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Genome Modification in Human Embryonic Stem Cells

TOYOAKI TENZEN*, FILIP ZEMBOWICZ AND CHAD A. COWAN*

Stowers Medical Institute, Harvard Stem Cell Institute, Center for Regenerative Medicine, Cardiovascular Research Center, Massachusetts General Hospital, Boston, Massachusetts

Induced pluripotent stem cell (iPSC) technology has emerged as the most promising method for generating patient-specific human embryonic stem (ES) cells and adult stem cells (Takahashi et al., 2007, Cell 131:861–872; Wernig et al., 2007, Nature 448:318–324; Park et al., 2008, Nature 451:141–146). So far, most studies of direct reprogramming have been done by using lentiviruses/retroviruses encoding the reprogramming factors. This represents a major limitation to therapeutic applications since viral integration in the host genome increases the risk of tumorigenicity, and low-level residual expression of reprogramming factors may alter the differentiation potential of the human iPSCs (hiPSCs). As a result, more attention has been paid to developing new techniques to manipulate the human genome, with the goal of making safer hiPSCs that have fewer or no lesions or alterations in the genome. Additionally, the efficiency of reprogramming and of homologous recombination in gene therapy must be improved, if iPSC technology is to be a viable tool in regenerative medicine. Here, we summarize the recent developments in human genome manipulation for generating hiPSCs and advances in homologous recombination for gene targeting.

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The derivation of human embryonic stem (ES) cells drew attention from the medical and scientific communities in the hope that these cells could be used for therapeutic purposes (Thomson et al., 1998; Cowan et al., 2004). Human ES cells have been considered to be powerful and necessary starting materials for regenerating defective tissues in patients without the risk of immune rejection. However, derivation of patient-specific ES cells is not an easy process and there have been ethical issues. To address these issues, a completely different approach was taken to make human ES cells by the Yamanaka group (Takahashi and Yamanaka, 2006). They successfully made induced pluripotent stem cells (iPSCs) from mouse somatic cells by expressing reprogramming transcription factors implicated in pluripotency. The human version (hiPSCs) was reported 1 or 2 years later (Takahashi et al., 2007; Yu et al., 2007; Maherali et al., 2008; Park et al., 2008).

hiPSCs have been regarded as the most promising way to make stem cells since they are easy to make and are less ethically objectionable. Although the initial articles demonstrated the great promise of making iPSCs as tools in regenerative medicine, and have shown their capacity to even become germline-competent, there have been concerns regarding the safety of using the cells differentiated from hiPSCs. The hiPS cells are easily made by modulating the human genome to ectopically express the four transcription factors c-Myc, Klf4, Oct4, and Sox2, but since overexpression of these transcription factors has been associated with tumorigenesis (Foster et al., 2005; Hochedlinger et al., 2005), there is a risk that the differentiated cells might be also tumorigenic when transplanted into patients. Insertion of transgenes into functional genes of the human genome can be detrimental (Nair, 2008). Furthermore, although the transcription factors are mostly silenced following reprogramming, it has been reported that residual transgene expression may be responsible for some of the differences between ESCs and iPSCs such as the altered differentiation potential of iPSCs into functional cell types (Soldner et al., 2009). Not surprisingly, research has turned to development of new technologies to make safer stem cells for their eventual therapeutic use. There are a few ways of making iPSCs—genomic modification, protein introduction, and treatment with chemical reagents (Maherali and Hochedlinger, 2008). Here we focus on the recent progress in modulating human genomic DNA for making hiPSCs. Following reprogramming, correction of a defective gene in patients by homologous recombination is also an essential technique if hiPSCs are to fulfill their promise as a tool in therapeutic medicine. Thus, we review recent developments in homologous recombination techniques as well.

Lentivirus/Retrovirus

The first hiPSCs were made by using lentiviruses, successfully demonstrating that ectopic expression of the four transcription factors c-Myc, Klf4, Oct4, and Sox2 can transform somatic cells into iPSCs (Takahashi et al., 2007; Yu et al., 2007; Maherali et al., 2008; Park et al., 2008). The resulting transduction efficiency was better than that of other methods because the viruses could integrate directly into the genome and the constructs coding for reprogramming factors are continuously present in the cell (Fig. 1A). Lentivirus/retrovirus vectors will continue to be useful research tools for disease models and basic research studies due to their ease of use and high reprogramming efficiency. However, they are not practical for eventual therapeutic uses for the reasons mentioned above.

