

AAV hits the genomic bull's-eye

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Adeno-associated virus mediates correction of genetic mutations in mouse liver.

Most gene therapy approaches have sought to deliver normal copies of a gene—usually as many as possible—into the nucleus of mutant cells with little regard for where the genes land. More recently, researchers have begun to develop methods for making specific changes in the genomic DNA sequence, enabling correction of mutant genes. Of these gene targeting approaches, one of the most promising relies on adeno-associated virus (AAV) vectors, which have been shown to correct faulty genes at reasonable efficiency in cultured cells. In this issue, Miller *et al.*¹ extend this work by demonstrating hepatic gene correction by AAV vectors *in vivo*, using both a mutant *LacZ* reporter mouse and a murine model of mucopolysaccharidosis type VII. These studies provide a foundation for the development of AAV vector systems for targeted gene therapy in humans.

Gene therapy approaches that modify the genome can be classified as either gene addition or gene correction (Fig. 1a). Gene addition seeks to add copies of an expression cassette (containing cDNA of the normal gene, a promoter and a poly-A sequence) into the genome. The cassettes may randomly integrate into the genome (e.g., retroviral and lentiviral vectors) or remain predominantly episomal in the nucleus (e.g., plasmids, adenoviral and AAV vectors).

The advantages of gene addition include simplicity and generally high levels of gene expression. However, the approach has drawbacks, including occasional silencing of the expression cassette, ectopic expression of the transgene at levels and cellular sites differing from those of the endogenous gene, and the potential for insertional mutagenesis. Although

the use of cell- and gene-specific promoters can theoretically overcome the obstacle of nonphysiologic expression, in practice this approach has proved challenging owing to the complexity of most gene promoters. Furthermore, gene addition approaches cannot reverse dominant genetic mutations, as they do not alter the endogenous mutant gene.

Gene correction seeks to directly revert specific genomic mutations to normal and is considered the 'Holy Grail' of gene therapy. Advantages of this approach include both a decrease in mutant gene product and correct regulation of the repaired gene by its endogenous promoter (Fig. 1a). However, the search for genetic agents capable of accurately correcting 1 among 3 billion base pairs in the human genome has been a daunting pursuit, now over a decade old.

Vectors and approaches used for gene correction include small-fragment homologous recombination², RNA/DNA oligonucleotides³, triplex-forming oligonucleotides⁴, single-stranded oligonucleotides⁵ and AAV⁶. Although most of these vectors are effective at some level in cell lines, proof of their *in vivo* efficacy has been difficult for the field to consistently establish⁷. One hurdle in assessing gene correction approaches *in vivo* has been the lack of appropriate animal models in which correction efficiency can be accurately quantified at the histological and molecular levels.

Miller *et al.* sought to develop such a mouse model, based on the ROSA26-*LacZ* transgenic mouse line in which the *LacZ* transgene is located downstream of the endogenous housekeeping ROSA26 promoter. In their study, two derivative ROSA26-*LacZ* transgenic mice were generated, one bearing a wild-type *LacZ* transgene and the other a nonfunctional *LacZΔ4* transgene with 4 bp deleted. Two additional features, useful for assessing gene targeting at the molecular level, were also incorporated: a *lac* operator sequence (*lacO*) to enrich for target locus DNA by affinity capture on magnetic

beads, and a prokaryotic transcriptional promoter enabling a blue and white (corrected versus mutant) colony assay for functionality of the *LacZ* target locus in bacteria.

Using X-gal histocytochemical analysis of Rosa26-*LacZΔ4* liver infected with an AAV vector harboring a partial segment of the wild-type *LacZ* gene, the authors found β-Gal positive foci in the liver at a frequency in the range of 1 in 10⁵ hepatocytes. Molecular analysis of recovered target loci in bacteria demonstrated a similar frequency of gene correction, and this was confirmed by sequencing. Given that 0.04–1.5 vector genomes per cell were detected in the liver by Southern blot analysis, the efficiency of correction by the AAV genome was in the range of 1 in 10,000.

The correlation between gene correction frequencies obtained using histological and molecular quantitative methods is a major strength of these studies, especially considering that previous reports on *in vivo* gene targeting have relied heavily on artifact-prone, PCR-directed methods for molecular confirmation of targeting and less standardized methods for assessing expression and/or function of the corrected protein product. Hence, the ROSA26-*LacZΔ4* transgenic mouse line is likely to aid the field in evaluating the efficacy of other gene correction vectors and will be a useful platform for comparative studies.

Miller *et al.* provide additional proof of principle for *in vivo* AAV gene targeting using a naturally occurring *GusB* gene mutant (1 bp deletion) mouse model of mucopolysaccharidosis (MPS) type VII. AAV vectors harboring a 4.5-kb genomic fragment encompassing the wild-type *GusB* exon 10 sequence were evaluated for the extent of hepatic correction after tail vein injection. Gene correction was assessed using a histochemical stain for β-glucuronidase (the gene product of *GusB*). The frequency of correction (10⁻⁴) in these experiments was slightly higher than that seen in the ROSA26-*LacZΔ4* transgenic mouse model, and approximately

