

## Quantitation of Tissue-specific Target Gene Modulation Using Circulating RNA

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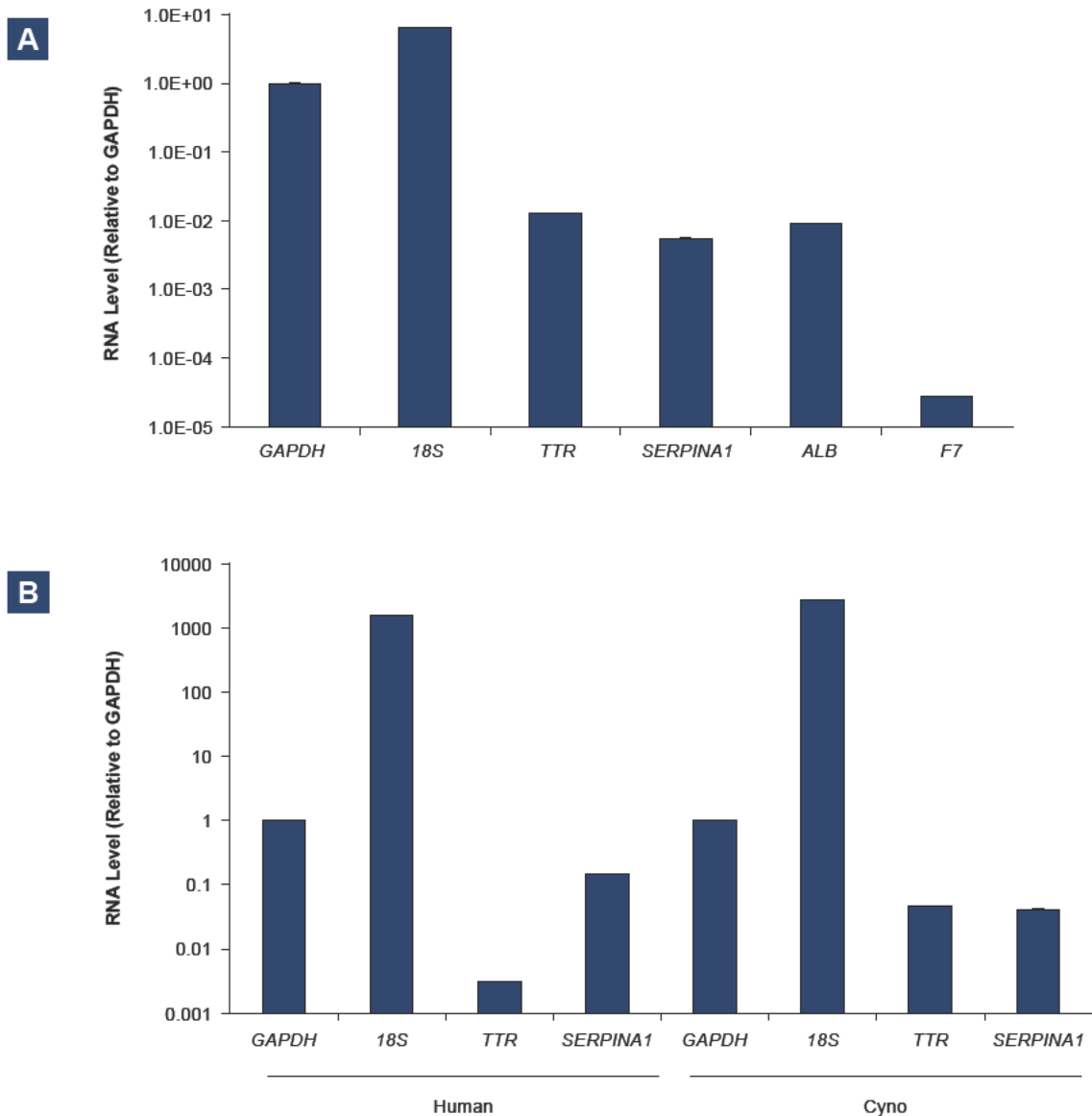