To address the issues caused by the permanent integration of transgenes, non-integrating vectors (plasmids and adenoviruses) were used for transient expression of reprogramming factors in mouse to successfully make iPSCs (Okita et al., 2008; Stadtfeld et al., 2008). However, the efficiency of these techniques is extremely low (100–1,000-fold lower than with integrating vectors). Recently, vector- and

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*Correspondence to: Toyoaki Tenzen and Chad A. Cowan, Stowers Medical Institute, Harvard Stem Cell Institute, Center for Regenerative Medicine, Cardiovascular Research Center, Massachusetts General Hospital, 185 Cambridge Street, CPZN-4234, Boston, MA 02114.
E-mail: totenzen@gmail.com, chadacowan@gmail.com

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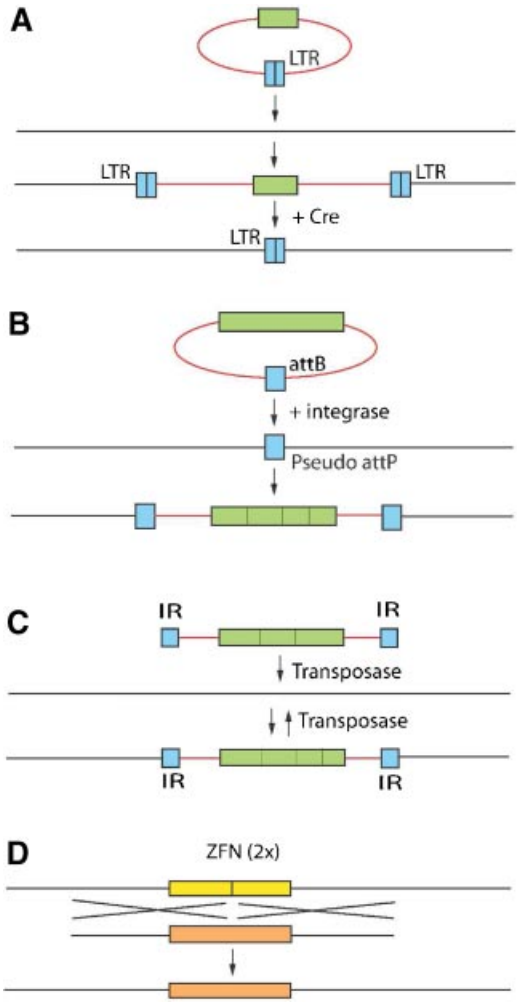

Fig. 1. Schematic representation of methods for introducing reprogramming factors (A–C) and for homologous recombination (D). A: The lentiviral/retroviral system. This lentiviral/retroviral system is commonly used for the generation of iPSCs. The donor vector has one copy of long terminal repeat (LTR), which is duplicated upon integration. There is no removal of the transgene once it integrates in the human genome. If loxP site is added to the LTR, most of the construct can be removed from the human genome with Cre recombinase. However, one copy of the LTR remains. B: The phiC31 system. The phiC31 vector has an attB site, which can recombine with pseudo attP sites in the human genome. Since the phiC31 donor vector has a large cargo space, all of the reprogramming factors can be separated by 2A peptides and inserted as a single transcript in the donor vector. This reaction is unidirectional and there is no removal of transgene after integration. C: PiggyBac system. A PiggyBac derivative that lacks the transposase gene has a large cloning space where all of the reprogramming factors, separated by 2A peptides, can be inserted as a single transcript. Transient expression of transposase can integrate and excise the transgene without footprint. D: Homologous recombination. Homologous recombination in human stem cells is used for correcting a mutation. If a pair of zinc finger nuclease (ZFN) is used to induce a single break at the site of recombination in the human chromosome, recombination efficiency increases significantly. Blue boxes are recombination sites when integration occurs. Thin lines in the LTR represent loxP sites. Green boxes are reprogramming factor(s). Thin lines in the green box represent 2A peptide enabling separate production of proteins from a single transcript. The yellow box is a defective exon of a gene. The line in the exon represents the single break point induced by a pair of ZFNs. The orange box is a functional exon corresponding to the defective exon.

transgene-free human iPSCs have been produced (Yu et al., 2009). The researchers used six factors (OCT4, SOX2, NANOG, LIN28, c-Myc, and KLF4) and the SV40 large T gene, which counteracts the possible toxic effects of c-Myc. Those genes were encoded in three episomal vectors, which are derivatives of the Epstein–Bar virus (Yu et al., 2009). One of the disadvantages of using conventional lentiviral vectors is that they each integrate in multiple sites making it impossible to control the copy number of each transgene, which is important for efficient production of iPSCs. Addressing this issue by the most efficient ratio of those vectors (although it may not precisely control the absolute copy number of each gene), they produced hiPSCs more efficiently.

