.4.3E). Furthermore, the resulting plasmid map will be used to facilitate the ensuing analysis of sequencing results of plasmid clones. If SnapGene is unavailable, use equivalent software for in silico analysis of DNA sequences and simulation of molecular cloning procedures.

8. Order top and bottom oligonucleotides as desalted purified products from Sigma-Aldrich or from an alternative preferred commercial supplier.

   A production scale of 0.05 µmol is sufficient. PAGE purification of the oligonucleotides is not required in our experience but could be beneficial if the subsequent cloning step proves inefficient.

GENERATION OF INDUCIBLE shRNA TARGETING VECTOR

In this second part of the procedure, the oligonucleotides obtained from Basic Protocol 1 are used to generate an AAVS1 targeting vector for inducible shRNA expression. First, the oligonucleotides are annealed, phosphorylated, and ligated to a pre-digested plasmid. Secondly, the recombinant DNA is transformed into *E. coli* for clonal propagation.
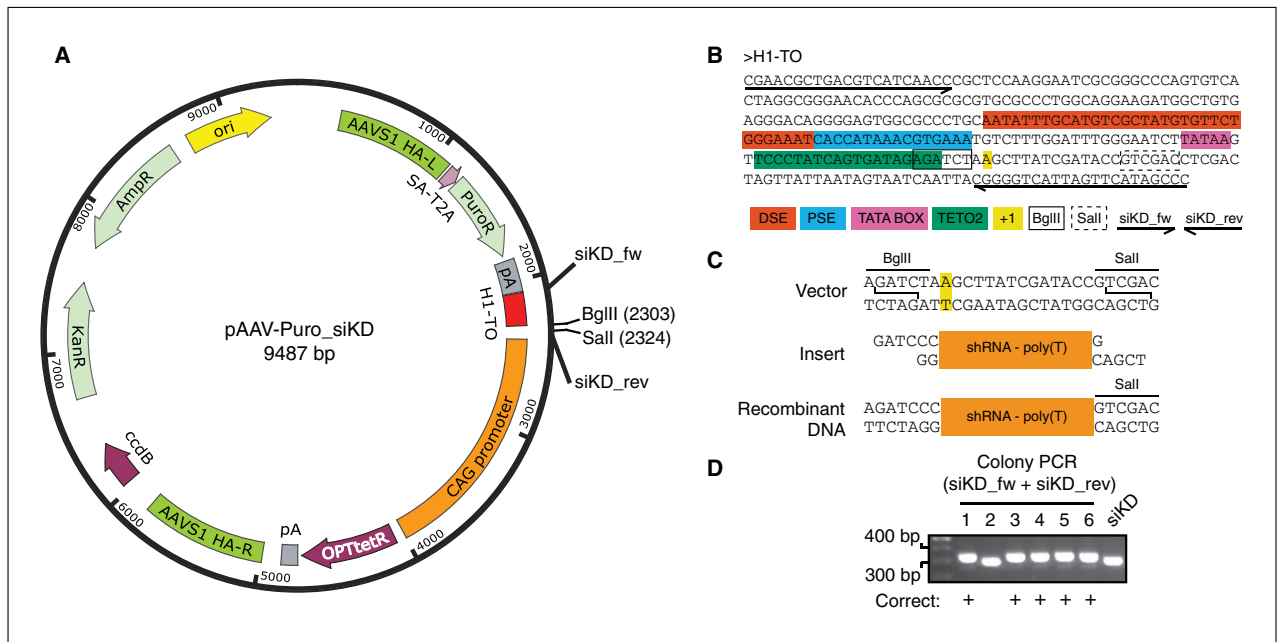
Figure 5C.4.4 Cloning of shRNAs into the OPTiKD plasmid. (**A**) Map of the plasmid used to generate OPTiKD hPSCs. The position of restriction sites and primers relevant to the protocol is shown. HA-L/R: left/right homology arm; SA: splice acceptor; T2A: self-splicing T2A sequence; PuroR: puromycin resistance cDNA; pA: polyadenylation sequence; H1-TO: tetracycline-inducible H1 RNA Polymerase III promoter containing a tet operator; CAG: CMV early enhancer, chicken β-actin and rabbit β-globin hybrid promoter; OPTtetR: codon-optimized tetracycline-responsive repressor protein cDNA; ccdB: ccdB toxin (truncated and thus inactive); KanR: kanamycin resistance; AmpR: ampicillin resistance; ori: high-copy origin of replication. (**B**) Nucleotide sequence of the tetracycline-inducible H1-TO RNA Polymerase III promoter. Key sequence features are color coded, and the locations of primers are show with arrows indicating their 5′-3′ directionality. The restriction enzyme cut sites used for shRNA cloning are shown in boxes. DSE: distal sequence element; PSE: proximal sequence element; TETO2: tet operator; +1: start position of RNA transcription. (**C**) Schematics of the shRNA cloning procedure described in Basic Protocol 2. The portion of the vector being cut is shown on top, and the restriction enzyme sites and resulting overhangs are indicated. The +1 position is in yellow (panel B). The double-stranded oligonucleotide containing the shRNA (Figure 5C.4.3E) is schematized in the middle. The product resulting from ligation of the cut vector and insert is on the bottom. Note that the *Bgl*I site is destroyed after ligation. (**D**) Representative example of colony PCR results from six screened bacterial clones. Clones with the expected larger amplification product are indicated. siKD: control PCR performed on pAAV-Puro_siKD (indicating the size of the empty vector).

Finally, bacterial clones are screened to identify correct recombinants that will be used for plasmid preparation and subsequent gene targeting.

2.A: The OPTiKD plasmid

This OPTiKD method is based on usage of pAAV-Puro_siKD (Figure 5C.4.4A), a bacterial plasmid which allows selective gene targeting of an all-in-one tetracycline-inducible shRNA cassette into the human AAVS1 locus (*PPP1R12C* gene, located on chromosome 19). The gene targeting step is described in detail in Basic Protocol 3. Briefly, this is achieved by the generation of a genomic DNA double-strand break by obligate heterodimer zinc-finger nucleases (ZFN) that target the first intron of *PPP1R12C* (Bertero et al., 2016; Hockemeyer et al., 2009). Such DNA damage is resolved via homologous directed-repair (HDR) based on the presence of two homology arms on pAAV-Puro_siKD that map to the 5′- and 3′-end of the double-strand break. This leads to the insertion of the DNA cassette present between the 5′- and 3′-end homology arms. This DNA cassette contains an antibiotic drug resistance gene trap that facilitates the selection of gene targeted hPSCs, and is followed by the all-in-on-inducible shRNA cassette proper.

The antibiotic drug resistance gene used is puromycin-N-acetyltransferase (PAT), which inactivates the protein synthesis inhibitor puromycin, a drug commonly used to rapidly

and specifically select eukaryotic cells. The PAT gene is driven by the endogenous *PPP1R12C* promoter through a gene-trap approach rather than relying on an autonomous promoter (Hockemeyer et al., 2009). This strategy relies on a strong splice acceptor that "hijacks" the *PPP1R12C* transcripts and splices in the PAT cDNA. This design strongly increases the probability that drug-resistant hPSCs are the result of correct gene targeting to the AAVS1 locus. Indeed, random integration of the pAAV-Puro_siKD plasmid will most often not result in expression of the PAT gene due to the lack of a dedicated promoter (the exception to this is if the plasmid is integrated in an intronic region of an expressed gene, but this is a rare event that in our experience occurs with less than 5% frequency; see Basic Protocols 3 and 4). Note that random integration of the targeting plasmid is an event that happens with comparable if not higher frequency to site-specific gene targeting through HDR. Therefore, the use of a gene-trap approach for the drug resistance gene is key to obtaining correctly targeted cells with high frequency (in our experience this is more than 95%; see Basic Protocols 3 and 4).

The inducible shRNA is placed under the transcriptional control of an H1 Pol III promoter modified to include a tet operator sequence (TO) after the TATA box (H1-TO promoter, Figure 5C.4.4B; Brummelkamp, Bernards, & Agami, 2002). Under control conditions (absence of the drug tetracycline) this promoter is efficiently bound by the tetR protein, which prevents expression of the shRNA. The tetR protein is expressed from the same targeting vector and is under the transcriptional control of the strong constitutive CAG promoter, which is highly expressed and resistant to silencing in hPSCs and differentiated cell types. The tetR protein was subjected to multi-parameter codon and RNA optimization for expression in human cells (Fath et al., 2011), which increased its steady-state expression by approximately one order of magnitude (Bertero et al., 2016). We named the resulting product OPTtetR, for codon OPTimized tetR, and demonstrated that when expressed in the all-in-one inducible shRNA OPTiKD cassette it was sufficient to virtually abolish "leaky" expression of the shRNA in the absence of tetracycline, a problem which was shown to markedly plague traditional TET-OFF-inducible shRNA methods based on the wild-type bacterial tetR (Bertero et al., 2016; Henriksen et al., 2007). We also showed that the OPTtetR is fully derepressible by addition of tetracycline, thus maximizing the dynamic range of inducible knockdown. The beneficial effects of using the OPTtetR are most likely due to its higher protein expression, which is expected to ensure higher binding efficiency to the cognate TO site and thus more consistent repression (Gray et al., 2007). Note that in contrast to the PAT gene the OPTtetR expression is driven by a constitutive promoter independent from the integration site. This strategy ensures stable expression even upon differentiation of hPSCs into lineages that might show low transcriptional activity of the *PPP1R12C* gene. A final point of note is that the orientation of the inducible shRNA and constitutive OPTtetR expression cassettes is key to the functionality of the all-in-one approach described here (A. Bertero unpub. observ.).

Transcription of the two Pol II-dependent transgenes (PAT and OPTtetR) is terminated by the strong bovine growth hormone polyadenylation signal, while the shRNA is terminated by a poly(T) tract (see Section 1.B in the introduction to Basic Protocol 1). The plasmid backbone contains a pUC bacterial origin of replication for high-copy plasmid production in *E. coli*, and an ampicillin resistance gene to allow selective propagation of bacteria containing the recombinant plasmid (a kanamycin resistance gene is also present but not used for the procedure described here). Note that a partial *ccdB* gene (which encodes for a bacterial toxin that poisons DNA gyrase) is present on the backbone, but this is not functional, as it lacks a start codon (this represents a remnant from the original backbone used to generate pAAV-Puro_siKD). Therefore, usage of special *E. coli* strains that tolerate expression of the ccdB toxin is not required for propagation of the plasmid.

2.B: Cloning of shRNA into the OPTiKD plasmid

The shRNA is cloned into pAAV-Puro_siKD by means of a simplified restriction enzyme–based molecular cloning step. The plasmid is cut with two different restriction enzymes (*Bgl*II and *Sal*I), thus generating distinct and non-compatible sticky 5′- and 3′-end overhangs that allow directional cloning (Figure 5C.4.4C). As single- and double-cut plasmids cannot be distinguished based on their size by traditional agarose gel DNA electrophoresis, the digested plasmid is also dephosphorylated. This is done to reduce the rate of self-ligation of contaminating molecules in which only one of the two restriction sites was successfully digested (and that are thus presenting compatible sticky ends). This means that the double-stranded DNA encoding for the shRNA obtained from annealed oligonucleotides must be phosphorylated prior to ligation. As mentioned in Section 1.B (see introduction to Basic Protocol 1), the *Bgl*II site is destroyed upon successful ligation, which facilitates screening of recombinant plasmids by diagnostic restriction digestion (Figure 5C.4.4C). Following successful ligation, the shRNA sequence will be placed so that the first base to be transcribed (a guanine or alanine; see Section 1.B in the introduction to Basic Protocol 1) is on the +1 position (indicating the known transcriptional start site of the H1 promoter, which is 26 bp downstream the end of the TATA box; Figure 5C.4.4B). The poly(T) stretch after the shRNA sequence functions as transcriptional terminator for Pol III. Recombinant plasmids isolated from bacterial clones are screened by colony PCR and/or diagnostic restriction digestion, and correct ligation products are further screened by Sanger sequencing to confirm that no mutations are present in the shRNA sequence. The inducible shRNA plasmid is finally expanded into *E. coli* in preparation to the gene targeting step (Basic Protocol 3).

Materials

pAAV-Puro_siKD plasmid [Addgene, cat. no. 86695; alternatively contact the corresponding authors (*abertero@uw.edu*; *lv225@cam.ac.uk*)]

FastDigest *Bgl*II (ThermoFisher, cat. no. FD0083)

FastDigest SalI (ThermoFisher, cat. no. FD0644)

FastAP Thermosensitive Alkaline Phosphatase (1 U/µl; ThermoFisher, cat. no. EF0654)

10× FastDigest Green Buffer (ThermoFisher, cat. no. B72)

Ultrapure DNase/RNase-free distilled water (ThermoFisher, cat. no. 10977015)

6× gel loading dye (New England Biolabs, cat. no. B7024S)

1% (w/v) agarose gel in TBE containing 0.5 µg/ml ethidium bromide (see recipe; use molecular biology grade agarose; ThermoFisher, cat. no. 17850)

1 Kb plus DNA ladder (ThermoFisher, cat. no. 10787018)

QIAEX II Gel Extraction Kit (Qiagen, cat. no. 20021)

"Top" single-stranded oligonucleotide for shRNA (custom oligonucleotide; Basic Protocol 1, step 8)

"Bottom" single-stranded oligonucleotide for shRNA (custom oligonucleotide; Basic Protocol 1, step 8)

T4 DNA Ligase Reaction Buffer (New England Biolabs, cat. no. B0202S)

T4 Polynucleotide Kinase (New England Biolabs, cat. no. M0201S)

4% (w/v) agarose gel in TBE containing 0.5 µg/ml ethidium bromide (see recipe; use UltraPure Low Melting Point Agarose; ThermoFisher, cat. no. 16520050)

Rapid DNA Ligation Kit (ThermoFisher, cat. no. K1422)

α-select Gold Efficiency *E. coli* (Bioline, cat. no. BIO-85027)

SOC medium (ThermoFisher, cat. no. 15544034)

LB agar plates containing 100 µg/ml ampicillin (see recipe)

siKD_fw primer (custom oligonucleotide, see Table 5C.4.1)

siKD_rev primer (custom oligonucleotide, see Table 5C.4.1)

dNTP mix (Promega, cat. no. U1511)

Table 5C.4.1 Oligonucleotides for Cloning Experiments

Primer name	Sequence (5′ → 3′)
Generation of inducible shRNA targeting vector (Basic protocol 2)	
siKD_fw	CGAACGCTGACGTCATCAACC
siKD_rev	GGGCTATGAACTAATGACCCCG
Generation of vector with multiple inducible shRNAs (Support protocol 1)	
5′_fw[a]	TGCGGTGGGCTCTATGGGTCAATTCGAACGCTGACGTCATCAAC
BL1_fw[a]	GCTGTGTCTTGACAGCAGACCTCGTCGAACGCTGACGTCATCAAC
BL2_fw[a]	ACACAAGTACTGTCGGCAACCACACCGAACGCTGACGTCATCAAC
BL1_rev[b]	GTCTGCTGTCAAGACACAGCATAGTGTAACGCGGAACTCCATATATGG
BL2_rev[b]	GTTGCCGACAGTACTTGTGTGTCCAGTAACGCGGAACTCCATATATGG
3′_rev[b]	GACCCCGTAATTGATTACTATTAATAACTAGTCGAGGTCGTAACGCGGAACTCCATATATGG
MsiKD_fw	TGGTGCATGACCCGCAA
BL1seq_fw	GCTGTGTCTTGACAGCAGAC
BL2seq_fw	ACACAAGTACTGTCGGCAAC
MsiKD_rev	GGCGTTACTATGGGAACATAC

[a]The 3′-most common portion of each forward primer is: 5′-CGAACGCTGACGTCATCAAC-3′. The 5′-most portion is specific to the desired overlap to be created following PCR amplification (Table 5C.4.2).
[b]The 3′-most common portion of each reverse primer is: 5′-GTAACGCGGAACTCCATATATGG-3′. The 5′-most portion is specific to the desired overlap to be created following PCR amplification (Table 5C.4.2).

