## MINI-REVIEW

# Find and replace: editing human genome in pluripotent stem cells

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## ABSTRACT

**Genetic manipulation of human pluripotent stem cells (hPSCs) provides a powerful tool for modeling diseases and developing future medicine. Recently a number of independent genome-editing techniques were developed, including plasmid, bacterial artificial chromosome, adeno-associated virus vector, zinc finger nuclease, transcription activator-like effecter nuclease, and helper-dependent adenoviral vector. Gene editing has been successfully employed in different aspects of stem cell research such as gene correction, mutation knock-in, and establishment of reporter cell lines (Raya et al., 2009; Howden et al., 2011; Li et al., 2011; Liu et al., 2011b; Papapetrou et al., 2011; Sebastiano et al., 2011; Soldner et al., 2011; Zou et al., 2011a). These techniques combined with the utility of hPSCs will significantly influence the area of regenerative medicine.**

**KEYWORDS**   gene targeting, gene editing, gene correction, pluripotent stem cell

## INTRODUCTION

Gene manipulation by classic homologous recombination is inefficient and laborious in human pluripotent stem cells (hPSCs), which has hampered the development of hPSCs-derived disease model and therapeutic applications. In 2003, Thomson group successfully knocked out *HPRT1* gene and knocked in a reporter at *POU5F1* locus in human embryonic stem cells (hESCs) using an electroporation approach (Zwaka and Thomson, 2003). A few other research groups also successfully obtained gene-modified cell lines with this conventional approach, nevertheless with extremely low efficiency (Urbach et al., 2004; Costa et al., 2007; Irion et al., 2007; Davis et al., 2008; Di Domenico et al., 2008; Bu et al., 2009; Kamei et al., 2009; Ruby and Zheng, 2009; Xue et al., 2009; Bu et al., 2010; Buecker et al., 2010; Fischer et al., 2010; Goulburn et al., 2011). To overcome technical limitations, novel gene editing strategies have been developed to enhance targeting efficiency in hPSCs, such as bacterial artificial chromosome (BAC) (Song et al., 2010), adeno-associated virus vector (AAV) (Khan et al., 2010; Asuri et al., 2011), zinc finger nuclease (ZFN) (Lombardo et al., 2007; Hockemeyer et al., 2009; Zou et al., 2009), transcription activator-like effecter nuclease (TALEN) (Cermak et al., 2011; Hockemeyer et al., 2011; Miller et al., 2011; Mussolino et al., 2011; Zhang et al., 2011), and helper-dependent adenoviral vector (HDAdV) (Suzuki et al., 2008; Li et al., 2011; Liu et al., 2011b). Here we focus on comparing cons and pros of the above novel techniques to explore their potential for efficient and safe gene targeting in hPSCs. The applications and properties of novel gene editing tools are summarized in Table 1.

## BAC

Recently, two independent groups reported successful gene targeting in hPSCs using BAC-based strategy (Song et al., 2010; Howden et al., 2011). Song et al. constructed BAC vectors containing long homologous DNA fragments to target human *ATM* and *TP53* alleles with a relative high efficiency (~27%) after 2 rounds of electroporation and drug selection (Song et al., 2010). Thomson group used a similar BAC-based approach and corrected a single base-pair mutation in human induced pluripotent stem cells (hiPSCs) from a patient

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**Table 1**  Summary of recently developed gene editing tools

