Homologous recombination in human iPS and ES cells for use in gene correction therapy

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The emergence of induced pluripotent stem (iPS) cell technology has shifted gene correction therapy toward reality. Crucial issues are ensuring the safety of using iPS cell technology in patients and discovering how best to transfer genetically manipulated iPS cells back into patients. One key issue that has hindered progress of gene correction therapy, however, is the inability to achieve efficient homologous recombination in human iPS cells. This review focuses on recently developed technologies that aim to improve homologous recombination in human embryonic stem cells and on their application to iPS cells.

The emergence of induced pluripotent stem (iPS) cell technology has dramatically changed the outlook for many rare sporadic genetic diseases, such as primary immunodeficiency diseases [1–6], making treatment strategies such as gene correction therapy feasible in practice. The advantage of gene correction therapy is that the methodology can be applied to various genetic diseases, regardless of the number of causative gene targets. The same protocol can be used to repair mutated DNA located in any part of any gene in which the abnormal gene is replaced with a normal gene by means of homologous recombination.

This review focuses on recently developed technologies that aim to improve homologous recombination in human embryonic stem (ES) cells and how these technologies can be applied to iPS cells and gene correction strategies. For an overview of gene correction therapy, or gene replacement therapy, readers are directed to the 2008 review by Townes [7].

What is gene therapy?

When a foot is injured, the foot is cured; when a bone is broken, the bone is repaired. Thus, when a gene in a patient's genome is compromised, the logical response is to fix the gene. This is the basic idea behind gene therapy. Generally, the term 'gene therapy' is used to describe the procedure of inserting a normal gene into a patient that has an abnormal-functioning gene to treat the patient's disease. There are two types of gene therapy: traditional gene therapy and gene correction therapy. In traditional gene therapy, normal genes are inserted into a patient's cells and/or tissues but the abnormal gene remains intact. In gene correction therapy, an abnormal gene in the genome of a patient's cells is replaced with a normal gene [7].

In 1989, David Melton and colleagues reported the first gene correction therapy approach in a mouse model in which they replaced and repaired a mutation in the HPRT gene in a Lesch–Nyhan syndrome mouse model [8]. Thereafter, Johnson and colleagues successfully disrupted targeted genes via homologous recombination, modifying the genes in ES cells that were ultimately used to prepare knockout mice [9].

Traditional gene therapy versus gene correction therapy

The advantage of traditional gene addition therapy over gene correction therapy is that it has much higher efficiency. In traditional gene therapy, a virus vector gene-delivery system is used to introduce a normal-functioning gene into recipient cells. With retroviral vector systems, genes of interest are randomly integrated into a patient's genome. Gene expression is integration-site-dependent and is often subject to gene silencing. The drawback of this method is that it is prone to insertional mutagenesis, increasing the risk of cancer [10]; however, the risk of transformation via insertional mutagenesis is mainly determined by the

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function of the therapeutic gene. Generally, a strong constitutive promoter is used to express the therapeutic gene. Strong constitutive promoters are useful for supplying enough protein to a patient, but constitutive promoters differ from intrinsic promoters in that they are 'on' all the time. By contrast, intrinsic promoters turn 'on' when the protein is needed and 'off' when the protein is no longer needed. Thus, intrinsic promoters are preferable because in such systems gene expression is finely regulated, as it is *in vivo*.

In gene correction therapy, only the mutated site of a patient's gene is replaced with a normal-functioning gene. Thus, the patient's genome undergoes minimal manipulations. Moreover, an intrinsic promoter is used to express the therapeutic gene. When the mutated gene is replaced by means of homologous recombination, an antibiotic-resistant gene is introduced into the genome of a patient's cells temporarily. The antibiotic-resistant gene is then removed through a site-specific recombination system such as Cre/loxP or Flp/FRT. Besides the repaired region, only an additional 34 bp sequence in the intron of the patient's genome remains. The minimal manipulations involved in gene correction therapy thus should be safer than introducing an entire gene through an expression cassette (strong promoter and polyA additional signal), which is used in traditional gene therapy.

iPS cell technology: progress toward realizing gene correction therapy in humans

The advent of iPS cell technology has made performing gene correction therapy in humans more realistic. In 2007, through collaboration, Townes and Jaenisch elegantly substantiated the concept of using combination iPS cell technology and gene therapy to cure inherent diseases in mice [11]. Through homologous recombination, they corrected the defect by introducing a therapeutic gene into mice iPS cells containing a human sickle hemoglobin allele. The cured iPS cells were differentiated into hematopoietic progenitors. The cells were transplanted into irradiated sickle mice. This example exemplifies that, at present, there are no technical limitations for carrying out gene correction therapy in mice. Thus, with this in mind, researchers have shifted the focus toward carrying out this procedure in humans. There are four methodological challenges, however, that need to be addressed if gene correction therapy is to be carried out successfully in humans: the low efficiency of homologous recombination in human iPS cells, the safety of human iPS cells, the complete differentiation of iPS cells to desired cell types and the transfer of genetically 'cured' iPS cells to patients. This review addresses the first issue (overcoming the low efficiency of homologous recombination in human iPS cells). For a discussion of the remaining issues, readers are directed to other reviews [7,12,13].

