Tissue-specific gene silencing monitored in circulating RNA

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Tissue-specific gene silencing monitored in circulating RNA

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ABSTRACT
Pharmacologic target gene modulation is the primary objective for RNA antagonist strategies and gene therapy. Here we show that mRNAs encoding tissue-specific gene transcripts can be detected in biological fluids and that RNAi-mediated target gene silencing in the liver and brain results in quantitative reductions in serum and cerebrospinal fluid mRNA levels, respectively. Further, administration of an anti-miRNA oligonucleotide resulted in decreased levels of the miRNA in circulation. Moreover, ectopic expression of an adenoviral transgene in the liver was quantified based on measurement of serum mRNA levels. This noninvasive method for monitoring tissue-specific RNA modulation could greatly advance the clinical development of RNA-based therapeutics.

Keywords: RNAi; circulating RNA; exosome; gene silencing; in vivo delivery

INTRODUCTION
Previously, it has been shown that messenger RNAs and micro RNAs derived from various tissues can be detected in circulation (Kamm and Smith 1972; Hunter et al. 2008). The circulating RNA is associated with vesicular structures, such as exosomes, and other ribonucleoprotein particles and is thereby protected from nucleolytic degradation (Smalheiser 2007). While the function of circulating RNA remains incompletely described, it has been suggested that it plays a role in intercellular signaling in normal and diseased states (Smalheiser 2007; Valadi et al. 2007; Hood et al. 2011; Record et al. 2011). In addition, it has been proposed that circulating RNA can be useful in diagnostic applications in different disease settings (Chan et al. 2003; O’Driscoll 2007; Conde-Vancells et al. 2008; Raimondo et al. 2011). However, the quantification of circulating mRNA and micro-RNA (miRNA) as a method for monitoring tissue-specific RNA modulation remains to be incompletely described,

RESULTS AND DISCUSSION
To confirm that liver-specific mRNAs can be detected in serum, filtered rat serum was subjected to high-speed centrifugation, and total RNA was isolated from the resulting pellet. To maximize recovery of RNA, LiCl was added to a final concentration of 1 M prior to centrifugation. As shown in Figure 1A, mRNAs corresponding to the liver-expressed genes Ttr, Serpin1, Alb, and FVII could be detected by reverse-transcription quantitative PCR (RT-qPCR). In addition, mRNA derived from the ubiquitously expressed gene Gapdh was detected. Similarly, TTR, SERPINA1, and GAPDH mRNA could be detected in RNA isolated from cynomolgus monkey (Macaca fascicularis) and human serum processed in a comparable manner (Fig. 1B). Between fourfold and 15-fold higher levels of Gapdh, Alb, Actb, and Ttr mRNA were detected in rat serum samples prepared with versus without, LiCl addition, indicating the importance of this step in the procedure (Fig. 1C). We could also measure levels of liver- and tumor-specific transcripts in serum obtained from patients

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with liver tumors, suggesting that this method can be extended into diseased tissues (Fig. 1D). Both α-fetoprotein (AFP) and glypican-3 (GPC3) are commonly expressed in hepatocellular carcinoma and serve as diagnostic serum protein markers (Bertino et al. 2012). As expected, the mRNA for AFP and GPC3 were not detected in normal human plasma samples (data not shown).

