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Gene Repair

Pointing the finger at genetic disease

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The ability to put a patch over the mutation responsible for an inherited genetic disorder would be a dream for gene therapy. Now exciting data, from groups led by Holmes and Porteus, illustrate that genetic mutations can indeed be directly corrected in the human genome.[1]

Most gene therapy strategies do not aim to modify the mutated gene, but rather deliver an additional correct copy of the gene. To cure inherited diseases this therapeutic gene must be stably maintained. One way of doing this is to use viral vectors that harness the ability of certain viruses to integrate their genome into the host chromosome. However, inherent to this strategy is the risk of insertional mutagenesis, with detrimental effects on gene expression and potentially cancer.[2]

Genetic manipulations that replace precisely mutated sequences would solve some of the dilemmas that gene addition strategies pose, including unregulated gene expression, transgene silencing and insertional mutagenesis. In principle, the cellular machinery of homologous recombination (HR) can be used to replace precisely mutant genome sequences. However, the low frequency of HR in mammalian cells (10^{-5} to 10^{-7}) and the high levels of random integration of the targeting vector (10^{-2} to 10^{-4}) have limited its development for therapeutic gene correction.

One way to stimulate HR is through a DNA double-strand break (DSB) in the target locus.[3] HR between the broken chromosome and its sister chromatid leads to homology-directed repair of the broken ends (alternatively a direct joining mechanism called nonhomologous end-joining can process these ends). The cellular DNA repair system also accepts extrachromosomal DNA as a donor template for repair. To exploit the enhanced HR that a DSB activates, one would need a way to direct nucleases to cleave DNA at specific sites in the genome. To achieve this aim the authors of the new study[1] developed artificial nucleases that are targeted to a disease gene: in this case the *IL2R*γ gene responsible for X-linked severe combined immunodeficiency (X-SCID).

The strategy the authors used relied on the DNA-binding specificity of zinc-finger proteins. The C_2H_2 zinc-finger motif consists of about 30 amino acids with two cysteine and two histidine residues, which zinc stabilizes. The fingers all have the same structural framework but variations in a few residues allow them to bind to distinct DNA sequences. Multiple fingers are assembled in a modular tandem fashion to recognize a specific DNA sequence. A combination of rational design and selection techniques has allowed a repertoire of zinc-fingers, each binding to a specific DNA triplet, to be defined. So in order to design sequence-specific zinc-finger proteins, the appropriate DNA binders to be linked together can be selected from this library of characterized modules.

Zinc-finger proteins could have an enormous impact on gene therapy applications. The DNA-binding protein can be hooked up to a multitude of effector domains to direct their activity. For example, the addition of domains that activate or repress expression of endogenous genes can allow these proteins to be used as transcription factors with exquisite specificity.[4] Moreover, zinc-finger proteins have been used to inhibit virus infections[5,6] and to alter integration site selection for viral vectors.[7]

In this new study, Urnov *et al*[1] attached zinc-fingers to the catalytic domain of *Fok*I endonuclease to create custom nucleases. This study builds on recent developments that established custom nucleases as valuable tools for genome engineering.[8,9] Because the *Fok*I nuclease domain must dimerize to become active,[10] they generated two separate nucleases, each with a DNA-binding domain that contains four zinc-fingers that recognize a 12-bp half site. The complete target sequence contained two 12-bp binding sites in opposite orientation separated by a short spacer (see Figure 1). The two DNA-binding domains were designed to bind sequences that surround a mutation hotspot in the *IL2R*γ gene, and an elegant targeting strategy allowed the frequency of gene repair to be determined. Strikingly they found that the mutation in the *IL2R*γ gene was repaired in almost 20% of cells in cell lines transfected with the repair plasmid and the custom nucleases.

The impressive features of the gene correction include the high frequency, lack of selection, and the ability to achieve bi-allelic gene modification to generate homozygous corrected cells. Unfortunately, none of the critical information about procedures used to optimize binding and cleavage was supplied, which makes it difficult for other investigators to build upon the general principles used.

The most critical aspect of this approach is clearly the specificity of DNA cleavage. In view of the adverse events in the X-SCID gene therapy trial,[2] prevention of genotoxic side effects is paramount for human gene therapy. It will be important to demonstrate that individual zinc-finger proteins maintain their sequence-specificity when they are incorporated into artificial nucleases. Gene repair strongly depends on insertion of a DSB at a specific site, however, this study was not designed to detect surplus DSBs at sites other than the target locus. Prevention of random integration into the host genome of individual system components is another challenge. Both events will change the genetic make up of cells, thus posing an inherent cancer risk. Although the development of a malignant phenotype requires multiple cooperating steps, every genetic manipulation poses a risk, especially in stem and progenitor cells with high proliferative potential.

Despite the exciting promise of this new study, homology-directed repair for gene therapy still faces a number of significant obstacles. A daunting challenge will be delivery of the required components. In this study, transient transfection was

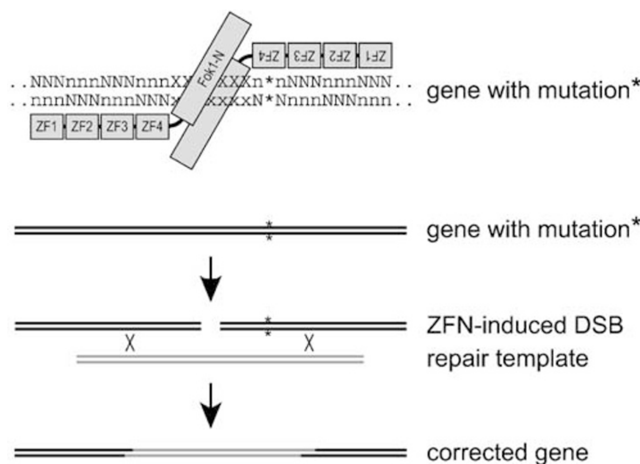

Figure 1 *Gene repair stimulated by zinc-finger nucleases. The custom nucleases comprise four zinc-finger DNA-binding modules (ZF1 through ZF4) that each recognize 3 bp of the target sequence. The zinc-fingers are fused to the catalytic domain of FokI endonuclease (Fok1-N), which has to dimerize in order to become active. Consequently, the full target sequence is made up of two 12 bp-half sites (NNNnnnNNNnnn), which are separated by a short spacer (X_n). The two custom nucleases are designed to bind close to the mutation*. Upon heterodimerization, the custom nucleases cut the target sequence within the spacer between the two half sites. The resulting DSB stimulates homology-directed repair and the repair template is used as a substrate for homologous recombination.*

used to deliver the repair plasmid and custom nucleases. However, this is unlikely to be optimal for human gene therapy and will not allow *in vivo* applications. Viral vector systems provide efficient systems for delivering genetic material. Probably the most attractive are vectors based on adeno-associated virus (AAV), as these have proven to be good templates for gene repair.[11,12] The issues of immunogenicity and cytotoxicity of the zinc-finger nucleases also need rigorous assessment. Precise regulation of gene expression will be required, as long-term expression of nucleases is not desirable.

Optimization of these designer nucleases for cytotoxicity and specificity, might ultimately enable their routine application as genetic scissors to introduce breaks that specifically enhance gene repair for genetic diseases. Combined with a repair template, these tools will allow us to patch up mistakes in the human genome, presenting a novel and potentially safer form of gene therapy. ■

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