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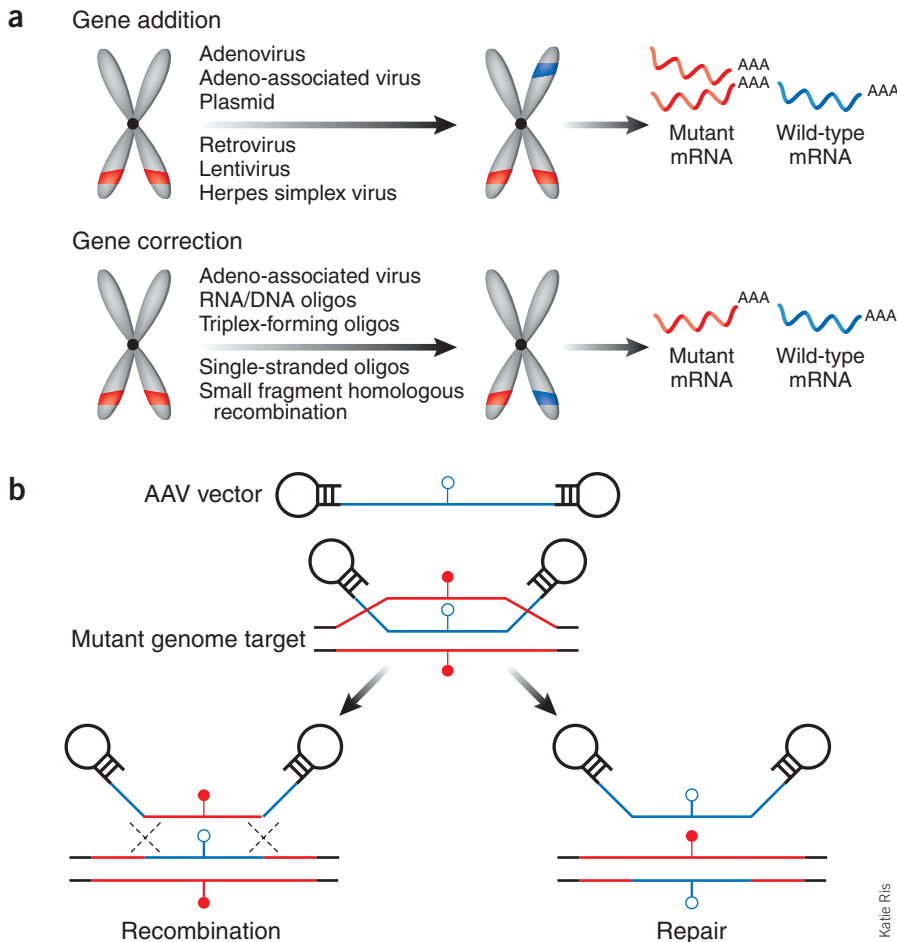


Figure 1 (a) Two general approaches to treat inherited genetic defects by altering genomic DNA. Gene addition involves adding one or more copies of a wild-type gene (blue) without altering the endogenous mutant genes (red). The levels of mutant mRNA and protein remain unchanged, and the transgene can either integrate (usually randomly) into the chromosome or remain episomal. Gene correction alters the sequence of a mutant gene to produce a wild-type sequence. As one or both of the endogenous genes are corrected, the levels of mutant mRNA and protein are reduced proportionately. Examples of major vector systems used in gene addition and gene correction are listed. Several of the gene correction approaches are significantly enhanced by zinc finger nuclease-induced double-stranded breaks at the target locus¹⁰. (b) Two potential mechanisms of AAV-mediated gene correction are recombination and repair (modified from ref. 6). In recombination, homologous pairing of vector-derived and genomic DNA sequences is followed by two recombination events, yielding an AAV vector harboring the mutant gene sequence and a corrected chromosomal gene. In repair, homologous pairing of vector-derived and genomic DNA sequences is followed by correction of the mutant gene, leaving the vector genome intact. This pathway would require synthesis of new chromosomal DNA using the vector-derived sequence as a template. Current data support homologous recombination as the most likely mechanism for AAV-mediated gene correction.

those caused by defects in genes with complex patterns of regulation, such as developmental diseases involving transcription factors. In these cases, gene correction would make it possible to maintain cell type-specific expression and/or tight regulation of the target gene required for normal function. Whether AAV or some other technology is the best vector to hit genomic targets remains to be worked out. In this regard, comparative studies of these vectors using the ROSA26-*LacZ*Δ4 model described by Miller *et al.* would be informative.

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100-fold higher than the spontaneous reversion frequency for this mutation (1 in 10⁶ hepatocytes). The reason for slightly higher targeting efficacies at the *GusB* locus is currently unclear, but could reflect locus-dependent effects or the size of the deletion being corrected (4 bp for *LacZ* versus 1 bp for *GusB*).

Despite the success of Miller *et al.* in demonstrating definitive gene correction *in vivo* with AAV vectors, several important questions remain unanswered. For example, the mechanism whereby the unique single-stranded AAV genome facilitates genomic alterations remains unresolved (Fig. 1b), and it is unclear how chromatin structure at the target locus may influence gene correction efficiency with AAV. Although the frequency of correction reported is below the level required for clinical benefit in most diseases, mechanistic insight will undoubtedly lead to improvements in this technology.

In this regard, evidence that double-stranded breaks at the target locus significantly enhance gene correction with AAV^{8,9} support

homologous recombination as the primary mechanism for gene correction with this vector. Also of interest is the fact that AAV genomes can remain extrachromosomal, randomly integrate and also mediate gene correction. Mechanistic studies suggest that the pathways controlling these properties of AAV genomes may be distinct. Hence, it may be possible to modify the AAV genome to target gene correction and prevent random integration. Such developments could be useful for preventing potential untoward events caused by random integration of the vector genome.

Gene correction using AAV or other gene targeting vectors will likely have a significant impact on gene therapies for diseases with complex etiologies. Included in this disease group are dominant genetic disorders such as skin diseases caused by keratin defects, osteogenesis imperfecta caused by collagen defects, and certain forms of amyotrophic lateral sclerosis caused by dominant mutations in *SOD1*. A second class of genetic disorders that would benefit from these technologies are

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