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 described.

Here we demonstrate that RNAi-mediated target gene silencing in the liver by systemic administration of siRNA results in quantitative reductions in serum mRNA levels that closely corroborate with the degree and kinetics of tissue mRNA silencing, including proof of the RNAi mechanism of action. Further, administration of an anti-miRNA oligonucleotide directed against a liver-specific miRNA was found to result in decreased levels of the miRNA in circulation.

Another application of this technique was demonstrated by quantification of liver expression of an adenoviral transgene using circulating mRNA. Finally, this technique was extended to a different tissue, where silencing of a brain-expressed mRNA was monitored and quantified in cerebrospinal fluid (CSF) following intraparenchymal CNS infusion of a specific siRNA.

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*, *Serpina1*, *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
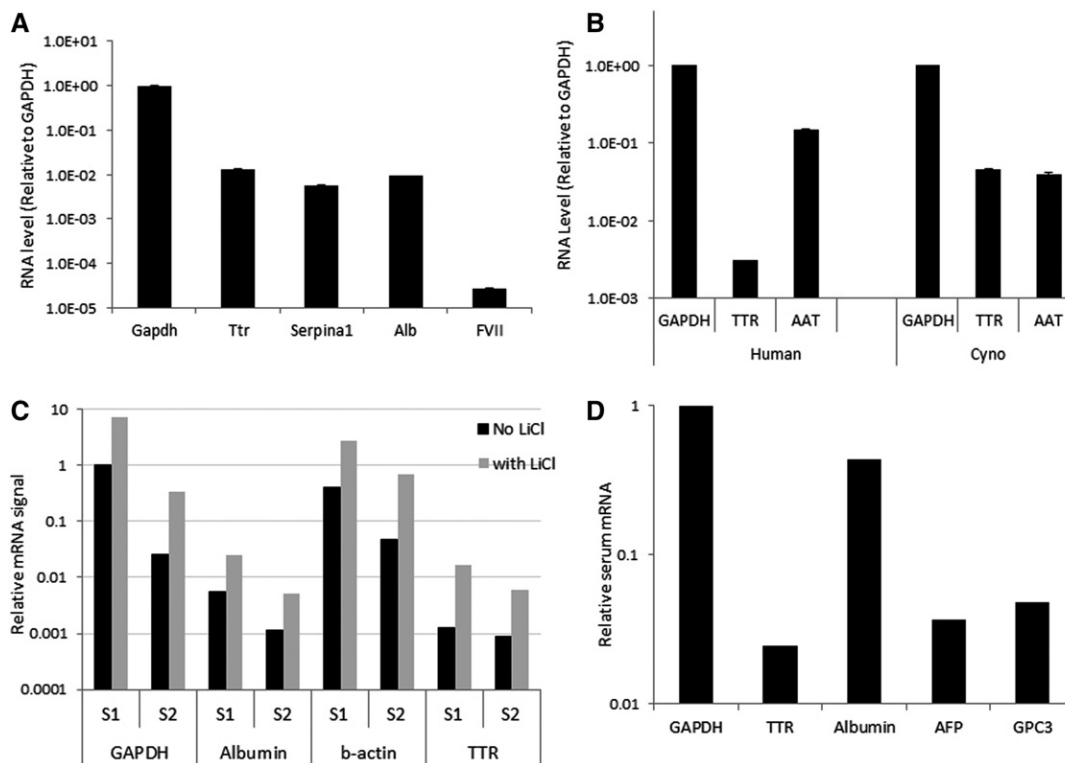
FIGURE 1. Detection of specific RNAs in serum from multiple species. (*A*) Relative levels of liver-specific (*Ttr*, *Serpina1*, *Alb*, *FVII*) and ubiquitous (*Gapdh*) mRNAs, measured by qPCR in RNA isolated from filtered, centrifuged rat serum. Values are relative to *Gapdh* levels. Rat serum was pooled 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.