Since liver-specific mRNAs were found in circulation, we next asked whether siRNA-mediated gene silencing in liver would result in corresponding reductions in serum mRNA levels for the specific genes targeted. Accordingly, rats were treated with a single intravenous dose of 0.3 mg/kg lipid nanoparticle (LNP) formulated siRNA targeting \(Ttr\), a liver-expressed gene with a well-established role in human disease (Saraiva 1995). Control animals received LNP-formulated luciferase siRNA. As shown in Figure 2A, treatment with LNP-formulated \(Ttr\) siRNA resulted in 96 ± 1% (mean ± SD) inhibition of liver \(Ttr\) mRNA, normalized to \(Gapdh\), in treated animals 24 h following administration. Regarding mRNA levels from serum, administration of the LNP-formulated \(Ttr\) siRNA resulted in a 94 ± 2% reduction in circulating \(Ttr\) mRNA (normalized to \(Serpina1\)). These results were extended to a second liver-specific target gene, \(Tmprss6\) (Finberg et al. 2008), which encodes a transmembrane protein whose levels cannot be measured in blood. Specifically, a single intravenous dose of 0.3 mg/kg LNP-formulated siRNA targeting \(Tmprss6\) resulted in reductions of 90 ± 1.3% in \(Tmprss6\) mRNA in the liver and 95 ± 2.5% in serum 24 h after administration (Fig. 2B). Thus, siRNA-mediated silencing of liver-expressed genes results in a concomitant reduction in circulating mRNA levels that can be readily detected by analyzing total RNA obtained by high-speed centrifugation of serum. These coordinated tissue- and target-specific mRNA reductions validate the method of circulating extracellular mRNA detection (cERD). In addition to mRNAs, miRNAs have been detected in circulation (Hunter et al. 2008). To test the applicability of cERD to monitor the activity of miRNA-targeting oligonucleotides, rats were treated with an LNP-formulated oligonucleotide directed against the liver-specific miRNA \(miR-122\) (anti-miR) (Krutzhof et al. 2005; Esau et al. 2006); control animals received a mismatched oligonucleotide (MM). Three days later, \(miR-122\) levels were measured in total RNA isolated from liver and serum. As shown in Figure 2C, treatment with the \(miR122\)-specific oligonucleotide reduced liver \(miR-122\) levels by 88 ± 5% compared with treatment with MM. In serum, a similar decrease

**FIGURE 1.** Detection of specific RNAs in serum from multiple species. (A) Relative levels of liver-specific (\(Ttr\), \(Serpina1\), \(Alb\), \(FVII\)) and ubiquitous (\(Gapdh\)) mRNAs, measured by qPCR in RNA isolated from filtered, centrifuged rat serum. Values are relative to \(Gapdh\) levels. Rat serum was pooled from three animals for this analysis; error bars, SD in experimental replicates. (B) Similar analysis of human and cynomolgus monkey (\(Macaca fascicularis\)) circulating RNA. Data shown are from one pooled human or monkey sample; error bars, SD among experimental replicates. (C) Two rat serum samples (S1, 3 mL; S2, 3.5 mL) were processed for circulating RNA isolation with or without LiCl addition. Relative levels of four different mRNAs were measured by qPCR (normalized to \(Gapdh\) level in the “no LiCl” sample S1). (D) Relative mRNA levels of liver-specific genes \(TTR\) and albumin (\(Alb\)) as well as tumor-specific genes \(AFP\) and \(GPC3\) as measured by qPCR in RNA isolated from a 75-yr-old male patient with metastatic liver cancer.
FIGURE 2. Silencing of liver mRNA is reflected in circulating mRNA levels. (A) Silencing of rat liver and serum Ttr 48 h after intravenous administration of 0.3 mg/kg LNP-formulated Ttr siRNA (si-TTR) or Luc siRNA (si-Luc). Levels of Ttr were normalized to Gapdh (liver) or Serpin1 (serum) levels. Group averages, relative to the si-Luc group, are shown (n = 6 per group). (B) Silencing of rat liver and serum Tmprss6 48 h after intravenous administration of 0.3 mg/kg LNP-formulated Tmprss6 siRNA (si-Tmprss6) or Luc siRNA (si-Luc). Levels of Tmprss6 were normalized to Gapdh (liver) or Serpin1 (serum) levels. Group averages, relative to the si-Luc group, are shown (n = 6 per group). (C) Relative levels of miR-122 detected by qPCR in equal amounts of total RNA isolated from rat liver or from filtered, centrifuged rat serum obtained 3 d following intravenous administration of 1 mg/kg LNP-formulated mismatch control oligonucleotide targeting miR-122 (anti-miR). Group averages compared to a control group receiving 1 mg/kg LNP-formulated mismatch control oligonucleotide (MM) are shown (n = 5 per group). Significance was determined by Student’s t-test (*P < 0.05). (D) Dose-dependent silencing of cynomolgus monkey (M. fascicularis) liver and serum TTR 48 h after intravenous administration of indicated doses (mg/kg) of LNP-formulated TTR siRNA (si-TTR). Levels of TTR were normalized to GAPDH (liver) or SERPINA1 (serum) levels. Group averages, relative to a 3 mg/kg LNP-Luc siRNA-treated control group (si-Luc), are shown (n = 3 per group). Significance relative to the Luc siRNA treated control was determined by ANOVA (*P < 0.005, †P < 0.05). (E) Relative levels of Snca measured by qPCR in total RNA isolated from the striatum, or from centrifuged cerebrospinal fluid (CSF) of rats following intraparenchymal CNS infusion of Snca siRNA (siSNCA; n = 27). Snca levels were normalized to Gapdh. Group averages relative to naïve animals (n = 4) are shown. Significance was determined by Student’s t-test (*P < 0.001, †P < 0.05). Error bars, SDs for each group.

