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COMMENTARY

Virus-mediated Genetic Surgery: Homologous Recombination With a Little "Helper" From My Friends

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The sequence-specific modification of genomic DNA is an important goal of gene- and stem cell-based therapies.[1,2] Since the demonstration that transgenic animals could be generated by targeting specific genomic loci by homologous recombination,[3,4] numerous investigators have focused on developing gene-targeting strategies that will efficiently and reliably correct mutations in genomic DNA.[1,5–7] Three papers have recently appeared,[8–10] including one in the early online edition of *Molecular Therapy*, that describe an efficient, helper-dependent adenovirus (HDAV)-based system for targeted modification of specific sequences in human embryonic and induced pluripotent stem (hES and hiPS, respectively) cells. These studies lay the foundation not only for the development of isogenic human pluripotent stem cells for basic research, but also for the generation of therapeutic, autologous, patient-derived stem cells in which the disease-causing mutation has been corrected.

The derivation of hES cells in 1998[11] and hiPS cells in 2007[12,13] prompted researchers to develop approaches to introduce sequence-specific genomic modifications into the DNA of these pluripotent cells. Early reports showed some promise and used classical gene-targeting vectors,[14,15] but few publications indicated that this strategy was sufficiently robust in pluripotent cells. By 2009, relying on the observation that double-strand breaks (DSBs) enhance homologous recombination in mammalian cells,[16–18] several groups reported the successful targeting of multiple genes in hES and hiPS cell lines by combining the expression of zinc-finger nucleases (ZFNs) with the delivery of homologous plasmid[19,20] or with oligo/polynucleotide donor DNA.[7,21] ZFNs[5,6] and more recently, the transcription activator-like effort nucleases (TALENs)[22,23] have provided researchers the tools to carry-out "genetic surgery" at specific genomic loci based on an increased efficiency of homologous recombination.[19,20] A primary concern with these targeted endonuclease systems is their potential to generate nonspecific off-site cleavage that could destabilize the genome of the targeted cells.[5,21]

The report in *Molecular Therapy* by Aizawa *et al.*,[8] describes the use of an HDAV system to deliver sequences essentially homologous to the genomic target and both overcomes some of the limitations of more classical approaches to homologous recombination as well as minimizes the potential for random integration into the genomic DNA. The HDAV system is effectively a "gutted" adenovirus from which all viral genes have been deleted, retaining only those viral sequences required for direct packaging of the DNA vector into the virus capsid.[24] The HDAV vector is packaged by cotransfection into a producer mammalian cell line with a "helper" virus genome that contains all of the viral genes necessary for vector replication, capsid production, and vector packaging.[25] An advantage of the recombinant HDAV vector is that it can accommodate between 25–35 kb of DNA, as opposed to, for example, the ~4.7 kb limitation imposed on the adeno-associated virus (AAV) system. This capacity is more than sufficient to accommodate large homologous regions of genomic DNA and drug selectable markers to stimulate homologous recombination and to allow enrichment of cells that have been targeted.

Because certain viruses have evolved precise mechanisms to deliver DNA to the nucleus, viral transduction tends to be much more efficient than that mediated by chemical or physical methods. Recombinant viral vectors also have less of a negative effect upon cell viability than nonviral methods that disrupt the cellular and nuclear membranes or sequester the DNA by binding it too tightly and interfering with delivery to the nucleus.[26,27] Consistent with this, the HDAV system described by Aizawa *et al.*[8] appears to give rise to little cytotoxicity, exhibits a higher overall efficiency of DNA delivery when compared to electroporation or chemical-based systems, and avoids the degradation and catenation of the vector often associated with the latter systems.[27] For all practical purposes, HDAV delivery can be viewed as functionally equivalent to nuclear microinjection of DNA. The availability of strategies to modify adenovirus vector tropism suggests that it might be possible to adapt this strategy to specifically target multiple types of cells or tissues.

The study uses a classical "positive–negative selection" strategy to enrich for homologous recombinants.[28] Initial

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selection for G418 (neomycin) resistance ($G418^R$) enriches for cells that have incorporated the recombinant targeting vector somewhere in their genomic DNA. The homologous targeting sequence is fused to the herpes simplex virus-thymidine kinase (*HSV-TK*) gene, which is lost during homologous recombination. Cells that have undergone homologous recombination are thus not sensitive to killing by HSV-TK activation following exposure to the pro-drug, gancyclovir (GANC). In contrast, $G418^R$ cells containing random integrations will retain the *HSV-TK* gene and will therefore be sensitive to GANC-induced killing. The hES or hiPS cells were exposed to individual HDAV vectors targeting one of five different native genes (*HPRT1, LIG1, LIG3, KU80,* and *HB9*). *HPRT1, LIG1, LIG3,* and *KU80* are expressed genes that are involved in either DNA synthesis (*HPRT1*) or the non-homologous end-joining (NHEJ) DNA repair processing (*LIG1, LIG3,* and *KU80*) pathways. The *HB9* gene is only transcriptionally active in motor neurons and is not expressed in pluripotent cells. The absolute gene-targeting efficiency ranged from 5.6×10^{-5} to 2×10^{-7} homologous recombinants per cell, with relative recombination efficiencies ranging from 7% to an impressive 81% of the $G418^R$ colonies. These results are consistent with what has been observed in mouse ES cells and in other somatic cells as well as when using ZFN-mediated homologous recombination and further confirms that the hES and hiPS cells are not recalcitrant to vector-mediated homologous recombination. Whether the range of efficiency is dependent on the genetic target, the cell line or the transcriptional status of the target gene will require further statistical analysis as well as a detailed analysis of the culture conditions at the time of the experiment.

HDAV-mediated gene targeting has been recently reported by an independent group at other gene targets: the *laminin A* gene (*LMNA*) in hES and hiPS cells[10] and the human β-*globin* gene.[9] As with the Aizawa study[8] the cells that had undergone HDAV-mediated homologous recombination maintained the expression of markers diagnostic for hES/hiPS cells, a normal karyotype, and the capacity to differentiate.[9,10]

There are a few caveats common to all these studies, some of which are inherent to HDAV-based gene targeting. If applied *in vivo*, there is the potential for such vectors to elicit an immune response to the adenovirus capsid proteins. This is less likely to be an issue for *ex vivo* applications, since the residual capsid proteins would likely be lost over several cell divisions. While random integration of intact targeting vectors was not detected in these studies, it is possible that a fragmented targeting vector might integrate into the host cell genome without detection. While this is beyond the scope of the present study and would require extensive sequencing analysis, it will be an important issue to address before the contemplation of clinical applications.

The possibility of removal of the positive drug selectable maker (*neomycin* gene) was achieved by flanking the latter with loxP or FRT sequences. Removal of the *neomycin* gene in some of the homologous recombinant hiPS cell lines was achieved by subsequent transfection of the Cre or FLP recombinase to catalyze site-specific recombination between the loxP or FRT sequences that flank the selectable gene. However, this process leaves behind some loxP or FRT sequences that are sometimes referred to as a footprint or scar. Demonstrating removal of the drug selectable marker in these cells is another important step towards defining protocols that will mitigate potential risks associated with patient-specific cell lines that could be used for therapeutic purposes. Whether the short loxP or FRT DNA footprint will have any detectable biological activity that might compromise the corrected cell lines for therapeutic application, remains to be seen.

These studies describe new tools for modifying and editing the genomic DNA in an efficient sequence-specific fashion. While the therapeutic potential of the HDAV system remains to be tested, it is a promising addition to the gene and cell therapy toolbox that could facilitate gene targeting for the treatment of inherited diseases.[1,3]

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