# Abstract

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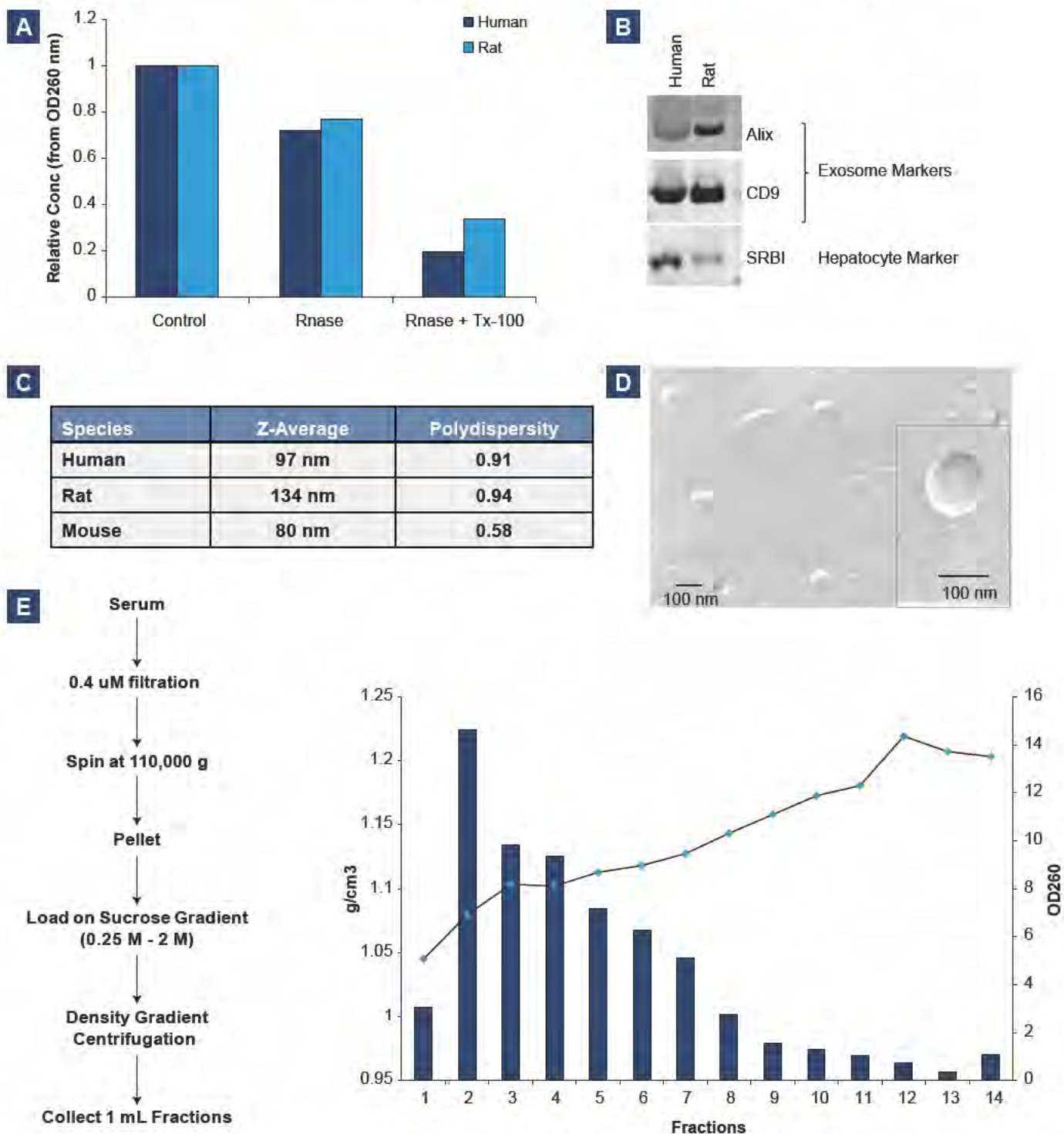
Pharmacologic target gene modulation is the primary objective for RNA antagonist strategies, including small interfering RNA (siRNA),<sup>1</sup> microRNA (miRNA) therapeutics, and antisense oligonucleotide therapeutics. In clinical applications, monitoring tissue-specific target mRNA modulation requires tissue procurement from patients that is highly limited in availability, if even justified. Here we show that circulating RNAs encoding tissue-specific gene transcripts can be detected in biological fluids of humans and experimental animals. Surprisingly, we demonstrate that RNAi-mediated target gene silencing in the liver by systemic administration of siRNA results in quantitative reductions in serum mRNA levels which closely corroborate 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. This technique was extended to a different tissue, where silencing of a brain-expressed mRNA was monitored and quantified in cerebrospinal fluid following intraparenchymal infusion of a specific siRNA. This non-invasive method for monitoring tissue-specific RNA modulation could greatly advance clinical development of gene therapy and RNA-based therapeutics.

