Published in final edited form as:
Cell Stem Cell. 2013 April 4; 12(4): 393–394. doi:10.1016/j.stem.2013.03.006.

Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs

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Transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems are new classes of genome-editing tools that target desired genomic sites in mammalian cells (Miller et al., 2011; Hockemeyer et al., 2011; Cong et al., 2013; Mali et al., 2013; Jinek et al., 2013). TALENs bind as a pair around a genomic site, in which a double-strand break (DSB) is introduced by a dimer of FokI nuclease domains. Recently published type II CRISPR/Cas systems use Cas9 nuclease that is targeted to a genomic site by complexing with a synthetic guide RNA that hybridizes a 20-nucleotide DNA sequence ("protospacer") beginning with G and immediately preceding an NGG motif recognized by Cas9—constituting a $G(N)_{19}NGG$ target DNA sequence—resulting in a DSB three nucleotides upstream of the NGG motif (Jinek et al., 2012). However it is generated, the DSB instigates either non-homologous end-joining (NHEJ), which is error-prone and conducive to frameshift mutations (indels) that knock out gene alleles, or homology-directed repair (HDR), which can be exploited with the use of an exogenously introduced double-strand or single-strand DNA repair template to knock in or correct a mutation in the genome.

We recently reported the use of a TALEN genome-editing system to rapidly and efficiently generate mutant alleles of 15 different genes in human pluripotent stem cells (hPSCs) as a means of performing rigorous disease modeling (Ding et al., 2013); the proportions of clones bearing at least one mutant alelle ranged from 2%–34%. Although one example of the use of CRISPRs in hPSCs has been reported (Mali et al., 2013), the efficiency of allele targeting was only 2%–4% (albeit in unsorted cells, in contrast to our system; see below).

We sought to compare the relative efficacies of CRISPRs and TALENs targeting the same genomic sites in the same hPSC lines with the use of the same delivery platform as we described previously (Ding et al., 2013). In the TALEN genome-editing system, we used the CAG promoter to cotranslate (via a viral 2A peptide) each TALEN with green fluorescent protein (GFP) or red fluorescent protein (RFP). For CRISPRs, we subcloned a human codon-optimized Cas9 gene with a C-terminal nuclear localization signal (Mali et al., 2013) into the same CAG expression plasmid with GFP, and we separately expressed the guide

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The authors report no relevant conflicts of interest.

RNA (gRNA) from a plasmid with the human U6 polymerase III promoter (Mali et al., 2013). The 20-nucleotide protospacer sequence for each gRNA was introduced using polymerase chain reaction (PCR)-based methods. Whether using TALENs or CRISPRs, equal amounts of the two plasmids were co-electroporated into hPSCs—either 25 μg of each plasmid, or 15 μg of each plasmid along with 30 μg of a DNA repair template if attempting knock-in—followed by fluorescence-activated cell sorting (FACS) after 24–48 hours, clonal expansion of single cells, and screening for mutations at the genomic target site via PCR.

We designed gRNAs matching $G(N)_{19}NGG$ sequences in seven loci in six genes—*AKT2*, *CELSR2*, *CIITA*, *GLUT4*, *LINC00116*, and *SORT1*—that we had previously successfully targeted with TALENs (Ding et al., 2013) and one locus, in *LDLR*, that we had not. We found that in our system CRISPRs consistently and substantially outperformed TALENs across loci and hPSC lines (see Table S1). The TALENs yielded clones with at least one mutant allele at efficiencies of 0%–34%, but matched CRISPRs yielded mutant clones at efficiencies of 51%–79% (Table S1). Just as with TALENs, CRISPRs produced a variety of indels of sizes ranging from one nucleotide to several dozen nucleotides in size, centered on the predicted cleavage sites, suggesting that NHEJ mutagenesis occurs in the same way regardless of whether CRISPRs or TALENs are used. We also found that CRISPRs readily generated homozygous mutant clones (7%–25% of all clones; Table S1) as discerned by sequencing. We also attempted to knock in E17K mutations into *AKT2* using a 67-nucleotide single-stranded DNA oligonucleotide as previously described (Ding et al., 2013). Although the predicted CRISPR cleavage site lay 11 and 13 nucleotides from the point mutations, respectively, the CRISPR yielded knock-in clones at a rate of 11%, whereas TALENs yielded only 1.6% (Table S1).

We speculate that the superior performance of CRISPRs in our system is due to the Cas9 protein being more highly expressed and better tolerated than TALENs in hPSCs, as we routinely observed earlier (<24 hours vs. 48 hours) and more robust (5%–10% of cells vs. <1%–2% of cells) GFP expression following electroporation. Other factors may include intrinsic DNA-unwinding activity of Cas9 and impaired TALEN binding on methylated DNA. It is possible that further optimization of the TALEN system that we developed could improve its efficiency and reduce the differential that we observe.

Two potential disadvantages of CRISPRs are worth noting. First, the requirement for a $G(N)_{19}NGG$ target sequence somewhat limits site selection. Because either DNA strand can be targeted, a target sequence occurs on average every 32 basepairs. This is no barrier for gene knockout, where any coding sequence can be targeted, but it may present difficulties when trying to knock in or correct a mutation at a specific location. However, the requirement for a G at the start of the protospacer is dictated by the use of the U6 promoter to express the gRNA, and alternative CRISPR/Cas systems can relieve this requirement (Cong et al., 2013).

Second, the extent of CRISPR off-target effects remains to be defined. Previous analyses have suggested that one-nucleotide mismatches in the first half of the protospacer are better tolerated than mismatches in second half (Jinek et al., 2012; Cong et al., 2013). None of the genomic sequences we targeted with CRISPRs have perfectly-matched or one-mismatch sequences elsewhere in the genome. For the *AKT2* sequence, there is a two-mismatch sequence differing at nucleotides 1 and 3, in the more “tolerant” half of the protospacer; we obtained zero clones with mutations at this potential off-target site, as compared to 61% at the on-target site (Table S1), suggesting that at least in this instance off-target effects are not likely to be a significant concern. Judicious selection of target sites may well be able to minimize systematic off-target effects. Nevertheless, clear-cut determination of the relative risk for both TALEN- and CRISPR-based approaches will require a systematic analysis.

It is important to highlight that all of these genome-editing technology approaches are still very much in development, and more detailed and comprehensive studies will be needed to determine their relative merits in different experimental circumstances. From a practical standpoint, CRISPRs are easier to implement than TALENs, as each TALEN pair must be constructed *de novo*, whereas for CRISPRs the Cas9 component is fixed and the gRNA requires only swapping of the 20-nucleotide protospacer. Given this consideration and our observations of substantially increased efficiency through replacing TALENs with CRISPRs in an otherwise identical system, we would suggest that CRISPRs might well prove to be a very powerful and broadly applicable tool for the stem cell community.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a Roche Postdoc Fellowship (Q.D.), grants R00-HL098364 (K.M.), R01-HL118744 (K.M.), and R01-DK097768 (K.M., C.A.C.) from the United States National Institutes of Health (NIH), a Cardiovascular Program Pilot Grant from the Harvard Stem Cell Institute (K.M.), and Harvard University. We thank Adrian Veres for advice and George Church and Prashant Mali for generously sharing plasmids.

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