(86 ± 9%) was measured, thus establishing the use of cERD to monitor miRNA inhibition.

A TTR silencing study was conducted in the cynomolgus monkey (M. fascicularis) to extend these results to higher species. Animals were administered LNP-formulated, TTR-specific siRNA by intravenous infusion at doses of 0.3, 1, and 3 mg/kg; control animals received LNP-formulated luciferase siRNA (3 mg/kg). Forty-eight hours after LNP-siRNA administration, blood was drawn, and then liver samples were obtained for analysis. As shown in Figure 2D, liver TTR mRNA levels were decreased by 34 ± 18% (P < 0.005), 47 ± 15% (P < 0.005), and 59 ± 4% (P < 0.05) following treatment with 0.3, 1, and 3 mg/kg TTR siRNA, respectively. As monitored by cERD, circulating TTR mRNA levels (normalized to SERPINA1) were reduced by 40 ± 6% (not significant) and 70 ± 8% (P < 0.005) in the 1 mg/kg and 3 mg/kg dose groups, respectively, but were unchanged in the 0.3 mg/kg dose group. Taken together with the rat studies, these data establish the applicability of the cERD method in higher species and thus the general utility of measuring circulating serum mRNA to confirm liver gene silencing induced by administered siRNA.

Circulating RNA has also been detected in CSF (Harrington et al. 2009). To determine whether cERD could be applied to CSF to monitor gene silencing in the brain, we infused siRNA targeting a Parkinson’s disease–associated gene, Snca (Spillantini et al. 1997), bilaterally into the striatum of rats. At the end of the 7-d infusion period, brains and CSF were collected for analysis. Consistent with previous studies demonstrating gene silencing in neuronal cells by intraparenchymal CNS infusion of Snca siRNA (siSNCA; n = 27), Snca levels were normalized to Gapdh. Group averages were not subjected to high-speed centrifugation, and total RNA was isolated from the pellets. A 61 ± 17% reduction in Striatum Snca mRNA was measured in animals receiving the Snca siRNA compared with naïve animals. To analyze CSF RNA, pooled samples were subjected to high-speed centrifugation, and total RNA was isolated from the pellets. A 61 ± 17% reduction in Striatum Snca mRNA was measured in CSF from Snca siRNA-treated animals versus controls (Fig. 2E). The greater relative degree of Snca mRNA reduction in the CSF compared with the striatum could be due to variability in the release of RNA by different
regions or cell types of the brain. Regardless, these results confirm the suitability of cERD to monitor silencing of a brain-expressed gene by assaying CSF RNA.

Previous studies have established that administration of a single dose of LNP-formulated siRNA leads to rapid and durable target gene silencing in the livers of rodents and primates (Frank-Kamenetsky et al. 2008; Akinc et al. 2010; Love et al. 2010). To compare the kinetics of siRNA treatment on levels of circulating and liver mRNA, rats were given a single dose of 0.1 mg/kg LNP-formulated Ttr siRNA, and serum and livers were collected 1, 2, 5, 8, 10, and 14 d later; control animals received LNP-luciferase siRNA. As shown in Figure 3A, maximal inhibition of Ttr mRNA in the liver (95 ± 2%, normalized to Gapdh) and in serum (92 ± 8%, normalized to Serpina1) as monitored by cERD was observed 1 d following administration of LNP-siRNA targeting Ttr. This effect was maintained 2 d post-treatment and returned to near control levels (as assessed by LNP-siRNA targeting luciferase) by day 10. In a second study, reductions in liver and serum Ttr mRNA were measured at earlier time points following siRNA administration. Within 3 h of treatment, both liver and serum Ttr mRNA levels were reduced by 40%–50%, with peak target reductions of ∼90% achieved in liver and serum by 12 h (Fig. 3B). Thus, there is a remarkable concordance of the relative Ttr mRNA levels in the liver and in serum as measured over time following treatment with LNP-siRNA, with respect to both onset and duration of target gene silencing. These results support the conclusion that monitoring circulating mRNA levels of a liver gene by cERD provides an accurate representation of tissue-specific target gene silencing.