Site-Specific Recombination

Cre/lox

Another way to avoid the problem caused by permanent and random integration of viruses coding for reprogramming factors is the use of the Cre/loxP recombination system. The Jaenisch group has shown that fibroblasts with idiopathic Parkinson's disease can be efficiently reprogrammed into iPSCs with this technique (Soldner et al., 2009). A single LoxP site was inserted into the LTR of the transgenic construct, which was then expressed in cells with traditional viral methods. After the iPSCs were obtained, the reprogramming factors used were excised by Cre recombinase expression and most of the transgenic region was removed with the exception of a single LTR remaining at the integration site (Fig. 1A). Interestingly, these factor-free hiPSCs showed a global gene expression profile more closely related to hESCs that to hiPSCs that retain the transgene (Soldner et al., 2009). This suggests that low-level transgene expression in hiPSCs affects the molecular characteristics of pluripotent cells.

phiC31

Another strategy was used by Thyagarajan et al., who used the bacteriophage phiC31 integrase to introduce transgenes into human ES cells. PhiC31 integrase works to carry out site-specific recombination at attachment (attP) sites in host bacteria. The human genome has a very small number of sequences called pseudo-attachment (pseudo-attP) sites that bear some resemblance to the native attP sequence (Chalberg et al., 2006) (Fig. 1B). Integration of the transgene occurs selectively at these sites. Importantly, among these sites there exist hot spots for integration, most frequently within introns of transcriptionally active genes, making the risk of gene disruption lower than with random integration. Additionally, there will be less suppression of the transgene through epigenetic modifications such as association with transcriptionally inactive chromatin domains. A large fraction of the integration events (30–60%) occur in fewer than 10 hot spots in human ESCs. Thus, this system allows more predictable integration sites than with traditional viral transgenic technology. In addition, this novel tool appears to have a larger cloning capacity than that of vectors used as part of traditional transgenic technology. PhiC31 integrase is a unidirectional recombinase that has no excision activity. This feature ensures stable integration but may not satisfy the demand for integration-free cells for use in regenerative medicine.

Transposons

Recently, there have been a few reports wherein *piggyBac* transposons were used in order to circumvent the problems caused by lingering expression of reprogramming transcription factors and permanent integration of viruses into the host genome (Fig. 1C) (Kaji et al., 2009; Woltjen et al., 2009; Yusa et al., 2009). In all of these studies, a similar construct was used.

piggyBac can have a large fragment (about 10 kb) inserted inside its expression cassette. The coding regions of the four reprogramming factors are driven by a single relatively strong promoter, separated by 2A peptides, which enables production of all four proteins from a single transcript. The construct is flanked by inverted repeats which are excision sites for host-factor independent *piggyBac* transposase, which is transiently expressed by another vector (Ding et al., 2005). The beauty of this system is that after somatic cells are transformed into pluripotent cells and expression of reprogramming factors is no longer necessary, the whole construct can be removed by transposase without any "footprint." Thus, the resultant pluirpotent cells and differentiated cells derived from them have few or no lesions at the sites of integration. Furthermore, this method ensures that all four reprogramming factors are expressed strongly since they are coded in a single vector that integrates into the host. These studies mainly used mouse cells; human cells were used to confirm integration and transformation into pluripotent cells but seamless excision by transposase has not yet been demonstrated. Considering that *piggyBac* transposition has been shown to occur in human cells (Ding et al., 2005), it is expected that seamless removal of the construct will occur in hiPSCs too.

Another transposon, *Sleeping beauty*, has been shown to transpose in human ES cells, suggesting that it may be a useful vector for expressing transgenes (Wilber et al., 2007). However, *Sleeping Beauty* integrates at 5′-TA-3′ sequences and leaves a footprint when excised (Ivics et al., 1997), and its cargo space is more limited than that of *piggyBac*.

Homologous Recombination

Classical

Homologous recombination must be used in order to correct genetic defects and repair diseased tissue in patient-derived human stem cells (Fig. 1D). Significant differences between mouse and human cells have hampered homologous recombination in human ES cells (Thomas and Capecchi, 1987). So far there are only several reports of gene modification in human ES cells by homologous recombination (Zwaka and Thomson, 2003; Urbach et al., 2004; Costa et al., 2007; Irion et al., 2007; Lombardo et al., 2007; Davis et al., 2008; Di Domenico et al., 2008; Zou et al., 2009), and the reported targeting efficiency is extremely low in both hES cells and hiPSCs (0.1–0.2%) (Zou et al., 2009). There are several problems with human ES cells that result in low targeting efficiency. Firstly, human ES cells cannot be cloned efficiently from single cells (Amit et al., 2000). Secondly, highly stable transfection of constructs has been difficult to achieve since the electroporation conditions used for mouse ECSs do not work well in human ESCs (Eiges et al., 2001). Integrase-defective lentiviral vectors (Leavitt et al., 1996; Naldini et al., 1996; Vargas et al., 2004; Nightingale et al., 2006; Philippe et al., 2006; Yanez-Munoz et al., 2006) provide solutions to many of these problems and are expected to be useful for delivery of constructs for homologous recombination into human ES cells (see below).

