Genome Editing in Stem Cells

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Abstract Progress in the derivation, expansion and differentiation of human embryonic stem cells in vitro has led to the development of a valuable platform for studying disease as well as providing an inexhaustible resource for cell replacement therapies. Targeted modification of stem cells utilising synthetic nucleases provides a means to genetically alter the genome in an efficient, site-specific manner. Editing with this approach offers a possibility to improve disease modelling in both patient-derived induced pluripotent stem cells (iPSCs) and embryonic and adult stem cells. It is possible that in the future, patients with monogenic diseases could be treated or cured by transplantation of autologous cells that have been altered to have the corrected genetic sequence. Currently, genetic modification of stem cells in vitro is most often achieved using siRNA or viral transduction; however, this can result in only transient modulation or raise the possibility of insertional mutagenesis, respectively. Targeted genome editing using zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and more recently clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR-associated protein 9 (Cas9) have been developed to overcome the drawbacks of gene transfer. In this review, we mechanistically outline these technologies before focusing on their expanding utility within the biomedical sciences.

Keywords Genome editing · Gene insertion · Gene disruption · Induced pluripotent stem cells · Cell therapy · Stem cell models

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Introduction: Mechanisms of Genome Editing

Targeted nuclease technology is dependent on the use of engineered molecules containing a sequence-specific DNA binding domain fused to a non-specific *FokI* endonuclease enzyme that must dimerize for efficient site-specific cleavage. When applied, a double-strand break occurs in the DNA, and the break is repaired by conventional DNA repair mechanisms: non-homologous end joining (NHEJ) or homologous recombination (HR). C_2H_2 zinc fingers (ZF), originally described in *Xenopus laevis* [1], are one of the most common types of DNA binding motifs found in the human genome and are considered the first generation of engineered nuclease DNA binding domains. They are approximately 30 amino acids in length, and each architecturally simple domain (a $\beta\beta\alpha$ -fold) has the capacity to bind to 3 base pairs of DNA [1]. To overcome problems that may arise from ZF targeting non-unique sequences, a number of groups have worked on developing polydactyl ZF proteins utilising a linker sequence to tether multiple ZF together, binding up to 18 base pairs of DNA. This confers specificity in a genome of up to 68 billion base pairs [2–4]. Furthermore, Maeder et al. developed oligomerized pool engineering ('OPEN'), a publicly available highly efficient approach to design multi-finger arrays that can be customised to a DNA fragment of choice [5].

Fifteen years later, an alternative second-generation genome editing platform to ZFNs was established and rapidly came to prominence in the literature. Similar to ZF proteins, TALENs consist of modular DNA binding motifs that target contiguous DNA sequences and are also linked to the *FokI* enzyme to execute site-specific double-strand breaks. However, these proteins, naturally found in plant pathogenic bacteria *Xanthomonas*, are considered to be more accessible to researchers aiming to engineer innovative fusion proteins as a tool for altering the genome [6]. The DNA binding motif of

transcription activator-like (TAL) effector proteins consist of ~35 tandem repeats, and each repeat (consisting of two amino acids) appears to be specific for a single nucleotide [6]. Therefore designing TALENs is more straightforward than ZFNs whilst maintaining similar diversity in sequence design. Moreover, when generating TALENs, there is no requirement for re-engineering the linker or spacer regions between the residues as is necessary when producing ZFNs. Consequently, TALEN production is faster, more economically viable and highly specific. Several plasmid sets are now openly available from repositories (www.addgene.org/) to aid the development of desirable fusion molecules including fast ligation-based automatable solid-phase high-throughput (FLASH) [7], plasmid-based methods such as ‘TAL Plasmids Sequence Assembly Tool’ (Golden Gate) [8] and ligation-independent cloning [9].

The most recent, and arguably most exciting, method for genome editing, the CRISPR/Cas system, was originally described in a broad spectrum of bacteria and archaea as an RNA-mediated defence mechanism via genetic silencing [10]. Collectively, a number of research groups [11–13] deciphered the mechanism and ultimately demonstrated that it could be manipulated to direct sequence-specific DNA cleavage in many model systems including zebrafish [14, 15], mouse [16, 17] and human [18, 19]. The CRISPR/Cas pathway is akin to the RNA interference pathway in eukaryotes as both methods recognise long RNA precursors that are further processed to hinder translation. In the CRISPR system, a guide RNA (gRNA) sequence can be designed to target the genomic locus of choice. Directly following the target sequence is a protospacer adjacent motif (PAM). PAM, along with a seed sequence contained within the gRNA, allows for stable and efficient DNA binding causing a double-strand break mediated by the Cas endonuclease enzyme (Cas9 is most commonly used) [20]. The CRISPR/Cas9-based technology makes genome editing fast (desirable target gRNA plasmids are easily produced), simple and relatively cheap; many component plasmids are also available from freely available repositories. Mali et al. showed that CRISPR/Cas9 genome editing was more efficient than TALENs and ZFNs in combination with the ease of retargeting the system to another locus in the genome [18]. However, the major caveat to this speedy technology is that the target RNA sequence is shorter than those of TALENs and ZFN; thus, it is more difficult to prevent off-target genomic disruption.

