Germline transmission of RNAi in mice

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MicroRNA molecules (miRNAs) are small, noncoding RNA molecules that have been found in a diverse array of eukaryotes, including mammals. miRNA precursors share a characteristic secondary structure, forming short 'hairpin' RNAs. Genetic and biochemical studies have indicated that miRNAs are processed to their mature forms by Dicer, an RNAse III family nuclease, and function through RNA-mediated interference (RNAi) and related pathways to regulate the expression of target genes (reviewed in refs. 1,2). Recently, we and others (reviewed in ref. 3) have remodeled miRNAs to permit experimental manipulation of gene expression in mammalian cells and have dubbed these synthetic silencing triggers 'short hairpin RNAs' (shRNAs). Silencing by shRNAs requires the RNAi machinery and correlates with the production of small interfering RNAs (siRNAs), which are a signature of RNAi.

Expression of shRNAs can elicit either transient or stable silencing, depending upon whether the expression cassette is integrated into the genome of the recipient cultured cell (reviewed in ref. 3). shRNA expression vectors also induce gene silencing in adult mice following transient delivery[4,5]. However, for shRNAs to be a viable genetic tool in mice, stable manipulation of gene expression is essential. Hemann and colleagues[6] have demonstrated long-term suppression of gene expression *in vivo* following retroviral delivery of shRNA-expression cassettes to hematopoietic stem cells. Here we sought to test whether shRNA-expression cassettes that were passed through the mouse germline could enforce heritable gene silencing.

We began by taking standard transgenesis approaches[7] using shRNAs directed against a variety of targets with expected phenotypes, including the genes encoding tyrosinase (albino), myosin VIIa (shaker), Bmp-5 (crinkled ears), Hox a-10 (limb defects), homogentisate 1,2,-dioxygenase (urine turns black upon exposure to air), Hairless (hair loss) and melanocortin 1 receptor (yellow). Three constructs per gene were linearized and injected into pronuclei to produce transgenic founder animals. Although we noted the presence of the transgene in some animals, virtually none showed a distinct or reproducible phenotype that was expected for a hypomorphic allele of the targeted gene.

Therefore, we decided to take another approach: verifying the presence of the shRNA and its activity toward a target gene in cultured embryonic stem (ES) cells and then asking whether those cells retained suppression in a chimeric animal *in vivo*. We also planned to test whether such cells could pass a functional RNAi-inducing construct through the mouse germline. For these studies, we chose to examine a novel gene, *Neil1*, which is proposed to have a role in DNA repair. Oxidative damage accounts for 10,000 DNA lesions per cell per day in humans and is thought to contribute to carcinogenesis, aging and tissue damage following ischemia[8,9]. Oxidative DNA damage includes abasic sites, strand breaks and at least 20 oxidized bases, many of which are cytotoxic or pro-mutagenic[10]. DNA *N*-glycosylases initiate the base excision repair pathway by recognizing specific bases in DNA and cleaving the sugar base bond to release the damaged base[11].

The *Neil* genes are a newly discovered family of mammalian DNA *N*-glycosylases related to the Fpg/Nei family of proteins from *Escherichia coli*[12,13] (T.A.R., E. Zaika, A.S. Fernandes, D.O. Zharkov, H. Miller and A.P. Grollman, submitted). Neil1 recognizes and removes a wide spectrum of oxidized pyrimidines and ring-opened purines from DNA, including thymine glycol (Tg), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 4,6-diamino-5-formidopyrimidine (FapyA)[12,13] (T.A.R., E. Zaika, A.S. Fernandes, D.O. Zharkov, H. Miller and A.P. Grollman, submitted). Tg, FapyG and FapyA are among the most prevalent oxidized bases produced by ionizing radiation[10] and can block replicative DNA polymerases, which can, in turn, cause cell death[14,15].

