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Genome Editing in Human Pluripotent Stem Cells: Approaches, Pitfalls, and Solutions

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Abstract

Human pluripotent stem cells (hPSCs) with knockout or mutant alleles can be generated using custom-engineered nucleases. Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 - nucleases are the most commonly employed technologies for editing hPSC genomes. In this Protocol Review we provide a brief overview of custom-engineered nucleases in the context of gene editing in hPSCs with a focus on the application of TALENs and CRISPR/Cas9. We will highlight the advantages and disadvantages of each method and discuss theoretical and technical considerations for experimental design.

Introduction

The isolation of human embryonic stem cells (hESCs) and the discovery of human induced pluripotent stem cell (hiPSC) reprogramming has sparked a renaissance in stem cell biology, *in vitro* disease modeling and drug discovery (Grskovic et al., 2011; Takahashi et al., 2007; Thomson et al., 1998). In general, hPSC-based disease models are well-suited to study genetic variation (Karagiannis and Yamanaka, 2014). Studies commonly compare patient-derived hiPSCs, e.g. with a disease-causing genetic mutation, and (age-matched) control subject-derived hiPSCs, typically differentiated to the disease-affected cell type, e.g. neurons or hepatocytes (Ding et al., 2013a; Sterneckert et al., 2014). A major caveat of this disease modeling strategy is the variability of differentiation propensities and phenotypic characteristics, even in hPSCs derived from the same donor (Bock et al., 2011; Boulting et al., 2011). Still, even if the cellular phenotype of a given mutation is strong and highly

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penetrant, it may be lost due to confounding effects of differences in genetic background of unrelated hPSC lines (Merkle and Eggan, 2013; Sandoe and Eggan, 2013). A very powerful approach to overcome this hurdle is to use custom-engineered endonucleases that enable precise and programmable modification of endogenous hPSC genomic sequences (Kim and Kim, 2014). This genome engineering strategy will prove invaluable for studying human biology and disease (Merkle and Eggan, 2013; Sterneckert et al., 2014).

Upon delivery in the cell, custom-engineered nucleases introduce site-specific double-strand breaks (DSBs) in the DNA that are repaired either through error-prone non-homologous end-joining (NHEJ) or precise homology-directed repair (HDR; reviewed in (Heyer et al., 2010; Jasin and Rothstein, 2013)). DSB repair through NHEJ will typically result in small insertions and/or deletions (indels) in the target locus. These indels cause frame shift mutations resulting in functional knock-out of protein coding genes (Ding et al., 2013a). Larger deletions can be introduced to create two DSBs simultaneously to knock out genes, regulatory regions or non-coding genetic loci (Canver et al., 2014). Dual DSBs will be repaired through NHEJ, deleting the complete intervening sequence (Mandal et al., 2014; Zhang et al., 2015). Precise genetic modifications such as nucleotide substitutions or deletions are achieved by co-delivery of an exogenous DNA donor template with engineered nucleases for integration though HR (Byrne et al., 2015; Hockemeyer et al., 2011).

Most engineered endonucleases comprise a customizable, sequence-specific DNA binding domain fused to a (non-specific) DNA endonuclease domain. Although naturally occurring homing endonucleases or meganucleases have been successfully used for genome engineering (Silva et al., 2011), their application in genome editing of hPSCs has been very limited. The first custom-engineered, site-specific endonucleases successfully used for genome editing in hPSCs were Zinc-Finger Nucleases (ZFNs; (Hockemeyer et al., 2009; Zou et al., 2009)). ZFNs are fusion proteins composed of several tandem Zinc-finger DNA binding domains coupled to the FokI endonuclease catalytic domain. The DNA binding domain of ZFNs consists of three to six zinc finger DNA-binding domains (ZFDBD) assembled in an array. This arrayed construction of the ZFN allows for specific targeting of genetic loci, as each ZFDBD binds to a specific nucleotide triplet. FokI endonuclease is only active when homodimerized, further complicating ZFN construction (Bibikova et al., 2003; Urnov et al., 2005). ZFNs are relatively difficult to engineer and their design and construction in the laboratory remain technically challenging.

An alternative custom-engineered endonuclease is the Transcription Activation-Like Effector Nuclease derived from the plant pathogen *Xanthomonas* (TALEN; (Boch et al., 2009)). Like ZFNs, TALENs consist of a customized TALE DNA binding domain fused to a non-specific FokI nuclease domain. The TALE DNA binding domain comprises arrays of 33–35 amino acids where the amino acids in position 12 and 13 of each array determine nucleotide binding specificity. TALEN-mediated genome editing in hPSCs has been used for generation of hPSC gene reporter lines, biallelic knock out of genes, and repair and introduction of point mutations (Ding et al., 2013a; Luo et al., 2014; Soldner et al., 2011). As with the design of ZFNs, each DNA target sequence requires re-engineering of the TALEN DNA binding domain. Recently, an increasingly popular RNA-guided endonuclease has been developed for genome editing in eukaryotes (Cong et al., 2013; Mali

et al., 2013). First derived from *Streptococcus pyogenes* (SpCas9; referred to in this Review as Cas9 unless otherwise noted), the Cas9 system consists of the Cas9 nuclease and short non-coding CRISPR RNA sequences referred to as single guide RNAs (sgRNAs). These sgRNAs contain a customizable 20 nucleotide sequence that guides a co-expressed Cas9 nuclease to the sgRNA target sequence for creation of a site-specific DSB (Jinek et al., 2012).

In this Protocol Review, we will discuss TALEN- and CRISPR/Cas9-mediated genome editing protocols for genome engineering in hPSCs that follow a general workflow, shown in Figure 1, and highlight problems, pitfalls, and solutions associated with each. Many of the gene editing approaches described in this Protocol Review have been first validated and established in other cell types, but wherever possible we reference their application in hPSCs.