DNA cleavage either by Cas9 or *FokI* results in a double-strand break, inducing activation of internal DNA repair mechanism to preserve genomic integrity. The two most common mechanisms by which DNA is religated are NHEJ or homologous recombination. If repair is carried out by NHEJ, the Ku70/Ku80 end-binding protein complex religates the DNA. However, this repair mechanism is executed in an error-prone manner, often resulting in insertions, deletions

Fig. 1 Mechanisms of genome editing by ZFNs, TALENs and CRISPR/Cas9. **a** ZFN bind to target DNA (one zinc finger to 3 bp of DNA) where the *FokI* endonuclease enzyme dimerizes to induce a non-specific double-strand break. TALENs behave in a similar manner to ZFNs; however, one TALEN binds to one base pair of DNA. The CRISPR guide RNA binds to the target DNA and is cleaved by the Cas9 endonuclease enzyme, aided by PAM sequences and a ‘seed sequence’. **b** The resulting cleaved DNA can undergo repair by two cellular mechanisms, NHEJ or homologous recombination. **c** NHEJ is error prone so often introduces ‘indels’. Homologous recombination can be manipulated to insert a transgene, provided the transgene contains flanking recombination sites

and substitutions at the break site [21]. This strategy is exploited during genome editing to mutate or silence target genes. For gene insertion and correction, homologous recombination is manipulated to incorporate donor DNA (most likely a gene) exogenously introduced to the cell on a separate construct, provided the flanking regions contain recombination sites (Fig. 1).

Applications of Genome Editing in Stem Cells

Gene Insertion

There is great potential to treat human monogenic diseases such as spinal muscular atrophy, sickle cell and X-linked adrenoleukodystrophy (X-ALD) by gene complementation or correction. Gene therapy has been used widely to treat monogenic diseases and there have been many recent successes [22–25]. Achieving widespread gene delivery to ameliorate disease is a major challenge beyond proof of concept and has largely been based on improving gene delivery vector technology and long-term transgene expression. To date, some of the most effective gene therapy approaches have involved ex vivo gene therapy of blood cells to treat Wiskott-Aldrich syndrome (WAS) [26], X-linked severe combined immunodeficiency (X-SCID) and adenosine deaminase deficiency (ADA) [27, 28]. X-SCID is caused by mutations in the *IL2RG* gene situated on the X chromosomal at Xq13.1 and affects males by causing defective maturation of T cells and NK cells resulting in severe immunodeficiency. Hacein-Bey-Abina et al. showed that an ex vivo gene complementation strategy using retroviral-mediated gene therapy targeting haematopoietic stem cells resulted in long-term phenotypic correction [27]. Although most patients received therapeutic benefit, two developed leukaemia due to insertional mutagenesis; a nearby oncogene (*LMO2*) became deregulated as a result of random integration [29]. The occurrence of leukaemia was not isolated to the X-SCID patients treated in this study. Between 1999 and 2009, 5 of the 20 participants across 5 independent X-SCID gene therapy trials in Europe developed leukaemia due to enhancer activity of the provirus inserted

into an oncogenic gene region [30]. In the WAS trial, most patients also developed leukaemia [26]. Notwithstanding

oncogene deregulation associated with non-specific genomic integration, there are other impediments to this method of