GoTaq Flexi DNA Polymerase (Promega, cat. no. M8291)
Luria Bertani (LB) broth containing 100 µg/ml ampicillin (see recipe)
1.5% (w/v) agarose gel in TBE containing 0.5 µg/ml ethidium bromide (see recipe; use standard molecular biology grade agarose)
QIAprep Spin Miniprep Kit (Qiagen, cat. no. 27104)
Glycerol for molecular biology, >99% (Sigma-Aldrich, cat. no. G5516-100ML)
QIAfilter Plasmid Midi Kit (Qiagen, cat. no. 12243)

UV transilluminator
PCR tubes
Thermocycler with heated lid
Heated water bath
Humidified bacterial incubator
Sanger sequencing facility (or commercial provider)
Bacterial culture orbital shaker

Additional reagents and equipment for agarose gel electrophoresis (Voytas, 2001)

2.C: Preparation of cloning vector

1. One-step digest and dephosphorylate pAAV-Puro_siKD by preparing the following reaction:

a.	pAAV-Puro_siKD	Variable volume (5 µg)
b.	FastDigest *Bgl*II	3 µl
c.	FastDigest *Sal*I	3 µl
d.	FastAP (1 U/µl)	3 µl
e.	FastDigest Green Buffer (10×)	9 µl
f.	Ultrapure ddH$_2$O	to 90 µl

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Incubate for 1 hr at 37°C.

A shorter incubation of 5 to 30 min can alternatively be performed, as these enzymes are fast cutters, but we recommend 1 hr to maximize efficiency since a large amount of plasmid is being digested. No significant restriction enzyme star activity should be observed for up to 3 hr of incubation, and a longer incubation time is not recommended. The above reaction can be scaled down to digest 1 to 2 μg of plasmid if desired. However, we find it is best to prepare a larger batch of plasmid to be used for multiple ligations.

2. Add 18 μl of gel loading dye, mix and run on a 1% (w/v) agarose gel in TBE containing 0.5 μg/ml ethidium bromide. Include the DNA ladder in one separate well as molecular weight control. Visualize the DNA using a UV transilluminator, excise the 9466-bp band with a clean scalpel, and perform DNA gel extraction using QIAEX II Gel Extraction Kit according to the manufacturer's instructions and aiming for a final concentration of 25 to 100 ng/μl.

Essential protocols for agarose gel electrophoresis are included in Voytas (2001).

It is advisable to run 500 ng of uncut plasmid in a separate well as control for complete digestion. Should undigested plasmid be contaminating the preparation, we advise repeating step 1 with a longer incubation time and/or reducing the amount of plasmid being digested. When a large plasmid batch is being prepared, divide the gel-extracted vector in 5- to 10-μl aliquots and store for later use at –20°C for at least 6 months. Ethidium bromide is a known carcinogen and should be handled with care while wearing appropriate personal protective equipment and in accordance with local safety regulations. Alternative products carrying lower risk for carcinogenesis are available on the market and are an advisable alternative wherever possible.

2.D: Preparation of shRNA inserts

3. Assemble the following reaction in a PCR tube:

a.	Top oligo (200 μM in ddH$_2$O) (Basic Protocol 1)	5 μl
b.	Bottom oligo (200 μM in ddH$_2$O) (Basic Protocol 1)	5 μl
c.	T4 DNA Ligase Reaction Buffer (10×)	2 μl
d.	T4 Polynucleotide Kinase	1 μl
e.	Ultrapure ddH$_2$O	7 μl

4. Phosphorylate, denature, and anneal the shRNA oligonucleotides by incubating the reaction from step 3 in a thermocycler according to the following program (lid kept at 95°C):

a.	37°C	1 hr
b.	95°C	5 min

Ramp down to 80°C at –0.1°C/sec:

c.	80°C	4 min

Ramp down to 75°C at –0.1°C/sec:

d.	75°C	4 min

Ramp down to 70°C at –0.1°C/sec:

e.	70°C	4 min

Ramp down to 10°C at –0.1°C/sec:

f.	10°C	hold

The phosphorylated and annealed double-stranded oligonucleotides can be stored at 4°C for maximum of 24 hr or at –20°C for at least 2 weeks. This annealing protocol has been optimized for shRNAs, as cooling from 95°C to 70°C is very slow, with extensive pauses to facilitate annealing of oligonucleotides that have a high melting temperature and propensity to self-anneal.

5. *Optional:* Verify the annealing by running 10 pmol of the annealed double-stranded oligonucleotides side-by-side to single-stranded oligonucleotides (mixed as described in step 3) on a high-percentage (4%, w/v) agarose gel (use low-melting-point agarose; also see step 2).

   The annealed oligonucleotides should show a band at a higher molecular weight compared to the single-stranded oligonucleotides. Note that single-strand oligonucleotides will not resolve at the expected size, as they form secondary structures under non-denaturing conditions. Estimate the proportion of annealed versus non-annealed oligonucleotides: this should be >70%. Repeat steps 3 to 4 if necessary (see Troubleshooting).

6. Dilute the annealed double-stranded oligonucleotides from step 3 1: 500 in ddH$_2$O by performing serial dilutions of 1:10 and 1:50. Store on ice.

   The diluted double-stranded oligonucleotides are not stable and should be stored on ice or at 4°C for not more than 1 hr.

2.E: Generation of recombinant DNA

7. Assemble the following ligation reaction in a PCR tube using the Rapid DNA Ligation Kit:

a.	Cut vector from step 2	Variable volume (50 ng)
b.	Diluted oligo from step 6	4 μl
c.	5× Rapid Ligation Buffer	2 μl
d.	T4 DNA Ligase, 5 U/μl	1 μl
e.	Ultrapure ddH$_2$O	to 10 μl

   Incubate for 30 min at room temperature (22° to 25°C).

   Longer incubation times are usually not required, but overnight incubation at room temperature can be attempted if the ligation according to this protocol proves inefficient (see Troubleshooting). An additional negative-control ligation omitting the oligo (all the reagents above but 'b', its volume to be replaced by ddH$_2$O) can help in determining the success of the molecular cloning experiment, and is recommended for first-time user or as a troubleshooting experiment.

8. Transform 5 μl of the ligation product from step 7 into 50 μl of α-select *E. coli* according to manufacturer's instructions using a heated water bath for heat shock.

   If preferred, use an equivalent highly competent and recombinase-deficient strain suitable for plasmid propagation. Recovery of transformed bacteria at 37°C in SOC medium is optional when using plasmids carrying an ampicillin-resistance gene, but we strongly advise investigators to perform it, as it has been shown to improve the yield by at least a factor of 2. Use a minimal volume of medium (100 to 250 μl) to facilitate bacterial plating in step 9.

9. Plate all of the transformed bacteria onto a previously prepared LB agar plate containing 100 μg/ml ampicillin and incubate overnight at 37°C in a humidified incubator.

   To facilitate the spreading of all transformed bacteria, we advise briefly centrifuging the tube for 15 sec at 6000 × g, room temperature, discarding the supernatant by gently pouring, and resuspending the bacterial pellet in the remaining medium (50 to 100 μl) before plating.

2.F: Colony PCR screening of bacterial clones

10. Prepare the following PCR master mix using GoTaq Flexi DNA Polymerase (volumes for one reaction; adjust as needed and include one extra sample for a negative control):

a.	siKD_fw primer (10 μM in ddH$_2$O); Table 5C.4.1	0.5 μl
b.	siKD_rev primer (10 μM in ddH$_2$O); Table 5C.4.1	0.5 μl
c.	dNTP mix (10 mM)	0.5 μl
d.	MgCl$_2$ (25 mM)	2 μl
e.	5× Green GoTaq Flexi Buffer	5 μl
f.	GoTaq Flexi DNA Polymerase	0.125 μl
g.	Ultrapure ddH$_2$O	11.375 μl

Aliquot 20 μl of the reaction into PCR tubes and store on ice.

Screening of six to eight colonies per construct is recommended. If a negative control ligation was performed during step 7, this can be used to estimate the level of background due to self-ligated vector. If this is really low, screening of two to four colonies should be sufficient. Since the efficiency of shRNA cloning for most sequences is very high (>90%), colony PCR screening might be entirely bypassed (skip steps 10 to 15). If this is the case, it is recommended to isolate six to eight clones for plasmid miniprep and confirm successful cloning by BglII digest followed by DNA sequencing (steps 16 to 18).

11. For each clone to be analyzed, prepare two sets of sterile PCR tubes containing 200 μl of LB broth with 100 μg/ml of ampicillin, and 10 μl of ultrapure ddH$_2$O, respectively (use a 96 well plate if analyzing a large number of clones).

12. Using a micropipettor set on 5 μl and a sterile tip with an aerosol-barrier filter, pick a single bacterial colony from the plate from step 9. Pipet up and down five times into the tube with ddH$_2$O to break down the colony, transfer 5 μl of the bacterial suspension to the PCR tube, and pipet the remaining 5 μl to the tube with LB broth. Change tip and repeat this step for all clones to be analyzed. At the end of this step transfer the tubes with LB broth to 4°C storage for later use.

13. To the tube prepared for the negative control, add 5 μl of uncut pAAV-Puro_siKD plasmid diluted to 0.2 ng/μl. This reaction will facilitate determining positive clones based on the size of the PCR band (step 15).

14. Perform the PCR according to the following protocol:

a.	95°C	5 min
b.	95°C	30 sec
c.	60°C	30 sec
d.	72°C	1 min

Repeat steps b to d 34 times:

| e. | 72°C | 2 min |
| f. | 10°C | hold |

15. Run 7 μl of the PCR reaction on a 1.5% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. Run at 100 V for at least 30 min, image the gel using an UV transilluminator, and identify the clones giving a product which size is approximately 350 bp (depending on the size of the shRNA sequence; Figure 5C.4.4D).

Self-ligated vectors will result into a product of 295 bp, which can be identified from the negative control from step 13 (Figure 5C.4.4D). If the size difference between the shRNA containing plasmids and the empty control vector is unclear, run the gel for a longer time until the size resolution is sufficient.

16. Inoculate the bacterial clones containing the correct ligation product using the liquid culture from step 12 by transferring 100 μl of the bacterial suspension to a microbiology tube containing 4 ml of LB broth with 100 μg/ml of ampicillin. Incubate in a bacterial culture orbital shaker at 37°C overnight (16 hr) while shaking at 225 rpm.

We recommend expanding two to four clones in order to have some backup should the subsequent sequencing results demonstrate any mutation in the shRNA sequence (step

19). Store the bacterial plate from step 9 at 4°C for up to 2 weeks. This can be reused to repeat steps 12 to 15 if required.

2G: Isolation and quality control of the plasmid

17. Isolate plasmids from bacterial cultures (step 16) using the QIAprep Spin Miniprep Kit following manufacturer's instructions. Save 200 µl of the bacterial culture at 4°C.

18. *Optional:* Confirm shRNA cloning by performing a diagnostic restriction digestion with *Bgl*II using pAAV-Puro_siKD as control.

    Correct recombinants containing the shRNA will not be linearized by BglII (provided no new BglII site is found in the shRNA sequence), as this restriction site is destroyed during the cloning. If the plasmids have been pre-screened by colony PCR (steps 10 to 15), this step is redundant and can be omitted.

19. Perform Sanger DNA sequencing using the siKD_fw primer to confirm the presence of the shRNA and its correct sequence.

    The strong hairpin secondary structure of certain shRNAs can lead to difficulties in Sanger sequencing. In this case, try sequencing with siKD_rev primer and ask the sequencing company to use conditions appropriate for sequencing of GC-rich templates or sequences with a strong secondary structure (see Troubleshooting). We recommend using the plasmid map generated in step 7 of Basic Protocol 1 as reference to align the sequencing results.

20. Using the liquid culture from step 17, expand the bacterial clones containing the correct shRNA in 50 ml of LB broth with 100 µg/ml of ampicillin. Incubate in a bacterial culture orbital shaker at 37°C overnight (16 hr) while shaking at 225 rpm.

    The liquid culture from step 17 should be used within a week of storing at 4°C. Otherwise we recommend re-transforming the plasmid from step 17, picking a fresh bacterial colony, pre-inoculating it in 2 ml of LB broth with 100 µg/ml of ampicillin for 6 hr, and then diluting it 1:1000 in 50 ml of LB broth to be incubated as described above.

21. Prepare a glycerol stock to be stored at –80°C for long-term backup by mixing 200 µl of sterile autoclaved 50% (v/v) glycerol in ddH$_2$O with 200 µl of the bacterial culture from step 20.

    Glycerol stocks can be stored for up to 10 years and used to recover the plasmid. For this, streak the glycerol stock as in step 9 then repeat step 20.

22. Use the remaining culture to isolate the plasmid using the QIAfilter Midiprep Kit. Resuspend the purified plasmid in elution buffer (10 mM Tris·Cl, pH 7.5 in ddH$_2$O) at a concentration of 500 to 1000 ng/µl and store at –20°C for up to a year.

    Avoid repeated freeze-thaw cycles of the plasmid. ddH$_2$O can be used instead of elution buffer, but the shelf life of the plasmids will decrease. We did not notice interference of elution buffer with the subsequent gene targeting steps. We advise using freshly prepared plasmids (less than a month of storage) to increase the efficiency of gene targeting. We strongly advise repeating step 19 to confirm the shRNA sequence before proceeding with gene-targeting experiments.

GENERATION OF SEVERAL INDUCIBLE shRNA TARGETING VECTOR IN PARALLEL

This alternate protocol can be followed instead of Basic Protocol 2 when several inducible shRNA vectors are to be prepared simultaneously. The method is largely similar to Basic Protocol 2 but includes a few modifications that facilitate and streamline the procedure including: (1) the use of 96-well reaction plates and of a multichannel pipettor for preparation of most reactions and dilutions; and (2) plating of bacteria onto

multi-compartment well plates (Figure 5C.4.2). The protocol relies on the same back-ground information provided for Basic Protocol 2 (see Sections 2.A and 2.B in the introduction to Basic Protocol 2) and requires the same materials and equipment, aside from those specifically indicated below.

Additional Materials (*also see Basic Protocol 2*)

> MicroAmp Optical 96-Well Reaction Plate (ThermoFisher, cat. no. N8010560)
> MicroAmp Clear Adhesive Film (ThermoFisher, cat. no. 4306311)
> 8 or 12-channel pipettor appropriate for volumes between 1 and 200 µl
> Sterilin 100-mm Square Petri Dishes, 25 Compartments (Dynalon, cat. no. 8700-0357
> ColiRollers Plating Beads (Novagen, cat. no. 71013-3)

1. Digest, dephosphorylate, and gel extract the pAAV-Puro_siKD plasmid exactly as described in Section 2.C (steps 1 and 2 of Basic Protocol 2).

2. Phosphorylate, anneal, and dilute the shRNA oligonucleotides as described in Section 2.D (steps 3 to 6 of Basic Protocol 2), but prepare all reactions and dilutions in a 96-well reaction plate sealed with an adhesive film. For step 6 (oligonucleotide dilution) use a multichannel pipettor to facilitate the procedure.

3. Perform the ligation reactions as described in step 7 of Basic Protocol 2, but pre-pare them in a 96-well reaction plate and use a multichannel pipettor to transfer oligonucleotides. Seal the plate with an adhesive film.

4. Transform the ligations in α-Select Gold *E. coli* according to the following modifica-tions of the manufacturer's instructions.

    a. Thaw the bacteria on ice and aliquot 10 µl of bacterial suspension into an ice cold 96-well plate using ice-cold tips; keep the plate on ice.
    b. Using a multichannel pipettor, add 1 µl of the ligation product from step 3. Seal the plate with an adhesive film, gently mix by tapping the plate three times, and incubate for 30 min on ice.
    c. Heat shock the bacteria by submerging the bottom of the plate in a heated water bath set at 42°C for exactly 15 sec. Transfer on ice for 2 min, then place at room temperature.
    d. Gently remove the adhesive film, add 90 µl of SOC medium to each well using a multichannel pipettor, and re-seal the plate. Incubate at 37°C for 1 hr (no agitation required).

    > *E. coli from a different source can be used if desired. In this case, we recommend optimizing the heat-shock step timing, as in our experience this differed from the supplier's recommendation when using only 10 µl of bacterial suspension.*

5. Plate the recombinant bacteria from step 4 onto previously prepared 25-compartment petri dishes containing LB agar with 100 µg/ml of ampicillin. Place 20 µl and 80 µl of each bacterial suspension into two separate compartments, add two to three glass plating beads per compartment, and shake the mix in a side-to-side motion to evenly distribute the cells on the surface. Remove the beads by inverting the dish and pouring them over from the lid, and incubate the dish overnight at 37°C in a humidified incubator.

    > *It is expected that by plating 1/5 and 4/5 of the bacterial suspension at least one of the two conditions will result into 5 or more distinct bacterial colonies.*

6. Screen the bacterial clones by colony PCR as described in Section 2.F (steps 10 to 16 of Basic Protocol 2) but prepare the reactions in a 96-well reaction plate sealed with an adhesive film.

Depending on the number of plasmids to be generated, screen two to four colonies each.

