

Efficient delivery of siRNA for inhibition of gene expression in postnatal mice

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It has recently been shown that RNA interference can be induced in cultured mammalian cells by delivery of short interfering RNAs (siRNAs). Here we describe a method for efficient *in vivo* delivery of siRNAs to organs of postnatal mice and demonstrate effective and specific inhibition of transgene expression in a variety of organs.

A relatively new technique using RNA interference (RNAi) allows for the experimental inhibition of gene expression¹. RNAi is triggered by the presence of double-stranded RNA (dsRNA) in the cell and results in the rapid destruction of the mRNA containing identical or nearly identical sequence². Use of RNAi by means of long dsRNAs (greater than 50 bp) has been limited in mammalian systems (exceptions being mouse oocytes, pre-implantation embryos and a few cell lines derived from embryos) because long dsRNAs also induce a nonspecific inhibitory response resulting from the interferon pathway^{3–7}. Studies of the RNAi pathway in invertebrates, however, have demonstrated that cleavage of long dsRNAs to lengths of 21–25 bp results in dsRNAs that are active in eliciting RNAi^{8–11}. It has recently been shown that RNAi can be accomplished in cultured mammalian cells with siRNAs of 21–23 bp, thus circumventing induction of the interferon response^{7,12}.

We and others have developed a technique to efficiently deliver plasmid DNA to organs of postnatal mice using rapid injection of a large volume of physiological solution into the tail vein^{13,14}. Here we tested whether this ‘high-pressure’ delivery technique could deliver siRNAs for RNAi induction using as a reporter gene the modified luciferase gene, *luc*⁺, of the firefly *Photinus pyralis*. We co-injected postnatal mice with 10 µg of a plasmid containing *luc*⁺ and 1 µg of a plasmid containing the unrelated luciferase gene of the sea pansy *Renilla reniformis* (as quantitative control) along with 5 µg of a synthetically prepared siRNA duplex targeted against firefly *luc*⁺ (siRNA-*luc*⁺). We used as controls mice receiving plasmid alone and mice receiving siRNA targeted against *EGFP*, encoding enhanced green fluorescent protein (siRNA-*EGFP*), in place of siRNA-*luc*⁺. (See Web Notes A and B for detailed descriptions of the siRNAs and the injection protocol used in this study, respec-

tively.) One day after injection, we collected the organs, prepared homogenates and screened these for the activity of target firefly luciferase⁺ and control *R. reniformis* luciferase. In mice receiving siRNA-*luc*⁺, expression of firefly luciferase⁺ was inhibited by 80–90% in the liver, spleen, lung, kidney and pancreas, compared with that in mice injected with the plasmids alone (Fig. 1). We observed no significant inhibition in mice injected with the control siRNA-*EGFP*. Expression of *R. reniformis* luciferase was unaffected by the presence of siRNA, suggesting that inhibition is specific to the target gene and that the interferon response is not activated to an appreciable extent in these tissues (data not shown). Our results thus indicate that siRNA can be delivered using this method and can thereby inhibit expression of genes in a specific manner in organs of postnatal mice.

Because the liver is central to metabolism and is the target of many drugs, we examined further our ability to deliver siRNA to the liver and to determine its effects on gene expression. We first tested the amount of siRNA needed to induce RNAi in the liver by carrying out a titration of siRNA-*luc*⁺. High-pressure injection of as little as 0.05 µg into the tail vein led to detectable inhibition of firefly luciferase⁺ expression (36 ± 17%) one day after injection. Injection of 0.5 µg and 5 µg resulted in increasingly greater inhibition (78% ± 5% and 88% ± 3%, respectively). These results indicate that inhibition of target gene expression by siRNA is dose-dependent and show that the high-pressure method efficiently delivers siRNA.

To determine the longevity of RNAi induced by delivery of siRNA, we co-delivered 10 µg of a liver-specific long-term expression plasmid (pSEAP) containing the gene encoding a secreted form of the human placental alkaline phosphatase (SEAP), and 5 µg of an siRNA targeted to the coding region of the gene (siRNA-SEAP). We used as controls mice injected with pSEAP alone and mice injected with a control siRNA targeted to the plasmid backbone (siRNA-*ori*). Five mice were included in each experimental group. We monitored SEAP expression by measuring SEAP activity in the serum at various times after injection. One day after injection (day 1), SEAP expression was highly inhibited; SEAP activity was only 17 ± 7% of that in mice receiving

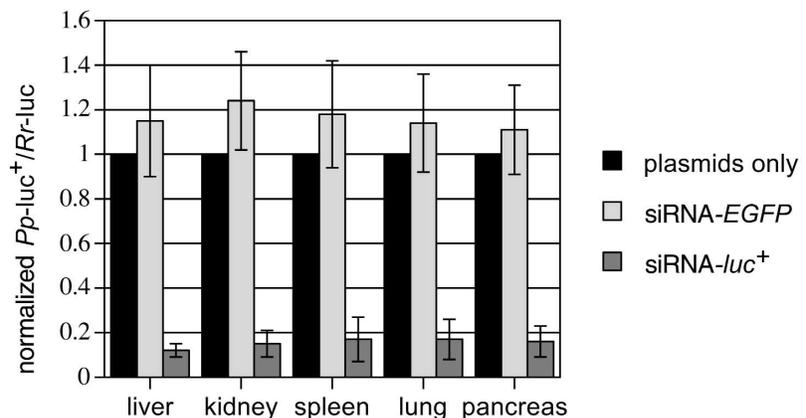
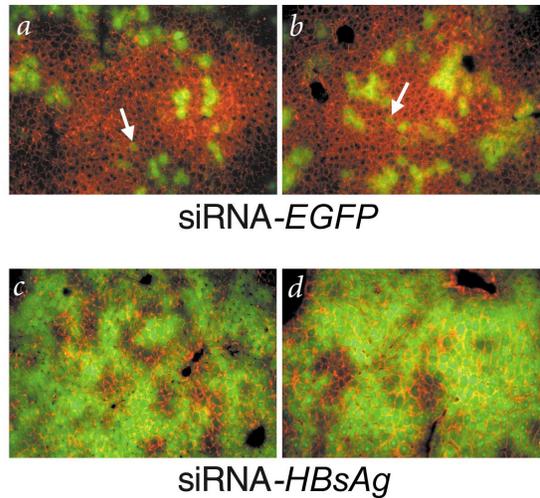


