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Effective RNAi-mediated gene silencing without interruption of the endogenous microRNA pathway

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Abstract

Systemic administration of synthetic small interfering RNAs (siRNAs) effectively silences hepatocyte gene expression in rodents and primates[1–3]. Whether or not *in vivo* gene silencing by synthetic siRNA can disrupt the endogenous microRNA (miRNA) pathway remains to be addressed. Here we show that effective target-gene silencing in the mouse and hamster liver can be achieved by systemic administration of synthetic siRNA without any demonstrable effect on miRNA levels or activity. Indeed, siRNA targeting two hepatocyte-specific genes (apolipo-protein B and factor VII) that achieved efficient (~80%) silencing of messenger RNA transcripts and a third irrelevant siRNA control were administered to mice without significant changes in the levels of three hepatocyte-expressed miRNAs (miR-122, miR-16 and let-7a) or an effect on miRNA activity. Moreover, multiple administrations of an siRNA targeting the hepatocyte-expressed gene *Scap* in hamsters achieved long-term mRNA silencing without significant changes in miR-122 levels. This study advances the use of siRNAs as safe and effective tools to silence gene transcripts in animal studies, and supports the continued advancement of RNA interference therapeutics using synthetic siRNA.

Recently, it was reported that adeno-associated viral (AAV)-expressed short hairpin RNA (shRNA), when administered to mice, can result in profound toxicity, presumably by saturation of the cellular miRNA pathway[4]. AAV expression of shRNA seemed to elicit toxicological effects by interference of cellular pathways for miRNA biogenesis, including

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transport of nuclear miRNA precursors to the cytoplasm by the nuclear karyphorin exportin-5. shRNA-mediated toxicological effects were evident by marked reductions in cytoplasmic levels of mature miRNA, and occurred in a manner independent of both 'on-target' silencing and shRNA sequence. The findings of ref. [4] can be explained by viral vector expression of shRNA at high levels, because lower-level shRNA expression seems to be tolerated[4,5]. However, the implications for cellular delivery of synthetic siRNA that acts downstream of miRNA biogenesis have remained undetermined.

We interrogated the biochemical and functional status of the miRNA pathway after *in vivo* administration of synthetic siRNA formulated in liposomal nanoparticles such as those recently described[2,6]. Formulated, synthetic siRNA targeting two hepatocyte-expressed gene transcripts (si-Apob targeting apolipoprotein B (*Apob*) and si-FVII targeting factor VII (*F7*)) and an irrelevant gene (si-Luc targeting luciferase) were administered by a single intravenous bolus injection at different dose levels in groups of three mice. At both 2 and 30 days, silencing of on-target gene transcripts in liver homogenates was measured by the branched DNA assay[2], and levels of endogenous miRNA (miR-122, miR-16 and let-7a) were assessed by both northern blot and nuclease protection assays.

Administration of high-dose (5 mg kg^{-1}) si-Apob and si-FVII resulted in marked silencing of the hepatocyte-expressed genes to 22 ± 3% and 17 ± 2% relative to placebo-treated mice at the 2-day time point, respectively (Supplementary Fig. 1). The siRNA effects were dose-dependent, with intermediate silencing effects observed in the animals treated with a low dose (2 mg kg^{-1}) at the same time point. Consistent with previous findings[1,2], the RNA interference (RNAi)-mediated silencing effect was selective, with no measurable effect on *Apob* mRNA levels with si-FVII or si-Luc treatment. Furthermore, there was no significant effect on *F7* mRNA levels in mice treated with si-Apob or si-Luc siRNA. At the 30-day time point, *Apob* and *F7* mRNA returned to near normal levels. Treatment with siRNA was not associated with any observed toxicities over the 30-day time period.

To determine whether either ~80% silencing of two different hepatocyte-expressed genes or hepatocyte delivery of three distinct siRNAs results in dysregulation of miRNA biogenesis, levels of the liver-specific miRNA, miR-122, and the broadly expressed miRNAs miR-16 and let-7a were measured in liver tissue samples at both time points for all treatment groups at all dose levels. As shown in Fig. 1, there was no significant reduction in miRNA levels for individual-siRNA-treated animals, as assessed by northern blot assay. To quantify more accurately miRNA levels, a nuclease protection assay was used to measure simultaneously each miRNA together with the small nuclear RNA *U6*. Inclusion of the *U6* control allowed for normalization of the total amount of RNA in each assay. As summarized in Supplementary Table 1, no significant differences were measured in miRNA levels in siRNA-treated animals. Expression levels ranged from 80% to 110% of the levels measured in PBS-treated animals, and were not associated with any trend related to the siRNA dose level or degree of gene silencing. Certainly, no reduction of miRNA levels to match the >80% level previously reported in ref. [4] was observed with administration of synthetic siRNA.