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) (Krutzfeldt 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
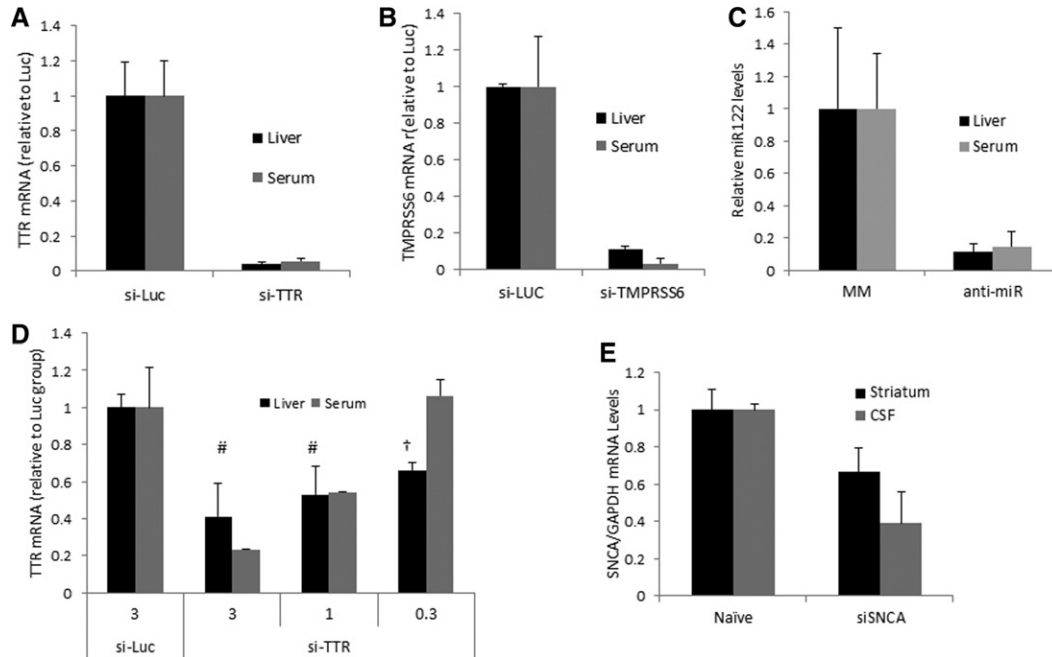
FIGURE 2. Silencing of liver mRNA is reflected in circulating mRNA levels. (*A*) Silencing of rat liver and serum *Ttr* 48 h after intravenous administration of 0.3 mg/kg LNP-formulated *Ttr* siRNA (si-TTR) or Luc siRNA (si-Luc). Levels of *Ttr* were normalized to *Gapdh* (liver) or *Serpina1* (serum) 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 *Serpina1* (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 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 controls 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.

($86 \pm 9\%$) 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 $34 \pm 18\%$ ($P < 0.005$), $47 \pm 15\%$ ($P < 0.005$), and $59 \pm 4\%$ ($P < 0.05$) 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 $40 \pm 6\%$ (not significant) and $70 \pm 8\%$ ($P < 0.005$) 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 siRNA infusion (Lewis et al. 2008; Querbes et al. 2009), a $33 \pm 13\%$ reduction in striatal *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 $61 \pm 17\%$ reduction in *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
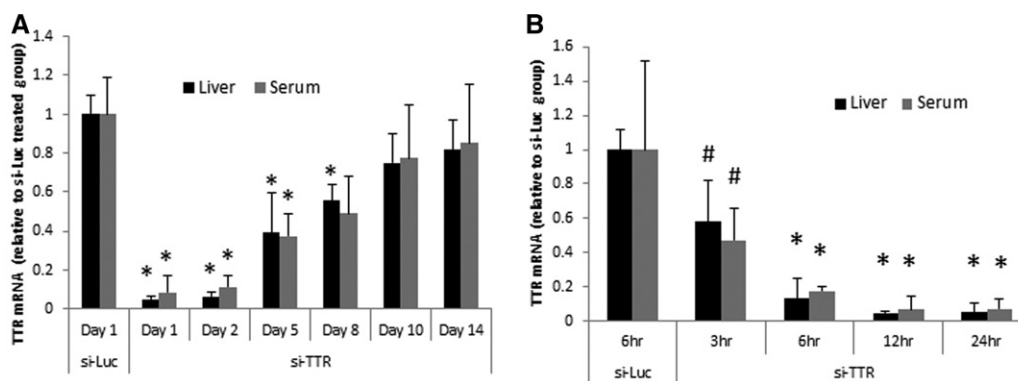
FIGURE 3. Concordance of silencing in liver and circulating RNA. (*A*) Time course of rat liver and serum *Ttr* silencing following intravenous administration of 0.1 mg/kg LNP-formulated *Ttr* siRNA (si-TTR). Levels of *Ttr* were normalized to *Gapdh* (liver) or *Serpina1* (serum) levels. Group averages, 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.
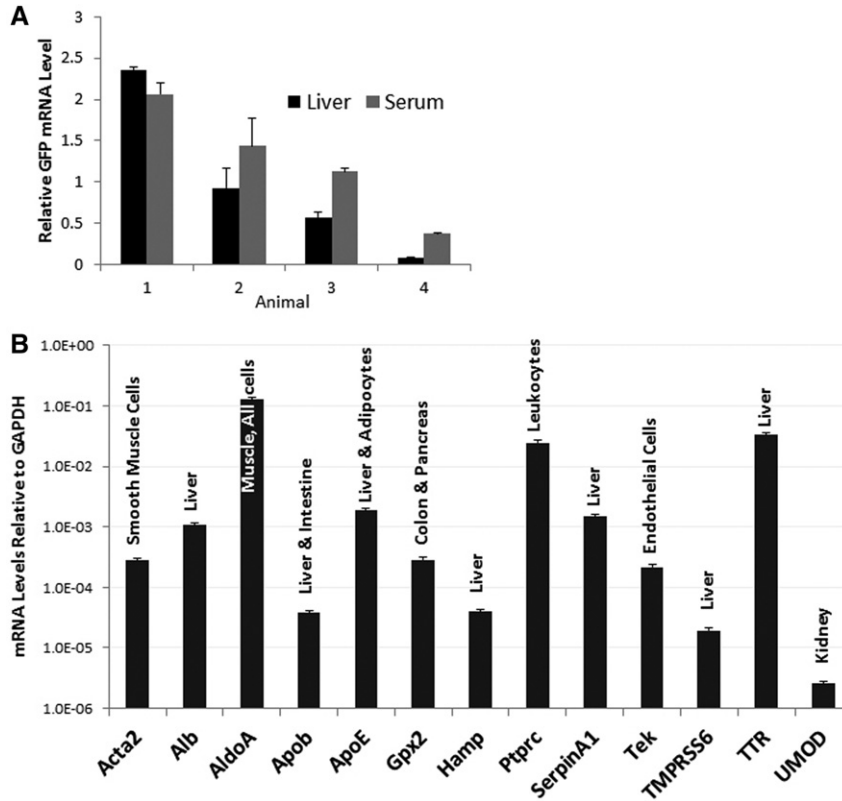
FIGURE 4. Extending circulating RNA measurements beyond liver gene silencing. (*A*) Relative levels of GFP mRNA measured in total RNA isolated from liver and filtered centrifuged serum of four individual rats 4 d following intravenous injection of 10^{11} pfu Adeno-GFP. Values presented are the means of two technical replicates for serum and two technical replicates each from two separate pieces of liver from each animal. (*B*) Relative levels of tissue-specific mRNAs (smooth muscle actin [Acta2], albumin [Alb], aldolase A [AldoA], apolipoprotein B [ApoB], apolipoprotein E [ApoE], Gpx2, hepcidin [Hamp], Ptprc, SerpinA1, Tek, TMPRSS6, transthyretrin [TTR], uromodulin [UMOD]) measured by qPCR in RNA isolated from rat serum (normalized to GAPDH). The tissue of origin, as verified from NCBI and http://biogps.org, is marked *above* each bar.