Low efficiency of homologous recombination in human ES and iPS cells

The exact reason why the efficiency of homologous recombination in mouse ES cells is very high is unknown. Although the efficiency of homologous recombination in human pre-B cells of the immune system is also high [14], homologous recombination in human ES cells is low [15]. Because human iPS cells are extremely similar to human ES cells, the efficiency of homologous recombination in human iPS cells is also believed to be as low as that of human ES cells.

The potential problems affecting homologous recombination in human iPS cells are as follows. First, when the same method used to induce mouse ES cells is applied to human ES or iPS cells, the efficiency of homologous recombination is too low. Second, the conditions used to culture human ES cells differ from those used to culture mouse ES cells. Mouse ES cells can be cultured as dispersed cultures in which cells do not aggregate [16]; thus, transformed and untransformed cells can be easily separated before subculturing. By contrast, human ES cell cultures cannot be maintained under dispersed culture conditions because of dissociation-induced apoptosis [17]; thus, transformed and untransformed cells cannot be easily separated before subculturing. As a result, some cell aggregates containing both transformed and untransformed cells are often subcultured. For this reason, it is difficult to isolate a single colony derived from a single recombinant cell. In addition, in contrast to mouse ES cells, human ES cells cannot be cloned efficiently from single cells, making it difficult for rare recombination events to occur.

Increasing the efficiency of homologous recombination in human ES and iPS cells

Culturing conditions and positive and negative selection

The problem of low cloning efficiency, which results from the diminished cell viability of human ES cells after dissociation, could be solved by adjusting the initial culture conditions, as described by Yoshiki Sasai's laboratory [18]. In this novel approach, the addition of Y-27632, a Rho-associated kinase inhibitor, to human ES cell cultures greatly increases viability by decreasing dissociation-induced apoptosis [18]. This, in turn, improves cloning efficiency, facilitating subcloning after gene transfer.

Another strategy might involve incorporating positive selection genes (neomycin-resistance gene) or negative selection genes (*Herpes simplex* thymidine kinase or diphtheria toxin A subunit genes) into target ES or iPS cells. Even if some cell aggregates composed of recombinant cells and parental cells are grown together, with long growing times the nonrecombinant cells should eventually die and be selected out without adversely affecting the recombinant cells. One possible class of candidate negative selection molecule is engineered apoptosis-related factors [19,20]. Expression of apoptosis-related factors might promote the removal of apoptotic cells without adversely affecting ambient cells during negative selection.

Modified electroporation to promoterless reporters

In 2003, James Thomson's laboratory described efficient methods for inducing homologous recombination in human ES cells [15]. They used a modified electroporation method to introduce targeting vectors into human ES cells. Because human ES cells are notably larger than mouse ES cells (~14 μm diameter versus ~8 μm diameter), they used electroporation parameters (a single 320 V, 200 μF pulse) suitable for larger cells. In addition, instead of electroporating the cells in PBS, they placed the cells in an isotonic, protein-rich solution (standard cell culture medium) and performed electroporation at room temperature. In their experiments targeting the hypoxanthine phosphoribosyltransferase-1

(HPRT1) gene, they used a neomycin-resistance gene for positive selection and a thymidine kinase gene for negative selection. After transfecting 1.5×10^7 cells with a linearized HPRT1-targeting vector, they obtained 350 G418-resistant clones [15]. Of these, 50 were resistant to gancyclovir, and 7 of these were true clones, which translates to a homologous recombination efficiency of 14% (7/50) [15].

In their experiments targeting the Oct4 gene, Zwaka and Thomson used two promoterless reporter (EGFP and neo) selection cassettes containing two internal ribosomal entry sites and introduced the reporter-selection cassettes into the 3′ untranslated region of Oct4 by homologous recombination [15]. A promoterless reporter increases efficiency similarly to the gene-trap strategy. They obtained 103 G418-resistant clones, and 27% (28) of these clones were positive for homologous recombination [15]. Using a second targeting vector with a longer 3′ homologous arm, they obtained a higher rate of homologous recombination of almost 40% (22 homologous clones out of 56 G418-resistant clones) [15].