DNA binding domain, nuclease and template design

When choosing the most suitable custom-engineered endonuclease for any given hPSC gene targeting experiment, target site accessibility (chromatin state or methylation state) and the type of desired genetic modification must be considered. Target site binding is influenced by methylation state and is an important consideration when using TALENs since conventional TALE DNA binding domains cannot bind and cleave targets that contain methylated cytosines, usually found within CpG islands (Bultmann et al., 2012; Kim et al., 2013). The TALE DNA binding affinity for its cognate DNA sequence also determines the TALEN activity. Low binding affinity results in reduced TALEN activity and a very strong affinity reduces TALEN specificity (Guilinger et al., 2014a; Jankele and Svoboda, 2014; Meckler et al., 2013). Hyper-methylated DNA sequences may be more efficiently targeted using a CRISPR/Cas9 approach as the CRISPR guide RNA is able to bind methylated DNA (Hsu et al., 2013), but it has been suggested that CRISPR guide RNA activity is partly dependent on DNaseI hypersensitivity of the locus targeted. On the other hand, TALEN DNA binding domains have less stringent design rules and in general are less mismatch-tolerant than CRISPR/Cas9 (Hockemeyer et al., 2011; Miller et al., 2011; Miller et al., 2015). Whereas CRISPR/Cas9 target sites are limited to loci harboring a protospacer-adjacent motif (PAM), TALEN DNA binding domains can be designed to target any sequence, offering substantially higher targeting densities compared to CRISPR guide RNAs (Miller et al., 2015; Reyon et al., 2012; Tsai et al., 2014). It has been estimated that there is a dimeric target site per 3 bp of random DNA sequence for TALENs based on the requirement for a 5′ T and the range of compatible spacing. The targeting range of TALENs is essentially unrestricted given the flexibility of these parameters (Dr. S. Tsai, Joung lab MGH, personal communication; (Reyon et al., 2012)).

Both TALEN and CRISPR/Cas9 genome editing methods have been used to target a wide variety of genomic loci for the creation of NHEJ-mediated gene knock out hPSC lines (Ding et al., 2013a; Frank et al., 2013; Li et al., 2013). CRISPR/Cas9 is generally more efficient than TALENs for NHEJ-mediated knockout of target genes (Ding et al., 2013b). The efficiency of HDR-mediated gene editing in hPSCs is comparable between CRISPR/Cas9 and TALEN (Yang et al., 2013) and both gene editing approaches have been used to

successfully generate hPSC gene knock-in lines (Byrne et al., 2015; Chen et al., 2014; Ding et al., 2013a; Hockemeyer et al., 2011; Hou et al., 2013; Osborn et al., 2013). For both CRISPR gRNA and TALEN DNA binding array design online design tools are available (Table 1).

Precise editing of point mutations or SNPs, generation of gene reporters, and precise gene deletions or insertions are HDR-dependent and require an exogenous DNA template (Choulika et al., 1995). The two most common DNA templates for HDR-mediated gene editing are single-stranded oligodeoxynucleotides (ssODNs) and plasmid DNA templates. Single-stranded ODNs are most often used for introduction or repair of point mutations, whereas plasmid DNA templates are primarily used for larger gene insertions. Whereas the generation of targeting plasmids may require substantial cloning and inclusion of homology arms on 5′ and 3′ ends that ideally measure more than 400 base pairs (Hendel et al., 2014), ssODNs are typically 100 – 200 nucleotides long with at least 40 base pairs of homology on either side (Byrne et al., 2015; Ran et al., 2013b) and readily obtained from commercial vendors.

Design of DNA binding domains - TALENs

The DNA binding domain of TALENs can be engineered to target any DNA sequence. The TALE DNA binding domain comprises 33 to 35 amino acid repeats, of which the amino acids in position 12 and 13 in each repeat recognize a single DNA base. These two amino acids constitute the variable di-residues (RVDs). There are four canonical RVDs, NN, NI, HD and NG that recognize and bind guanine, adenine, cytosine and thymine, respectively (Boch et al., 2009; Moscou and Bogdanove, 2009). TALE DNA binding domains that target a DNA sequence containing one or more 5-methylated cytosines (5mC), often found in CpG islands in promoter regions and proximal (5′) exons, bind their target DNA less efficiently (Bultmann et al., 2012; Reyon et al., 2012). This 5mC sensitivity can be overcome by using the demethylating agent 5-aza-dC during cell culture, though this compound has pleiotropic effects and can result in substantial cytotoxicity (Palii et al., 2008). To avoid the use of potentially harmful demethylating compounds, Valton *et al.* studied 5mC binding of the alternative cytosine binding RVD N* (Boch et al., 2009), and found a 2–17 fold increase in 5mC binding compared to the canonical cytosine binding RVD HD (Valton et al., 2012). CRISPR/Cas9, on the other hand does bind methylated sequences and is more efficient than TALEN in generating DSBs at methylated target sites (Hsu et al., 2013; Miller et al., 2011). The TALE DNA binding array allows for more DNA sequence targeting flexibility than CRISPR gRNAs, since the only requirement is a thymine (T) immediately upstream of the DNA binding domain (Boch et al., 2009; Moscou and Bogdanove, 2009). Recent re-engineering of synthetic RVDs has allowed for any 5′ nucleotide recognition by the N-terminal TALE domain (Lamb et al., 2013), further increasing the targeting flexibility of TALENs.

Generating a TALE DNA binding domain is relatively straight forward but each DNA target sequence requires re-engineering of the TALE array. In general, it is recommended to design a pair of TALENs that, including the spacer region, spans about 45–60 nucleotides (Figure 2A). Although, theoretically this strategy would avoid off-target sites with homologous

sequences as the target sequence is longer, hence more specific, TALEN off-target activity has been reported (Guilinger et al., 2014a). If the goal is to knock out a gene, ideally the first exon should be targeted and sites that reside in the 3′ end of the coding sequence should be excluded. It is also recommended choosing a target site that resides in a common exon, in case a single gene is expressed as multiple splice variants (Kim et al., 2013). Modular multimer TALE DNA binding arrays can be built from RVD monomers using a hierarchical or Golden Gate cloning method (Cermak et al., 2011; Engler et al., 2009; Zhang et al., 2011). A less labor intensive and time consuming method to assemble TALE DNA binding arrays is the Fast Ligation-based Automatable Solid-phase High-throughput (FLASH) system, although this method does rely on a pre-existing library of plasmids containing one to four TAL effector repeats consisting of all possible combinations of the canonical RVDs (Reyon et al., 2012). A ligation-independent cloning method has also been described to generate TALENs in a high-throughput manner (Schmid-Burgk et al., 2013). The advantage of these methods is that they are relatively fast and provide flexibility in the length of the TALE DNA binding arrays. Although more costly, gene synthesis represents an easy way to generate codon-optimized TALENs as has been shown for their use in hPSCs (Yang et al., 2013). Our and other labs have built libraries of plasmids containing multimer TALE DNA binding modules that can either be easily digested and sequentially ligated into a TALEN backbone (Ding et al., 2013a) or assembled in a one-step Golden Gate cloning reaction (Kim et al., 2013). The advantage of these latter libraries is that any laboratory with basic molecular biology capabilities can cost-effectively build TALENs for genome editing purposes.