## Figure 1. Relative RNA Levels in Serum



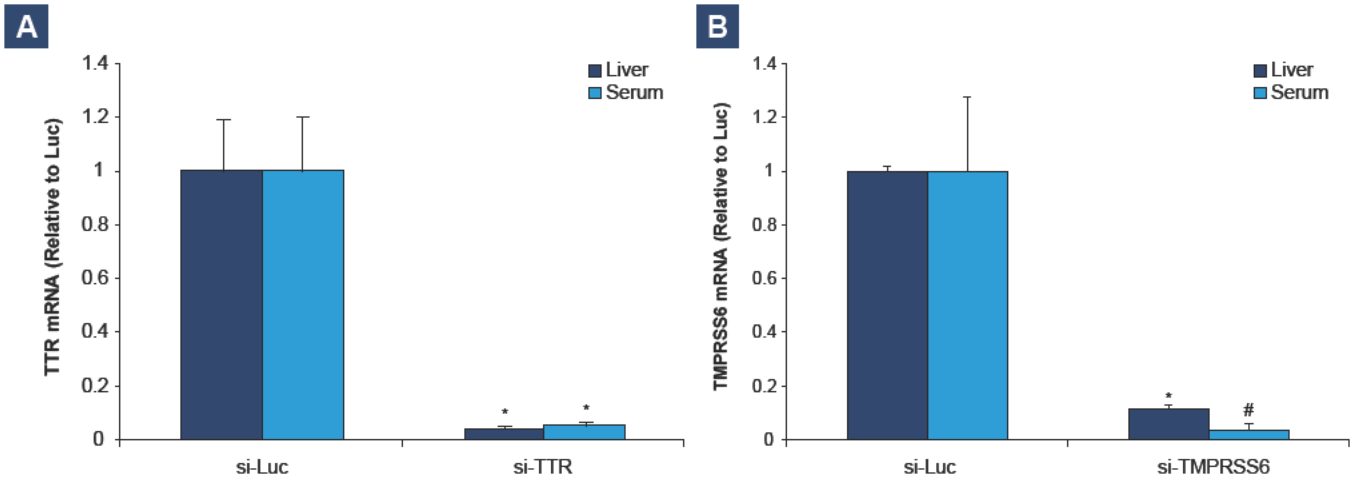
**Figure 1. Liver specific RNA is present in serum. A.** Relative levels of liver-specific (*Ttr*, *Serpina1*, *Alb*, *F7*) and ubiquitous (*Gapdh*) mRNAs, and 18S rRNA measured by qPCR in RNA isolated from filtered, centrifuged rat serum. Values are relative to *Gapdh* levels. **B.** Similar analysis of human and cynomolgus monkey (*Macaca fascicularis*) circulating RNA.