| Name | Targeted genomic loci | Cell type | Mutation/Insertion/Correction | References |
|---|---|---|---|---|
| Plasmid | *HPRT1* | hESCs, hiPSCs | Mutation/Correction /Insertion | Zwaka and Thomson, 2003; Urbach et al., 2004; Di Domenico et al, 2008; Sakurai et al., 2010; Buecker et al., 2010 |
| | *OCT3/4* | hESCs | Insertion | Zwaka and Thomson, 2003; Braam et al., 2008; Kamei et al., 2009 |
| | Three loci | hESCs | Insertion | Costa et al., 2007 |
| | *ROSA26* | hESCs | Insertion | Irion et al., 2007 |
| | *MIXL1* | hESCs | Insertion | Davis et al., 2008 |
| | *ISL1* | hESCs | Insertion | Bu et al., 2009 |
| | *Fezf2* | hESCs | Insertion | Ruby and Zheng, 2009 |
| | *Olig2* | hESCs | Insertion | Xue et al., 2009 |
| | *BAF250a* (*ARID1A*) | hESCs | Mutation | Bu et al., 2010 |
| | *NANOG* | hESCs | Insertion | Fischer et al., 2010 |
| | *NKX2-5* | hESCs | Insertion | Elliott et al., 2011 |
| Baculoviral vector | *AAVS1* | hESCs | Insertion | Ramachandra et al., 2011 |
| AAV | *HPRT1* | hESCs, hiPSCs | Insertion/Mutation/ Correction | Mitsui et al., 2009; Khan et al., 2010 |
| | *NANOG* | hESCs, hiPSCs | Insertion | Mitsui et al., 2009 |
| | *HGMA1* | hESCs | Insertion | Khan et al., 2010 |
| | *Type I collagen* | Mesenchymal stem cells | Correction | Deyle et al., 2011 |
| BAC | *ATM* | hESCs | Mutation | Song et al., 2010 |
| | *TP53* | hESCs | Mutation | Song et al., 2010 |
| | *HPRT1* | hESCs | Mutation | Song et al., 2010 |
| | *OAT* | hiPSCs | Correction | Howden et al., 2011 |
| ZFN | *GFP* | hESCs | Correction | Zou et al., 2009 |
| | *PIG-A* | hESCs, hiPSCs | Mutation / Correction | Zou et al., 2009 |
| | *OCT3/4* | hESCs | Insertion | Hockemeyer et al., 2009 |
| | *AAVS1* | hESCs, hiPSCs | Insertion | Hockemeyer et al., 2009; Lombardo et al., 2011 |
| | *PITX3* | hESCs, hiPSCs | Insertion | Hockemeyer et al., 2009 |
| | *CCR5* | Hematopoietic K-562 cells, Jurkat cells, lymphoblastoid cells, cord blood CD34+ hematopoietic progenitor cells, hESCs | Insertion | Lombardo et al., 2007; Lombardo et al., 2011 |
| | *IL2RG* | K-562 cells, lymphoblastoid cells, HEK293 | Insertion/Correction | Lombardo et al., 2007 |
| | *TP53*, DNMT1 shRNA | hESCs | Insertion | DeKelver et al., 2010 |
| | gp91[phox] | hiPSCs | Correction | Zou et al., 2011b |
| | α-Synuclein (α-*SYN*) | hESCs, hiPSCs | Correction/ Mutation | Soldner et al., 2011 |
| | β-globin (*HBB*) | hiPSCs | Correction | Zou et al., 2011a |
| | α$_1$-antitrypsin (*A1AT*) | hiPSCs | Correction | Yusa et al., 2011 |
| HDAdV | *HPRT1* | hESCs | Mutation | Suzuki et al., 2008 |

| Name | Targeted genomic loci | Cell type | Mutation/Insertion/Correction | References |
|------|----------------------|-----------|------------------------------|------------|
| | *LMNA* | hiPSCs, Olfactory ectomesenchymal stem cells | Correction | Liu et al., 2011b |
| | β-globin (*HBB*) | hiPSCs | Correction | Li et al., 2011 |
| TALEN | *CCR5* | HEK293T, K562 | Insertion/Mutation | Miller et al., 2011; Mussolino et al., 2011 |
| | *IL2RG* | HEK293T | Insertion | Mussolino et al., 2011 |
| | *NTF3* | HEK293 | Mutation | Miller et al., 2011 |
| | *OCT4* exon 1 | hESCs, hiPSCs | Insertion | Hockemeyer et al., 2011 |
| | *OCT4* STOP codon | hESCs, hiPSCs | Insertion | Hockemeyer et al., 2011 |
| | *AAVS1* | hESCs, hiPSCs | Insertion | Hockemeyer et al., 2011 |
| | *PITX3* exon 1 | hESCs, hiPSCs | Insertion | Hockemeyer et al., 2011 |
| | *PITX3* STOP codon | hESCs, hiPSCs | Insertion | Hockemeyer et al., 2011 |
| | *HPRT1* | HEK293T | Mutation | Cermak et al., 2011 |