gene transfer; host immune responses may counteract the benefit conferred with transgene expression and it is not uncommon to encounter long-term transgene silencing as a result of promoter methylation [31]. Artificial nuclease-mediated genome editing could potentially alleviate viral vector-associated risks. Moreover, the levels and frequency of expression of the transgene are under the regulation of the endogenous promoter, so the level of functional protein produced should be equivalent to that of non-patient controls [32]. In 2005, Urnov et al. successfully used ZFNs to target and cleave the endogenous IL2RG gene in cells in vitro [33]. Two years later, Lombardo et al. combined the infectious properties of an integration-deficient lentiviral vectors (IDLV) with the site-specific properties of ZFN to increase the efficiency (13–39 % across different cell types) of DNA insertions at the same locus. A green fluorescent protein (GFP) reporter contained within the homology arms of the IL2RG donor sequence was co-transduced with the ZFN-expressing IDLV, and PCR analysis of the GFP⁺ cells sorted by fluorescence-activated cell sorting (FACS) showed the anticipated integration junctions between the cassette and the IL2RG locus [34]. In the same study, the authors also implemented this system to insert GFP into the CCR5 locus of cord blood CD34⁺ haematopoietic progenitor cells and two human embryonic stem (ES) cell lines, achieving gene targeting at higher levels than previously observed in human stem cells. CCR5 is a chemokine receptor found on the surface of white blood cells that acts as a co-receptor to HIV-1, facilitating viral entry into the host. Individuals carrying a 32-bp deletion within the coding region produce a non-functional receptor and are subsequently resistant to HIV-1 infection [35].

A follow-up study also by the Naldini group in 2014 further supports the clinical translation of ZFN-based correction of monogenic disorders. Using the same IDLV/ZFN system, haematopoietic stem cells (HSCs) from the bone marrow of a 4-month-old X-SCID patient were modified to incorporate the fully functional IL2RG gene, in vitro. They saw long-term reconstitution of IL2RG expression in the lymphoid progeny of the HSCs [36]. This exciting approach has great clinical potential if integration-deficient lentiviral vectors pass relevant safety assessments, and the appropriate stem cell population can be isolated from patients for modification.

Gene Disruption

Although introducing a transgene via HR is an excellent way to exploit the cell internal repair mechanisms to deliver therapeutic benefit, recombination efficiency is poor and may vary with regard to the cell type. It is thought that HR events are dependent on the cell cycle, preferentially occurring in the late S, G₂ and M stages [37]. In the absence of a donor plasmid for HR, NHEJ is the dominant method for double-strand break

repair. Hans Clevers' group optimised gene disruption by NHEJ via the CRISPR/Cas9 system in murine and human intestinal stem cells in 2013. They used a short guide RNA to target the *APC* gene—a negative regulator of the Wnt pathway—and found that only stem cells in which biallelic inactivation occurred had the capacity to proliferate in the absence of the Wnt agonist R-spondin [38].

In 2008, Perez et al. permanently modified the HIV co-receptor CCR5 in CD4⁺ T cells by designing ZFNs to target the first transmembrane domain of the gene and allowing NHEJ mechanisms to introduce random mutations. They noted that 30 % of all sequences analysed had a 5-bp insertion-duplication of the region between the ZFN binding sites [39]. However, they also observed a wide range of other deletion and insertion mutations at the break site. So, although this paper shows great potential to impart HIV-1 resistance in T lymphocytes or bone marrow haematopoietic stem cells from patients, translation to the clinic as a therapy may be hindered by the unpredictable nature of NHEJ repair as a tool for gene disruption.

Designing an artificial nuclease with a recognition sequence targeted to a locus, where the only difference between the endogenous sequence and the disruption donor sequence is the exact bases intended for modification, would allow for the introduction of missense or nonsense mutations in a regulated manner. In recent years, there has been a shift towards this type of genome editing, particularly in the context of pluripotent stem cells.

Genome Editing in Pluripotent Stem Cells

The pioneering work of Yamanaka and co-workers on somatic reprogramming and induction of pluripotency by the forced expression of four transcription factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*) [40] allows for iPSC lines to be generated from patient-derived fibroblasts. This methodology has led to the establishment of a plethora of disease-specific human iPSC lines from patients [41–44]. Targeted differentiation of such iPSC means that the biology of disease can be studied in patient-derived cell types that would otherwise be impossible to obtain, such as those of the central nervous system (CNS) and heart [45, 46]. This remarkable research platform now facilitates the unification of genomics, transcriptomics and biochemistry in disease relevant cells in the lab. Last year, a breakthrough research article was published by Jiang et al. where the authors used ZFNs to model Down's syndrome (trisomy 21). The study demonstrated that it was possible to correct the chromosome 21 gene dosage imbalance by recombining a single non-coding RNA, *XIST* (X-inactivation gene), into the *DYRK1a* locus (21q22) [47•]. *XIST* is responsible for compensating for the difference in dosage of X-linked genes between males (XY) and females (XX). Located on the inactive X chromosome, the non-coding RNA