7. Prepare glycerol stocks for long-term storage from small-scale bacterial cultures as described in step 21 of Basic Protocol 2, then isolate the plasmids as described in step 17 of Basic Protocol 2. Confirm the shRNA sequence by Sanger sequencing as described in step 19 of Basic Protocol 2.

To simplify the workflow when many plasmids are generated in parallel, minipreps can be used directly for gene targeting experiments at the expense of a reduced targeting efficiency. Alternatively, perform plasmid midipreps to obtain DNA of better quality (steps 20 to 22 of Basic Protocol 2).

GENERATION OF TARGETING VECTOR WITH MULTIPLE INDUCIBLE shRNAs

This optional protocol outlines a method to produce a single OPTiKD AAVS1 targeting vector carrying multiple inducible shRNAs to be co-expressed in the same cell (Figure 5C.4.2 and Figure 5C.4.5A). Such an approach allows simultaneous knockdown of multiple genes in order to study complex biochemical pathways involving more than one gene. Alternatively, the method can be applied to co-express several distinct shRNAs targeting different portions of the same transcript in order to increase the level of knockdown.

S1.A: Assembly of the multiple shRNA vector

This approach relies on having first cloned the desired shRNA sequences in the pAAV-Puro_siKD vector as described in Basic Protocol 2 or the Alternate Protocol. Then, inducible shRNA cassettes are assembled into a single plasmid by a one-step seamless Gibson cloning assembly reaction (Gibson et al., 2009). This method involves isothermal joining of multiple DNA fragments sharing specific sequence overlaps at the 5′- and 3′-ends (Figure 5C.4.6A). Individual inducible shRNA expression cassettes (consisting of the H1-TO promoter and the shRNA sequence) are amplified by DNA polymerase chain reaction (PCR) using primers that introduce specific sequences at the 5′ and 3′-end to create 20- to 39-bp-long sequence overlaps with the plasmid or the various fragments to be assembled. Such overlaps are designed to allow directional assembly of the multiple shRNA cassettes in the desired order. Using this approach, we have successfully assembled OPTiKD plasmids carrying two or three inducible shRNAs, a procedure described in the following protocol, but we anticipate that the method could be scaled up to assemble up to six to eight shRNAs. After the assembly, each pair of joined fragments is separated by a 105-bp spacer sequence consisting of a 75-bp common fragment resulting from PCR amplification of the plasmid backbone plus a pair-specific 30-bp sequence introduced by the primers used for PCR (Table 5C.4.1). By physically separating the shRNA cassettes, such a spacer sequence minimizes transcriptional interference. We refer to the pair-specific 30-bp sequence as "block" or "BL," with BL1 separating the first and second shRNA in a 2- or 3-way assembly, and BL 2 separating the second and third shRNAs in a 3-way assembly (Table 5C.4.2). The nucleotide compositions of BL1 and BL2 are 5′-ACTATGCTGTGTCTTGACAGCAGACCTCGT-3′ and 5′-TGGACACACAAGTACTGTCGGCAACCACAC-3′, respectively (Table 5C.4.1). Usage of alternative block sequences would allow assembly of more than three shRNAs (Wang, Shi, Liu, Jules, & Feng, 2006).

Materials

pAAV-Puro_siKD plasmid [Addgene, cat. no. 86695; alternatively contact the corresponding authors (*abertero@uw.edu*; *lv225@cam.ac.uk*)]
FastDigest *Bsp*119I (ThermoFisher, cat. no. FD0124)
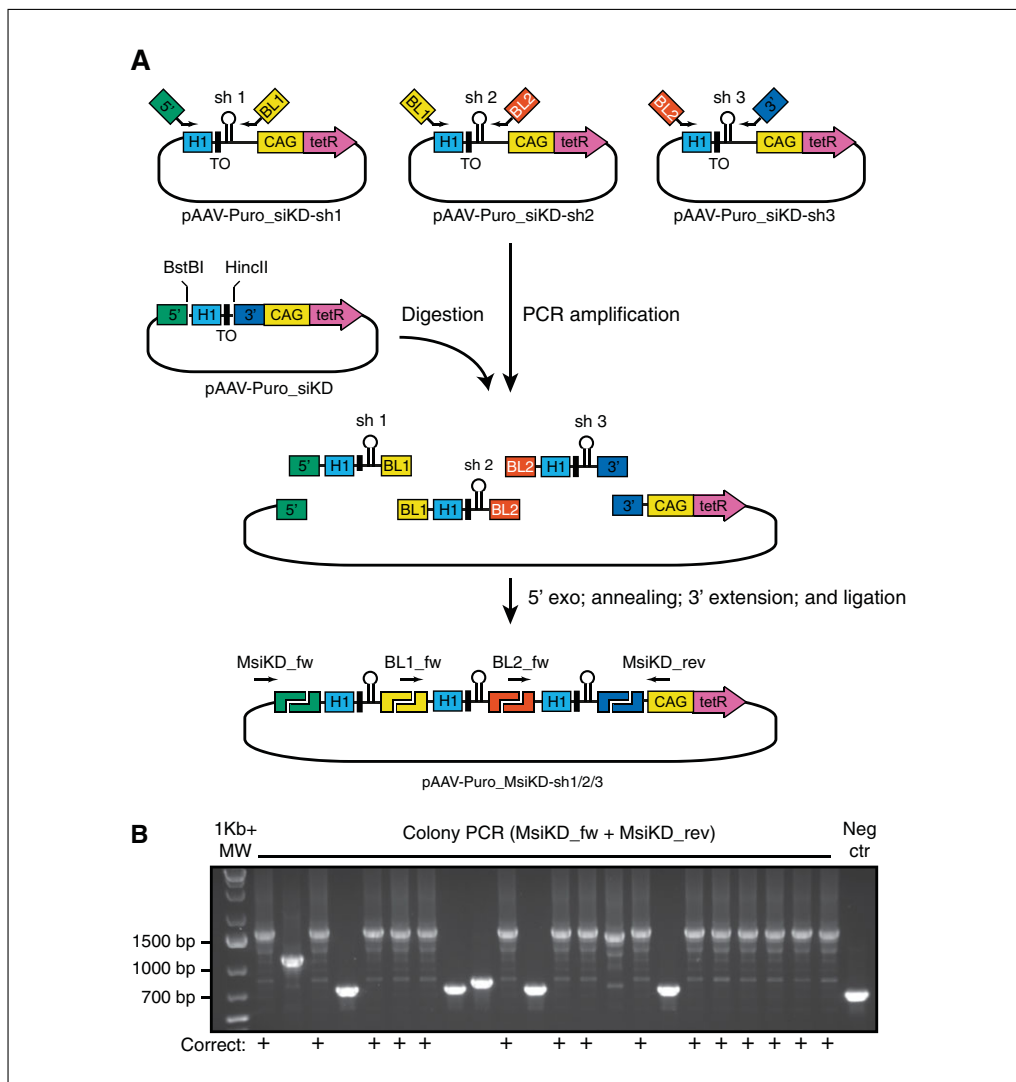FastDigest *Hin*cII (ThermoFisher, cat. no. FD0494)

Figure 5C.4.5 Generation of OPTiKD vectors containing multiple shRNAs. (**A**) Schematic of the cloning procedure described in Support Protocol 1. On the top, the location of primer annealing portions is shown with arrows that indicate their 5′-3′ directionality, while the non-annealing portions are shown in colored boxes. 5′/3′: overlap to the 5′- and 3′-portion of the cut vector; BL1/2: block sequences providing the necessary overlaps for directional Gibson assembly. The steps involved in Gibson assembly of the PCR-amplified shRNA cassettes with the cut vector are indicated. On the bottom, the location of primers for colony PCR and sequencing are shown on the final construct. (**B**) Representative example of colony PCR results from 22 screened clones after assembly of three shRNA cassettes. Clones with the expected amplification product are indicated. Neg control: PCR performed on pAAV-Puro_siKD (indicating the size of vectors with only one shRNA cassette). Note that the second screened clone from the left likely integrated only two of the three shRNA cassettes due to erroneous assembly.

pAAV-Puro_siKD-shRNA (with custom shRNAs of interest; Basic Protocol 2)
Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs, cat. no. M0494S)
5′_fw (custom oligonucleotide, see Table 5C.4.1)
BL1_fw (custom oligonucleotide, see Table 5C.4.1)
BL2_fw (custom oligonucleotide, see Table 5C.4.1)
BL1_rev (custom oligonucleotide, see Table 5C.4.1)
BL2_rev (custom oligonucleotide, see Table 5C.4.1)
3′_rev (custom oligonucleotide, see Table 5C.4.1)
QIAquick PCR Purification Kit (Qiagen, 28104)

Table 5C.4.2 Oligonucleotide Combinations for PCR of Inducible shRNA Cassettes to be Assembled by Gibson Assembly

Assembly of 2 shRNAs			
shRNA	Primer fw	Primer rev	Fragment
First shRNA (sh1)	5′_fw	BL1_rev	5′_sh1_BL1
Second shRNA (sh2)	BL1_fw	3′_rev	BL1_sh2_3′

Assembly of 3 shRNAs			
shRNA	Primer fw	Primer rev	Fragment
First shRNA (sh1)	5′_fw	BL1_rev	5′_sh1_BL1
Second shRNA (sh2)	BL1_fw	BL2_rev	BL1_sh2_BL2
Third shRNA (sh3)	BL2_fw	3′_rev	BL2_sh3_3′

1.5% (w/v) agarose gel in TBE containing 0.5 μg/ml ethidium bromide (see recipe)
NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, cat. no. E5520S)
MsiKD_fw (custom oligonucleotide, see Table 5C.4.1)
BL1seq_fw (custom oligonucleotide, see Table 5C.4.1)
BL2seq_fw (custom oligonucleotide, see Table 5C.4.1)
MsiKD_rev (custom oligonucleotide, see Table 5C.4.1)
FastDigest *Nde*I (ThermoFisher, cat. no. FD0583)

Additional reagents and equipment for molecular cloning experiments (see Basic Protocol 2)

S1.B: Preparation of cloning vector

1. Digest pAAV-Puro_siKD by preparing the following reaction:

a.	pAAV-Puro_siKD	Variable volume (5 μg)
b.	FastDigest *Bsp*119I	5 μl
c.	FastDigest *Hinc*II	5 μl
d.	FastDigest Green Buffer (10×)	10 μl
e.	Ultrapure ddH$_2$O	to 100 μl

   Incubate for 1 hr and 30 min at 37°C.

   Bsp119I is a isoschizomer of BstBI, which can be alternatively used if preferred. Refer to step 1 of Basic Protocol 2 for additional considerations on restriction enzyme digestion and storage of the cut plasmid that also apply to this step.

2. Gel-extract the digested plasmid following electrophoretic run as described in step 2 of Basic Protocol 2, but excising the 9246-bp band.

   A fainter 241-bp band corresponding to the excised H1-TO promoter should be visible upon successful plasmid digestion. Refer to step 2 of Basic Protocol 2 for additional considerations that also apply to this step.

S1.C: Preparation of shRNA inserts

3. Refer to Table 5C.4.2 to identify the primer combinations to generate the PCR fragments with the required overlaps for directional Gibson assembly.

   Table 5C.4.2 lists primer pairs for fragments to be used in assembly of two or three shRNAs. If four or more shRNAs are to be assembled, additional primers will need to be designed according to the indications from Section S1.A (see introduction to this protocol).

4. For each shRNA cassette to be amplified, use the corresponding pAAV-Puro_siKD-shRNA vector (Basic Protocol 2) as template in the following reaction mix:

a.	pAAV-Puro_siKD-shRNA	Variable volume (4 ng)
b.	Q5 Hot Start High-Fidelity 2× Master Mix	100 µl
c.	Forward primer (5 µM in ddH₂O), Table 5C.4.1	10 µl
d.	Reverse primer (5 µM in ddH₂O), Table 5C.4.1	10 µl
e.	Ultrapure ddH₂O	to 200 µl

5. Aliquot the reaction into four PCR tubes with 50 µl each and run the PCR in a thermocycler according to the following program (lid kept at 95°C):

a.	98°C	1 min
b.	98°C	10 sec
c.	65°C	30 sec
d.	72°C	30 sec

Repeat steps b to d for 34 cycles

e.	72°C	30 sec
f.	10°C	hold

6. Pool the four reactions, purify the DNA using the QIAquick PCR Purification Kit according to the manufacturer's instructions, and confirm successful amplification by running 200 ng of the resulting DNA on a 1.5% agarose gel (Basic Protocol 2, step 2).

A sharp band at approximately 400 bp (depending on the fragment and shRNA sequence) should be visible. Fainter nonspecific amplification bands at lower molecular weights and some residual primer sequences might be present, but are not problematic provided that they can be clearly distinguished from the fragment of interest.

7. Gel-extract 2 µg of DNA for each fragment following the electrophoretic run as described in step 2 of Basic Protocol 2, but using a 1.5% (w/v) agarose gel and excising the band at approximately 400 bp.

If nonspecific amplification bands or residual primers are present make sure to run the electrophoresis long enough so to clearly separate them from the fragment of interest. Co-extraction of such unwanted DNA will significantly reduce the efficiency of the assembly step.

S1.D: Generation of recombinant DNA

8. Prepare the following Gibson assembly reaction in a PCR tube using the NEBuilder HiFi DNA Assembly Cloning Kit:

a.	Cut vector from step 2	variable volume (150 ng)
b.	shRNA cassette 1 PCR fragment from step 7	variable volume (35 ng)
c.	shRNA cassette 2 PCR fragment from step 7	variable volume (35 ng)
d.	shRNA cassette 3 PCR fragment from step 7	variable volume (35 ng)
e.	NEBuilder HiFi DNA Assembly Master Mix (2×)	10 µl
f.	Ultrapure ddH₂O	to 20 µl

Incubate for 1 hr at 50°C in a thermocycler

In the case where only two shRNAs are to be assembled, omit reagent d from the above mix. For assemblies of more than four shRNAs, the conditions might need to be adjusted according to the supplier's recommendations regarding the maximum pmol of DNA to be used in each reaction. A larger reaction volume might also be required. A negative control assembly omitting reagents b, c, and d can be performed to evaluate the potential background from contaminating undigested plasmid, but this is only recommended as potential troubleshooting.

9. Transform 2 µl of the assembly product from step 8 into 50 µl of NEB 5-alpha *E. coli* (provided with the NEBuilder HiFi DNA Assembly Cloning Kit) according to manufacturer's instructions using a heated water bath for heat shock.

10. Plate 10% of the transformed bacteria onto a previously prepared LB agar plate containing 100 µg/ml of ampicillin and incubate overnight at 37°C in a humidified incubator

    A yield of more than 50 colonies is to be expected. Store the remaining bacterial suspension at 4°C as backup.

S1.E: Colony PCR screening of bacterial clones

11. Assemble colony PCR reactions according to steps 10 to 13 of Basic Protocol 2, but using MsiKD_fw and MsiKD_rev primers (Table 5C.4.1).

    Screening of 8 to 12 colonies is recommended. As an alternative to colony PCR, plasmids can be screened by restriction digestion (step step 16, below). In this case, proceed directly to step 14, below, but inoculate 8 to 12 randomly chosen bacterial colonies.

12. Perform PCR according to the protocol described in step 14 of Basic Protocol 2, but increase the timing of step d (extension) to 2 min to accommodate for the larger product.

13. Run PCR reaction product on a 1.5% agarose gel as described in step 15 of Basic Protocol 2, but identify clones giving a product size of approximately 1200 bp (assembly of two shRNAs) or 1600 bp (assembly of three shRNAs; Figure 5C.4.6B).

    Plasmids resulting from rare events of inappropriate assemblies or from contaminating undigested plasmid will result in products of approximately 700 to 800 bp (single shRNA cassette) or 1200 bp (two shRNA cassettes) and should be discarded (Figure 5C.4.6B).

14. Inoculate the bacterial clones containing the correct ligation product as described in step 16 of Basic Protocol 2.

S1.F: Isolation and quality control of the plasmid

15. Isolate plasmids from bacterial cultures as described in step 17 of Basic Protocol 2.

16. *Optional:* Confirm the success of Gibson assembly by performing a diagnostic restriction digestion with *Bsp*119I and *Nde*I.

    Correctly assembled plasmids will release a restriction fragment of approximately 1000 bp (assembly of two shRNAs) or 1350 bp (assembly of three shRNAs). If the plasmids have been pre-screened by colony PCR (steps 11 to 13 of this protocol), this control is redundant and can be omitted.

17. Perform Sanger sequencing analyses using MsiKD_fw, BL1seq_fw, and BL2seq_fw (Table 5C.4.1; Figure 5C.4.6A) to confirm the presence of the shRNA cassettes and their correct sequence.

