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RNA interference gene therapy

RNA interference gets infectious

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Reports that short hairpin RNAs (shRNAs) expressed from plasmids could also trigger RNA interference (RNAi) offered the promise of RNAi gene therapy using viral vectors.[1] Two manuscripts by Rubinson *et al*[2] and Hemann *et al*[3] in Nature Genetics deliver on this promise by demonstrating that RNAi delivered by retroviral and lentiviral vectors can silence genes in mice.

RNAi is a conserved surveillance system that responds to double-stranded RNA by silencing mRNAs with homology to the double-stranded RNA trigger (reviewed in Hannon[4]). Since the discovery that 21 nucleotide synthetic RNA duplexes (siRNAs) can trigger RNAi in mammalian cells, the field has exploded, with new developments emerging at an astonishing rate.

RNAi is now commonly used to establish knockdown phenotypes in cultured cells, but application of this technology in mammals has been slowed by difficulty in transfecting siRNA *in vivo*.[5] However, these two new studies have made significant inroads into this problem. Notably, Rubinson and co-workers also created transgenic mice expressing shRNAs.

These manuscripts complement recent reports by Xia *et al*,[6] who used adenovirus to deliver siRNAs *in vivo*, and Hasuwa *et al*,[7] who also created transgenic mice expressing shRNAs. Momentum in this area is building, so expect to see a flood of manuscripts using similar approaches.

Two groups[8,9] have previously expressed shRNAs from retroviral vectors in cultured cells. Rubinson *et al* and Hemann *et al* extend this approach by transducing cells *ex vivo* with lentivirus and retrovirus, respectively, before introducing them into mice. These experiments should be of interest to gene therapists since *ex vivo* gene transfer is used extensively in gene therapy trials.

To show that their shRNA-expressing retrovirus could silence genes in cultured cells, Rubinson *et al* infected activated $CD8^+$ T cells derived from T-cell receptor transgenic mice with a virus expressing a $CD8^+$ shRNA (as well as GFP). After 3 days, they sorted the cells for GFP and found that $CD8^+$ expression was reduced by 14-fold. They also used two other lentiviruses to infect noncycling murine dendritic cells and primary OTI T cells, silencing the proapoptotic Bim and CD25 genes, respectively.

The authors next infected murine hematopoietic stem cells (HSCs) with their $CD8^+$-targeting lentivirus. After sorting for GFP-positive (transduced) cells, they injected these cells into lethally irradiated congenic mice. After 8 weeks, the infected HSCs had reconstituted all blood cell lineages and the frequency of splenocytes expressing CD8 was reduced by 10-fold. The authors confirmed that they had indeed infected HCSs by serially passaging bone marrow cells from these mice.

Excitingly, Rubinson and co-workers were able to transduce embryonic stem cells and single-cell embryos with their CD8-targeting lentivirus in order to create transgenic mice that express CD8 shRNAs. GFP^+ ES cells were injected into RAG-deficient blastocysts that would not normally produce B and T cells. Developing T cells in the thymus of the resulting mice showed a nine-fold reduction in the expression of CD8, with very few $CD8^+$ cells detected in the thymus and spleen. Transduction of single-cell embryos with the CD8-targeting lentivirus gave similar results that were maintained into adulthood. Furthermore, transduction of single-cell embryos with a lentivirus expressing p53 shRNAs resulted in reduced expression of this protein in liver and brain. Clearly, this approach could have wide application as an alternative to the time-consuming process of making knockout animals. Multiple simultaneous knockdowns may also be possible using this technique.

Hemann and co-workers used retroviral vectors to express three different shRNAs targeting the *Trp53* tumor suppressor gene (p53-A, p53-B and p53-C). Transient transfection with plasmids expressing these shRNAs resulted in varying degrees of p53 inhibition. p53-C worked the best followed by p53-B, with p53-A showing only modest suppression. In a colony-forming assay with mouse embryonic fibroblasts infected with the three retroviruses, the ability to form colonies correlated with the degree of p53 suppression.

To examine the effects of p53 suppression on tumorigenesis, Hemann *et al* infected HSCs isolated from Eμ-Myc fetal liver and transplanted them into lethally irradiated mice. Mice reconstituted with Eμ-Myc HSCs normally form B-cell lymphomas by 4–6 months of age due to expression of Myc. The p53 protein promotes apoptosis in response to hyperproliferative signals; therefore, inactivation of the *Trp53* gene accelerates lymphomagenesis.

In contrast to control mice, mice reconstituted with HSCs transduced with p53-A, p53-B and p53-C developed palpable lymph nodes. In some respects, it is surprising that transduction with p53-A resulted in this phenotype, since it very weakly suppresses p53 production in culture. There was, however, a dramatic difference in the survival of the three groups of mice. Mice infected with the least effective virus, p53-A, showed no overall decrease in survival compared to control mice, while mice infected with p53-B and p53-C developed B-cell lymphomas that reached a terminal stage in 95 and 67 days, respectively.

Pathological analyses also revealed differences in the recipients of the three viruses. p53-A mice did not develop lymphomas, while p53-B developed massive lymphomas that showed high levels of apoptosis. Tumors in p53-C-treated mice were similar to those in mice bearing *Trp53* null lymphomas.

Presumably, the differences in outcome between the treatments with the three viruses result from varying suppression of p53. The authors suggest that being able to decrease gene expression by variable amounts using shRNAs with varying activities could be useful, offering an advantage over knockout mice. It is clear from their data that even a powerful knockdown of p53 expression by p53-C does not have the same effect as a gene knockout.

Although the lymphomas of p53-C recipient mice were histologically similar to $Trp53^{-/-}$ lymphomas, analysis of DNA content showed important differences. $Trp53^{-/-}$ lymphomas are highly aneuploid, while neither p53-B nor p53-C tumors had aberrant DNA contents. Thus, even small amounts of p53 can maintain chromosome stability, although it cannot cause apoptosis. This is interesting biologically, but it should also serve as a cautionary note to researchers who might think of RNAi as a simplified replacement for gene knockout approaches.

One of the most appealing aspects of RNAi is that, at least conceptually, it plugs so easily into established gene therapy methods. The gene therapy field has focused most on introducing therapeutic genes. It may now be feasible to routinely shut genes off as well.

Retroviruses and lentiviruses are relatively easy to make; so the approaches used in these two manuscripts could be easily accessible to many researchers. Expression of shRNAs from viruses should also facilitate the use of RNAi in cell lines that are hard to transfect. It may also enable wider application of RNAi *in vivo*. Generation of knockdown transgenic mice by transduction with shRNA viruses could be an especially powerful approach (with the caveat that it is not exactly equivalent to a gene knockout). And as Hemann *et al* demonstrate, it is possible to construct an 'epiallelic series of hypomorphic mutations' using a series of shRNAs with various potencies. This may be useful in systems where gene dosage is critical. Viral delivery of shRNAs will clearly be a useful research tool, and it is also obvious from these reports that RNAi gene therapy has finally arrived. ■

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