accumulates across the chromosome, dampening gene activity [48]. One of the remarkable achievements of this study is the size of the doxycycline-controlled transgene that was targeted to the locus of male patient-derived iPSC—21 kb of transgene was successfully integrated along with the doxycycline control construct in an allele-specific manner. This is the largest transgene to date to be integrated in a site-specific manner. In nearly all clones (98.5 %) positive for *XIST* RNA, the insertion was localised to *DYRK1A*, and high-resolution cytogenetic banding on 6 chosen clones suggested that significant abnormalities were found only on chromosome 21. Within 5 days of *XIST* activation, the inactivated chromosome developed into a Barr body, carrying repressive histone markings, and analysis of chromosome 21 in nuclei showed that the DNA became markedly condensed. In addition, 3 weeks after induction, 97 % of CpG island containing genes on chromosome 21 demonstrated increased promoter methylation. Notably, when the pluripotent stem cells were subjected to neural induction culture conditions, the *XIST* clones had the capacity to develop into rosettes, a hallmark of multipotent neural stem cells. The uninduced lines, however, showed a delay in rosette formation when compared to the corrected counterparts. This ZFN-based study provides an excellent platform therefore to examine a number of developmental and cellular aberrations in Down's syndrome patients [47].

Recent iPSC studies comparing genetics and biochemistry, particularly in complex multifactorial diseases, have been somewhat compromised by correlating patients with normal or heterozygote siblings as the sample genomes are not identical [49]. Furthermore, research in both mouse [50, 51] and human [52, 53] models also suggest that early iPSCs retain some epigenetic memory from the cells that they originate from, and the differentiation capacity of the iPSCs is often

biased [50–53]. An individual gene mutation's specific contribution to disease can only realistically be evaluated in comparison to a truly isogenic control. This can be achieved using artificial nuclease technology to revert the known mutation back to normal; thus, the only genetic difference between the lines is the disease-associated mutation. Designing an artificial nuclease with a recognition sequence targeted to a locus, where the only difference between the endogenous sequence and the disruption donor sequence is the exact bases intended for modification, allows for the introduction of missense or non-sense mutations in a regulated manner. Such approaches are particularly useful for diseases that are late onset and/or manifest discrete functional or morphological changes when cultured in vitro. An et al. reported a study employing the CRISPR-based genome editing strategy to model Huntington's disease (HD) in patient-derived iPSC cells [54]. Homologous recombination was performed to produce a HD isogenic allelic series using two Cas9 enzymes, wild-type and Cas9 D10A, and found that the mutant version reduced the number of off-target events associated with the wild-type enzyme.

In a reversal of the isogenic control principle, hES cell lines generated from genetically normal embryos can be subjected to disease-causing mutagenesis using genome editing. In 2011, Soldner et al. performed a simple but elegant ZFN study to introduce two point mutations (A53T/E46K) in the α -synuclein gene in hES cells, generating a Parkinson's disease model. Conversely, the group then repaired the A53T mutation in patient-derived iPSCs, successfully altering one base pair only, without altering the rest of the genome [55]. To do this, as described above, an exogenous double- or single-stranded sequence of template DNA was introduced with the locus-specific mutations to serve as a template for DNA repair by homologous recombination. More recently, Ding et al.

Table 1 Comparison of ZFN, TALEN and CRISPR/Cas technologies

Technology	Advantages	Disadvantages
ZFN	<ul style="list-style-type: none"> -Commercially available for academic research from Sigma-Aldrich -Many proteins already available -Publicly available strategy, OPEN, to aid with ZFN construction -Efficient delivery of large transgenes -Progress made in translation to the clinic. Sangamo have ZFNs in phase 1 and phase 2 trials to treat HIV-1 by targeting the CCR5 gene 	<ul style="list-style-type: none"> -Expensive to purchase -Difficult to construct if not obtained commercially -Depending on the target sequence, some off-targeting may occur
TALEN	<ul style="list-style-type: none"> -Unlike ZFNs, TALEN production does not require re-engineering of the linkage between molecules -Rapid assembly using Golden Gate cloning, high throughput solid phase assembly and ligation-independent cloning -The ease of production allows for genome editing at multiple target sites in a cell -Fewer reported off-target events compared to CRISPR 	<ul style="list-style-type: none"> -Delivery can be more difficult as TALENs are large proteins -Some sensitivity to methylation
CRISPR/Cas9	<ul style="list-style-type: none"> -Easy to design and highly versatile -Relatively cheap -Paired single-strand break mutants (nickases) have recently been developed to reduce off-target effects of CRISPRs -High frequency of editing achieved 	<ul style="list-style-type: none"> -Off-target effects at high frequency have been recorded [63] as the target sequence of CRISPRs is generally shorter than ZFN and TALENs -Some target design constraints (e.g. a GG sequence must directly succeed the short guide RNA target)