A modified RACE-PCR technique has been used to confirm the RNA interference mechanism by identification of the predicted siRNA cleavage product in a number of preclinical and clinical studies (Zimmermann et al. 2006; Frank-Kamenetsky et al. 2008; Querbes et al. 2009; Davis et al. 2010), including detection of circulating siRNA cleavage in a recent clinical trial (Coelho et al. 2013). To investigate whether RNAi in the liver could be demonstrated in circulating RNA, serum was collected from rats 24 h after treatment with LNP-formulated Ttr siRNA (or luciferase siRNA control) and processed as described above to obtain circulating RNA. Products generated by the modified RACE-PCR method were cloned, and individual colonies were selected for sequencing. Out of 45 clones derived from the luciferase siRNA-treated animals, none corresponded to the predicted Ttr siRNA cleavage site. In contrast, 15 of 51 clones obtained from the Ttr siRNA treatment group were found to terminate precisely at the predicted siRNA cleavage position (P = 0.002) (for representative sequence traces, see Supplemental Fig. S1A,B). Similar results were obtained with serum from cynomolgus monkeys treated with LNP-formulated TTR siRNA (Supplemental Fig. S1C). Therefore, the RNA interference mechanism occurring in liver was molecularly verified by analysis of circulating RNA in serum.

To extend the analysis beyond endogenously expressed genes, rats were injected with an adenoviral vector to induce GFP expression at various levels in the liver (Herrmann et al. 2004). The relative levels of GFP mRNA measured in circulation were in good agreement with the corresponding liver mRNA levels for four animals analyzed 5 d after adenoviral injection (Fig. 4A). Thus, measurement of mRNA levels in serum can be applied to the analysis of gene therapy delivery of exogenous genes.

We have shown that analysis of RNA in biological fluids can be used to quantify the effects of RNA antagonists, such as siRNA- or miRNA-targeted oligonucleotides, and gene-expression vectors, such as adenoviral constructs, on RNA levels in rodent and nonhuman primate tissues. Circulating RNA was found to correspond closely with tissue RNA levels and

**FIGURE 3.** Concordance of silencing in liver and circulating RNA. (A) Time course of rat liver and serum Ttr silencing following intravenous administration of 0.1 mg/kg LNP-formulated Ttr siRNA (si-TTR). Levels of Ttr were normalized to Gapdh (liver) or Serpina1 (serum) levels. Group averages, relative to a 0.1 mg/kg LNP-Luc siRNA-treated control group (si-Luc), are shown (n = 7 per group, for each time point). (B) Levels of rat Ttr mRNA measured by qPCR at the indicated times following intravenous administration of 0.3 mg/kg LNP-formulated Ttr siRNA (si-TTR). Ttr levels were normalized to levels of Gapdh (liver) or Serpina1 (serum), and group averages were expressed relative to control animals receiving 0.3 mg/kg si-Luc analyzed at 6 h (n = 5 per group). Significance relative to the Luc siRNA-treated control group was determined by ANOVA (P < 0.001; **P < 0.01). Error bars, SDs for each group.
The tissue of origin, as verified from NCBI and http://biogps.org, is marked modulin (UMOD) measured by qPCR in RNA isolated from rat serum (normalized to GAPDH).