    Refer to step 19 of Basic Protocol 2 for advice on sequencing shRNAs. If required, also use the primer MsiKD_rev. To facilitate the analysis of sequencing results, we recommend creating a reference plasmid map by using SnapGene (or an equivalent software) to simulate in silico the cloning steps performed in steps 1 to 8.

18. Propagate, purify, and create a long-term stock of the plasmid (named pAAV-Puro_MsiKD-shRNAs) as described in steps 20 to 22 of Basic Protocol 2.

GENERATION OF OPTIKD hPSCs

In this third part of the procedure, the targeting vector with the inducible shRNA(s) is integrated into the AAVS1 locus to generate OPTiKD hPSCs (Figure 5C.4.6A). First, hPSCs are co-transfected with the targeting vector and two AAVS1 ZFN plasmids. Correctly gene-targeted hPSCs are then selected by addition of puromycin to eliminate
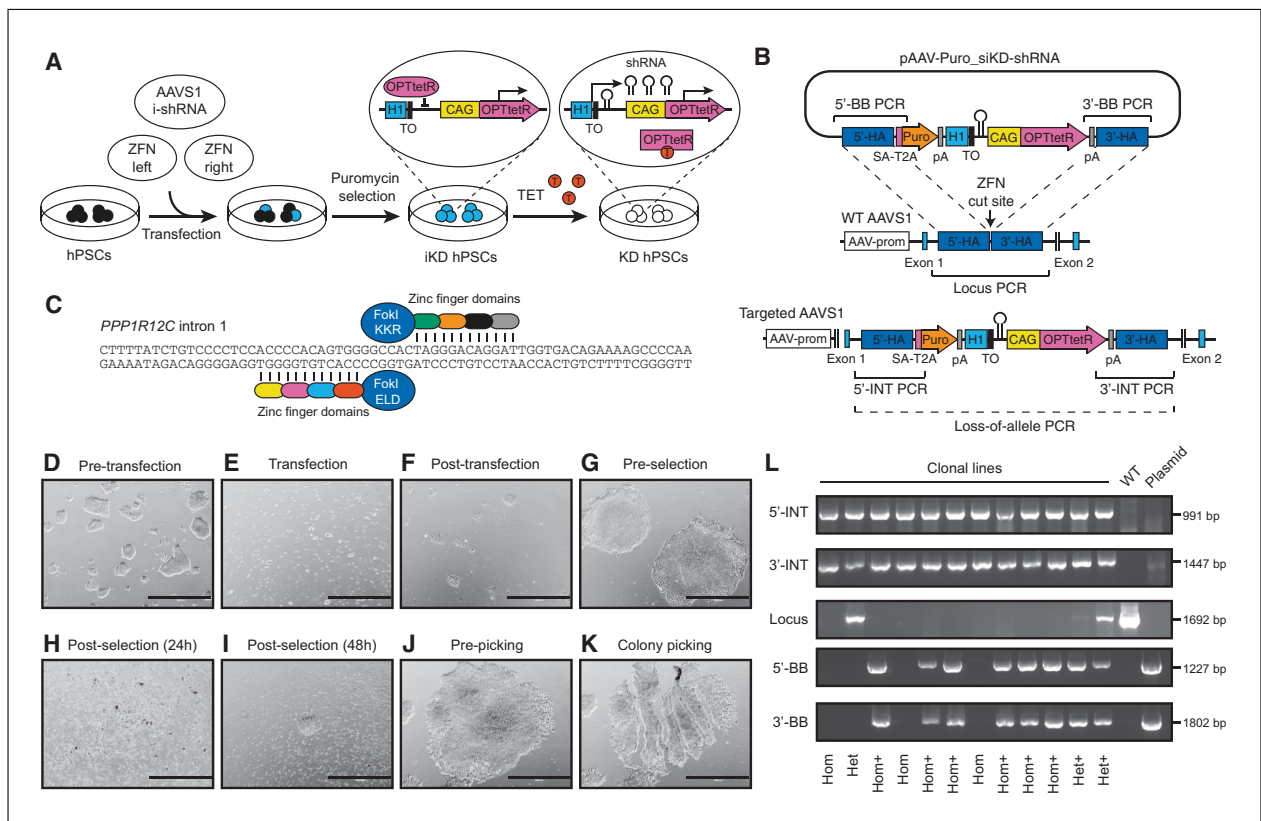
Figure 5C.4.6 Generation of OPTiKD hPSCs. (**A**) Schematic of the gene targeting procedure described in Basic Protocol 3. The resulting OPTiKD transgenic allele and its functionality in presence or absence of tetracycline (TET) are shown. ZFN: zinc finger nuclease; i-shRNA: inducible shRNA; iKD: inducible knockdown; KD: knockdown. (**B**) Schematic of the AAVS1 gene-targeting event that generates the OPTiKD transgenic allele through homologous recombination of the donor plasmid. The genotyping strategies used to identify correctly targeted hPSCs are shown (Support Protocol 2). Locus PCR: PCR product of wild-type AAVS1 locus (indicating a non-targeted allele); Loss-of-allele: potential PCR amplification that fails onto the targeted allele due to large size and high GC-content; 5′/3′-INT PCR: PCR product of transgene 5′/3′-end integration region (indicative of expected transgene targeting); 5′/3′-BB PCR: PCR product of vector backbone 5′/3′-end (indicative of nonspecific off-target plasmid integration). Refer to the legend of Figure 5C.4.2 for additional abbreviations found in panels A-B. (**C**) Location of the binding sites for the AAVS1 ZFN. FokI ELD/KKR: mutant obligate heterodimer *Fok*I endonuclease domains (Section 3.A; see Basic Protocol 3 introduction). (**D-K**) Representative phase-contrast images of hPSCs at the indicated stages of the gene targeting procedure. Scale bars: 1000 μm. (**L**) Representative example of genotyping results from 12 screened OPTiKD clonal sublines (refer to panel B and Table 5C.4.4). The inferred genotypes are indicated (Table 5C.4.3). Hom: homozygous; Het: heterozygous; +: off-target plasmid integration. WT: control PCR from wild-type hPSCs; Plasmid: control PCR from pAAV-Puro_siKD plasmid.

untargeted cells. The resulting OPTiKD cells can be used for experimental purposes either directly as a pool arising from various targeting events or after clonal selection of individual sublines (Support Protocol 2).

3.A: Gene targeting of the AAVS1 locus

As introduced in Section 2.A (see introduction to Basic Protocol 2), selective integration of the targeting vector into the AAVS1 locus is facilitated by ZFNs, which induce site-specific double-strand breaks that are then repaired by HDR using the targeting vector as template (Figure 5C.4.6B). This leads to integration of the transgenic cassette placed between the two homology arms, which contains all functional elements of the OPTiKD method. The ZFN-based gene targeting procedure was originally described by the Jaenisch laboratory (Hockemeyer et al., 2009), which also developed a similar approach based on Transcription Activator-Like Effector Nucleases (TALENS; Hockemeyer et al., 2011). More recently, analogous strategies based on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 gene editing have been also

reported (Mali et al., 2013; Oceguera-Yanez et al., 2016; Sim, Cardenas-Diaz, French, & Gadue, 2015). In all cases, the engineered nucleases recognize a very similar (if not identical) sequence between exons 1 and 2 of the *PPP1R12C* gene on human chromosome 19.

Our protocol relies on a modified version of the original ZFN-based method by Jaenisch and colleagues (Hockemeyer et al., 2009). ZFNs consist of two functional domains: a DNA-binding domain, which is classically constituted by two or more two-finger modules each recognizing a hexamer sequence, and a DNA-cleaving domain comprising the nuclease domain of *Fok*I (Gaj et al., 2013). Such a domain must dimerize in order to cleave the DNA, and thus a pair of adjacent ZFNs each targeting opposite DNA strands are required in order to induce a double-strand break (Figure 5C.4.6C). In our method, the original ZFN plasmids have been modified in the following ways: (1) the two *Fok*I domains carry respectively the ELD (Q486E, I449L, and N496D) and KKR (E490K, I538K, and H537R) mutations, which provide superior cleavage activity while minimizing nonspecific cleavage by suppressing homodimerization (Doyon et al., 2011); (2) the ZFN cDNAs were codon-optimized for mammalian expression, which increases the steady-state protein expression; (3) the ZFNs are expressed under the control of the strong cytomegalovirus (CMV) promoter, which ensures rapid and transient expression upon transfection. The resulting plasmids (pZFN_AAVS1-L-ELD and pZFN_AAVS1-R-KKR) are approximately 4 kbp long, and should be expanded in transformed bacteria cultured in presence of 50 μg/ml kanamycin. These recommended plasmids are available upon request from the corresponding authors. The plasmid repository Addgene offers alternative ZFNs (#60915 and #60916; Sim et al., 2015) and TALENs (#59025 and #59026; González et al., 2014) that are compatible with our targeting vector, as they induce double-strand breaks in the same genomic region. Plasmids for CRISPR/Cas9 editing of the AAVS1 locus are also available from Addgene, but in this case modifications of the targeting vector might be required to ensure that these will not be cut by the Cas9 protein (for instance by mutating the protospacer adjacent motif sequence). If using nucleases different from the ones we recommend, the gene editing procedure might need to be re-optimized, in particular the ratio of nuclease plasmids to targeting vector.

3.B: Plasmids delivery and selection of gene targeted lines

The two AAVS1 ZFN plasmids and the targeting vector must be co-expressed to initiate gene targeting (Figure 5C.4.6A). For this, we recommend the use of GeneJuice, a non-lipid-based chemical transfection reagent which is minimally toxic to hPSCs (Figure 5C.4.6F). With the cost-effective and simple method described in the following protocol we routinely achieve 20% to 40% transfection efficiency with >80% survival in a number of hPSC lines. Alternatively, we have also successfully used cationic-lipid-based transfection reagents such as Lipofectamine 2000 (Bertero et al., 2016), albeit the toxicity of this method proved significant in certain hPSC lines. Finally, nucleofection represents another valid option that allows higher efficiency of plasmid delivery (up to 70%; Bertero et al., 2016) at the expense of decreased cell survival (50% or less), need for specialized equipment, and increased costs. Therefore, we only recommend nucleofection as a backup approach to improve plasmid delivery should initial transfection experiments prove inefficient in a certain hPSC line. Regardless of the method of preference, it is of crucial importance to achieve plasmid delivery in at least 20% of the cells in order to maximize the efficiency of gene targeting (see Commentary).

Following transfection, hPSCs are grown for a few days to allow successfully targeted cells to form a small sub-colony of 8 to 64 cells, as this will promote their survival during the subsequent drug selection step (Figure 5C.4.6G). Gene-targeted hPSCs are then selected by adding puromycin to the culture medium. As described in Section 2.A

(see introduction to Basic Protocol 2), the OPTiKD transgenic cassette includes a gene trap–based puromycin resistance gene (PAT) whose expression is driven by the endogenous promoter found in the AAVS1 locus (*PPP1R12C* gene). In our experience, the dosage of puromycin required to complete selection while inducing minimal toxicity in gene-targeted cells is similar across different hPSC lines (0.4 to 1.2 µg/ml). However, we recommend testing this aspect in each new line by performing a drug-sensitivity experiment. In addition to puromycin, the ROCK inhibitor Y-27632 can be added during the first 2 days of selection in order to promote survival of the small colonies of gene targeted cells (Watanabe et al., 2007). The vast majority of hPSCs will die following puromycin selection, and a number of small colonies should appear and gradually grow over the subsequent days of culture (Figure 5C.4.6H to J). Since the efficiency of targeting using this method is extremely high, with more than 95% of clonally isolated lines showing the expected transgene in the AAVS1 locus (Bertero et al., 2016), the resulting OPTiKD hPSCs can be used directly for experimental purposes. Alternatively, individual clonal sub-lines can be isolated to generate a homogeneous population (Figure 5C.4.6K), an optional procedure described in Support Protocol 2. Once drug selection is complete, addition of puromycin to the medium is not required, and depends upon user preference. Indeed, we have not observed silencing of the OPTiKD transgene even upon prolonged culture for up to 20 passages.

3.C: Culture conditions

The following protocol was optimized for hPSCs maintained in Essential 8 (E8), a commonly used and commercially available medium for hPSC culture in feeder-free, chemically defined, and xeno-free conditions (Chen et al., 2011). The protocol further relies upon seeding hPSCs on tissue culture dishes pre-coated with recombinant human Laminin-521, a physiological, chemically defined, and xeno-free substrate that supports optimal attachment and growth of genetically stable hPSCs (Lu et al., 2014; Rodin et al., 2014). In our experience, these conditions maximize both the speed and efficiency when generating OPTiKD cells. We have also successfully generated feeder-free OPTiKD hPSCs in cells maintained in a chemically defined media containing bovine serum albumin (CDM-BSA) or polyvinyl acid (CDM-PVA) and seeded upon culture dishes coated with FBS and gelatin (Bertero et al., 2016; Vallier, 2011). Thus, we anticipate that the procedure described below will be applicable in a wide range of feeder-free hPSC culture conditions following potential minor optimizations. Should feeder-dependent culture of hPSCs be preferable to the user, more extensive modifications to the protocol might be required, including the use of puromycin resistant mouse embryonic fibroblasts (MEFs) such as DR4 MEFs.

The protocol below describes the procedure to generate OPTiKD hPSCs from cells cultured and transfected in a 6-well plate format. Successful transfection of a single well of 6-well plate should result in at least five colonies following drug selection (range of 5 to 100 depending on the cell line). However, when possible, we recommend transfecting two to three wells for each inducible shRNA in order to maximize the chances of success. In this case, the different wells can serve as biological replicates during subsequent experiments. If clonal isolation of OPTiKD sub-lines is preferred, having multiple wells each containing a few (5 to 20) clearly distinguishable colonies facilitates picking while minimizing potential for multiclonality in any given colony. Nevertheless, the protocol can be scaled to different plate formats (both larger and smaller) with minimal optimization. For instance, we have successfully scaled down volumes and plasmid quantities in the procedure described below to generate OPTiKD hPSCs in a 12-well plate format, which simplifies the generation of multiple OPTiKD lines in parallel.

Genetic Manipulation of Stem Cells

5C.4.23

Materials

Human pluripotent stem cells (hPSCs; user specific; see appropriate units in this
  manual)
Complete Essential 8 (E8) medium (see recipe)
Opti-MEM reduced serum medium with GlutaMAX (ThermoFisher, cat. no.
  51985034)
Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium
  (ThermoFisher, cat. no. 14190250)
Versene solution (ThermoFisher, cat. no. 15040066)
10 mM Y-27632 stock solution (see recipe)
0.4% trypan blue (ThermoFisher, cat. no. 15250061)
GeneJuice transfection reagent (EMD Millipore, cat. no. 70967)
pZFN_AAVS1-R-KKR [contact the corresponding authors (*abertero@uw.edu*;
  lv225@cam.ac.uk)]
pZFN_AAVS1-L-ELD [contact the corresponding authors (*abertero@uw.edu*;
  lv225@cam.ac.uk)]
pAAV-Puro_siKD-shRNA or pAAV-Puro_MsiKD-shRNAs (Basic Protocol 2,
  Alternate Protocol, or Support Protocol 1)
AAVS1-CAGGS-EGFP (Addgene, cat. no. 22212)
10 mg/ml puromycin dihydrochloride (Sigma Aldrich, cat. no. P9620-10ML)

Recombinant human Laminin-521-coated 6-well culture plates (see recipe)
15-ml conical centrifuge tubes
Inverted microscope equipped with fluorescent lamp and filters for FITC (EGFP)
  detection
Hemacytometer

Additional reagents and equipments for culture of human pluripotent stem cells (Costa
et al., 2008)

3.D: Preparatory steps

1. Grow hPSCs to 50% to 60% confluence in complete E8 medium without antibiotics
   on 6-well plates coated with Laminin-521 (Figure 5C.4.6D). Maintain cells in an
   incubator with 5% CO_2 and 95% humidity.

   *Cells to be used for transfection should be subconfluent and within their exponential
   growth phase for optimal transfection. Estimate the necessary cell amount based on the
   number of wells to be transfected (300,000 cells per well; step 8). Antibiotics are toxic
   during transfection due to the increased cell membrane permeability. Therefore, it is
   recommended to omit them from the culture medium for at least 2 days both before and
   after transfection.*

2. On the day before setting up the transfection, pre-coat overnight new 6-well plates
   with Laminin-521 as described in Reagents and Solutions.