hESCs, human embryonic stem cells; hPSCs, human pluripotent stem cells; hiPSCs, human induced pluripotent stem cells; AAV, adeno-associated virus vector; BAC, bacterial artificial chromosome; ZFN, zinc finger nuclease; HDAdV, helper-dependent adenoviral vector; TALEN, transcription activator-like effecter nuclease.

with gyrate atrophy after two rounds of homologous recombination (Howden et al., 2011). BAC vectors seem to represent a simple and feasible method to edit genome in hPSCs. They are more efficient to transfer long homologous arms with better accuracy relative to classic homologous recombination in hPSCs. However, there are certain limitations of BAC-based gene targeting including low delivery efficiency due to its large size, potential intracellular degradation of long naked BAC DNA, and difficulty to design probes or primers to confirm gene targeting by Southern blot and long PCR.

## ZFN

ZFNs are a class of artificial nucleases that stimulate frequency of gene editing by producing double-strand breaks at specified DNA sequence. Theoretically, ZFNs could be used to create gene knock-ins and knock-outs into any gene loci in various somatic cell types. In 2007, Lombardo et al. used a vector based on integrase-deficient lentivirus (IDLV) to transfer ZFN and specific donor DNA into hESCs and human lymphoblastoid cells and successfully integrated 800-bp cDNA into *IL2RG* locus, mutations of which lead to X-linked severe combined immunodeficiency (SCID-X1). In this study, they observed 5% targeting efficiency in hESCs without drug selection (Lombardo et al., 2007). Thereafter, ZFNs were widely used to create or correct specific mutations in hPSCs (Hockemeyer et al., 2009; Zou et al., 2009; Asuri et al., 2011; DeKelver et al., 2010; Lombardo et al., 2011; Mussolino et al., 2011; Sebastiano et al., 2011; Soldner et al., 2011; Yusa et al., 2011; Zou et al., 2011a, 2011b). In these studies, hPSCs retained characterizations of 'stemness' and normal cell karyotype while achieving permanent and heritable gene modifications at specific endogenous loci. Recently, Jaenisch group corrected an *α-SYNUCLEIN* point mutation in

Parkinson's disease patient-derived hiPSCs (Soldner et al., 2011). In this study, > 1% efficiency with corrected *α-SYNUCLEIN* at both alleles was achieved in the same settings without any genomic "scar" caused by selection markers or molecular footprints. More recently, a novel gene targeting method by combining a piggyBAC transposon and ZFN was reported to correct defected *α₁-ANTITRYPSIN* gene in patient-derived hiPSCs in the absence of genomic "scars" (Yusa et al., 2011). Compared to plasmids- or BAC-based gene targeting strategies, which require two rounds of homologous recombination to modify both alleles, ZFNs-mediated homologous recombination allows biallelic edits at the same time. Therefore, ZFN strategy circumvents the necessity of repeated editing process, improves editing efficiency and saves time for researchers. In addition, ZFNs are able to efficiently target silenced genomic loci in hPSCs (Hockemeyer et al., 2009).

ZFNs-based gene targeting techniques are not flawless. One of the main concerns is the off-target effects. Two studies showed that ZFNs cleaved off-target sites in genome besides the *in vitro* predicted DNA sequences, which might cause chromosomal instability and genotoxicity (Gabriel et al., 2011; Pattanayak et al., 2011). In addition, even on-target effect of ZFNs can cause genotoxicity, as repair of ZFN cleavage sites also leads to frequent mutations (Lombardo et al., 2007; Cathomen and Joung, 2008) and abnormal integrations of donor DNA (Hockemeyer et al., 2009).