Their modulation by antagonists, suggesting that RNA levels from biological fluids provide an accurate “real-time” representation of tissue RNA status. Importantly, the RNAs detectable in serum correspond to genes that are very highly expressed (Alb, TTR/Ttr, SERPINA1/Serpina1) as well as moderately expressed (Tmprss6, F7) in the liver (http://www.ncbi.nlm.nih.gov/UniGene). Moreover, we were able to identify several other tissue-specific transcripts in circulating RNA isolated from rat serum (Fig. 4B). We envision that this cERD method will have broad applicability in clinical studies since it allows the routine, accurate, and frequent measurement of organ-specific target gene modulation without the need for tissue biopsies.

MATERIALS AND METHODS

siRNAs, oligonucleotides, and formulations

The LNP formulations used in the rat and cynomolgus monkey studies were prepared using methods and chemical compositions similar to those previously described (Zimmermann et al. 2006; Akinc et al. 2010). Chemically modified siRNAs were synthesized at Alnylam (2′-O-methyl-modified nucleotides are in lowercase): rat Trt, sense AGuGucucAgcuAuAAdTsTdT and antisense UuuGuuAGAcAAGAaCAUCdTsTdT; rat Tmprss6, sense uGGuuAuuccuAGGGuAuAAdTsTdT and antisense UuGuACCUcAgAAAuAcCA dTsTdT; rat Snca, sense AcAucAAGAuGcuAc ccAcCdTsTdT and antisense GGUuAGuAcCUuAGGGuGdTsTdT; cynomolgus monkey TTR, sense GGuuuuuuAuGucAcAAGGA and antisense GGUuAGuACuCuUAGGGuUdTdTsTdT and luciferase, sense cuuAcGucAGuAc cuucGAdTsTdT and antisense GGUuAGuACuUAGGGuUdTdTsTdT. The oligonucleotide directed against miR-122 and the mismatch control oligonucleotide were provided by Regulus Therapeutics, Inc. The sequences have been described (Krutzfeldt et al. 2005).

Animal experiments

All studies were conducted in accordance with animal welfare regulations under IACUC-approved research protocols.

Male Sprague-Dawley rats were administered LNP-formulated siRNA or LNP-formulated oligonucleotide as a single injection via the tail vein at a dose volume of 3 mL/kg body weight. For the adenovirus studies, rats received tail vein injections of 1 × 10¹¹ pfu Adeno-GFP (Viraquest) in a volume of 2 mL. Animals were killed at various time points. To prepare serum, blood was collected via caudal venacava into serum separation tubes and allowed to clot at room temperature for ~30 min prior to centrifugation at 4°C. Livers were collected, frozen in liquid nitrogen, and stored at −80°C.

For brain infusion studies, male Sprague-Dawley rats were anesthetized and placed into a stereotaxic frame (Benchmark Digital Stereotaxic, myNeuroLab). A 30-gauge osmotic pump infusion cannula (Plastics One) was inserted into each hemisphere, targeting the striatum (AP 0.5, ML 3, and DV 5.1 relative to bregma; incisor bar 3.3 mm below the interaural line). A 30-gauge osmotic pump infusion cannula was implanted into the lateral ventricle for continued infusion of a vehicle solution. After 7 d of infusion, rats were anesthetized and mounted in a stereotaxic frame. CSF was collected using a syringe with a 30-gauge needle through the atlanto-occipital membrane. After CSF collection, rats were killed, and brains were removed. Coronal slices, 1 mm thick, from each slice were snap-frozen in liquid nitrogen, and stored at −80°C.

The nonhuman primate study was conducted at Covance Laboratories (Madison, WI). Male cynomolgus monkeys (M. fascicularis) received 15-min infusions of LNP-formulated siRNA via the
saphenous vein at a dose volume of 5 mL/kg body weight. Two days later, animals were anesthetized, and blood was collected from a femoral vein into serum separator tubes without anticoagulant. Blood was incubated for 30–60 min at room temperature and centrifuged. Serum was harvested and stored at −80°C. Animals were killed, and liver samples (~1 g) were collected, frozen in liquid nitrogen, and stored at −80°C.

Serum sample from healthy donors and patients with liver tumors were obtained from Bioreclamation.