   *We recommend preparing two to three wells for each inducible shRNA targeting vector
   to be transfected (Section 3.C; see introduction to Basic Protocol 3). A mock transfection
   without any plasmids is recommended as a negative control to monitor the efficiency of
   drug selection (step 13). Finally, a transfection using AAVS1-CAGGS-EGFP (to be used
   instead of pAAV-Puro_siKD-shRNA) is also recommended as positive control, as it will
   allow monitoring transfection and gene targeting efficiency by visual inspection of EGFP
   fluorescence (steps 11 to 13). For both these controls, a single well is sufficient.*

3. Pre-warm the new Laminin-521 coated plates in the incubator, allow Opti-MEM
   and E8 to reach room temperature, thaw and gently mix the plasmids and Y-27632,
   and pre-warm Versene at 37°C in a heated water bath.

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3.E: Collection of hPSCs

4. Aspirate hPSC culture medium, rinse the cells with 2 ml of DPBS without calcium and magnesium, and incubate in 2 ml of Versene for 3 to 6 min at 37°C in the incubator until colonies are ready for mechanical passaging.

   Volumes are for one well of 6-well plate. The EDTA contained in Versene chelates divalent ions such as calcium and magnesium, thus inhibiting cell-cell and cell-matrix contacts. The timing of Versene incubation is dependent on the cell line and growth conditions and must be adjusted empirically. When monitored under the microscope, the edges of hPSC colonies must be lifting while cells at the center of the colonies must be rounding up. However, do not wait until the whole colony is lifting.

5. Gently aspirate Versene and mechanically lift cells by adding 1 ml of complete E8 supplemented with 10 μM Y-27632 (E8+Y). Gently pipet up and down five to ten times to mechanically triturate the colonies and transfer to a 15-ml conical tube. Wash the well once with 1 ml of E8+Y to collect residual cells and transfer to the same conical tube.

   Before lifting the cells, confirm that the colonies are readily detaching from the plate by gently tapping it on the side. If necessary, add 100 μl of Versene and incubate the cells at room temperature for up to 5 min. When lifting the cells, add medium gradually and proceed from the bottom to the top of the well to prevent the calcium found in E8 from inactivating Versene in cells that have not been yet mechanically detached.

6. Inspect an aliquot of the cell suspension under the microscope to confirm dissociation to near single cells. If necessary, triturate again by gently pipetting 5 to 10 times.

   Aim to generate a suspension with approximately 50% single cells while the rest are found in small clumps of 2 to 10 cells (Figure 5C.4.6E). Do not over-triturate and minimize mechanical stress on the cells.

7. Dilute 50 μl of cell suspension in an equal volume of trypan blue solution and perform live cell count using a hemacytometer. Dilute the cell suspension to 150,000 live cells/ml in E8+Y.

   hPSC viability should be >95%. Prepare sufficient volume for the number of wells to be plated (plus some extra to account for pipetting errors); 300,000 cells are required for each condition.

3.F: Transfection of hPSCs

8. Remove the excess Lamin-521 coating solution from pre-coated 6-well plates (step 1) and immediately aliquot 300,000 hPSCs per well in a volume of 2 ml. Gently transfer the plate to the incubator.

   Do not let the coated surface dehydrate, as this will inactivate the Laminin-521 coating. When plating the cells, avoid creating turbulences that can lead to uneven plating by gently pipetting the cell suspension. Do not shake the plate to prevent cell clumping in the middle of the well.

9. For each well to be transfected, prepare the transfection mix according to the following steps:

   a. Add 6 μl of GeneJuice to 100 μl of Opti-MEM in a microcentrifuge tube.
   b. Immediately mix by vortexing at maximum speed for 3 sec.
   c. Incubate at room temperature for 5 min.
   d. Add the following plasmids:

pZFN_AAVS1-R-KKR	667 ng
pZFN_AAVS1-L-ELD	667 ng
pAAV-Puro_siKD-shRNA	667 ng

a. Immediately mix by pipetting 3 to 5 times. Do not vortex

b. Incubate at room temperature for 15 min.

Once prepared, the transfection mix is stable for up to 30 min at room temperature. When transfecting multiple wells with the same inducible shRNA plasmid, prepare a master mix by scaling the volumes above accordingly. We recommend performing a negative control transfection omitting all plasmids and a positive control transfection using AAV-CAGGS-EGFP instead of pAAV-Puro_siKD-shRNA (step 1). Finally, a transfection with pAAV-Puro_siKD-shRNA alone can be performed to validate the functionality of ZFN plasmids. This should result in no or very few colonies, which are the result of rare events of random plasmid integration into an active gene, compared to the large number of colonies obtained when including the ZFN plasmids, the result of correct AAVS1 gene targeting events. However, we recommend this control only as a potential troubleshooting measure.

10. Pipet the transfection mix drop-by-drop onto the hPSCs from step 8. Cover all the well surface by following a spiral pattern from the outside to the inside to ensure homogeneous dispersion. Transfer the plate to the incubator and gently rock back and forth, left and right two to three times to further distribute the transfection mix. Incubate overnight (16 to 20 hr).

hPSCs readily adhere to Laminin-521 and should be firmly attached to the bottom of the plate at this stage.

3.G: Selection of gene targeted hPSCs

11. On the day following transfection, aspirate the culture medium, gently rinse the cells once with 2 ml of DPBS without calcium and magnesium, and add 2 ml of fresh complete E8 medium.

From here onwards, the culture medium must be replaced daily without any cell wash unless stated otherwise.

Inspect the cells to confirm lack of bacterial contamination. There should be very little toxicity due to the transfection reagent, with most of the cells being attached and forming small colonies (Figure 5C.4.6F). hPSCs might look spindle-shaped at this stage, but the morphology will improve following removal of ROCK inhibitor from the medium. If a positive control transfection with AAV-CAGGS-EGFP has been performed (step 9), EGFP fluorescence should be readily detectable in at least 20% of the cells.

12. After approximately 48 hr (on the third day following transfection), begin selection by adding 1 µg/ml puromycin to the culture medium.

Selection should be started when the cells reach approximately 50% to 70% confluence [Section 3.B (see introduction to Basic Protocol 3); Figure 5C.4.6G]. If a positive-control transfection with AAV-CAGGS-EGFP has been performed (step 9), EGFP-positive clusters of 8 to 32 cells should be visible. The optimal dose of puromycin should be determined for every new cell line (Section 3.B; see introduction to Basic Protocol 3). Puromycin should be added to the medium from now on except when passaging the colonies (step 14), in order to avoid excessive stress on single cells. Once the OPTiKD hPSC line has been established and banked puromycin can be withdrawn (Section 3.B; see introduction to Basic Protocol 3). Addition of 10 µM Y-27632 (ROCK inhibitor) during the first 48 hr of selection can increase survival of gene targeted colonies.

13. After approximately 48 hr (on the fifth day following transfection) selection should be completed (Figure 5C.4.6H to I).

All cells should be dead in the negative control transfection without the targeting plasmid (step 9). If a positive control transfection with AAV-CAGGS-EGFP has been performed (step 9), colonies with homogeneous EGFP expression should be visible.

14. After approximately 2 to 5 days (on the seventh to tenth day following transfection), individual hPSC colonies will reach an appropriate size for passaging (500 to 1000 μm; Figure 5C.4.6J). The resulting OPTiKD hPSCs can be directly used for validation and experimental purposes (Basic Protocol 4). Alternatively, they can first be clonally isolated (Support Protocol 2).

> *The growth rate will be cell-line dependent and certain hPSCs might require up to 20 days following transfection to reach this stage. If multiple wells have been transfected with the same inducible shRNA vector, we advise keeping them separate as biological replicates. The OPTiKD line should be expanded and banked for long-term storage in liquid nitrogen as soon as possible. Furthermore, it is recommended to perform standard quality controls such as karyotyping and testing to exclude mycoplasma contamination.*

CLONAL ISOLATION AND GENOTYPING OF OPTIKD hPSCs

This optional protocol describes the generation of clonal OPTiKD hPSC lines in which the AAVS1 gene targeting event is confirmed at the genomic level while off-target random integration of the targeting vector is excluded. Individual gene-targeted colonies are first mechanically picked and expanded into clonal sublines, and then subjected to extensive DNA genotyping by PCR.

S2.A: Advantages and disadvantages of clonal isolation

As introduced in Section 3.B (see introduction to Basic Protocol 3), this step is not strictly required given the high efficiency in generating correctly gene-targeted lines according to Basic Protocol 3. However, we recommend considering this additional procedure if the cells will be used for experiments at the single-cell level and it will be impractical or impossible to distinguish correctly targeted inducible knockdown cells from contaminating wild-type hPSCs. Furthermore, this procedure can be advantageous if the level of knockdown observed following induction of the shRNA in non-clonal OPTiKD hPSCs is less than 75%, as clonal isolation allows selection of the OPTiKD hPSCs sublines with maximal knockdown efficiency across the targeted pool. Finally, this additional step is most useful to maximize experimental success when gene knockdown is to be initiated at a stage of hPSC differentiation that is subsequent to the one in which the efficiency of gene knockdown has been initially validated. Indeed, only copies of the inducible shRNA transgene that have been integrated into the AAVS1 locus will support consistent inducible gene knockdown across a wide range of lineages (Bertero et al., 2016). On the contrary, randomly integrated transgenic copies can be transcriptionally active at early but not late stages of hPSC differentiation due to silencing events associated to chromatin remodeling initiated by cell-fate choices. Therefore, not considering the potential for random integration of the targeting plasmid could result in an overestimation of the level of knockdown that can be achieved only by means of the correctly targeted inducible shRNA transgenes.

On the other hand, clonal isolation of hPSCs has one main disadvantage in that it increases the likelihood of observing line-to-line variability across sublines due to distinct genetic and epigenetic features. This drawback is somewhat mitigated in OPTiKD lines since uninduced cells maintained in the absence of tetracycline will serve as internal controls that allow evaluation of such potential intrinsic line-to-line variability (Basic Protocol 4). Nevertheless, we recommend that when clonal isolation is preferable for one of the reasons listed above, at least two clones for each inducible shRNA should be functionally analyzed to ensure that any given phenotype is reproducible across multiple sublines. Finally, karyotyping of clonally isolated sublines is of utmost importance to identify potential aneuploid lines and exclude them from experimental analyses. Indeed, the appearance of karyotypic abnormalities following gene-targeting experiments is

unfortunately not uncommon due to both the strong selective pressure that is imposed on the cells during drug selection and the single-cell growth requirement of the procedure.

S2.B: Clonal isolation of OPTiKD sublines

OPTiKD sublines can be isolated by manually picking gene-targeted colonies obtained from the procedure described in Basic Protocol 3. For this, it is best to transfect cells in the 6-well format recommended by the protocol or even to use a larger plate, both to simplify picking and to maximize the spacing among colonies. Even so, it should be noted that this approach does not formally ensure clonality of the derived sublines, since any colony could have resulted from two independent targeting events in cells that were in close proximity. Moreover, hPSCs can extensively migrate across nearby colonies over prolonged culture. Nevertheless, both of these events are relatively rare provided that the colonies are monitored immediately after puromycin selection in order to mark for exclusion those that are found in too close proximity (less than 1 to 2 mm). Therefore, in this protocol, we still use the word "clonal" when referring to the pseudo-clonal lines that can be isolated using this method. A more stringent way to generate clonal lines would be to seed hPSCs into 96- or 384-well plates at limiting dilution of one cell per well or following cell sorting of individual cells. However, these approaches are very time consuming, and thus we recommend them only as a backup option should there be any doubts regarding the clonality of the sublines. Another alternative to the approach outlined below is to seed a small number of single cells into a large petri dish, for instance 1,000 to 5,000 cells in a 100-mm-diameter dish, so that they might grow into individual colonies. While this method suffers from similar limitations to the one we propose and the resulting colonies are still pseudo-clonal, it can prove advantageous if the colonies from Basic Protocol 3 are too densely packed to ensure robust clonal isolation. Moreover, such strategy can allow subsequent clonal isolation of lines that have not been initially processed according to the protocol below.

S2.C: Genotyping of OPTiKD sublines

Following clonal isolation, we recommend that OPTiKD hPSCs be genotyped by PCR. Indeed, while functional validation of OPTiKD hPSCs by quantitative real-time PCR (qPCR) and/or western blot will serve in most circumstances (Basic Protocol 4), the advantage of having a clonally isolated line is maximized when the genotype is known (Section S2.A, above, in the introduction to this protocol). As introduced earlier, two potential events must be screened for: (1) site-specific on-target integration of the transgene into the AAVS1 locus; (2) random integration of the targeting plasmid elsewhere in the genome. It should be noted that while cells characterized only by the second type of modification are very rare, as in most cases random integration of the plasmid will not support expression of the gene trap–based puromycin resistance gene (see Section 2.A in the introduction to Basic Protocol 2), off-target integration events are observed in 25% to 80% of correctly targeted lines (Bertero et al., 2016). Both on- and off-target integration events can be efficiently and easily screened by PCR using genomic DNA as template (Figure 5C.4.5B; Table 5C.4.3).

Site-specific transgene insertion is detected by using a primer pair in which one primer is located on the transgene while the second maps to the genomic locus outside of the homology arm (Figure 5C.4.6B). We recommend performing this type of PCR to confirm that both the 5′- and 3′-end of the transgene have been correctly integrated. Thus, the presence of the expected product in both of these "transgene integration" PCRs, which we refer to as 5′-INT and 3′-INT PCR, indicates that at least one of the two alleles of the AAVS1 locus has been targeted (Table 5C.4.3). Furthermore, performing another PCR using the two primers mapping to the genomic locus outside of the homology arms allows determining if only one or both of the two alleles have been edited (Figure 5C.4.6B). While an unmodified allele will result in a PCR product corresponding to the wild-type

Table 5C.4.3 Inferring the Genotype of OPTiKD Clonal Lines from PCR Results

5′-INT and 3′-INT	Locus	5′-BB and 3′-BB	Possible genotype[a]
Both bands at expected size	No band	No bands	Homozygous
Both bands at expected size	Band at expected size	No bands	Heterozygous[b]
Both bands of expected size	No band	At least one band at expected size	Homozygous with additional plasmid copies[c]
Both bands at expected size	Band at expected size	At least one band at expected size	Heterozygous[b] with additional plasmid copies[c]
One or more band absent or at incorrect size	Any	Any	Incorrect targeting
No bands	Band at expected size	At least one band at expected size	Untargeted

[a]Only the most common results are described. Refer to Section S2.C (see introduction to Support Protocol 2) to interpret other possible genotyping results.
[b]Alternatively the line could a mix of two of more clones with at least one wild-type allele.
[c]Alternatively the targeting plasmid is still present in the cells as an episome.

AAVS1 locus, insertion of the transgene leads to loss-of-allele PCR in which no product is observed. This is not only because the transgene is very long, but also because the CAG promoter contains an extremely GC-rich portion, which together result in PCR amplification failure. Therefore, when no band is detected in this PCR, which we refer to as locus PCR, homozygous targeting of the locus can be inferred (Table 5C.4.3). The amplification of a PCR product can indicate: (1) a rare wild-type clone; (2) a heterozygous clone; (3) a non-clonal population that contains a mixture of targeted and untargeted alleles (see Section S2.B, above in the introduction to this protocol). While the first option can be ruled out based on the result of 5′-INT and 3′-INT PCR, the last two possibilities cannot easily be teased apart. Therefore, we recommend that only clones showing homozygous targeting by locus PCR be selected for further analyses: not only will this ensure higher expression of the inducible shRNA, but it also reinforces the notion that the lines are most likely truly clonal.

Once on-target recombination of the transgene has been established, it is then advisable to monitor possible off-target random integration. For this, we recommend a PCR reaction using a primer specific to the transgene and a second primer located on the targeting plasmid backbone outside of the homology arms (Figure 5C.4.6B). While this reaction will readily reveal if any plasmid has been randomly integrated in the genome, it will not result in amplification in case of on-target integration. In fact, during HDR, only the homology arms will be incorporated in the AAVS1 locus, while the rest of the plasmid backbone will be lost. Since random integration does not always involve the whole plasmid, we recommend performing two such PCRs to monitor the 5′- and 3′-end of the transgene cassette. The presence of a band in either of these two "backbone" PCRs, which we refer to as 5′-BB and 3′-BB PCR, indicates that the targeting plasmid has likely been stably integrated in a random genomic region. One possible exception to this is if the plasmid is still found as an episome in the cells. However, if the genotyping is performed according to the timeline suggested in the protocol below, this is an extremely unlikely event. In fact, transiently transfected plasmids will be serially diluted following each cell division as well as gradually degraded by hPSCs. Thus, in our experience no residual

episomal plasmid will be detectable by PCR 2 weeks post transfection. It is important to mention that the PCRs described here will not detect random integration of the plasmid if the transgene portion has been lost during such an event. However, as the primary goal of this procedure is to identify OPTiKD lines with additional copies of the transgene outside of the AAVS1 locus, this is not a significant limitation. Finally, it should be noted that while 5′-BB and 3′-BB PCRs provide a rapid and sensitive method to detect random integration of the transgene, the gold standard analysis for this would be to perform Southern blotting with a DNA probe specific to the targeting vector. However, this is a complex and time-consuming procedure that involves the use of radioactive reagents. Thus, we suggest this only as a secondary screening and/or troubleshooting step.