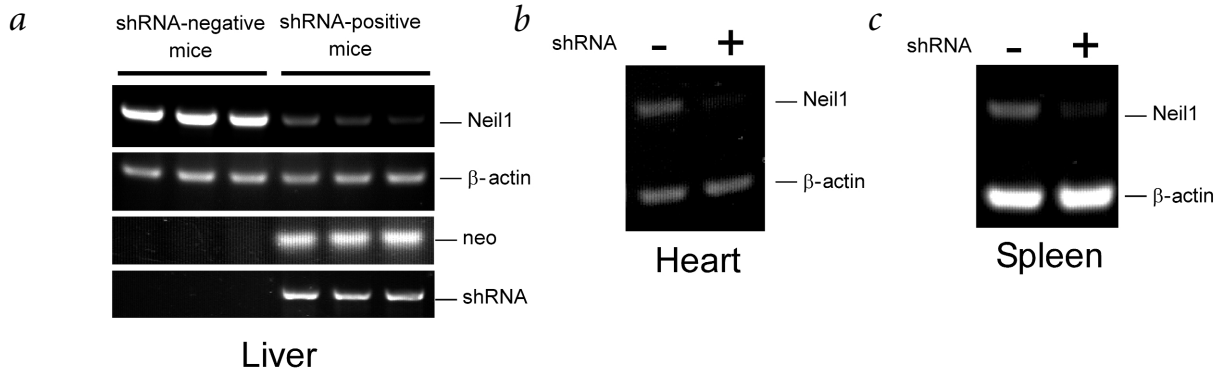

Fig. 1 Heritable repression of Neil1 expression by RNAi in several tissues. ***a***, Expression of *Neil1* mRNA in the livers of three mice containing the *Neil1* shRNA transgene (shRNA-positive) or three siblings lacking the transgene (shRNA-negative) was assayed by RT-PCR (top row is Neil1). An RT-PCR of β-actin was done to ensure that equal quantities of mRNAs were tested for each mouse (second row). Expression of the neomycin resistance gene (neo), carried on the shRNA vector, was tested similarly (third row). Finally, the mice were genotyped using genomic DNA that was PCR-amplified with vector-specific primers (bottom row). Similar studies were performed in the ***b***, heart and ***c***, spleen. Animal procedures have been approved by the SUNY, Stony Brook Institutional Animal Care and Use Committee (IACUC).

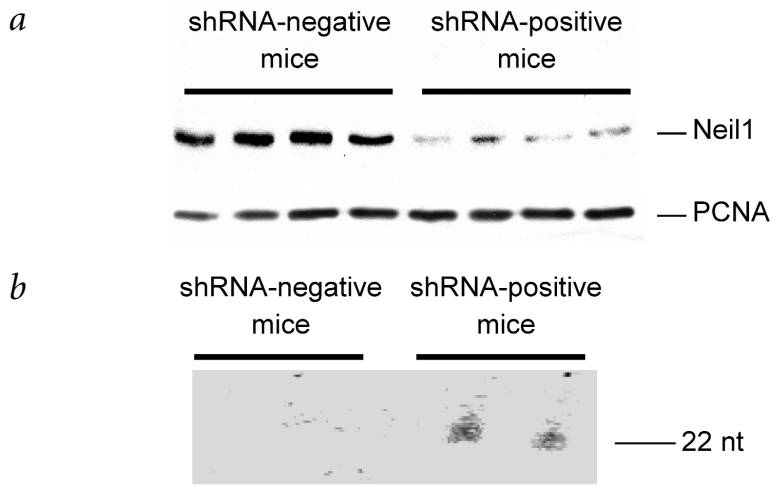

Fig. 2 Reduction in Neil1 protein correlates with the presence of siRNAs. ***a***, Expression of Neil1 protein was examined in protein extracts from the livers of mice carrying the shRNA transgene (shRNA-positive) or siblings lacking the transgene (shRNA-negative) by western blotting with Neil1-specific antiserum. A western blot for PCNA was used to standardize loading. ***b***, The presence of siRNAs in RNA derived from the livers of transgenic mice as assayed by northern blotting using a 300 nt probe, part of which was complementary to the shRNA sequence. We note siRNAs only in mice transgenic for the shRNA expression cassette.

The Nth1 and Ogg1 glycosylases each remove subsets of oxidized DNA bases that overlap with substrates of Neil1 (refs. 16–18). However, mice with null mutations in either *Nth1* (refs. 19,20) or *Ogg1* (refs. 21,22) are viable, raising the possibility that Neil1 activity tempers the loss of Nth1 or Ogg1. Recently, a residual Tg-DNA glycosylase activity in *Nth1*$^{-/-}$ mice has been identified as Neil1 (ref. 23).

We constructed a single shRNA expression vector targeting a sequence near the 5′ end of the *Neil1* coding region. This vector was introduced into mouse embryonic stem cells by electroporation, and individual stable integrants were tested for expression of the Neil1 protein (detailed procedures are available at *http://www.cshl.edu/public/SCIENCE/hannon.html*). The majority of cell lines showed an ~80% reduction in Neil1 protein, which correlated with a similar change in levels of *Neil1* mRNA (data not shown). These cells showed an approximately two-fold increase in their sensitivity to ionizing radiation (T.A.R., E. Zaika, A. S. Fernandes, D. O. Zharkov, H. Miller and A. P. Grollman, submitted), consistent with a role for Neil1 in DNA repair. Two independent ES cell lines were injected into BL/6 blastocysts, and several high-percentage chimeras were obtained. These chimeras were out-crossed, and germline transmission of the shRNA-expression construct was noted in numerous F_1 progeny (13/27 for one line and 12/26 for the other).

To determine whether the silencing of *Neil1* that had been observed in ES cells was transmitted faithfully, we examined *Neil1* mRNA and protein levels. Both were reduced by approximately the same extent that had been observed in the engineered ES cells (Figs. 1,2). Consistent with this having occurred through the RNAi pathway, we detected the presence of siRNAs corresponding to the shRNA sequence in F_1 animals that carry the shRNA expression vector but not in those that lack the vector (Fig. 2*b*).

The aforementioned data demonstrate that shRNAs can be used to create germline transgenic mice in which RNAi has silenced a target gene. These observations open the door to using of RNAi as a complement to standard knock-out methodologies and provide a means to rapidly assess the consequences of suppressing a gene of interest in a living animal. Coupled with activator-dependent U6 promoters (P. Paddison, J. Du, E. Julien, W. Herr and G.J.H., unpublished data), the use of shRNAs will ultimately provide methods for tissue-specific, inducible and reversible suppression of gene expression in mice.

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Competing interests statement
The authors declare that they have no competing financial interests.

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