**Isolation and analysis of tissue RNA**

Total RNA was extracted from frozen rat liver or striatum using the Qiagen RNeasy kit. For mRNA quantification, cDNA was prepared with the high-capacity cDNA reverse transcription kit (Applied Biosystems) using random primers. For miRNA quantification, cDNA was prepared with the Taqman MicroRNA reverse transcription kit using a miR-122 specific stem–loop primer (Applied Biosystems). Quantitative PCR was performed on a Roche LightCycler 480 using Applied Biosystems Taqman gene expression miRNA assays or miRNA assays (rat Ttr, Rn01406102; rat SerpinA1, Rn00574670; rat Tmprss6, Rn01504810; rat Alb, Rn00592480; rat F7, Rn00596104; rat Sica, Rn01425140; rat Gpdh, 4352338E; miR-122, 002245). A custom Taqman assay was used to quantify GFP expression (forward, GACAACCACTACCTGAGCAC; reverse, ACCATGTGATGACAAACCAACGA).

Cynomolgus monkey TTR and GAPDH liver mRNA levels were measured in Proteinase K–digested liver lysates using gene-specific branched DNA assays (Panomics).

**Isolation and analysis of RNA from serum and CSF**

Human serum was obtained from volunteer donors under an IRB-approved protocol. Rats were killed, and blood was collected from the vena cava for serum isolation 2.5–3.5 mL serum per rat was used for each sample. Rat CSF was collected from multiple rats and pooled for a total volume of 2 mL. Serum (rat, cynomolgus monkey, and human) and CSF (rat) were either filtered (0.4 μm) or centrifuged at 10,000 g for 10 min prior to mixing with LiCl (final concentration 1 M diluted from 8 M stock; Ambion) and incubated for 1 h at 4°C. Tubes were balanced with 1X PBS before spinning at 110,000 g for 100–120 min. Samples were spun in thick-wall polycarbonate tubes in MLA-55 or MLA-130 rotor in Beckman Coulter centrifuge. The combination of differential centrifugation to collect circulating RNA was intended to maximize total RNA yield. Total RNA was isolated from pellets by Trizol extraction (Invitrogen) and pooled for a total volume of 2 mL. Serum (rat, cynomolgus monkey serum, and isopropanol precipitation. Bioanalyzer (Agilent) analysis of selected RNA samples failed to detect a discrete rRNA band, thus yielding unreliable quantitation results. Synthesis of cDNA and Taqman gene expression analysis were performed as described above. Human and rat Taqman gene expression assays: TTR, Hs00174914; SERPINA1, Hs01097800; TMPRSS6, Hs00542184; ALB, Hs00910225; AFP, Hs00173490_m1; GPC3, Hs01018938_m1 GAPDH, 4326317E. Custom Taqman assays were used to quantify gene expression in cynomolgus monkey serum (TTR: forward, TGGTCAGCACAGCTTATGCAGCAG; reverse, GGAGATGTTGCTGTCCTGAA, probe, FAM AGCA TGCAGGTTGG; SERPINA1: forward, ACTAAGGTCTTCAGC AATGGG, reverse, GCTTCAGTGGCTTCTGATCG, probe, FAM- TGGTCAGCACAGCTTATGCAGCAG; GAPDH: forward, ATGGTCAGATGATCCACCCA, reverse, CATCGCCCACTTGTTT G, probe, FAM AGCTTCCGTTCTACCCCTTACAC). For the rat, the assays were the same as described above for tissues and in addition Acta2, Rn01759928_g1; Aldoa, Rn00820577_g1; ApoB, Rn01499054_m1; Apoe, Rn00593680_m1; Gpx2, Rn00822100_g1; Hamp, Rn00584987_m1; Ptprc, Rn00709901_g1; Tek, Rn0143337_m1; and UMOD, Rn00567180_m1.

Ligation-mediated RACE PCR to detect the Ttr siRNA-mediated cleavage product in rat serum was performed using the GeneRacer kit (Invitrogen). Nested PCR products were cloned, and individual clones were sequenced.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

**COMPETING INTEREST STATEMENT**

A.S., Q.C., and D.W.Y.S. are employees of Alnylam Pharmaceuticals, Inc.

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Author contributions: D.W.Y.S. and D.G. conceived the project. A.S., Q.C., D.W.Y.S., and D.B. designed the experiments and interpreted the results. A.S., Q.C., and D.B. performed the experiments. D.B. wrote the manuscript.

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