Collectively, by performing the five PCR reactions described above, it is possible to determine the most common genotypes found in clonal OPTiKD sublines (Table 5C.4.3). In general, we recommend that only clones with homozygous integration of the targeting plasmid and no off-target integration events be selected for further analysis in order to ensure maximal reproducibility (see Section S2.A, above, in the introduction to this protocol). Since in our experience these lines are derived with an efficiency of approximately 25% (range: 10% to 40%; Bertero et al., 2016), screening of at least 12 clones is recommended (Figure 5C.4.6L). Nevertheless, homozygous clones containing extra copies of the targeting plasmid can sometimes lead to a stronger level of inducible knockdown, which might be advantageous when using sub-optimal shRNAs. In this case, we strongly recommend validating the knockdown at the same stage of hPSC differentiation to be examined experimentally, in order to ensure that the randomly integrated transgenes are active in the cell type of interest (see Section S2.A, above, in the introduction to this protocol).

Materials

OPTiKD colonies (Basic Protocol 3)
DNeasy Blood & Tissue Kit (Qiagen, cat. no. 69504)
LongAmp *Taq* DNA Polymerase (New England Biolabs, cat. no. M0323S)
10 mM dNTP mix (Promega, cat. no. U1511)
Locus_fw (custom oligonucleotide, see Table 5C.4.4)
Locus_rev (custom oligonucleotide, see Table 5C.4.4)
OPTtetR_fw (custom oligonucleotide, see Table 5C.4.4)
Puro_rev (custom oligonucleotide, see Table 5C.4.4)
Puro_rev2 (custom oligonucleotide, see Table 5C.4.4)
Backbone_fw (custom oligonucleotide, see Table 5C.4.4)
Backbone_rev (custom oligonucleotide, see Table 5C.4.4)
Dimethyl sulfoxide (DMSO), PCR-grade (Sigma Aldrich, cat. no. D9170)
1% (w/v) agarose gel in TBE containing 0.5 µg/ml ethidium bromide (see recipe;
    use molecular biology grade agarose; ThermoFisher, cat. no. 10787018)

Recombinant human Laminin-521-coated 12-well culture plates (see recipe)
Permanent marking pen
8- or 12-channel pipettor appropriate for volumes between 2 and 7 µl
MicroAmp Optical 96-Well Reaction Plate (ThermoFisher, cat. no. N8010560)
MicroAmp Clear Adhesive Film (ThermoFisher, cat. no. 4306311)

Additional reagents and equipment for agarose gel electrophoresis and hPSC
    culture (see Basic Protocols 2 and 3; Voytas, 2001; Costa, 2008)

S2.D: Preparatory steps

1. Obtain OPTiKD colonies according to Basic Protocol 3. After initiating drug selection (step 12 of Basic Protocol 3), monitor the surviving colonies daily under the

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Table 5C.4.4 Primers for Genotyping of OPTiKD Clonal Lines

PCR type	Primer name	Primer location	Primer sequence (5′ → 3′)	Amplicon wild-type[a]	Amplicon target[b]	Amplicon plasmid[c]	Temp. ann[d]	Ext. time[d]
Locus	Locus_fw	Genomic, 5′ to 5′ HA	CTGTTTCCCCTTCC CAGGCAGGTCC	1692 bp	No band	No band	65°C	1′ 30″
	Locus_rev	Genomic, 3′ to 3′ HA	TGCAGGGGAACGG GGCTCAGTCTGA					
5′ INT	Locus_fw	Genomic, 5′ to 5′ HA	CTGTTTCCCCTT CCCAGGCAGGTCC	No band	991 bp	No band	65°C	1′
	Puro_rev	Puromycin resistance	TCGTCGCGGGTGGC GAGGCGCACCG					
3′-INT	OPTTetR_fw	OPTTetR cDNA	CCACCGAGAA GCAGTACGAG	No band	1447 bp	No band	60°C	1′ 30″
	Locus_rev	Genomic, 3′ to 3′ HA	TGCAGGGGAACG GGGCTCAGTCTGA					
5′-BB	Backbone_fw	Backbone, 5′ to 5′ HA	ATGCTTCCGGCTC GTATGTT	No band	No band	1227 bp	60°C	1′ 30″
	Puro_rev2	Puromycin resistance	TGAGGAAGAGTTC TTGCAGCTC					
3′-BB	OPTTetR_fw	OPTTetR cDNA	CCACCGAGAAAGC AGTACGAG	No band	No band	1802 bp	60°C	2′
	Backbone_rev	Backbone, 3′ to 3′ HA	ATGCACCACCGGG TAAAGTT					

[a]Result of PCR on wild-type AAVS1 allele.
[b]Result of PCR on OPTiKD-targeted AAVS1 allele.
[c]Result of PCR on targeting vector (positive control for off-target plasmid integration).
[d]Variable parameter in PCR protocol (step 14 of Support Protocol 2).

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microscope and use a permanent pen to mark on the bottom of the plate the colonies that are found in close proximity (less than 1 to 2 mm away). These will be excluded from picking.

2. On the day before picking, pre-coat 12-well plates overnight with Laminin-521 as described in Reagents and Solutions.

> *We recommend preparing at least one 12-well plate for each shRNA used to generate OPTiKD hPSCs (see Section S2.C above, in the introduction to this protocol). Volumes in this protocol are for plating picked colonies on 12-well plates. If another multi-well format is preferable, scale these volumes according to the surface area.*

3. When OPTiKD colonies are ready for picking (size of 500 to 1000 µm; step 14 of Basic Protocol 3), use a permanent marking pen to mark those that have not been previously excluded from picking (see step 1) and that show the expected hPSC morphology with little or no background differentiation (Figure 5C.4.6J).

4. Remove the excess Laminin-521 coating solution from pre-coated 12-well plates (see step 2) and immediately add 1 ml of complete E8 supplemented with 10 µM Y-27632 and 1× penicillin-streptomycin. Transfer the plates to the incubator to pre-equilibrate, and pre-warm Versene at 37°C in a heated water bath.

> *Addition of penicillin and streptomycin is optional but recommended if colonies will be picked outside of the tissue culture hood, to minimize the risk of bacterial contamination.*

S2.E: Picking

5. Aspirate the culture medium, rinse the cells once with 2 ml of DPBS without calcium and magnesium, and incubate with 1 ml of Versene at 37°C in the incubator for up to 2 min.

> *This gentle Versene pre-treatment step is critical to impair cell attachment to the extracellular matrix. In colonies directly picked from Laminin-521-coated plates, the carryover of extracellular matrix strongly inhibits attachment to the new plate. The timing of Versene incubation is dependent on the cell line and on the size of the colonies. Proceed to the next step as soon as the cells start to show early signs of disaggregation (see step 4 of Basic Protocol 3): prolonged treatment can lead to the colonies lifting off prematurely.*

6. Gently aspirate Versene and gently add 3 ml of DPBS without calcium or magnesium.

> *Cells are very prone to mechanical lifting at this stage, so proceed with extreme care.*

7. Mechanically pick the pre-selected colonies (see step 3, above) by using a micropipettor equipped with a sterile tip. Use a microscope to facilitate visualization during the procedure. Transfer each colony to a single well of a laminin-coated 12-well plate (step 2), then gently pipet up and down three to five times to triturate.

> *Before collecting the cells, aim to break up the colony into strips using the pipet tip to facilitate its disaggregation (Figure 5C.4.6K). After plating the cells, confirm that small clumps of 20 to 100 cells have been obtained, and if necessary triturate again by gently pipetting three to five times. If the colonies are very large (750 to 1000 µm), it is possible to split the cells into two wells and proceed directly to step 10. Alternatively, it is possible to use some of the cell suspension directly for genotyping PCR. However, we discourage this approach, since in our experience it can lead to a large number of false negative results.*

8. If any colony is left in the well used for picking, gently aspirate the DPBS and add fresh complete E8 medium supplemented with 1× penicillin-streptomycin.

> *The cells will recover and can be used for further picking if required.*

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9. On the next day, confirm that the picked colonies have attached, gently aspirate the culture medium, and replace with fresh complete E8. Culture the cells according to standard procedures until they are ready for passaging (see step 1 of Basic Protocol 3).

S2.F: Genotyping

10. Split each clonal OPTiKD line into two wells of a laminin-coated 12-well plate: one with approximately 1/3 of the cells and the second with the remaining 2/3. Cells in the first well will be grown while the second will be used for genotyping. Passage the cells according to steps 4 and 5 of Basic Protocol 3, above, but instead of transferring the lifted cells into a conical tube, directly aliquot them into the new wells.

11. After 24 to 48 hr, extract genomic DNA from the genotyping plate from the previous step using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions. Dilute the DNA to 50 ng/μl.

    Wait until the cells reach at least 30% confluence to ensure a sufficient yield. Samples with concentration lower than 50 ng/μl can be used, but the concentration should never be below 20 ng/μl to ensure a reliable result.

12. For each of the PCR reactions detailed in Table 5C.4.4 assemble a reaction master mix to be used for all samples plus three recommended controls (volumes are for one sample):

a.	LongAmp Reaction Buffer (5×)	2 μl
b.	dNTP mix (10 mM)	0.3 μl
c.	Forward primer (5 μM in ddH$_2$O), Table 5C.4.4	0.5 μl
c.	Reverse primer (5 μM in ddH$_2$O), Table 5C.4.4	0.5 μl
d.	DMSO	0.2 μl
e.	LongAmp *Taq* Polymerase	0.4 μl
f.	Ultrapure ddH$_2$O	to 8 μl

13. Aliquot the reaction from step 12 into a 96-well plate (8 μl of per well) and add 2 μl (100 ng) of genomic DNA for each sample in separate wells. Seal the plate with an adhesive film.

    We recommend also including the following controls: (1) wild-type hPSC DNA, 100 ng; (2) targeting plasmid, 1 ng; (3) no template control (Figure 5C.4.6L and Table 5C.4.4).

14. Run the PCR in a thermocycler according to the following program (lid kept at 95°C):

a.	94°C	5 min
b.	94°C	15 sec
c.	Annealing temperature (see Table 5C.4.4)	30 sec
d.	65°C	Extension time (see Table 5C.4.4)

    Repeat steps b to d for 34 cycles.

e.	65°C	3 min
f.	10°C	hold

15. Remove the adhesive film from the plate and, using a multichannel pipettor, add 2 μl of gel loading dye. Mix by vortexing at low speed and run 6 μl of each reaction on a gel for DNA electrophoresis as described in step 2 of Basic Protocol 2, using a 1.0% (w/v) agarose gel.

    If possible we advise to use a multichannel pipettor to facilitate loading the gel.

16. Visualize the gel using a UV transilluminator to determine the results of each PCR reaction. Taking into account all the results infer the possible genotype for each

OPTiKD line that was analyzed (Table 5C.4.3 and Section S2.C) and identify the clones to be selected for further expansion and experimental analysis.

17. Confirm that the clones of interest have been growing well in the second 12-well plate from step 10 of this protocol. Once they are ready to be passaged, split them for expansion, banking, and experimental analyses (step 14 of Basic Protocol 3).

   We recommend performing steps 11 to 16 within 24 to 48 hr in order to ensure that the cells maintained on the second 12-well plate will not grow to overconfluence by the time the genotypes have been determined.

VALIDATION OF OPTIKD hPSCs

In this last section, we describe the validation of OPTiKD hPSCs. Given that this aspect will vary broadly depending on the gene of interest, the reagents that are available to study such gene, and the experimental question being investigated, a conventional step-by-step protocol would be poorly suited to this task. Therefore, we instead present general considerations in a discursive form to help the user design the most appropriate validation experiments.

Materials

   Tetracycline hydrochloride (Sigma-Aldrich, cat. no. T7660; see recipe for stock solution)

4.A: Inducible knockdown using OPTiKD hPSCs

Knockdown can be induced by simple addition of the drug tetracycline the culture medium at a recommended concentration of 1 μg/ml, a dose that does not induce toxicity to hPSCs and does not interfere with hPSC differentiation. Note that the tetracycline analog doxycycline is equally effective for inducing knockdown in hPSCs, but we have not extensively tested potential side effects during hPSC differentiation. Tetracycline is a light-sensitive reagent and should be protected from direct illumination during preparation, storage, and use. Aqueous solutions of tetracycline are unstable and gradually become turbid due to hydrolysis and precipitation. Thus, aqueous tetracycline solutions should be stored as single-use aliquots at –80°C (see Reagents and Solutions). Once diluted in culture medium and maintained at 37°C, the half-life of tetracycline is approximately 24 hr. Therefore, we recommend performing medium changes at least every other day. It is best to add fresh tetracycline to the culture medium just before use, but, should it be preferable to prepare larger volumes of medium, these should be stored at 4°C and used within a week of preparation.

It is very important to keep in mind that tetracycline is a widely used antibiotic in livestock animals such as cows and horses. Therefore, animal-derived products such as fetal bovine serum (FBS) and bovine serum albumin (BSA) carry the risk of being contaminated with tetracycline. It is therefore important to batch-test animal-derived reagents (in particular FBS and derivatives) for absence of detectable tetracycline contamination before use in OPTiKD hPSCs. Wherever possible, we recommend substituting such reagents with tetracycline-free validated alternatives that are available from commercial suppliers.

4.B: Validation of OPTiKD hPSCs

We recommend validating newly generated OPTiKD hPSCs both at the transcript and protein level. Importantly, if expression of the target gene is low or absent in hPSCs, the cells should be first differentiated into an appropriate cell lineage expressing such gene. This will usually be the cell type to be functionally analyzed in follow-up experiments, but should differentiation into such lineage be expensive, laborious, and/or time consuming, validation can be instead performed in any alternative hPSC-derived cell

type expressing similar levels of the gene. Transcript levels can be readily monitored by quantitative real-time PCR (qPCR), which is our preferred validation method given its simplicity, cost-effectiveness, and wide applicability to any gene of interest. When using the $\Delta\Delta$Ct approach to quantify relative mRNA expression levels over control samples (Livak & Schmittgen, 2001), it is important to validate that the expression of the chosen housekeeping gene is not sensitive to the addition of tetracycline. Validation by qPCR can be performed after 24 to 48 hr following induction of knockdown.

Once transcript-level knockdown has been confirmed, it is important to validate loss of the protein product. This relies on the availability of a good antibody against the protein of interest and is therefore not always possible. If such an antibody exists, we recommend to initially validate OPTiKD cells by western blot, which allows for relatively simple and rapid semi-quantitative evaluation of protein knockdown. Flow cytometry can provide an alternative and more quantitative assessment with the added advantage that this is evaluated at the single-cell level and thus interrogates the heterogeneity in the population. For example, if a pseudo-clonal OPTiKD subline has been selected (Support Protocol 2), flow cytometry can confirm that the knockdown is homogeneous and that the selected subline is most likely clonal. If OPTiKD cells have not been clonally isolated, it is important to ensure that the majority of the population (>90%) undergoes inducible knockdown before proceeding with extensive follow-up experiments. Immunocytochemistry is an alternative way to assess population heterogeneity in OPTiKD cells, while it also allows for spatial and morphological analyses. However, immunocytochemistry is poorly quantitative and prone to false-positive signals, thus usually requiring extensive optimization. Regardless of the technique used, wherever possible it is important that protein-level validation be performed while including appropriate controls, one of the most important being a cell type not expressing the protein of interest. This type of validation is better performed after prolonged tetracycline treatment of at least 5 to 10 days in order to ensure evaluation of the maximal level of protein knockdown that can be achieved (see Commentary).

REAGENTS AND SOLUTIONS

Use deionized, distilled water (ddH$_2$O) or equivalent in recipes and protocol steps.