Design of DNA binding domains - CRISPR gRNA

The CRISPR/Cas9 (Type II CRISPR system; (Jinek et al., 2012)) genome editing system comprises the Cas9 nuclease, a CRISPR RNA (crRNA) array that encodes the short guide RNA (sgRNA) and a trans-activating crRNA (tracrRNA) that facilitates the processing of the crRNA array into discrete units. Each crRNA unit contains a 20 nucleotide guide sequence and a partial direct repeat, where the guide sequence directs the Cas9 to a 20 base pair DNA target through Watson-Crick base pairing (Jinek et al., 2012). Upon co-expression, complex formation of the sgRNA with Cas9 nuclease will introduce a genomic DSB at the target site. With the predominantly used conventional CRISPR-Cas9 system derived from *S. pyogenes*, the target DNA must immediately precede a 5′-NGG PAM (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013)(Figure 2B), although other Cas9 orthologues have different PAM requirements (Hou et al., 2013). CRISPR/Cas9-mediated targeting in hPSCs is more efficient than TALEN-mediated targeting (Ding et al., 2013b), and that efficiency can be further increased by multiplexing sgRNAs in a single genome-editing experiment (Mandal et al., 2014). Although the relatively short *S. pyogenes* PAM confers flexibility in target sequence selection (the PAM motif NGG is found every 8–12 base pairs on average for the human genome - (Cong et al., 2013; Hsu et al., 2013)), this targeting strategy is limited to NGG-proximal sequences. This may limit the use of CRISPR/Cas9 when target specificity is required, e.g. introducing a DSB at a precise sequence location for HDR-mediated repair of point mutations. Non-canonical PAM sequences and Cas proteins derived from alternative bacteria have recently expanded the number of potential target sites (Hsu et al., 2013; Kleinstiver et al., 2015; Zetsche et al., 2015; Zhang et

al., 2014). The efficacy of these alternative PAMs and Cas9 orthologues for hPSC genome editing remains to be determined.

Another important point to consider with the design of sgRNAs is the potential for off-target effects as the sgRNA is mismatch-tolerant (Fu et al., 2013). Off-target indels found in CRISPR/Cas9 gene editing experiments can be dramatically reduced using “truncated” sgRNAs that are 17 to 18 instead of 20 nucleotides long, without sacrificing on-target cutting efficiency. The utility of truncated sgRNAs may be target-dependent as it has been postulated that truncated sgRNAs have a reduced binding affinity for their cognate sequence. This reduced binding affinity may in some cases result in reduced on-target activity of truncated sgRNAs (Fu et al., 2014). The same group and others also reported the use of an N-terminal fusion of FokI to a catalytically inactive Cas9 (dCas9) that requires dimerization of FokI monomers for DSB introduction necessitating two separate sgRNAs (targeting orthogonal DNA sequences) to introduce a DSB (Guilinger et al., 2014b; Tsai et al., 2014). Although longer sgRNAs have been used as well, the increase in specificity is minimal (Cho et al., 2014; Ran et al., 2013b). Another approach to reduce the likelihood of off-target effects and to increase on-target specificity is the use of a mutant “nickase” variant of Cas9. The Cas9-D10A mutant protein introduces a single strand nick, instead of a DSB, which is repaired through HDR and not NHEJ. This will result in precise repair at the DNA nick without introduction of any indels (Cong et al., 2013; Mali et al., 2013) (Figure 2C). This strategy of Cas9 nickase-mediated targeting has been used to generate hPSC-reporter cell lines (Merkle et al., 2015). Merkle *et al.* did find a number of loci that were targeted unsuccessfully, suggesting that gene knock-ins mediated by Cas9-D10A nickase activity is sequence or locus dependent (Merkle et al., 2015). The use of a pair of Cas9-D10A nucleases, targeted to opposite DNA strands with separate gRNAs such that the nicks are less than 100 base pairs apart, essentially creates a DSB with 50- to 1500-fold fewer off-target indels than wild-type Cas9 (Ran et al., 2013a)(Figure 2C). Whole genome sequencing in CRISPR/Cas9 and TALEN genome edited hPSCs revealed very high specificity and minimal genome mutational load with TALENs and truncated gRNAs, where nearly all of the mutations accumulated during regular hPSC culture (Smith et al., 2014; Suzuki et al., 2014; Veres et al., 2014).

Design of DNA donor templates for gene knock-ins

In contrast to the generation of hPSC gene knockout lines based on NHEJ, precise editing of point mutations, gene addition or deletion, and generation of reporter lines require an exogenous DNA template used by HDR to precisely engineer the genotype of interest (Jasin and Rothstein, 2013). The DNA template comprises left and right homology arms and an intervening DNA sequence that contains the DNA insert of interest (Capecchi, 1989). For the editing of point mutations or SNPs, ssODNs are preferable as a DNA template, because the sequence to be edited is generally very small (typically < 5nt mismatch between wild type and repair template sequence). The homology arms can be kept short without affecting HDR-mediated gene editing efficiency (Ding et al., 2013a; Ding et al., 2013b; Yang et al., 2013), although others have reported variability in targeting efficiency using ssODNs (Radecke et al., 2010). Design of ssODNs is straightforward and they can be synthesized commercially, which is very cost-effective. Adeno-associated virus (AAV) vectors have also

been successfully used as HDR-repair templates to introduce mutations at multiple genomic loci in hPSCs (Khan et al., 2010; Li et al., 2012) and hematopoietic stem cells (Ellis et al., 2013).

There is a correlation between the size of the sequence load and the length of the homology arms that determines the efficiency of HDR-mediated genome editing (Radecke et al., 2010). HDR-mediated introduction of a bigger sequence load such as a reporter gene or gene replacement requires longer homology arms. It has been reported that HDR-mediated genome editing is improved by using homology arms of 400 base pairs or longer (Hendel et al., 2014; Merkle et al., 2015). Also, sequence divergence between homology arms of the repair template and chromosomal locus targeted should ideally be less than 2% to avoid decrease in HDR-mediated targeting (Elliott et al., 1998), making it important to sequence the homology regions spanning the locus in each cell line to be targeted. In order to prevent re-cutting of the edited sequence after successful insertion, it is recommended to introduce a silent mutation in the TALEN or sgRNA binding site.