## Figure 2. Circulating RNA Is Present in Exosomes



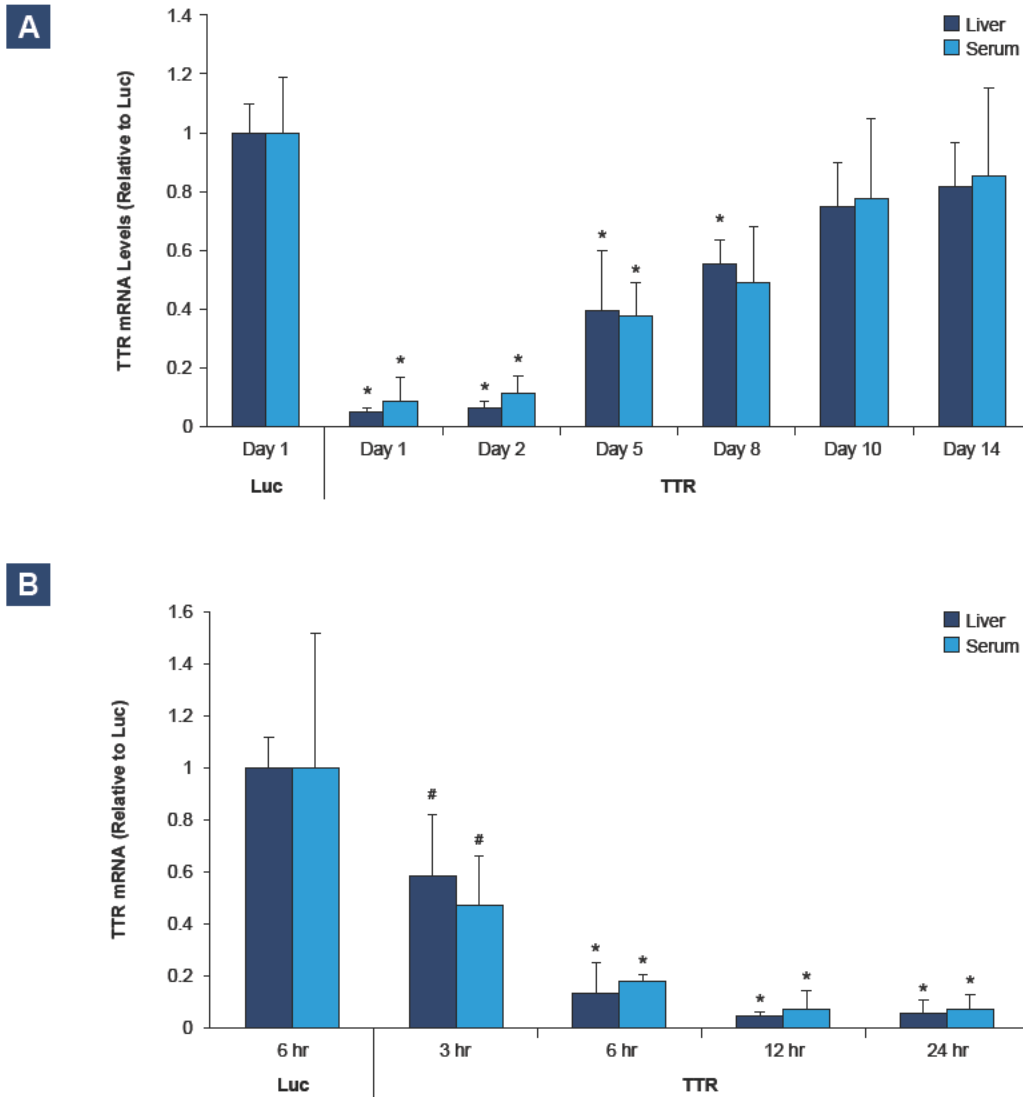
**Figure 2A.** Serum exosomes protect circulating RNA from nucleolytic degradation, and contain known exosome protein markers. Total RNA in material obtained by high-speed centrifugation of filtered human and rat serum was measured by UV absorbance following Ribonuclease A digestion (Rnase), or following Ribonuclease A digestion after pre-treatment with Triton-X-100 (Rnase + Tx-100). Measurements relative to untreated samples (Control) are shown. **B.** Detection of the exosomal markers ALIX and CD9, and the hepatocyte protein marker, SRBI by western analysis of material by high-speed centrifugation of filtered human and rat serum. **C.** Exosomes prepared by high-speed centrifugation of human, rat and mouse serum were analyzed by dynamic light scattering (Malvern, Inc.). Particle diameters (Z-Average) and polydispersity are shown. **D.** Freeze-fracture electron micrograph of exosomes obtained by high-speed centrifugation of filtered rat serum. **E.** Material obtained by high-speed centrifugation of filtered rat serum was fractionated on a sucrose density gradient. Density ( $\text{g}/\text{cm}^3$ , line) and nucleic acid content (OD260, bars) were measured in 1 mL fractions.