Agarose gel (1%, 1.5%, or 4%) in TBE for DNA electrophoresis

Dissolve the required amount of agarose I powder [molecular biology grade (ThermoFisher, cat. no. 17850) *or* UltraPure Low Melting Point Agarose (ThermoFisher, cat. no. 16520050) as called for in protocol] in 1×TBE buffer (see recipe) in a glass bottle. Place a cap on the bottle but leave loose. Incubate at room temperature for 15 min to pre-dissolve. Microwave for about 1 to 2 min or until all of the powder is fully dissolved, but do not let the solution boil. Allow the solution to cool at room temperature for 5 to 10 min (the temperature of the solution should not go below 65°C to prevent premature gelling) and add 10 mg/ml ethidium bromide (Sigma-Aldrich, cat. no. E1510-10ML) to a final concentration of 0.5 µg/ml. After mixing, pour the solution into a gel casting tray equipped with the appropriate combs and let the gel set for 15 to 30 min before use.

Also see Voytas (2001).

Essential 8 (E8) medium, complete

Add 10 ml of provided 50× Essential 8 supplement to a 500-ml bottle of Essential 8 medium (ThermoFisher, A1517001) and mix by gently inverting (avoid making bubbles, to prevent denaturation of the cytokines). Optionally, add 100× penicillin-streptomycin (10,000 U/ml; ThermoFisher, 15140122) to a 1× concentration. Store

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complete Essential 8 medium at 4°C and use within 7 days to prevent loss of potency of the associated cytokines.

Laminin-521-coated culture plates

Thaw Laminin-521 stock solution (BioLamina, cat.no. LN521-02) at 4°C overnight and mix well by inversion before use. To prepare 1 ml of coating solution for final coating at approximately 0.5 µg/cm^2, add 50 µl of the stock to 950 µl of DPBS with calcium and magnesium (ThermoFisher, cat. no. 14040133). Add 1 ml to one well of 6-well plate (Corning, cat. no. 3516) or 0.5 ml to one well of a 12-well plate (Corning, cat. no. 3513). Swirl to ensure that the bottom surface is entirely covered. Tightly seal and incubate at 4°C overnight. Alternatively, plates can be coated for 2 hr at 37°C. Pre-coated plates can be stored at 4°C for up to 2 weeks provided they do not dry out.

LB agar plates

Dissolve 20 g of agar powder (e.g., BD Difco) in 1 liter of LB broth and mix well. Autoclave on liquid cycle and let cool until it is warm enough to touch (approximately 50°C). Add the appropriate antibiotic [ampicillin (Sigma-Aldrich, cat. no. A5354-10ML)] to 100 µg/ml and swirl to mix (do not shake as this will create bubbles). Pour into 10-cm petri dishes to completely cover the bottom surface. Allow the plates to set for 2 hr at room temperature and store at 4°C up to 3 months.

Luria-Bertani (LB) broth

To prepare a 1 liter solution, mix:
1. 950 ml of ddH$_2$O
2. 10 g tryptone (e.g., BD Difco)
3. 10 g NaCl
4. 5 g yeast extract (e.g., BD Difco)

Dissolve and adjust the pH to 7.0 with 5 N NaOH. Bring the volume to 1 liter with ddH$_2$O. Sterilize in the autoclave on a liquid cycle and store at room temperature for up to 6 months. Let the medium cool to room temperature before use.

Tetracycline stock solution

Dissolve 50 mg of tetracycline hydrochloride in 5 ml of ultrapure ddH$_2$O. The resulting solution should have a mild yellow-orange color. Filter-sterilize using a 0.22-µm filter and prepare single-use 5- or 10-µl aliquots. Store at –80°C for up to 6 months.

Prepare this reagent protected from direct illumination.

Tris-borate-EDTA (TBE) buffer

To prepare a 1 liter 5× stock solution add:

1.   800 ml of ddH$_2$O
2.   54 g of Tris base
3.   27.5 g of boric acid
4.   20 ml of 0.5 M EDTA (pH 8.0)

Mix by stirring until dissolved then bring volume to 1 liter with ultrapure ddH$_2$O. The pH should be approximately 8.3. Store at room temperature for up to 6 months.

To obtain a 1× working solution, add one volume of 5× TBE to four volumes of ddH$_2$O. Dilute just before use and do not reuse.

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Y-27632 stock solution, 10 mM stock

To obtain a 10 mM solution dissolve 1 mg of Y-27632 powder (Tocris, cat. no. 1254) into 312.25 µl of DMSO. Prepare single-use aliquots and store at –20 °C for up to 6 months.

COMMENTARY

Background Information

Recent advances in our ability to perform high-throughput analyses of genomes, transcriptomes, and proteomes carry with them the key challenge of functionally annotating an ever-growing list of genes with potential links to human development, physiology, and disease (Cooper & Shendure, 2011). As a result, there is a high demand for simple, scalable, rapid and robust platforms for functional genetic analyses in multiple human cell types, including populations that are transitory during development, rare, or inaccessible. Loss-of-function experiments in hPSCs provide a unique opportunity to address this major challenge, as they can differentiate into any adult cell type of clinical interest (Trounson & De-Witt, 2016). However, high-throughput functional genetic applications of hPSCs require easy and efficient methods to conditionally manipulate gene expression in both hPSCs and hPSC-derived cells. Indeed, this is necessary both for the study of genes that are essential for hPSC self-renewal and for functional analyses at specific stages of differentiation.

Unfortunately, progress in this direction was hampered for years due to the difficulty in manipulating gene expression in hPSCs using most conventional methods (Cao et al., 2010; Costa et al., 2007; Krishnan et al., 2006; Ott et al., 2006; Stein et al., 2010; Yao et al., 2004; Zwaka & Thomson, 2003). This situation dramatically changed following the recent revolution in the field of genetic engineering due to the development of customizable nucleases such as ZFNs, TALENs, and, more recently, CRISPR/Cas9 (Cong et al., 2013; Jinek et al., 2012; Joung & Sander, 2013; Mali et al., 2013; Urnov, Rebar, Holmes, Zhang, & Gregory, 2010). These can be used to induce site-specific double-strand DNA breaks that are repaired either by the error-prone non-homologous end joining (NHEJ) pathway, resulting in random small insertions or deletions (indels) that can generate loss-of-function alleles, or by HDR mechanisms driving recombination of a donor DNA fragment carrying specific mutations, thus resulting in precise gene editing (Gaj et al., 2013; Kim & Kim, 2014). In particular, these methods can be applied to gene editing of human GSHs in order to overexpress transgenes, genetic reporters, and shRNAs (DeKelver et al., 2010; Gaj et al., 2013; Hockemeyer et al., 2011; Hockemeyer et al., 2009; Kun et al., 2014; Smith et al., 2008).

These technological improvements have recently provided the foundation of several methods for conditional loss-of-function studies in hPSCs. However, these technologies still have important drawbacks. First, even with the advent of CRISPR/Cas9, recombination-based inducible knockout is still quite complex and time consuming, as it relies on generation of gene-specific targeting vectors and on two gene-targeting steps (Chen et al., 2015). Secondly, the iCRISPR inducible CRISPR/Cas9 knockout approach relies on the introduction of random indels (González et al., 2014), and thus generates a mixed cell population carrying different mutations, including some that do not induce loss of gene function. Third, the CRISPRi-inducible CRISPR/Cas9 interference method relies on sgRNAs that are either delivered by transient transfection, which is poorly efficient and not fully reproducible, or by random integration, which can result in mosaic expression (Mandegar et al., 2016). Moreover, all of the current inducible CRISPR/Cas9-based methods involve conditional overexpression of the Cas9 or of Cas9 fusion proteins by using an inducible promoter (the tetracycline responsive element, or TRE) that is heavily silenced during hPSC differentiation into multiple lineages, even after targeting into GSHs (Bertero et al., 2016; Haenebalcke et al., 2013; Mandegar et al., 2016; Ordovas et al., 2015). Therefore, these systems are unlikely to work in a diversity of cell types.

On the contrary, the OPTiKD protocol described here relies on a transgenic cassette that has been extensively tested not to suffer from silencing during hPSC differentiation into a large number of cell types (Bertero et al., 2016). Moreover, OPTiKD is easy to implement (it relies on one straightforward and scalable cloning step, and on subsequent simple transfection of hPSCs), rapid (only one gene targeting step is involved), efficient (more than 95% efficiency in generating inducible knockdown lines), and scalable (given the high

efficiency the isolation of clonal lines can be entirely bypassed). We also recently reported a sister method to OPTiKD which we named OPTiKO, for optimized inducible knockout (Bertero et al., 2016). This approach takes advantage of the same cassette used for inducible shRNA expression in OPTiKD, but repurposes it to drive an inducible sgRNA for CRISPR/Cas9. By combining this with a constitutively overexpressed Cas9 protein, we were able to demonstrate tightly controlled and efficient induction of gene knockout in hPSCs and hPSC-derived cells (Bertero et al., 2016; Fogarty et al., 2017). This method shares many of the advantages of OPTiKD described above, but the two approaches present distinct features that make each most useful for certain applications. Indeed, full gene-knockout with OPTiKO allows more powerful studies of genes that are still functional even when expressed at low levels. Furthermore, OPTiKO is also applicable to non-coding regulatory regions of the genome, which represent a large portion of disease-associated loci (Zhang & Lupski, 2015). On the other hand, the ability to tune the knockdown level using OPTiKD permits studying genes whose complete loss of function is lethal, while it also facilitates the investigation of gene-dosage mechanisms. Finally, OPTiKD is fully reversible.

Collectively, OPTiKD not only overcomes the limitations of previous inducible shRNA methods (Zafarana et al., 2009) but also provides an advantageous alternative to currently available inducible knockout approaches for the study of gene function in hPSCs and hPSC-derived cells. Finally, while the current unit is focused on the application of OPTiKD in hPSCs, we expect that this method will be widely applicable with minor modifications to many cell types that are amenable to genetic manipulation, including adult stem cells and cancer stem cells (Drost et al., 2015; Mandal et al., 2014).

Critical Parameters and Troubleshooting

The most critical parameter to generate an effective OPTiKD hPSC line is the identification of a potent shRNA capable of inducing strong gene knockdown. Wherever possible, we recommend using pre-validated shRNAs found in the literature or from the TRC library (Section 1.A; see Basic Protocol 1 introduction). Choose shRNA with a validated knockdown level of at least 75% (ideally >90%). If no validated shRNAs are available for the gene of interest, we recommend choosing the

shRNAs with the highest "Adjusted Score" in the TRC library database. In the first instance, we recommend the selection of at least three validated shRNAs or six non-validated shRNAs for the gene of interest: this should result in having at least two good shRNAs (>75% knockdown), which is the minimum number of shRNAs that must be used in order to validate the specificity of phenotypes resulting from gene knockdown (see below). In the event that this is not the case, more shRNAs can be tested at a later stage. Should this be possible, it is advised to pre-screen non-validated shRNAs using alternative methods such as transient transfection into easy-to-transfect cells, or lentiviral infection.

The second critical parameter in the procedure is the efficiency of hPSC gene targeting. For this, using hPSCs of the best possible quality is of the utmost importance. Cells should be growing well in feeder-free conditions, have little or no background differentiation, and be amenable to near single-cell growth. For this, we recommend using Essential 8 medium and Laminin-521 coating, as they have proven widely applicable to a number of hPSCs that are hard to maintain in other feeder-free culture conditions. Aside from this, effective plasmid delivery is crucial, and transfection efficiency ought to be at least 20% to increase the chances of experimental success. Should a specific hPSC demonstrate reluctance to transfect even after extensive optimization, nucleofection can be a valid alternative.

Selected troubleshooting procedures are described in the protocols, and a comprehensive reference can be found in Table 5C.4.5.

Anticipated Results

OPTiKD hPSCs allow tightly controlled, potent, homogeneous, dose-responsive, and reversible inducible gene knockdown both in hPSCs and in hPSC-derived lineages (Figure 5C.4.7A-F). When a potent shRNA has been selected, this is expected to show a 75% to 95% reduction in both mRNA and protein levels (Figure 5C.4.7F). Furthermore, when compared to wild-type controls or OPTiKD cells expressing an shRNA targeting a different transcript (see below), mRNA levels in OPTiKD cells maintained in the absence of tetracycline should not be significantly different thanks to the robust transcriptional repression of the shRNA [Section 2.A (see introduction to Basic Protocol 2); Figure 5C.4.7A and 5C.4.7F]. We recommend culturing cells in the presence of 1 µg/ml tetracycline to ensure

Table 5C.4.5 Troubleshooting

Problem	Possible causes	Troubleshooting	Possible solutions
1. Few or no colonies after transformation of inducible shRNA vector (step 9 of Basic Protocol 2 or step 5 of the Alternate Protocol)	Wrong oligonucleotide design	Perform *in silico* simulation of the cloning procedure to check design (step 7 of Basic Protocol 1)	Re-design oligonucleotides (Basic Protocol 1)
	Inefficient oligonucleotide annealing	Monitor oligonucleotide annealing (step 5 of Basic Protocol 2)	Re-attempt annealing but increasing the incubations at 80, 75, and 70°C to 10 min and/or increasing the ionic strength of the annealing buffer (steps 3-4 of Basic Protocol 2)
	Inefficient ligation or transformation	Perform positive ligation and transformation controls recommended by supplier	Perform ligation overnight at room temperature and/or use new ligase enzyme (step 7 of Basic Protocol 2 or step 3 of the Alternate Protocol). Use new competent bacteria. Transform more ligation product (scale bacteria accordingly).
2. Few or no positive colonies containing expected inducible shRNA vector (step 15 of Basic Protocol 2)	Contamination with uncut or single-cut vector during ligation. Inefficient de-phosphorylation.	Perform negative control ligation omitting shRNA to monitor background (step 7 of Basic Protocol 2)	Re-prepare vector increasing incubation time and/or using new enzymes (step 1 of Basic Protocol 2)
3. Poor quality sequencing QC of inducible shRNA vector (step 19 of Basic Protocol 2)	Strong hairpin sequence interferes with sequencing reaction	-	Re-attempt sequencing using conditions appropriate for GC- and secondary structure-rich sequences (add DMSO up to 5%; increase template concentration; use a 7:1 molar ratio of dITP[a]/dGTP.
4. Mutation in shRNA sequence (step 19 of Basic Protocol 2)	Error during oligonucleotide synthesis	-	Screen additional bacterial clones and/or repeat cloning using PAGE-purified oligonucleotides
5. Few or no colonies after transformation of multiple inducible shRNA vector (step 10 of Support Protocol 1)	Inefficient Gibson assembly	Perform positive control Gibson assembly reaction provided with the kit	Re-assemble the vector increasing the incubation time at 50°C to 4 hr (step 8 of Support Protocol 1). Use a new assembly kit.

continued

Table 5C.4.5 Troubleshooting, *continued*

Problem	Possible causes	Troubleshooting	Possible solutions
6. Few or no positive colonies containing expected multiple inducible shRNA vector (step 13 of Support Protocol 1)	Incorrect overlaps in PCR-amplified shRNA cassettes	-	Double-check primer sequences; re-prepare primer stocks; re-prepare the shRNA cassettes (steps 4 to 5 of Support Protocol 1)
7. Mutation in multiple shRNA cassettes (step 17 of Support Protocol 1)	PCR-induced mutation	-	Screen additional clones. Repeat PCR reducing the number of cycles to 25-30 (step 5 of Support Protocol 1). Use new enzyme.
8. Few or no colonies following selection of gene targeted hPSCs (step 14 of Basic Protocol 3)	Poor transfection efficiency (<20%)	Perform control transfection with AAVS1-CAGGS-EGFP (step 9 of Basic Protocol 3) and quantify EGFP fluorescence by flow cytometry after 24 hr	Optimize transfection for specific hPSC line by varying plasmid amount (1-4 μg), GeneJuice to plasmid ratio (2-6 μg/μl), cell number (100,000-500,000), and timing (during plating or after 24-48 hr). Test alternative plasmid delivery methods such as nucleofection.
	Too stringent puromycin selection (step 12 of Basic Protocol 3)	-	Use minimal concentration required to kill wild-type hPSCs (step 12 of Basic Protocol 3). Decrease puromycin dose by half after 48 hr of selection. Add ROCK inhibitor for up to 72 hr during selection. Remove puromycin once selection is complete to promote cell growth.
	Inefficient homologous recombination	Perform gene targeting control omitting ZFN plasmids (step 9 of Basic Protocol 3)	Re-prepare the ZFN plasmids and confirm their identity by restriction digestion and sequencing. If other nucleases have been used switch to the plasmids recommended in the protocol.
	Differentiation of hPSCs	-	Use high-quality hPSCs with minimal no/background differentiation
	Culture conditions do not support growth of small hPSC colonies following selection	-	Prepare fresh complete Essential 8 medium daily. If other culture conditions have been used switch to Essential 8 and Laminin-521 as recommended in the protocol.