established a swift and efficient TALEN assembly platform and successfully re-engineered the genome of 2 hES cell lines, generating mutant alleles of 15 different genes, such as *AKT2*, *CFTR* and *PLIN1* [56]. In one example, they demonstrated that it was possible to determine functional effects of a modified *SORT1* gene in hES-derived hepatocyte-like cells. They also observed phenotypic changes in the other mutant lines generated (when compared to the isogenic control lines) that were directly linked to a number of pathologies when subjected to differentiation conditions including insulin resistance, lipodystrophy and hypoglycaemia [56].

An added advantage to genome editing over HR is that a selection or reporter cassette can be introduced in conjunction with the desired mutation to provide a means for isolating the modified cell clones. Chen et al. used a lox-P-flanked neomycin cassette when using TALENs to introduce point mutations introduced into the *SCN1A* gene responsible for causing severe myoclonic epilepsy of infancy (SMEI) [57]. The selectable marker was excised after generation, selection and characterisation of iPSCs lines. There are many strategically similar studies in iPSCs that use genome editing to either correct or disrupt a gene by introducing a point mutation via artificial nuclease technology, providing new models for studying diseases such as Barth syndrome, HIV and β -thalassaemia [58–60]. The technology is also becoming more innovative; research groups are combining the more desirable aspects of each artificial nuclease to further increase efficiency and develop highly customised iPSC/hESC lines [60–62]. The cutting edge approach by Gonzales et al. produced a TALEN-mediated Cas9 inducible hES cell line and then used CRISPRs for biallelic targeting of six different genes, thus producing a double and triple knockout line [61]. The authors of the above studies all stressed the rapidity with which the lines were generated. Isolating patient samples for reprogramming into an embryonic-like stem cell can be both time consuming and costly. Furthermore, multiplexed targeting such as this may provide a novel platform to study diseases that are polygenic for up to dozens of gene, rather than being restricted to treating monogenic disorders using viral vectors containing a single transgene.

Conclusion: Summary and Future Directions

To conclude, it is clear that novel genome editing technologies will dramatically aid in the progression of cell-based therapy and in our understanding, of many genetically linked diseases. As the field has expanded dramatically in recent years, it is now possible to generate permanent and specific multiple-hit pluripotent cell lines for differentiation into the disease-associated cell type. However, one of the limiting factors in this respect is the availability of well-established specification protocols. Another confining factor, particularly crucial when considering translation into the clinic, is the need for highly efficient and

sometimes biallelic editing events. Although there are few studies done to directly compare the relative efficiencies of ZFNs, TALENs and CRISPRs/Cas9, one research group, Ding et al., surmised that the CRISPR/Cas9 system is superior to TALEN-mediated genome editing in human pluripotent stem cells, possibly as a result of increased tolerance to the Cas9 enzyme [64]. Combination approaches are sometimes favoured over more traditional methods to increase efficiency such as transposon/CRISPR [60], TALEN/CRISPR (iCRISPR) [61] and IDLV/ZFN [34, 36] as mentioned. Table 1 outlines both the advantages and disadvantages of current ZFN, TALEN and CRISPR/Cas 9 technology.

To provide therapeutic benefit to patients, genome editing strategies to correct disease-linked mutations in either adult or iPSC-derived progenitor cells for autologous transplant must be deemed safe, i.e. not disrupt other loci in the genome. Excitingly, a whole genome analysis study by Veres et al. demonstrated that there is a very low incidence of off-target mutation events induced by both TALEN and CRISPR/Cas9 targeted to the *SORT1* gene in hES cells [65]. Currently, there is encouraging progress being made in gene correction for a variety of diseases. For example, Schwank et al. have recently published data that provides evidence of functional repair of the *CFTR* gene in adult stem cells isolated from cystic fibrosis patients, mediated by the CRISPR/Cas9 system. They also observed very few off-target effects associated with the treatment. Moreover, a forskolin-response assay implied that the function of the disease-associated transmembrane receptor was restored [38]. Although this disease affects multiple organs and so would be complex to treat using this method, this research is indicative of the potential for genome editing to advance cell replacement therapy.

Compliance with Ethics Guidelines

Conflict of Interest Lorna M. FitzPatrick and Tristan R. McKay declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any previously unpublished studies with human or animal subjects performed by any of the authors.

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