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Validation of AAV-mediated gene targeting

To the editor:
A series of letters to *Nature Biotechnology* have highlighted the controversy surrounding chimeric RNA/DNA oligonucleotide (RDO) technology[1–3], including a failed attempt to modify the human, X-linked hypoxanthine phosphoribosyltransferase (*HPRT*) locus that established specific criteria for validating gene-targeting experiments[3]. We believe an alternative gene-targeting approach based on single-stranded parvoviral vectors, such as adeno-associated virus (AAV), satisfies these criteria.

In our laboratory, we have shown that AAV vectors can introduce specific mutations (including insertions, deletions, and substitutions) into homologous chromosomal sequences at multiple chromosomal positions[4–7]. With this method, up to 1% of normal human cells undergo accurate gene targeting at a single-copy chromosomal locus without preselection for reporter gene expression, which is 4–6 logs higher than the targeting rates obtained in normal human cells with conventional techniques based on electroporation or transfection of plasmid constructs[8,9]. Although AAV-mediated gene targeting rates are lower than some of those reported for RDOs, the method is notable for its accuracy, flexibility, and success in human cells, so it should prove useful for both therapeutic and scientific applications requiring precise manipulation of the human genome. The four established validation criteria[3] for AAV-mediated gene targeting are addressed below.

First, the assay used must require conversion of a wild type to a rare mutant genotype to rule out spontaneous reversions. This has been shown for *HPRT* targeting experiments in which AAV vectors introduced a 4 bp insertion or several different single-base substitutions into coding exons of wild-type alleles, at frequencies well above background mutation rates and in a dose-dependent manner[4,7].

Second, the mutation should be absent in the cells used. This has been demonstrated both for *HPRT* gene targeting experiments[4,7] and for mutation correction experiments with reporter genes, such as neomycin phosphotransferase (*neo*)[4,5] or alkaline phosphatase[6]. In the *HPRT* experiments, cell lines containing the mutated alleles did not exist in the laboratory until created by gene targeting, ruling out cell culture contamination artifacts.

Third, gene targeting must be documented in expanded, individual clones rather than pooled cell extracts to avoid artifacts due to input targeting vector genomes. In both *HPRT* and *neo* experiments, we have documented AAV-mediated gene targeting in clones derived from single cells and expanded to over 10^7 cells, either by PCR-independent methods (such as Southern blot analysis[4,5] and sequencing of targeted alleles recovered as bacterial plasmids) or by sequencing of PCR amplification products[4,7].

Fourth, gene targeting should be confirmed at the protein level at time points that rule out the selection and expansion of rare spontaneous mutations. Protein expression was assessed in all our experiments, either by 6-thioguanine selection to detect the elimination of functional HPRT[4,7], G418 selection to detect corrected Neo protein[4,5], or histochemical staining to detect alkaline phosphatase activity[6]. These assays were performed two to ten days after infection with the AAV vector, too soon for significant expansion of cells with spontaneous mutations. We also followed the time course of corrected, functional alkaline phosphatase expression in unselected cell populations[6].

On the basis of these rigorous criteria, we feel that transduction by AAV vectors is a validated method for efficient gene targeting. However, we are not aware of any confirmatory publications by independent research groups. Given the contentious nature of the field of high-efficiency gene targeting, we encourage others to experiment with AAV, and to publish their successes and their failures.

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