## TALEN

TALEN was recently developed as a novel technique for gene targeting in hPSCs (Miller et al., 2011; Zhang et al., 2011). Similar to ZFNs, engineered TALENs were generated by fusing FokI catalytic domain capable of inducing double

strand breaks (DSBs) and TALE repeats domain to recognize specific DNA sequence (Kiefer, 2011). TALENs exhibited similar (Mussolino et al., 2011) or higher (Miller et al., 2011) cleavage activity for efficient gene targeting and fewer off-target effects to reduce nuclease-associated toxicity relative to ZFNs system (Mussolino et al., 2011). TALENs seem very promising for targeted genome engineering. Nevertheless, given that TALEN repeats are larger than that of zinc finger proteins (ZFPs), effective delivery methods should be developed to obtain higher gene targeting efficiency (Miller et al., 2011). Another unknown factor that should be considered is their off-target effects since TALENs employ a similar nuclease-based gene editing mechanism like ZFNs. Thus their potential off-target effects and genotoxicity need to be carefully examined in future studies.

### HDAdV

An alternative method based on HDAdV has been shown to be highly efficient for gene editing in hPSCs with transient gene transfer efficiency as high as ~100% (Suzuki et al., 2008; Liu et al., 2011b). HDAdV-based gene targeting was first reported by Suzuki et al. in 2008 (Suzuki et al., 2008), which provided a "proof of principle" evidence that this technology could be used for effective gene editing in hPSCs. Recently, we presented evidence for the advantage of HDAdV-based gene correction in patient-derived hiPSCs and mesenchymal stem cells (Liu et al., 2011b). Different from AAV-based method, HDAdV minimizes safety issues that virus vectors may bear, as the essential sequence for viral duplication and packaging is provided by a separate helper virus. By utilizing this system, we have successfully corrected mutated *LMNA* in progeria patients-specific hiPSCs with high efficiency and (epi-)genomic integrity (Liu et al., 2011a, 2011b). This new system offers several advantages for gene manipulation in human cells. First, toxic viral elements are removed from HDAdV, therefore allowing incorporation of long homologous DNA (up to 37 kb) into vector backbone (Suzuki et al., 2008). This high cloning capacity of HDAdV enables flexible use of homology arms, promoters/enhancers, selection markers, reporter genes or even combinations with other gene editing tools such as ZFNs or TALENS. Second, HDAdV gives a high efficiency in genomic editing in hPSCs (for *LMNA* locus, the gene targeting efficiency is about 90%), probably due to its efficient DNA delivery and long retention time in hPSCs (Liu et al., 2011b). Third, as demonstrated by successfully targeting two different mutations in *LMNA* and subsequent single-nucleotide polymorphism (SNP) analyses, a single HDAdV (*LMNA*-c-HDAdV) can be potentially applied to correct over 200 different laminopathies-associated *LMNA* mutations (Liu et al., 2011b). Fourth, similar to ZFNs, HDAdV exhibits excellent performance in editing transcriptionally inactive genomic loci (Li et al., 2011; Liu et al., 2011b). Disadvantages of the

HDAdV-based gene correction include targeting one allele upon one round of transduction and retaining a tiny molecular footprint (*FRT* site) in the genome, although no evidence shows that the *FRT* site could affect any gene function (Li et al., 2011; Liu et al., 2011b). Another concern is its relatively complicated process associated with HDAdV, which may hamper its broad range of applications.