The northern blot and nuclease protection results established that synthetic siRNAs do not inhibit the synthesis or processing of cellular miRNAs. We extended these findings to investigate whether systemically administered siRNAs interfere with endogenous miRNA function. Although *in vivo* data on miRNA function are limited to only a few published reports[7–9], it has been shown that inhibiting mouse miR-122 with antagomirs significantly increases the mRNA levels of a number of liver-expressed genes including *Aldoa*, *Hfe2*, *Tmed3*, *Lass6*, *Slc35a4*, *Tmem50b* and *Gpx7* (ref. [9], see Supplementary Fig. 2). Accordingly, liver mRNA levels for these seven putative miR-122 targets were quantified using branched

DNA assays (normalized to *Gapdh*) in mice treated with si-Apob, si-FVII and si-Luc (two days after injection), and compared to PBS-treated animals. Moreover, protein levels of total Aldolase and another miR-122 target, Iqgap1 (ref. [9]), were assayed by western analysis of liver lysates. Consistent with the lack of effect on mature miR-122 levels, there was no increase in the mRNA or protein levels of any of these genes that could be correlated with the dose of siRNA administered or the degree of gene silencing observed (Fig. 2 and Supplementary Fig. 3).

These studies in mice demonstrate that acute gene silencing of ~80% by single administration of siRNA is not associated with any changes in biosynthesis of miR-122, miR-16 or let-7a, nor with any increase in miR-122 target-gene expression. To explore whether prolonged gene silencing by multiple doses of siRNA would alter miRNA levels, and to extend our studies to another species, we targeted the hepatocyte-expressed gene SREBP cleavage-activating protein (*Scap*) in hamsters. To silence *Scap*, hamsters were given three, weekly injections of a formulated siRNA targeting *Scap* (si-Scap) or a mismatch control siRNA (si-Scapmm) at a dose of 2.5 mg kg^{-1}. Repeated treatment with si-Scap resulted in significant (~75%) silencing of *Scap* mRNA, as well as a very significant reduction in Scap protein on day 21 relative to saline-treated animals (Supplementary Fig. 4). As shown in Fig. 3, silencing of *Scap* for three weeks by multiple doses of si-Scap had no effect on levels of miR-122, nor did repeat-dosing of the mismatch control, si-Scapmm.

Our findings demonstrate that robust gene silencing, both acute and longer-term, can be achieved in the liver without any detectable alteration of cellular miRNA biogenesis or function. Certainly, additional studies are warranted such as those with longer ‘dicer substrate’ siRNAs (>21 nucleotides in length) that act upstream of 21-nucleotide siRNA and could interfere with miRNA biogenesis[10]. Meanwhile, effective gene silencing with siRNA, in which RNA interference is mediated downstream of miRNA biogenesis, can be dissociated from toxicities that may be intrinsic to DNA-expressed shRNA.

METHODS SUMMARY

Antagomirs and siRNAs were synthesized as described[1,9]. Formulation of siRNAs into liposomes was performed by extrusion. Antagomirs and formulated siRNAs were administered to mice and hamsters by intravenous injection. Expression levels of miRNAs were determined by northern blotting and nuclease protection assay of total RNA isolated from livers of treated animals. Levels of miR-122 target mRNAs were measured in liver lysates using the QuantiGene assay (Genospectra). Western blot analysis was used to measure Scap protein levels in hamster liver lysates and miR-122 target (Aldoa, Iqgap1) protein levels in mouse liver lysates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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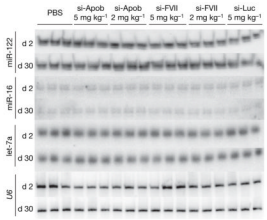

Figure 1. Northern blot analysis of mature miRNAs reveals no detectable differences among siRNA treatment groups

Northern blots of total RNA from mouse livers isolated two days (d 2) and 30 days (d 30) after intravenous injections of PBS, si-Apob (5 mg kg^{-1} and 2 mg kg^{-1}), si-FVII (5 mg kg^{-1} and 2 mg kg^{-1}) or si-Luc (5 mg kg^{-1}) hybridized to radiolabelled probes specific for miR-122, miR-16 and let-7a (one animal per lane). Hybridization to a radiolabelled probe specific for *U6* was used to confirm equal loading of RNA.

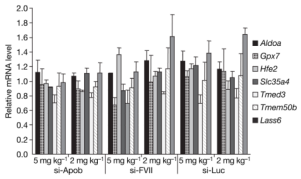

Figure 2. Liver mRNA levels of miR-122 targets are unaffected by siRNA treatment

Mouse liver *Aldoa*, *Gpx7*, *Hfe2*, *Slc35a4*, *Tmed3*, *Tmem50b* and *Lass6* mRNA levels normalized to *Gapdh* mRNA measured 2 days after intravenous injections of PBS or different doses (5 mg kg^{-1} and 2 mg kg^{-1}) of formulated siRNA targeting *Apob* (si-Apob), *F7* (si-FVII) or luciferase (si-Luc) (n = 3 per group). Data are shown as group mean ± s.d. normalized to the PBS group for each gene.

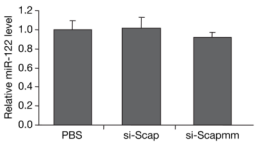

Figure 3. Long-term silencing of *Scap* in hamster liver by repeat administration of siRNA does not affect miR-122 levels

Hamster liver miR-122 levels measured by nuclease protection (normalized to *U6* RNA levels) 7 days after three, weekly intravenous injections of PBS or 2.5 mg kg^{-1} formulated siRNA targeting *Scap* (si-Scap) or a mismatch control siRNA (si-Scapmm). Data are shown as mean ±s.d.