Fig. 1 RNA interference in organs of mice after injection of siRNA. Mice (strain ICR, Harlan, 4–6 wk old) were co-injected with the pGL3 control vector and pRL-SV40 plasmids (Promega) alone or together with siRNAs. Both plasmids contain the SV40 enhancer/promoter to drive expression of the reporter gene. In all cases, the ratio of *P. pyralis* luciferase⁺ (Pp-luc⁺) to *R. reniformis* luciferase (Rr-luc) activities was calculated to compensate for differences in transfection efficiencies between mice. The ratios were then normalized to those observed in mice receiving no siRNA. Black bars indicate normalized Pp-luc⁺/Rr-luc activity ratios in mice receiving plasmids only; white bars indicate normalized Pp-luc⁺/Rr-luc activity ratios in controls co-injected with siRNA-EGFP; gray bars indicate normalized Pp-luc⁺/Rr-luc activity ratios in those co-injected with siRNA-luc⁺. The activity ratios of Pp-luc⁺/Rr-luc in plasmid-only controls varied between 1 and 4 before normalization and between various organs and mice. The plotted data show average ± s.d. from three independent experiments with five mice per group. All experiments carried out on live animals were approved by the Mirus Corporation Institutional Animal Care and Use Committee.

Fig. 2 Inhibition of EGFP expression in transgenic mice after delivery of siRNA. Mice (strain C57Bl/6-TgN(ACTbEGFP)10sb, Jackson Laboratories, 10 wk old) were injected with siRNA-EGFP (a, b) or a control siRNA, siRNA-HBsAg (c, d). Livers were collected 48 h after injection. Frozen sections were fixed and then counterstained with Alexa 568 phalloidin (red) to visualize cell outlines. Images were acquired using a Zeiss Axioplan 2 fluorescence microscope outfitted with a Zeiss AxioCam digital camera. There was some variability in the degree of EGFP expression across the liver. The images show areas that are representative of the level and distribution of EGFP expression across all sections examined. Examples of cells in mice treated with siRNA-EGFP, containing decreased but detectable amounts of EGFP, are indicated by arrows.



pSEAP alone. By day 4, the inhibition had decreased and SEAP expression was $68 \pm 39\%$ of that in the control mice. The relatively large standard deviation reflects the observation that recovery from RNAi as measured on day 4 can be somewhat variable among mice. By day 14, SEAP activity in mice receiving siRNA-SEAP had returned to the levels observed in the controls. That full recovery of SEAP expression occurred discounts the trivial explanation that siRNA targeted to SEAP coding sequence inhibits SEAP expression simply by negatively affecting cellular uptake of pSEAP. No significant inhibition of SEAP expression was observed at any time in mice injected with siRNA-ori. Thus, the effect of RNAi persists for several days after siRNA delivery, a time frame sufficient for many types of experimental studies.

To test the usefulness of RNAi for inhibiting the expression of a gene, we used siRNA to inhibit the expression of the gene encoding EGFP in a transgenic mouse strain that expresses this reporter gene in nearly all organs, including the liver¹⁵. High-pressure tail-vein injection of 50 μg of siRNA-EGFP resulted in substantial reduction of EGFP expression in a large percentage of hepatocytes at 48 hours after injection, as determined by fluorescent microscopy (Fig. 2). In some

cells, this inhibition seemed nearly complete, whereas in others, low or moderate levels of EGFP were observed. We saw similar effects in EGFP-expressing cells that were treated with siRNA *in vitro* (data not shown). These results may be due to incomplete inhibition in cells that take up lesser amounts of siRNA. High-pressure delivery of fluorescently labeled siRNA reveals that *in vivo* uptake is not equal in all hepatocytes when this method is used (data not shown). Injection of one-tenth as much siRNA-EGFP also inhibited EGFP expression, but both the number of cells affected and the degree of EGFP inhibition within a given cell were lower (data not shown). Injection of a control siRNA, siRNA-HBsAg, targeted to the gene encoding the hepatitis B virus surface antigen did not affect EGFP expression. The small percentage of EGFP-negative cells seen in liver sections injected with the control siRNA are probably not the result of nonspecific inhibition or of the injection procedure, as a similar percentage of EGFP-negative cells was also seen in uninjected mice (data not shown). These results indicate that siRNA is effective at inhibiting the expression of a transgene expressed from the genome and imply that delivery of siRNA to the liver by high-pressure tail-vein injection results in uptake of siRNA by at least a

majority of hepatocytes. This procedure will allow the use of siRNA in mice for gene function and drug target validation studies. Moreover, the demonstration that siRNA can effectively inhibit gene expression in mammals opens the door for exploring the use of siRNA in humans to treat disease.

GenBank accession numbers. pGL3 control vector, U47296; pRL-SV40 plasmid, AF025845.

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Note: Supplementary information is available on the Nature Genetics website.

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Competing interests statement

The authors declare competing financial interests: details accompany this paper on the Nature Genetics website (<http://genetics.nature.com>).

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