Table 5C.4.5 Troubleshooting, *continued*

Problem	Possible causes	Troubleshooting	Possible solutions
9. Few or no colonies showing expected gene targeting (step 16 of Support Protocol 2)	Incomplete puromycin selection (step 12 of Basic Protocol 3)	-	Use minimal concentration required for complete killing of wild-type hPSCs within 48 hr (step 12 of Basic Protocol 3). Start puromycin selection earlier
10. Few or no colonies showing homozygous gene targeting (step 16 of Support Protocol 2)	Picked colonies were not clonal	Perform clonal isolation by plating single cells in 96- or 384-well plates	Repeat targeting procedure and closely monitor resulting colonies daily (steps 1-3 of Support Protocol 2). Reduce the number of transfected cells (scale other reagents accordingly). Seed cells on larger plate format (scale volumes of culture media accordingly).
	Inefficient gene targeting	-	Optimize transfection for specific hPSC line as described in point 8 of this table
11. No or low level of gene knockdown in OPTiKD cells after induction with tetracycline	Gene targeting was inefficient and/or failed	Isolate clonal lines and confirm genotyping by genomic DNA PCR (Support Protocol 2)	Repeat gene targeting implementing optimizations described for Troubleshooting points 8 to 10. Include the recommended controls (step 9 of Basic Protocol 3)
	Mutation in shRNA sequence or wrong shRNA	Confirm correct transgene sequence by Sanger sequencing of 5′-INT genotyping PCR (Table 5C.4.4)	QC plasmid used for transfection (step 22 of Basic Protocol 2). Re-prepare plasmid.
	Inefficient shRNA	Test the shRNA by transient transfection in easy-to-transfect cell line (add tetracycline)	Design a new shRNA and/or find a validated shRNA. Generate vector containing multiple shRNAs against the same gene (Support Protocol 1).
	Tetracycline is ineffective	If available, test tetracycline batch on validated OPTiKD line	Prepare new tetracycline stock, protect from light and follow storage recommendations (Reagents and Solutions)
	Low transcriptional activity of the transgene following >20 passages	Knockdown was effective in previous experiments	Grow cell in presence of puromycin even after selection is completed. Thaw cell stock from earlier passage.

Table 5C.4.5 Troubleshooting, *continued*

Problem	Possible causes	Troubleshooting	Possible solutions
12. Gene knockdown in OPTiKD cells maintained in absence of tetracycline	Contamination of culture medium with tetracycline	Culture cells without animal-derived reagents (in particular FBS) to test for potential tetracycline contamination	Obtain tetracycline-free guaranteed animal-derived reagents
13. Cell death in tetracycline-treated cells	Tetracycline dose toxic in specific cell type and or culture conditions	Test toxicity of tetracycline in wild-type cells	Lower the concertation of tetracycline to sub-toxic levels that still induce full knockdown
	Knockdown induces cell death	Confirm that tetracycline-treated control OPTiKD are viable (unrelated or scrambled shRNA)	Analyze cells at shorter time points after tetracycline treatment
14. Inconsistent results using different shRNAs	shRNAs induce different level of knockdown of dosage-sensitive gene	Monitor phenotypic response to lower doses of tetracycline inducing intermediate knockdown	Focus only on more potent shRNAs (>75% knockdown) and if necessary test alternative shRNAs
	Off-target shRNA effects	Confirm shRNA specificity by genome wide transcriptome analyses	Exclude non specific shRNAs and if necessary test alternative shRNAs. Perform rescue experiments by overexpressing shRNA-resistant transcript. Cross-validate findings using alternative method such as CRISPR/Cas9-mediated knockout.

[a]2′-deoxyinosine-5′-triphosphate.

maximal induction of gene knockdown, but if preferable this dose could be lowered down by a factor of 10 without seeing a substantial decrease in efficiency (Figure 5C.4.7C). Nevertheless, doses between 10 and 100 ng/ml will lead to dose-dependent effects on the induction of gene knockdown (Figure 5C.4.7C), which can be advantageous in certain experimental settings. Less than 10 ng/ml tetracycline shows no detectable activity.

Induction of knockdown at the transcript level is extremely rapid and has proven consistent across the multiple genes we have tested, regardless of the cell type, with near-maximal decrease observed between 12 and 24 hr after addition of tetracycline (Figure 5C.4.7D). In fact, shRNAs induce active degradation of their target transcripts through the micro-RNA machinery, a process which is largely transcript-independent (Lambeth & Smith, 2013). On the contrary, protein loss is only consequential to reduced translation upon mRNA knockdown and is thus largely dependent on the steady-state level and the half-life of the protein, as well as on the cell type. We have observed near-maximal protein loss in OPTiKD hPSCs anywhere between 2 to 5 days after addition of tetracycline. However, hPSCs are rapidly dividing cells that, upon mRNA knockdown, will serially dilute the protein following each cell division. On the other hand, when knockdown is induced in cells that do not proliferate as rapidly as hPSCs, maximal knockdown of very stable proteins such as EGFP (whose half life is more than 24 hr) can require up to 4 weeks (Bertero et al., 2016). Therefore, if the timing of knockdown is important for the experimental
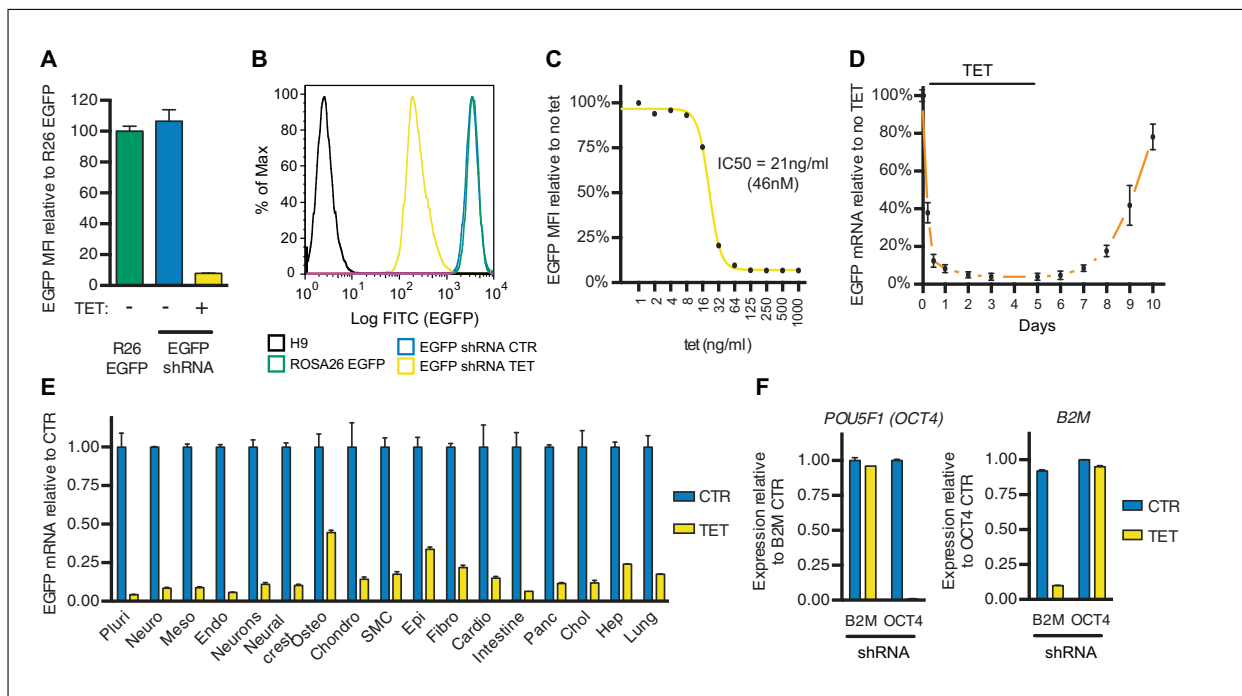
Optimized
Inducible Gene
Knockdown in
hPSCs

5C.4.42

Figure 5C.4.7   Performance of OPTiKD in hPSCs and hPSC-derived cells. Data presented in this figure has been previously presented in Bertero et al. (2016) and selectively reproduced here to provide representative examples of the expected results when using OPTiKD. (**A**) Quantification of EGFP median fluorescence intensity (MFI) in EGFP OPTiKD hESCs (clonal sublines). Homozygous ROSA26 CAG-EGFP hESCs (R26 EGFP; constitutive EGFP expression) were re-targeted with pAAV-Puro_siKD-EGFP (homozygous targeting). Cells were analyzed in absence (−) or presence (+) of tetracycline (TET) for 5 days. EGFP levels were compared to those in homozygous ROSA26 CAG-EGFP hESCs. Results are from two to three individual lines per condition. (**B**) Representative flow cytometry EGFP quantification in EGFP OPTiKD hESCs treated as described in panel A. (**C**) Tetracycline dose-response curve for EGFP knockdown in EGFP OPTiKD hESCs. The half-maximal inhibitory concentration (IC$_{50}$) is reported. Results are from two independent cultures per dose, and the mean is shown. (**D**) EGFP mRNA knockdown and rescue kinetics in EGFP OPTiKD hESCs. Results are from two independent cultures per time point. (**E**) EGFP mRNA expression measured by quantitative real-time PCR (qPCR) in absence (CTR) or presence of tetracycline for 5 days (TET) in the indicated cell types derived from EGFP OPTiKD hESCs. EGFP levels are reported relative to control conditions for each individual lineage. Pluri: undifferentiated hESCs; Neuro: neuroectoderm; Meso: lateral plate mesoderm; Endo: definitive endoderm; NC: neural crest; Osteo: osteocytes; Chondro: chondrocytes; SMC: smooth muscle cells; Epi: epicardium; Fibro: cardiac fibroblasts; Intestine: intestinal organoids; Panc: pancreatic cells; Chol: cholangiocytes; Hep: hepatocytes. (**F**) Representative qPCR validation of OCT4 and B2M OPTiKD hESCs (clonal sublines). Cells were analyzed in the absence (CTR) or presence of tetracycline for 5 days (TET). Clones were analyzed in duplicate.

design, this aspect must be verified for each gene of interest and in the specific cell type to be studied.

Withdrawal of tetracycline from knockdown OPTiKD cells allows rescue of the knockdown (Figure 5C.4.7D). For this, it is important to extensively wash the cells with fresh medium in order to remove all traces of tetracycline from the culture (two washes of 5 min each should suffice). Rescue of mRNA expression follows a slower kinetics compared to the induction of knockdown, since the shRNA persists for 2 to 3 days even after removal of tetracycline. In our experience, it takes between 5 and 7 days to see near-maximal recovery in gene expression in hPSCs. In this, the timing varies from gene to gene depending on the transcriptional activity, the half-life of the

mRNA, and the steady-state level that must be reached to attain the expression observed prior to the induction of knockdown. Rescue of protein expression follows a similar kinetics but is delayed by 1 to 3 days, depending on the translation rate and protein stability. Nevertheless, although these general guidelines can provide a rule of thumb applicable to most genes, this aspect will need to be determined in detail for every gene of interest if the precise timing is key to the experimental design.

Experiments for functional analyses using OPTiKD hPSCs must be designed to include a number of controls that allow robust evaluation of phenotypic consequences of gene knockdown. Most importantly, each knockdown sample ought to be paired with cells that have been maintained in the absence of

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tetracycline. This is the essential control to be used when measuring any potential change in cell behavior following knockdown, as it provides the closest biological comparison. Aside from this basic control, we also recommend including OPTiKD cells that express an shRNA targeting a different gene that is known not to be involved in the biological process of interest (unrelated shRNA control), or an shRNA that does not target any known human gene (scrambled shRNA control). These cells should be examined both in the absence of tetracycline and after addition of tetracycline. The first aspect allows an evaluation of line-to-line variability across OPTiKD cells, which is particularly important if a clonal OPTiKD subline is being used (Section S2.A; see Support Protocol 2 introduction). It also functions as control for any potential biological effects induced by the gene targeting procedure. Unrelated or scrambled shRNA controls treated with tetracycline are key to: (1) excluding that tetracycline has any side effects in the biological process of interest, as this drug can have metabolic effects in certain cell types (Chatzispyrou, Held, Mouchiroud, Auwerx, & Houtkooper, 2015; Moullan et al., 2015); (2) exclude non-specific effects due to cellular overexpression of shRNAs, which can, under certain conditions, compete with the endogenous miRNA processing pathways (Boudreau, Martins, & Davidson, 2009; Grimm et al., 2006). Of note, if multiple shRNAs are being expressed from the same OPTiKD vector (Support Protocol 1), we recommend that the unrelated or scrambled shRNA control also include an equal number of shRNAs in order to more closely mimic the level of shRNA overexpression induced under these conditions. Having clarified the importance of these additional controls, we would like to stress that our experience has revealed no obvious side effects of OPTiKD in the specification of any of the many hPSC-derived cell types we tested due to the gene targeting procedure, the treatment with tetracycline, or the overexpression of up to three shRNAs (Bertero et al., 2016). While we recommend including all these controls when a new cell type and/or biological process are being investigated for the first time, once nonspecific effects inherent in the method have been excluded, it is possible to include only the no-tetracycline control in order to simplify experimental procedures and analyses.

While shRNAs are a versatile, powerful, and easy-to-implement tool, as with any experimental technique there are some inherent limitations that need to be considered when interpreting the results. It is well established that certain shRNAs can have profound nonspecific effects by leading to down-regulation of unrelated transcripts that share partial sequence homology (Jackson et al., 2003). While over the years computational tools used to design shRNAs have been greatly improved to minimize such potential off-target effects (Pei & Tuschl, 2006), they are by no means perfect. Therefore, appropriate controls must be put in place to ensure that any observed effects following shRNA overexpression are truly specific to the knockdown of the gene of interest (Cullen, 2006). While the gold standard for this is to rescue the phenotype by overexpressing an shRNA-resistant transcript, this is technically quite challenging and time consuming, and therefore might not be widely applicable. Furthermore, interpretation of rescue experiments is not always straightforward, as the gene overexpression often exceeds physiological expression levels and thus can lead to yet other potential confounding effects. Therefore, as a more rapid and practical alternative, we recommend that at least two shRNAs, but ideally three or four, should be used in order to validate that the phenotype is consistently reproduced with multiple shRNAs. Should this not be feasible in a first instance, for example in a case where multiple genes are being screened at the same time, we recommend that any positive hit should be further validated with new independent shRNAs. Finally, cross-validation of the results using an independent method is also good practice.

A final aspect worth discussing is that, as with any experiment, it is important that sufficient biological and technical replication be performed to ensure the statistical significance of the results. This must follow established conventions set by the scientific community, and where appropriate should be determined according to power calculations to predetermine the amount of replication required to reliably determine an effect of a given magnitude given the expected degree of biological variability (Nakagawa & Cuthill, 2007; Vaux, Fidler, & Cumming, 2012). Specifically to OPTiKD, we recommend that multiple independently-derived hPSC lines should be used as biological replicates, in particular if clonal sub-lines have been isolated (Section S2.A; see Support Protocol 2 introduction). Furthermore, experiments should be performed in hPSCs or hPSC-derived cells at different passages to ensure reproducibility.

Time Considerations

Figure 5C.4.2 outlines the experimental procedure and the timelines associated with each of the protocols described in this unit. Collectively, an experienced user can complete the four basic protocols and generate OPTiKD hPSCs in as little as 3 weeks, while we estimate that a first-time user should allow up to 6 weeks. Inclusion of support protocols to assemble targeting vectors for multiple inducible knockdowns and/or to clonally isolate OPTiKD hPSCs will add approximately 2 to 3 weeks to the process.

The procedure can be stopped at the end of each protocol. Additionally, molecular cloning experiments described in Basic Protocol 2, the Alternate Protocol, and Support Protocol 1 are largely flexible: intermediate DNA products can be stored at 4°C overnight or at –20°C for up to 1 month, and bacteria can be stored at 4°C as liquid culture or LB agar plates for up to 1 week, or at –80°C as glycerol stocks for long-term storage. The gene targeting procedures are less flexible, and it is recommended to allow 1 to 2 weeks of consecutive work to complete Basic Protocol 3. If clonal isolation of OPTiKD lines is desirable, it is recommended to allow 2 to 4 consecutive weeks to complete both Basic Protocol 3 and Support Protocol 2. Nevertheless, if necessary, it is possible to freeze non-clonal OPTiKD hPSCs at the end of Basic Protocol 3 and then initiate Support Protocol 2 at a later stage by seeding individual cells into 96- or 384-well plates for clonal isolation. Finally, if OPTiKD cells are to be analyzed following hPSC differentiation, the timing required for generation of the cell type of interest must be taken into account, and this can vary from a week up to several months.

Acknowledgements

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Supplement 44

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