The homologous recombination efficiency reported by Thomson's group is not so bad. However, the two genes they introduced, HPRT1 and Oct4, are known to be efficiently incorporated. The HPRT1 gene is located on the X chromosome. HPRT-deficient cells are easily selected because they can grow in media containing 6-thioguanine (6-TG), which inhibits the growth of cells that express HPRT1. Oct4 gene is known to be one of the best genes. In mouse ES cells, its homologous recombination efficiency is extremely high, although the exact reason for this is unknown. The homologous recombination efficiency of human ES cells and the names of targeted genes are summarized in Table 1.

Recombinase-mediated cassette exchange method applied to human ROSA26 locus

Mouse ROSA26 locus is another gene that can be targeted with high efficiency for genetic modification [21]. Irion *et al.* [21] identified the human homolog of mouse ROSA26 locus and targeted a red fluorescent protein (tdRFP) cDNA to this locus through homologous recombination in human ES cells. Because tdRFP is flanked by loxP and mutant lox2272 sites, they were also able to exchange the tdRFP cassette with the cDNA of interest (flanked by heterotypic loxP sites) using a recombinase-mediated cassette exchange method [21]. The cassette exchange depended on site-specific Cre recombinase, a highly efficient Cre-protein-dependent transduction system. For homologous recombination of human ES cells, Irion *et al.* used a promoterless neomycin-resistance gene to increase the efficiency of obtaining true clones. Their targeting efficiency was ~2.3% (2/88) [21]. In the same 2007 report, Irion *et al.* commented: 'With only three previous reports of gene targeting in hES cells [15,22,23], crucial parameters for optimizing homologous recombination in these cells remain to be determined' [21].

Helper-dependent adenoviral vectors

In September of 2008, Mitani and colleagues reported highly efficient homologous recombination in human ES cells using a helper-dependent adenoviral vector system [24]. Unlike retroviruses and lentiviruses, adenoviruses do not integrate into the human genome. Helper-dependent adenoviral vectors have a lower cytotoxicity and elicit a smaller host immune response than standard E1-deleted adenoviral vectors because the gene transfer cassette contains only the packaging signal sequence and lacks all viral genes, which have been completely removed from the vector genome [25]. The virus system produces virus particles containing the foreign gene when the helper adenovirus is excised. The helper adenovirus itself cannot produce helper viruses when transfected into HEK293 cells expressing Cre protein because the packing signal sequence of the helper virus, which is flanked by two loxP sites, is removed from the helper virus genome [25]. Thus, only the foreign gene linked to a packaging signal is packaged into adenovirus particles.

TABLE 1

Summary of targeted genes and efficiency of homologous recombination in human ES cells

Targeted gene	Human ES cell line	Percent efficiency of HR[a] to random integration	Number of clones of successful HR/number of (double) positive clones	Comments	Refs
HPRT1	H1.1	14%	7/50	Checked resistance to 6-TG	[15]
Oct4	H1.1	27%	28/103	Used promoterless GFP + neo	
Oct4	H1.1	40%	22/56	6.3 kb and 6.6 kb homologous arms in 5′ and 3′ direction	
hROSA26	HES2	2.3%	2/88	Used promoterless neo	[21]
HPRT1	H1	2.3%	6/260	Used promoterless neo, 2% oxygen condition, nonisogenic DNA	[33]
MIXL1	HES3	–	Several clones/ not reported	Used promoterless GFP + neo	[34]
CCR5	HUES-3, HUES-1	5.3%[b]	–	Used ZFN-mediated gene editing by integrase-defective lentiviral vector; antibiotics selection-free system	[26]
HPRT1	KhES-1	45%	14/31	Used helper-dependent adenoviral vector; 13.4 kb and 9.2 kb homologous arms in 5′ and 3′ direction	[24]
HPRT1	KhES-3	33%	1/3	–	

[a] Homologous recombination.

[b] Percentage of GFP-positive cells in targeted integration of the GFP cassette into the CCR5 gene using zinc finger nucleases (ZFNs) and integrase-defective lentiviral vector delivery.

There are two advantages of using helper adenoviral systems for introducing foreign genes into cells: their low cytotoxicity makes it possible to use them at higher multiplicities of infection and their expanded cloning capacity permits the insertion of larger homology arms for homologous recombination. Suzuki *et al.* [24] used a neomycin-resistance gene and a thymidine kinase gene flanked on either side by longer homology arms of 14.3 kbp and 9.2 kbp. By using a helper-dependent adenoviral vector, they achieved a homologous recombination efficiency of 45%: indeed, 14 of 31 G418/ganciclovir double-resistant colonies had been targeted successfully at the HPRT1 gene by homologous recombination. In fact, the gene targeting frequency of the helper-dependent adenoviral vector system was ~300-fold higher than that achieved by electroporation [24]. Significantly, when the helper-dependent adenoviral vector system was used for homologous recombination, the targeted-to-random integration ratio was higher than that of nonviral electroporation [24]. They mentioned the possibility that blocking the DNA terminus might prevent random integration of adenoviral genomes into human chromosome because the termini of adenoviral genomes that enter nuclei are known to be protected by adenoviral terminal protein. This means that DNA with a naked terminus can easily integrate randomly into human chromosomes but DNA with a blocked terminus cannot. Nonetheless, the gene targeting frequency is no different and results in a higher targeted-to-random integration ratio.