Another important DNA template design consideration is the location of the intended mutation up- or downstream of the DSB. The so-called “conversion tract” or distance between mutation and DSB should be as short as possible and in general less than 50 base pairs, beyond which the HDR-mediated repair efficiency drops dramatically (Elliott et al., 1998; Yang et al., 2013). Introduction of a silent mutation which adds or removes a restriction endonuclease cut site in the ssODN can aid in mutation analysis and clone screening. To optimally pursue this strategy, this additional mismatch should also be less than 10 nucleotides away from the DSB site (Yang et al., 2013).

Analysis of nuclease cutting efficiency

When designing ideal NHEJ-mediated gene knock out or HDR-mediated SNP repair strategies, the percentage of nuclease activity as determined by gel-based assay of highly active nucleases should ideally be greater than 25% when assessed in HEK293T or U2OS cells and under optimal delivery conditions (Hendel et al., 2015b). Although the nuclease activity depends on multiple parameters such as cell type, target and delivery method, the 25% cutting efficiency determined in HEK293T or U2OS, in our hands generally translates to efficient nuclease activity in hPSCs. Should your engineered nucleases prove less efficient than this goal, one strategy for improvement of nuclease activity is to “cold-shock” the transfected cells at 30 °C for 24 hours. This treatment improved ZFN mediated DSB introduction in mammalian cell lines and has been used to improve TALEN activity in embryonic stem cells as well (Carlson et al., 2012; Doyon et al., 2010). We and others, however, do not routinely culture TALEN or CRISPR/Cas9 transfected hPSCs at this temperature as it also affects the growth of cells. Higher nuclease activity is generally better for successful genome editing experiments. In certain circumstances, especially for HDR-mediated editing events, the flexibility in TALEN or CRISPR gRNA design can be limited. Gene editing application and DNA sequence permitting, we generally design and test at least 3 guide RNAs or 2–3 TALEN plasmid pairs and use the most active engineered nuclease for hPSC targeting experiments.

The nuclease activity of any given TALEN or CRISPR/Cas9 determines the efficiency of site-specific DSB introduction and subsequent targeting events at loci of interest. Because delivery of TALENs or CRISPR gRNAs in hPSCs is inefficient (see below), evaluation of nuclease activity is typically tested in an easy to transfect cell line, such as the U2OS or HEK239T cell lines. Because transfection in these cell lines is more efficient, cleavage efficiency is much higher compared with hPSCs. In general, a mutation efficiency of at least 25% in a gel-based cleavage assay is necessary to successfully use the tested TALEN or sgRNA in an hPSC targeting experiment. This is dependent upon the target locus; we have found sgRNAs with high cleavage efficiency in HEK293T cells that did not introduce DSBs in hPSCs. This may be due to target locus accessibility, the target sequence itself or differences in the DNA damage response between hPSCs and immortalized somatic cell lines (Chari et al., 2015; Liu et al., 2014). The mutations generated by NHEJ after introduction of nuclease-induced DSBs usually range from one to tens of inserted or deleted nucleotides. Detecting these indels provides a quantitative (indirect) measurement of TALEN- or CRISPR/Cas9-mediated cleavage activity. Our labs primarily use a gel-based mutation detection assay with the CEL-I nuclease (Surveyor assay) or T7 endonuclease I (T7EI), which is rapid and cost-effective. These assays rely on *in vitro* melting and annealing of mutant and wild-type genomic DNA followed by recognition and cleavage of resultant mismatches by exogenous endonucleases. T7E1 endonuclease is more sensitive and has a lower detection limit for cleaved mutant alleles than does the Surveyor assay (Sakurai et al., 2014; Vouillot et al., 2015). Gel-based mutation assays cannot readily detect indels less abundant than 1–2% of the genetic population, and are unable to demonstrate the type of indel introduced (Hendel et al., 2015b). Digital droplet PCR (ddPCR)-based can also be used to accurately analyze NHEJ and HDR events as a measure for nuclease cutting efficiency (Miyaoka et al., 2014). This method uses a reference probe to detect a genomic region distant from the DSB and a second labeled probe designed to bind wild type DNA at the predicted cut site. The decrease in signal from the DSB targeted probe is a measure for nuclease cleavage efficiency (Berman et al., 2015, abstract #1915W, ASHG Annual Meeting). Sequencing-based techniques, such as Sanger and NGS sequencing are also used to analyze mutagenic NHEJ events and are more sensitive than gel-based mutation detection assays. A simple and cost-effective *in silico* method to evaluate CRISPR/Cas9-mediated cleavage efficiency is Tracking of Indels by Decomposition (TIDE; (Brinkman et al., 2014). Here, two Sanger sequence traces, one from a control and another from a sgRNA transfected genomic DNA PCR sample, are uploaded and analyzed by a decomposition algorithm (Brinkman et al., 2014). In addition, NGS approaches can detect mutation frequencies up to 0.007% and give direct information about the indel sequence (Chen et al., 2013; Yang et al., 2013). One disadvantage is that sequence-based assays use relatively short PCR amplicons to evaluate indel frequency that can lead to underestimation of the number of indels, especially when larger insertions or deletions are introduced that fall outside the PCR amplicon boundaries (due to read-length limitations).

An alternative technique to track genome engineering outcome in mammalian cells has been developed called the traffic light reporter, which generates a flow cytometric readout of HDR-mediated gene targeting and NHEJ-mediated gene disruption (Certo et al., 2011). Although this technique provides a simple, rapid and quantitative readout, the prerequisite

generation of a reporter gene prevents measurement at endogenous target loci. The use of the traffic light reporter system has not yet been reported in hPSCs. Some other less popular methods of indirect quantitative mutation assays are fluorescent PCR assays, DNA melting analysis and restriction fragment length polymorphism (RFLP) analysis (Hendel et al., 2015b), although the latter is often used for detecting HDR-mediated editing events (Ding et al., 2013a; Ran et al., 2013b). A recently developed method for analyzing gene editing outcomes in hPSCs, single molecule real-time (SMRT) sequencing, has been reported to allow quantification of HDR-mediated gene editing events using plasmid DNA templates with long arms of homology (Hendel et al., 2014).