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 *Ttr*, sense cAGuGuucuuGcucuAuAAdTdT and antisense UuAuAGAGcAAGAAcACUGdTdT; rat *Tmprss6*, sense uGGuAuuuccuAGGGuAcAdTsdT and antisense UGuACCCuAGGAAAuACcAdTsdT; rat *Snca*, sense AcAccuAAGuGAcuAccAcdTsdT and antisense GUGGuAGUcACUuAGGUGUdTsdT; cynomolgus monkey *TTR*, sense GGAuuucAuGuAAccAAGA and antisense GUGGuAGUcACUuAGGUGUdTsdT; and luciferase, sense cuuAcGcuGAGuAcuucGAdTsdT and antisense UCUUGGUuAcAUGAAAUCCdTdT. 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 1×10^{11} 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 vena cava 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 implanted into each hemisphere, targeting the striatum (stereotaxic coordinates AP 0.5, ML 3, and DV 5.1 relative to bregma; incisor bar 3.3 mm below the interaural line). Osmotic pumps (1 μL/hr flow rate, Alzet) containing 4 mg/mL siRNA dissolved in PBS were primed in 0.9% saline overnight at 37°C according to the manufacturer's instructions, and then connected to the cannula and implanted subcutaneously. 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, through the rat brain from anterior to posterior were obtained using a brain matrix (Braintree Scientific), and striatum was dissected from each slice, 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

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 detect 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

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 or miRNA assays (rat *Ttr*, Rn 01406102; rat *Serpina1*, Rn 00574670; rat *Tmprss6*, Rn01504810; rat *Alb*, Rn00592480; rat *F7*, Rn00596104; rat *Snca*, Rn01425140; rat *Gapdh*, 4352338E; *miR-122*, 002245). A custom Taqman assay was used to quantify GFP expression (forward, GACAACCACTACCTGAGCAC; reverse, ACCATGTGAT CGCGCTTC; probe, FAM-CCCTGAGCAAAGACCCCAACGA).

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 10,000g 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 1× PBS before spinning at 110,000g 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 RNA in circulating vesicles and of LiCl addition to precipitate free circulating RNA was intended to maximize total RNA yield. Total RNA was isolated from pellets by Trizol extraction (Invitrogen) 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 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, TGGCATCTCCCCATT CCA, reverse, CGGAATCGTTGGCTGTGAA, probe, FAM-AGCA TGCAGAGGTGG; *SERPINA1*: forward, ACTAAGGTCTTCAGC AATGGG, reverse, GCTTCAGTCCCTTTCTCATCG, probe, FAM-

TGGTCAGCACAGCCTTATGCACG; *GAPDH*: forward, ATGTT CCAGTATGATTCCACCC, reverse, CATCGCCCCACTTGATTTT G, probe, FAM-AGCTTCCCGTTCTCAGCCTTCAC). 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_gH; Hamp, Rn00584987_m1; Ptprc, Rn00709901_m1; Tek, Rn014 33337_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.

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