### Figure 3. Liver Gene Silencing Measured in Serum



**Figure 3. Silencing of Liver Gene Specific mRNA Is Reflected in Circulating mRNA Levels. A.** Silencing of rat liver and serum *Ttr* 48 hours after intravenous administration of 0.3 mg kg<sup>-1</sup> 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. Error bars represent standard deviations (n=6 per group). **B.** Silencing of rat liver and serum *Tmprss6* 48 hours after intravenous administration of 0.3 mg kg<sup>-1</sup> 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. Error bars represent standard deviations (n=6 per group).

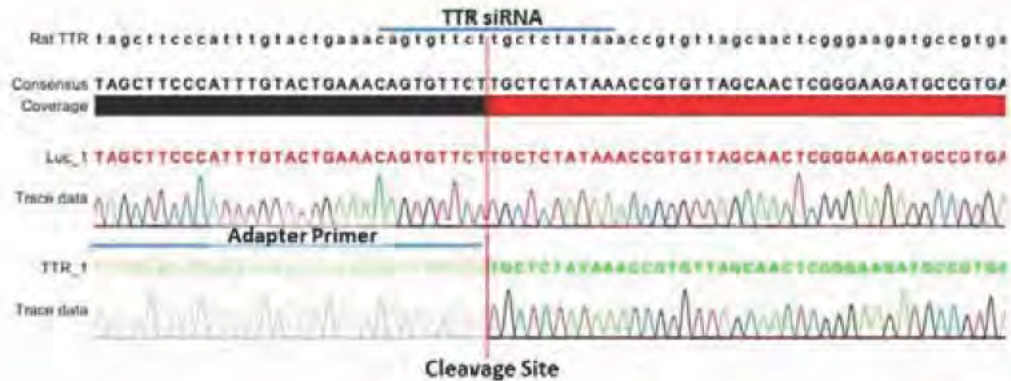
## Figure 4. Onset and Duration of Silencing



**Figure 4A.** Time course of rat liver and serum *Ttr* silencing following intravenous administration of 0.1 mg kg<sup>-1</sup> LNP-formulated *Ttr* siRNA (TTR). Levels of *Ttr* were normalized to *Gapdh* (liver) or *Serpina1* (serum) levels. Group averages, relative to a 0.3 mg kg<sup>-1</sup> LNP-Luc siRNA-treated control group (Luc), are shown. Error bars represent standard deviations (n=7 per group). **B. Onset of rat liver *Ttr* silencing is reflected in serum *Ttr* levels.** Levels of rat *Ttr* mRNA measured by qPCR at the indicated times following intravenous administration of 0.1 mg kg<sup>-1</sup> LNP-formulated *Ttr* siRNA (TTR). *Ttr* levels were normalized to levels of *Gapdh* (liver) or *Serpina1* (serum), and group averages were compared to control animals receiving 0.3 mg kg<sup>-1</sup> LNP-formulated Luc siRNA (Luc) analyzed at 6 hr. Error bars represent standard deviations (n=5 per group). Significance relative to the Luc siRNA treated control group was determined by ANOVA (\*, p<0.001; #, p<0.01).