Nuclease delivery into hPSCs

Having carefully designed and successfully generated active TALENs or CRISPR gRNAs, the next step in the hPSC genome editing workflow is delivery of the nucleases into hPSCs (Figure 1). This can be challenging and often involves selection or enrichment of successfully transfected cells, either by FACS or antibiotic selection (Ding et al., 2013a; Hockemeyer et al., 2011). TALE or CRISPR/Cas9 nucleases can be delivered to hPSCs in the form of DNA, RNA or protein.

Delivery as DNA

TALENs are primarily delivered as a combination of two DNA plasmids, with a 5′ TALE binding array fused to a FokI-nuclease monomer on one plasmid, while the other contains a 3′ TALE binding array also fused to a FokI-nuclease monomer (Christian et al., 2010; Miller et al., 2011). The CRISPR/Cas9 genome editing system delivered as DNA comprises either two plasmids, one containing the Cas9 nuclease and one the CRISPR gRNA (Mali et al., 2013) or one plasmid containing both the Cas9 nuclease and CRISPR gRNA in a single expression cassette (Ran et al., 2013b). The advantages of using DNA delivery of TALENs and CRISPR/Cas9 gRNA is the relatively straightforward cloning and the high efficiency in generating NHEJ-mediated knock out hPSC lines (Ding et al., 2013a; Ding et al., 2013b). On the other hand, the use of plasmid DNA nuclease delivery, especially with Cas9, has been associated with unwanted off-target indels (Merkle et al., 2015).

Delivery as RNA

TALEN mRNA delivery for gene editing in hPSCs has to our knowledge not yet been reported, although TALEN mRNA delivery to mouse embryonic stem cells has been successful in generation of transgenic (knock out) mice (Wefers et al., 2013). CRISPR/Cas9 delivered as mRNA has been used for targeting hPSCs (Kim et al., 2014). Although not yet reported in hPSC gene editing, chemically modified guide RNAs enhance CRISPR/Cas9 genome editing efficiency in human primary cells, such as T-cells and $CD34^+$ HSCs (Hendel et al., 2015a) and may further increase gene targeting efficiencies in hPSCs. Compared with plasmid delivery, mRNA transfection of CRISPR/Cas9 leads to faster expression and avoids unwanted integration of plasmid DNA encoding the nuclease(s) (Kim et al., 2014).

Delivery as protein

To our knowledge only one study used direct protein administration of TALENs fused to the protein transduction peptide TAT. This study targeted the CCR5 locus in hPSCs with an efficiency of 5% (Ru et al., 2013). On the other hand, direct protein delivery of Cas9 nuclease complexed with *in vitro* transcribed sgRNA (mRNA) has been widely successful in hPSC genome editing. The greatest advantage of protein delivery is quick degradation upon delivery resulting in a dramatic reduction of off-target indels (D'Astolfo et al., 2015; Kim et al., 2014; Liang et al., 2015; Zuris et al., 2015).

In addition to optimization of delivery vector (DNA, RNA, or protein) for custom-engineered endonucleases, the choice of delivery technique is equally important with hard to transfect cells like hPSCs. A few delivery techniques have been successfully applied in genome engineering of hPSCs. **Electroporation** as delivery technique for gene targeting in hPSCs has been successfully used in many studies (Ding et al., 2013a; Hockemeyer et al., 2011; Hou et al., 2013; Zwaka and Thomson, 2003). The most important disadvantage of this delivery technique is the massive amount of cell death that occurs after electroporation, necessitating a high input of hPSCs, usually around 1 x 10^7 cells per electroporation. Since the first electroporations of hPSCs for gene editing purposes (Zwaka and Thomson, 2003), methods have been improved, especially with regard to efficiency using single-cell hPSC suspensions (Costa et al., 2007) and improved survival using the Rho kinase (ROCK) inhibitor Y-23672 (Watanabe et al., 2007). **Nucleofection**, a modified electroporation technique, is an efficient method to deliver gene targeting and nuclease constructs to hPSCs (Byrne et al., 2015; Cai et al., 2007; Ran et al., 2013a; Sanjana et al., 2012; Yang et al., 2013). Recently, Cas9 protein and gRNA riboprotein complexes have been delivered using nucleofection resulting in efficient gene editing, while reducing off-target mutations (Kim et al., 2014; Lin et al., 2014). Fewer hPSCs are needed for nucleofection compared with conventional electroporation, typically in the range of 0.5 to 2.0 million cells per electroporation. The disadvantage is that nucleofection requires more optimization of the electrical parameters with cell-type specific reagents, which can be less cost-effective. **Cationic lipid-based transfection reagents** are widely used as a carrier for genetic material delivery into a variety of eukaryotic cells because of their efficiency and ease of use. Cationic lipid delivery of plasmid DNA into hPSCs has been very inefficient, though there are reports describing lipid-based transfection of hPSCs (Cai et al., 2007; Ma et al., 2012). We have recently developed an hPSC transfection protocol using a new lipid-based formulation, Lipofectamine 3000, that allows for efficient transfection and better cell survival of transfected hPSCs (Hendriks et al., 2015). Cas9 protein has been efficiently delivered to mouse ES cells using cationic lipids, though this method has not yet been applied to nuclease delivery in hPSCs. A novel protein transduction method based on osmolarity and a transduction protein inducing macropinocytosis, has recently been used to deliver gRNA and Cas9 protein to hPSCs. This method, "induced transduction by osmocytosis and propanebetaine" (iTOP) resulted in a more than 25% gene editing rate in H1 ES cells (D'Astolfo et al., 2015). Like other protein transduction methods, iTOP-mediated CRISPR/Cas9 expression is transient assuring that the gene editing system does not persist in the cell, avoiding off-target indels. **Viral vector-mediated delivery** of CRISPR/Cas9 gene editing constructs into hPSCs has been successful, especially non-

integrating viral vectors such as adenovirus and baculovirus (Zhu et al., 2013). In addition, the smaller *S. aureus* derived Cas9 has recently been packaged in an AAV vector that has a relatively small packaging capacity (4.7–4.8 kb). Lentiviral delivery of TALENs is inefficient, due in part to the viral reverse transcriptase that has difficulties transcribing the repetitive sequences within the TALE DNA binding array (Holkers et al., 2012). On the other hand, successful lentiviral delivery of CRISPR/Cas9 constructs and subsequent gene editing has been shown in a number of studies (Kabadi et al., 2014; Shalem et al., 2014).