### COMPARISON OF ZFN AND HDAdV

As mentioned above, ZFN and HDAdV are two main gene editing tools utilized in hESCs and hiPSCs. Recently, three laboratories independently reported successful gene corrections in hiPSCs derived from sickle cell disease (SCD) patients using ZFNs (Sebastiano et al., 2011; Zou et al., 2011a) as well as HDAdV (Li et al., 2011). SCD is a genetic disease caused by mutations in *β-GLOBIN* (*HBB*) gene, leading to severe anemia in adults. Zou et al. utilized ZFNs-based method to generate a precise correction of *HBB* gene in patient hiPSCs. Four hiPSC clones in which *HBB* mutation was precisely corrected were obtained after selection and careful examination (Zou et al., 2011a). In another study, Sebastiano et al. also applied ZFNs-based approach to correct the same mutation in hiPSCs derived from SCD patients. They obtained an average of 9.8% targeting efficiency and no random insertion was identified in all correctly targeted clones. The authors found no off-target effect within 12 most likely off-target genomic sites according to DNA sequencing, whereas complete genome should be examined to warrant a more accurate conclusion (Sebastiano et al., 2011). More recently, Belmonte group reported an efficient correction of *HBB* mutations in SCD-specific hiPSCs using an HDAdV-based strategy (Li et al., 2011). Indeed, all possible mutations in *HBB* can be precisely corrected with a single HDAdV correction vector (*HBB*-c-HDAdV) containing whole wild-type *HBB* genomic sequence (Li et al., 2011). More importantly, both gene-targeting and gene-correction efficiency at *HBB* locus in different hiPSCs lines were extremely high (80% to 100%), despite of the fact that *HBB* is transcriptionally inactive in hPSCs. In addition, it was demonstrated that no random integration of *HBB*-c-HDAdV occurred in genomic DNA, as determined by a fluorescence *in situ* hybridization (FISH) analysis (Li et al., 2011). All together, these novel gene-editing techniques have their own pros and cons. ZFNs-mediated gene editing strategy is capable of targeting both alleles in one targeting process without leaving molecular footprint, but may introduce unexpected DSBs and mutations in the genome. Due to a "one-step" gene knock-out or knock-in protocol, ZFNs (or TALENs) could probably be of a better choice for basic biomedical research (Kim and Svendsen, 2011). In contrast, HDAdV-based gene targeting technique exhibits a consistently high gene-targeting efficiency and leaves a possibility of modifying all potential mutations in a large genomic region, without random

integration and (epi-)genetic aberrance (Li et al., 2011; Liu et al., 2011b). Its disadvantages of laborious construction and complex procedure may hamper its broad use in basic biomedical research. Rather, HDAdV-based technique could be a superior choice for a robust and safe strategy to correct gene mutations in patient-specific hiPSCs prior to transplantation (Kim and Svendsen, 2011).

## CONCLUSION AND PERSPECTIVE

Employing genetically corrected mutation-free hiPSCs into clinical therapy is a promise of regenerative medicine (Sancho-Martinez et al., 2011). The development of robust and safe genome editing tools represents one of the most critical steps to make personalized regenerative medicine a reality. It is essential to confirm that there is no unexpected mutation in corrected hiPSCs prior to clinical transplantations. To this end, whole genome sequencing is strongly recommended for therapeutic purposes of corrected hiPSCs. It would be also interesting to examine whether it is possible to perform direct gene correction in patient somatic cells like fibroblasts with combined HDAdV and ZFN (or TALEN) strategies. In this case, desired cell types such as neurons obtained by trans-differentiation from gene-corrected somatic cells could be used for both disease study and therapeutic applications in the future (Jopling et al., 2011).

## ACKNOWLEDGEMENTS

## ABBREVIATIONS

AAV, adeno-associated virus vector; BAC, bacterial artificial chromosome; DSBs, double strand breaks; HDAdV, helper-dependent adenoviral vector; hESCs, human embryonic stem cells; hPSCs, human pluripotent stem cells; IDLV, integrase-deficient lentivirus; SCD, sickle cell disease; SCID-X1, X-linked severe combined immunodeficiency; SNP, single-nucleotide polymorphism; TALEN, transcription activator-like effecter nuclease; ZFN, zinc finger nuclease; ZFPs, zinc finger proteins

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