Zinc Finger Nucleases

Replacement of a defective gene with a functional gene by classical homologous recombination occurs with an extremely low efficiency. In order for gene replacement to be a viable clinical and experimental tool, the efficiency of gene therapy must be improved by developing a more effective homologous recombination strategy. It has been known that induction of a double strand break at the recombination site enhances homologous recombination significantly (up to 20%) in human cells (Porteus and Baltimore, 2003; Porteus and Carroll, 2005). However, it is impossible to induce a double strand break at a single specific site with a restriction enzyme since it cleaves at multiple sites in the genome. Zinc finger nucleases provide an attractive framework for cutting at a single specific site in the genome, allowing the design of novel DNA binding proteins with tailor-made sequence specificities (Fig. 1D) (Porteus and Carroll, 2005). One finger (a $\beta\beta\alpha$ structure) of a zinger finger protein can recognize and bind 3–4 bp along the major groove of DNA (Pabo et al., 2001). In theory, it is possible to design a novel zinc finger protein targeted to almost any desired site in the genome by aligning several different fingers in tandem. This specific DNA binding protein is then fused with the nuclease domain of the restriction enzyme *Fok*I. Since *Fok*I must dimerize in order to cut DNA (Bitinaite et al., 1998), it is necessary to design two different zinc finger DNA binding proteins whose binding sites are positioned closely and oppositely orientated. These two hybrid proteins can then be used to induce a single double stand break in the human genome with high specificity. Homologous recombination with the help of zinc finger nucleases is highly efficient and zinc fingers have been designed against multiple human gene targets, including VEGF-A, HoxB13, and CFTR (Maeder et al., 2008). Recently, zinc finger nucleases were used for homologous recombination in hiPSCs as well as in human ES cells, resulting in an increase in the targeting efficiency of 200–1,400-fold in these cells (Zou et al., 2009).

In order to introduce the constructs coding for the zinc finger nucleases and template DNA more efficiently, Lombardo et al. (2007) used an intergrase-defective lentiviral vector system, which allows high levels of gene correction (up to 50% in human cells and 5% in human ES cells).

Conclusion

Certainly, iPSC technology is a breakthrough for regenerative medicine, allowing facile derivation of patient-specific stem cells for research or clinical purposes. The lentiviruses/retroviruses used for the initial reports were the right choice for demonstrating the potential of hiPSCs, but recent research has

TABLE 1. Comparison of methods used in human genome modification

Method	Advantage	Disadvantage	References
Lentivirus/retrovirus	High efficiency easy to use	No removal of the transgene, incomplete suppression of transgene expression	Takahashi et al. (2007), Yu et al. (2007), Park et al. (2008), Maherali et al. (2008)
Cre/loxP	Removal of most of the transgenes	LTR remains in the genome	Soldner et al. (2009)
phiC31	More predictable integration sites	No removal of the transgene	Thyagarajan et al. (2008)
Transposon	Seamless removal of the construct	No detailed analysis in human, foot print remains in some cases	Yusa et al. (2009), Woltjen et al. (2009), Kaji et al. (2009)
Classical homologous recombination	Only DNA constructs are necessary and easy to set up experiments	Low efficiency of gene targeting	Costa et al. (2007), Davis et al. (2008), Di Domenico et al. (2008), Irion et al. (2007), Urbach et al. (2004), Zwaka and Thomson (2003)
Zinc finger nuclease	High efficiency of gene targeting	Need to design sequence-specific zinc finger domain	Lombardo et al. (2007), Zou et al. (2009)

focused on making iPSCs in a safer and more efficient way. Some of the methods seem to be safer indeed but still not ideal for the ultimate goal of regenerative medicine. More development is desired to make perfect iPSCs with no permanent genetic lesioning. So far, the use of *piggyBac* and a mixture of episomal vectors is most promising for making human iPSCs considering its relatively good efficiency and supposedly little or no alteration of human genome following reprogramming. However, we cannot rule out the possibility that some alterations occur somewhere in the human genome after transposon removal. For this purpose, it may be important to establish a method for checking the entire genome for abnormalities at the molecular level. Once iPSCs are derived, homologous recombination using zinc finger nucleases can facilitate homologous recombination and gene targeting significantly. Again, further work will be necessary to determine whether zinc finger nucleases create undesired genome instability and will allow the ultimate goal of generating safe, functional, and repaired patient-specific stem cells to be a reality (Table 1).

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