Genome-edited cell selection and genotyping

Upon successful delivery of custom-engineered nucleases into hPSCs, the final step in a typical hPSC gene editing workflow is the selection of clonal gene edited hPSCs. Depending on the type of targeting experiment this can be achieved several ways. The TALEN and CRISPR/Cas9 constructs commonly used in our labs for hPSC genome engineering contain fluorescent reporters which enables enrichment of transfected hPSCs by FACS (Figure 3A). Although a dedicated FACS sorter would be ideal to avoid potential Mycoplasma contamination often seen with shared FACS sorters, our labs do use FACS sorting core facilities. We typically add Mycozap or Plasmocin to the cell culture media for 24 hrs. post-FACS. Reporter-positive cells are plated at a limiting dilution, allowing the formation of single cell-derived colonies. These single cell-derived colonies should be carefully monitored during their growth to avoid merged colonies, which will result in mixed genotypes upon expansion. Each colony is picked when it becomes about >500 μm in diameter and plated into one well of a 96-well plate (Ding et al., 2013a; Hendriks et al., 2015; Peters et al., 2008). Upon reaching confluence, the plate is split into two plates, one clone recovery plate and one plate for genomic DNA isolation for subsequent clone genotyping (Figure 1). Clones are analyzed by PCR amplification of a region surrounding the nuclease target site (DSB-site) and subsequent analysis of PCR amplicons on a high percentage agarose gel to identify edited hPSC clones. Targeted clones with potential frameshift-causing indels are identified by a band shift, indicating indel production (Figure 3B). HDR-mediated precise introduction of (single) base substitutions can be detected by RFLP if the ssODN used as donor template introduces or removes a restriction endonuclease site (Ding et al., 2013a)(Figure 3B). Positive clones are then Sanger-sequenced for genotype confirmation and identification (Figure 3C).

An elegant method for isolating genome-edited hPSC lines containing point mutations or single base substitutions was reported recently. This method uses digital droplet PCR (ddPCR) and sib-selection followed by subdivision of the targeted hPSC population until the rare correctly targeted hPSC clone can be isolated (Miyaoka et al., 2014). Less hands-on time and no need for antibiotic selection are the main advantages of this method. DdPCR has been used to generate 20 targeted hPSC knock-in lines with single base substitutions in a relatively short period of time (Miyaoka et al., 2014). Another recently-developed hPSC genome editing assessment tool is based on next generation sequencing (Yang et al., 2013). This genome editing assessment system (GEAS) quantitates gene-editing efficiency with HDR being measured by the percentage of reads containing precise base pair mismatches, whereas NHEJ efficiency is measured by the percentage of reads carrying indels (Yang et al., 2013). The main advantage of this approach is its sensitivity as it can detect HDR rates

of down to 0.007% (Yang et al., 2013). In the sib-selection method (Miyaoka et al., 2014), with each round of ddPCR one well of a 96-well plate with the highest percentage of targeted cells is passaged to a new 96-well plate and so on. In contrast, the GEAS method relies on *a priori* HDR assessment in an hPSC population (Yang et al., 2013). This latter method has the advantage of not only detecting single base pair substitutions, but also other types of precisely engineered indels or knock-ins. In addition, the advantage of GEAS over gel-based analysis of hPSC gene knockout clones (NHEJ) is that GEAS is able to detect single base indels, whereas the gel based method has a much lower resolution of down to 5–10 bases. Genome edited cell lines can be frozen for preservation upon expansion from the 96-well plate into larger cell culture vessels, or as 96 colonies within the 96-well microplate itself.

Concluding remarks

The field of genome engineering is rapidly evolving due to new technological developments. The ability to combine human pluripotent stem cell-based technology with state-of-the-art gene editing technology is impacting basic and applied biology research by generating better *in vitro* disease models, chemical screens and cell-based therapies. Though genome editing in human pluripotent stem cells has historically been very difficult due to the inefficiency of HDR in hPSCs (Zwaka and Thomson, 2003), the development of custom-engineered endonucleases to precisely target DNA DSBs substantially increased the efficiency of HDR-based gene editing in hPSCs (Ding et al., 2013b; Hockemeyer et al., 2009; Hockemeyer et al., 2011). HDR-based gene editing can be further augmented by modulating the NHEJ pathway (Chu et al., 2015; Maruyama et al., 2015; Yu et al., 2015).

The desired type of gene mutation, insertion, or deletion dictates the type of custom-engineered nuclease to use as well as the design of its DNA binding domain (TALEN) or CRISPR guide RNA (CRISPR/Cas9), and DNA donor template (Table 2). Although TALENs have been used very successfully to genome engineer hPSCs, their popularity as a gene editing tool in hPSCs is dwindling mainly due to the ease and versatility of the CRISPR/Cas9 platform. Most importantly, the CRISPR/Cas9 system more efficiently generates indels at the target site than do TALENs targeting the same locus in hPSCs (Ding et al., 2013b; Merkle et al., 2015). One major disadvantage of the CRISPR/Cas9 system compared to TALENs is the bigger potential for off-target cutting because of mismatch tolerance of the guide RNAs (Fu et al., 2013). A number of guide RNA and Cas9 design adjustments decrease off-target cleavage of the CRISPR/Cas9 system dramatically (Fu et al., 2014; Hsu et al., 2013; Merkle et al., 2015; Ran et al., 2013a).

Sustained expression of CRISPR guide RNAs and Cas9 nuclease from transfected plasmid DNA can exacerbate both off- and on-target nuclease activity. Delivery of custom-engineered nucleases either as mRNA or protein is as efficient as plasmid DNA derived nuclease in targeting hPSC loci while decreasing off-target indel accumulation (Kim et al., 2014; Liang et al., 2015). Three studies have been published recently showing a near absence of off-target cleavage after targeting hPSCs with TALENs and CRISPR/Cas9, and subsequent genome-wide sequence analysis (Smith et al., 2014; Suzuki et al., 2014; Veres et

al., 2014). Most SNPs and indels found in these studies are attributed to prolonged culture of hPSC lines.

