RNA interference gene therapy

Allelic gene inactivation: pinpointing the problem

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Several promising results have recently been reported on the silencing of gene expression in mammalian cells by the use of RNA interference (RNAi). This silencing can be achieved either by transfecting short interfering RNAs (siRNAs), or by transcription of short hairpin RNAs (shRNAs) from expression vectors. The use of this exciting technique could be a powerful tool for the development of new therapeutic strategies. The critical question of the specificity of RNAi has been addressed by Miller *et al.*¹ in a recent edition of the Proceedings of the National Academy of Science, USA.

In this paper, the authors describe the allele-specific silencing of dominant inherited disease genes by RNAi in cellular models. This was achieved by targeting either a linked single nucleotide polymorphism (SNP) (in case of the *Ataxin-3* gene), or the disease mutation (*TAU* missense mutation) directly. siRNAs were delivered by the three current approaches: *in vitro*-synthesized duplexes, plasmid or viral expression of hairpin RNA. Using expression cassettes generated from patient material, they showed that with all the three approaches they were able to downregulate specifically the target proteins, as detected by Western blot analysis. In agreement with other studies, they show that the RNAi effect is capable of discriminating between the wild-type and mutant alleles, and remains local to the target sequence. The authors emphasize, however, that the targeting of a specific gene requires careful design and engineering. The siRNAs designed for silencing the *Ataxin-3* gene were all employed successfully, whereas only one region in the *TAU* gene seemed to be efficiently targeted to inhibit expression. In addition, one nucleotide difference between two alleles may not be

sufficient to confer allele specificity unless it is placed centrally in the corresponding siRNA.

A major consideration of this study is that changes in expression levels were only determined for transfected genes. The question remains, therefore, as to whether this silencing of gene expression can also be detected in endogenously expressed genes, or, indeed, in an *in vivo* situation. Unfortunately, there is no functional readout for the silencing of these genes in the system used, and the mechanism by which the mutant proteins cause neuronal injuries also remains unclear. It would therefore be very interesting to investigate the phenotypic changes produced by RNAi *in vivo*. Several studies have recently been published, showing that the specific inhibition of mutant genes could restore the function of the wild-type gene. For example, we have previously demonstrated that by specifically inhibiting the dominant mutant *K-RAS*^{V12} allele, which is involved in tumorigenicity, cells lost their tumorigenic phenotype.² Another study describes the inhibition of mutant *p53* using siRNAs with only one single base difference compared to wild-type *p53*. Inhibition restored the protein function of *p53*, as indicated by the expression levels of the target protein p21 and by functional assays.³

The importance of careful siRNA design was emphasized in a publication by Jackson *et al.*⁴ This group demonstrated the expression of non-targeted genes with as few as 11 contiguous nucleotides of identity to the siRNA in question. This off-target RNAi activity was observed using microarray analysis only, and phenotypic changes were not measured. Maybe the most potent way to increase reliability of the phenotypic observation and to reduce off-target

effects would be to use two or more siRNAs for a specific target in a study.

Although the results of allele-specific silencing seem promising, the *in vivo* delivery of the siRNAs has to be further optimized. Recently, two reports described the efficient intravenous delivery of RNAi *in vivo* in mice.^{5,6} After 72 and 24 h, respectively, a decrease in the cotransfected reporter gene could be detected. In addition, Lewis *et al.* describe the inhibition of an endogenously expressed gene using the same method. The inhibition in expression, however, was transient and decreased over time. This transient character could possibly be overcome by using self-inactivating siRNA-expressing retroviruses, as described previously.² The successful delivery of siRNAs into cycling and noncycling cells by a lentiviral-based system has recently been described by Robinson *et al.*⁷ Interestingly, the authors were able to generate RNAi transgenic mice with this system. Further development of the lentiviral vector by the inclusion of inducible or tissue-specific promoters could extend the range of cells affected by the RNAi.

siRNAs are thought to be too short to induce the cellular interferon response, although one recent paper by Bridge *et al.*⁸ demonstrates that a number of RNAi vectors are able to trigger this response. Following the infection of human lung fibroblasts with a lentiviral vector containing shRNA against genes involved in chromatin regulation, the expression of an interferon target gene increased by more than 50-fold. This effect, however, was observed using only one of the five shRNA constructs tested. An interferon response was also observed when this particular shRNA was cloned into different vectors and transfected into HeLa cells.

In conclusion, despite the uncertainty concerning the *in vivo* effect of the siRNA used to silence the mutant disease genes, the paper by Miller *et al.* once more shows that siRNAs can be used to inhibit gene expression specifically, discriminating a mutant allele from the wild type by means of a single nucleotide difference. With the use of newly developed delivery systems, it should be possible to test the therapeutic potential of these specific RNAi tools *in vivo*. ■

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