At the present time, the helper-dependent adenoviral vector system seems to be the best system available for introducing targeting vectors for homologous recombination into ES or iPS cells. For some researchers, however, unfamiliarity with viral manipulation techniques might prove to be a psychological barrier to using virus vectors for gene transfer.

Zinc finger nucleases

The zinc finger nuclease (ZFN) method for gene editing is another interesting approach that implements custom-designed restriction enzymes (ZFNs) to target specific DNA sequences and to alter specific genes in ES cells. C2H2-zinc finger DNA-binding domains engineered to bind a DNA sequence of a potentially unique chromosomal site are fused to the nuclease domain of restriction endonuclease, FokI. This engineered ZFN specifically induces a double-strand break in the targeted DNA sequence. FokI possesses a property that enables it to digest DNA after protein dimerization. To increase specificity for DNA recognition and for inducing a double-strand break, two different zinc finger DNA-binding domains are used at the same time. The recognition site of one set of ZFNs is composed of two binding sites in the opposite orientation with suitable spacing. A ZFN-induced double-strand break in the chromosome can make specific repair-mediated sequence alterations by stimulating homologous recombination between the chromosome and the delivered targeting vector. Using such ZFNs, Lombardo *et al.* [26] reported extremely high levels of gene targeting (~5%) in human ES cells, even without selection. Although this system is effective, the possibility of 'off-target' effects – in which ZFNs might destroy or injure unexpected regions in the human genome without detection – exists. More recently, two groups (Zou *et al.* and Hockemeyer *et al.*) have successfully performed gene targeting of human iPS cells using ZFNs [27,28].

Other methods for improving homologous recombination efficiency

Again, why is the efficiency of homologous recombination in human ES cells lower than that in mouse ES cells? Homologous recombination occurs at a higher rate in mouse ES cells than in any other species. This is reasonable because gene disruption experiments involving homologous recombination are performed mainly in mice. In spite of the importance of gene disruption experiments, it is surprising that most published experiments have focused primarily on only a few species. I believe that the development of a method or discovery of a compound capable of increasing the efficiency of homologous recombination in human ES and iPS to the levels observed in mouse ES cells is just on the horizon. The ideal method or compound should either increase enzyme activity that promotes homologous recombination *in vivo* or should inhibit host defenses that prevent unnecessary homologous recombination *in vivo*.

Jayathilaka *et al.* [29] recently identified a small molecule, RS-1, capable of increasing homologous recombination by elevating the activity of RAD51, which, along with other RecA family members, directs homologous recombination during the repair of double-strand breaks of DNA [30]. These proteins assemble on single-stranded DNA to form presynaptic filaments, which perform the central steps of homologous recombination [31,32]. Jayathilaka *et al.* screened a 10,000 compound library by measuring the binding activity of hRAD51 to oligonucleotides that were end-labeled with a fluorescent tag [29]. They identified RS-1 (3-[(benzylamino)sulfonyl]-4-bromo-*N*-(4-bromophenyl) benzamide), that enhanced the homologous recombination activity of hRAD51 by promoting the formation of active presynaptic filaments [29]. These authors commented that RS-1 might increase homologous recombination activity *in vivo*, in addition to enhancing the biochemical activity of hRAD51 [29]. To date, however, they have not demonstrated whether RS-1 can enhance homologous recombination in ES cells *in vitro*.

The findings of Jayathilaka *et al.* illustrate the value of screening for compounds that increase the efficiency of homologous recombination. Future endeavors should focus on developing more direct screening assays applicable to the human iPS cell context.

Concluding remarks

If homologous recombination in human iPS cells becomes as routine as it is in mouse ES cells, iPS cell technology can be used not only for gene correction therapy but also for basic biology applications, such as the development of knockin and knockout human cells for analyzing the human-specific function of certain genes. Although iPS cell technology is expected to progress smoothly, it will take several years before it can be implemented for therapeutic use. In the meantime, we will continue to learn more about homologous recombination in human iPS cells by attempting new strategies, such as knockout, knockin and gene correction of various human genes using human iPS cells.

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