Powerful applications of the CRISPR/Cas9 gene editing platform are under constant innovation. Whole genome gRNA libraries (genome-wide CRISPR knockout screen – GeCKO) have been used to dissect gene function in hPSCs (Shalem et al., 2014). The ubiquitous transcribed AAVS1 “safe harbor” locus has been used to introduce a Cas9-GFP cassette under a doxycycline inducible promoter in different hPSC lines, resulting in inducible genome editing with transient expression of Cas9 (Gonzalez et al., 2014). These and other innovative applications of the CRISPR/Cas9 system are changing the face of *in vitro* genetics studies.

In this Protocol Review we have described a general TALEN- and CRISPR/Cas9-based hPSC genome editing workflow and pointed out experimental considerations. Although this Review primarily focuses on the use of TALEN and the *S. pyogenes* Cas9 nuclease, a new repertoire of TALE RVDs (Miller et al., 2015) and new orthologues of Cas9 nucleases and their cognate PAM sites (Hou et al., 2013; Kleinstiver et al., 2015; Ran et al., 2015) will ultimately allow targeting any genomic locus for sequence engineering. In combination with improvements in nuclease delivery methods for hPSC engineering, the TALEN and CRISPR/Cas9 gene editing platforms now present a formidable molecular toolbox to study stem cell biology and improve hPSC-based disease models.

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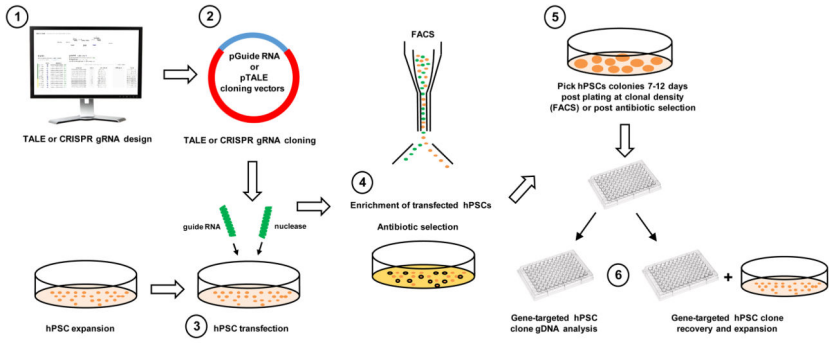

Figure 1. Human pluripotent stem cell gene editing workflow

(1) *In silico* design of the TALE array or CRISPR guide RNA. (2) Cloning and construction of nuclease and guide RNA vectors, either as DNA, mRNA or protein, (3) for transfection or transduction of single cell hPSCs. (4) After transfection or transduction, gene targeted cells can either be selected with FACS if the transfected vectors contain a fluorescent reporter, or antibiotics if there is a selection marker present in the nuclease vector. (5) One to two weeks after enrichment of targeted cells, hPSC colonies (6) are picked and expanded for genomic DNA analysis and for targeted clone recovery and expansion if genome editing was successful.

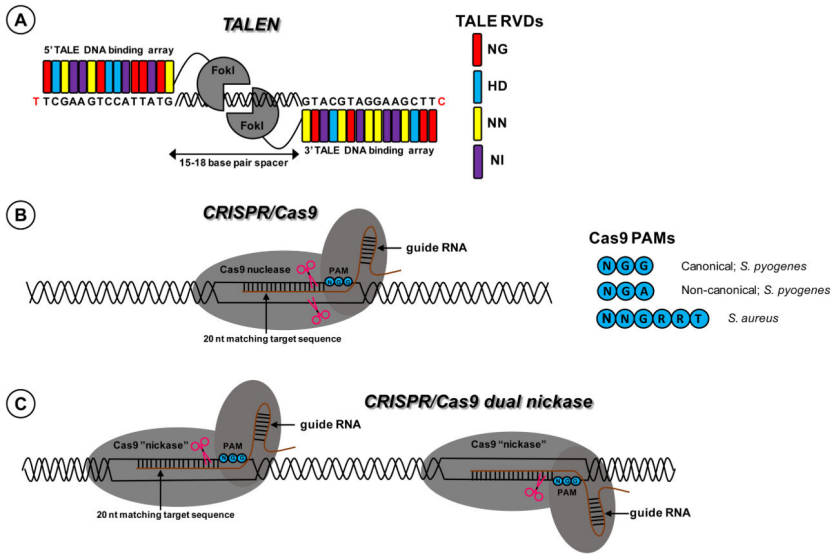

Figure 2.

Schematic showing (A) TALE DNA binding array in this example comprising 15 RVDs each binding their cognate nucleotide in the target sequence. The TALE binding arrays each are fused to a FokI monomer. In order for FokI dimerization and DSB to occur, there needs to be a DNA spacing sequence of 15 – 18 nucleotides between the 5′ and 3′ TALE binding array. The four canonical TALE RVDs are shown as well. (B) The CRISPR/Cas9 system consists of a guide RNA that contains a 20 nucleotide long target DNA matching sequence immediately upstream of a protospacer adjacent motif (PAM) that for *S. pyogenes* Cas9 is NGG. Without this PAM site the Cas9 nuclease is unable to form a complex with the guide RNA and introduce a double stranded break. (C) So called Cas9 nickase (Cas9D10A) is an engineered wildtype Cas9 that creates a DNA nick instead of a DSB. Co-expression of two sgRNAs in each other's vicinity with Cas9 nickase will create DNA nicks essentially forming a DSB. The dual nickase approach as shown in this schematic has been shown to increase specificity of gene editing (see text for details). Different Cas9 orthologues each with their own PAM requirements have been used for gene editing, where the *S. pyogenes* and *N. meningitides* have been successfully used in hPSC gene editing.

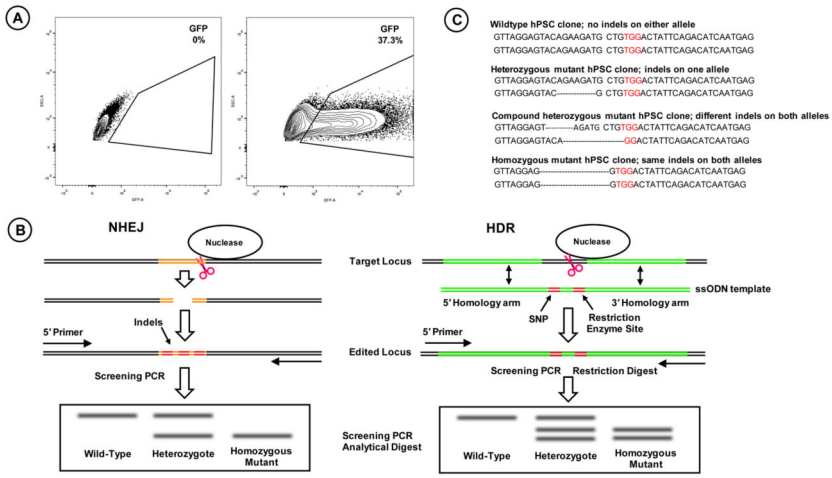

Figure 3.

(A) Example of FACS enrichment of transfected hPSCs. Left panel shows mock electroporated hPSCs with no GFP positive cells, whereas the right panel shows hPSCs electroporated with sgRNA and Cas9-GFP. (B) NHEJ-mediated indel formation in FACS enriched hPSCs creates heterozygous and homozygous frame-shift causing mutations resulting in gene knockout. Alternatively, co-expression of an ssODN containing a SNP and a restriction enzyme site, with Cas9-GFP and a sgRNA results in HDR-mediated introduction of the SNP in the hPSC genome. (C) Upon PCR screening of FACS enriched hPSCs, Sanger sequencing of positive clones confirms and identifies genotype. Shown are potential outcomes after gene editing with Cas9-GFP and a sgRNA.

Table 1

Online resources for *in silico* CRISPR/Cas9 and TALEN design

Website*	Application	Reference
http://crispr.mit.edu/	sgRNA/CRISPR design and off-target prediction	Hsu et al., 2013
https://tale-nt.cac.cornell.edu/	TALE design and off-target prediction	Doyle et al., 2012
http://zifit.partners.org/ZiFiT/	ZFN, TALEN and sgRNA/CRISPR design	Sander et al., 2010
http://www.e-talen.org/E-TALEN/	TALEN design	Heigwer et al., 2013
http://www.e-crisp.org/E-CRISP/	sgRNA/CRISPR design	Heigwer et al., 2014
https://chopchop.rc.fas.harvard.edu/	TALEN and sgRNA/CRISPR design and off target prediction	Montague et al., 2014
http://www.rgenome.net/	sgRNA/CRISPR design and off-target prediction	Bae et al., 2014 Bae et al., 2014
http://crispr.cos.uni-heidelberg.de/	sgRNA/CRISPR design and off-target prediction	Stemmer et al., 2015
https://crispr.bme.gatech.edu/	sgRNA/CRISPR off-target prediction	Cradick et al., 2014
http://bao.rice.edu/Research/BioinformaticTools/prognos.html	ZFN and TALEN off-target prediction	Fine et al., 2013
http://watcut.uwaterloo.ca/template.php	Design of silent mutations to introduce or remove restriction sites to aid hPSC clonal analysis	NA
http://tide.nki.nl/	CRISPR/Cas9 genome editing assessment tool	Brinkman et al., 2014†

*Most of these resources are for design of TALE DNA binding arrays and sgRNA sequences as well as for the design of restriction sites in ssODNs or plasmid DNA templates.

†This online tool quantifies indels after CRISPR/Cas9-mediated gene targeting, cannot be used to determine efficiency of indel generation after TALEN-mediated gene targeting

Table 2

Considerations and recommendations for hPSC gene editing using TALEN and CRISPR/Cas9

Locus accessibility	Locus accessibility for the TALEN or CRISPR/Cas9 nuclease is critical as it determines cleavage activity to a great extent. If permitted avoid 5mC present in CpG islands when using TALENs. For both TALEN and CRISPR/Cas9 nucleases avoid regions that are DNaseI hyposensitive.	Hsu et al., 2013 Chari et al., 2015 Valton et al., 2012
"Safe harbor" loci	Ubiquitous transcribed loci in hPSCs such as the AAVS1 on Chr. 19 and H11 on Chr. 22 are relatively easy to target for gene engineering without affecting hPSC growth and proliferation. These loci are often used to insert reporter genes.	Hockemeyer et al., 2011 Luo et al., 2014 Zhu et al., 2014
Gene editing event - Gene KO	Target start codon and avoid sites in the end of coding sequence. Choose target site residing in common exon	Kim et al., 2013
Gene editing event - Single base substitution	CRISPR/Cas9 or TALEN in combination with the use of ssODNs are very efficient to generate hPSC lines with single base substitutions. Inclusion of a silent mutation encoding or removing a RE site helps with screening gene edited clones using conventional PCR. If possible, design ssODNs that destroy the PAM site to avoid indel generation after successful gene editing	Ding et al., 2013a,b Miyaoka et al., 2014 Merkle et al., 2015
Gene editing event - larger deletions, generation of reporter lines	Efficient TALENs have been described for HDR-mediated integration of gene cassettes, in particular for the AAVS1 locus. CRISPR/Cas9 remains a very efficient nuclease for this purpose. Use of long homology arms with plasmid DNA templates is key for successful cassette integration. Physical separation of CRISPR/dual Cas9 nickase target sites through gene insertion will reduce on-target indel formation.	Hockemeyer et al., 2011 Merkle et al., 2015 Hendel et al., 2014
Nuclease cutting efficiency	Test 2–3 TALENs or sgRNAs in easy to transfect cell line. In general, at least25% cutting efficiency should be observed for successful subsequent gene editing in hPSCs*	Hendel et al., 2015 Hendriks et al., 2015
Nuclease delivery vector	Ideally, Cas9 nuclease should be delivered as protein (complexed with in vitro transcribed sgRNA) or mRNA to avoid on- and off-target cleavage due to prolonged Cas9 expression when delivered as plasmid DNA	D'Astolfo et al., 2015 Zuris et al., 2015 Kim et al., 2014
Nuclease delivery method	Electroporation of nucleases and sgRNA is efficient and relatively cost-effective but requires 10 times more hPSCs than nucleofection or transfection. Depending on the hPSC line used settings for both electroporation and nucleofection may require (extensive) optimization, whereas the newer generation lipid- based transfection reagents are cost-effective regarding their ease of use and amount of hPSCs/reagents needed.	Zwaka et al., 2003 Cai et al., 2007 Ma et al., 2012 Hendriks et al., 2015

* This is a general guideline; not all successful cleaving sgRNAs or TALENs tested in e.g. 293Ts, will cleave successfully in hPSCs (locus dependent).