## Figure 5. Cleavage Product Can Be Detected in Serum

**A**

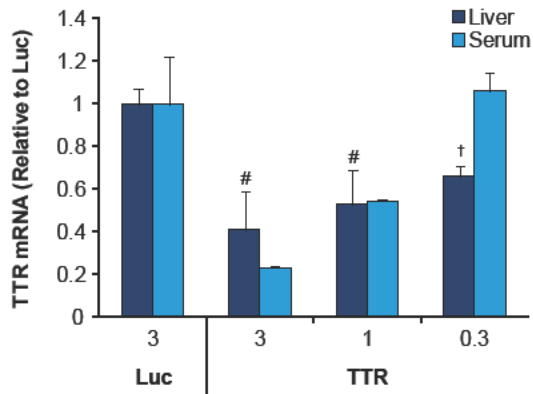


**B**

Treatment Group	# Correct Cleavage Site	Total
Luc	0	45
TTR	15	51

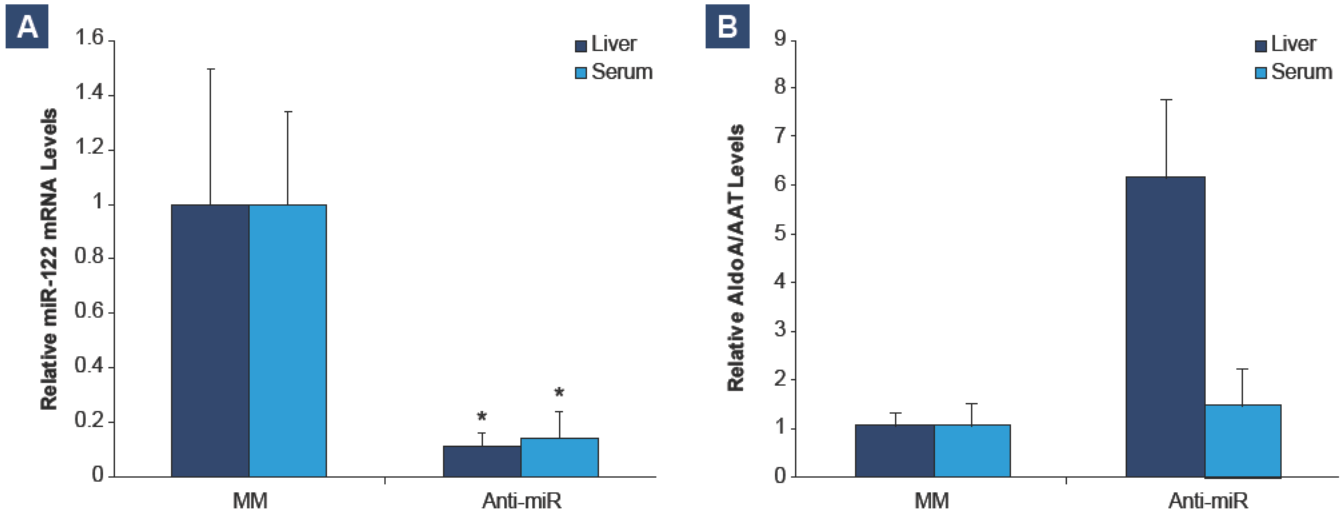
**Figure 5. Confirmation of RNAi mechanism by modified RACE-PCR analysis of circulating RNA.** **A.** Representative sequence traces of individual clones derived from circulating RNA from rats treated with LNP-formulated Luc siRNA (Luc\_1) or LNP-formulated *Ttr* siRNA (TTR\_1). The sequence obtained from the LNP-*Ttr* treated animal demonstrated cleavage at the predicted position. **B.** Summary of sequencing data showing that 15 of 51 sequenced clones from the LNP-*Ttr* group terminated at the predicted cleavage position compared to none of the 45 clones from the LNP-Luc group.  $p=0.002$  by chi-square analysis.

**Figure 6. Silencing of TTR Measured in Cyno Serum**



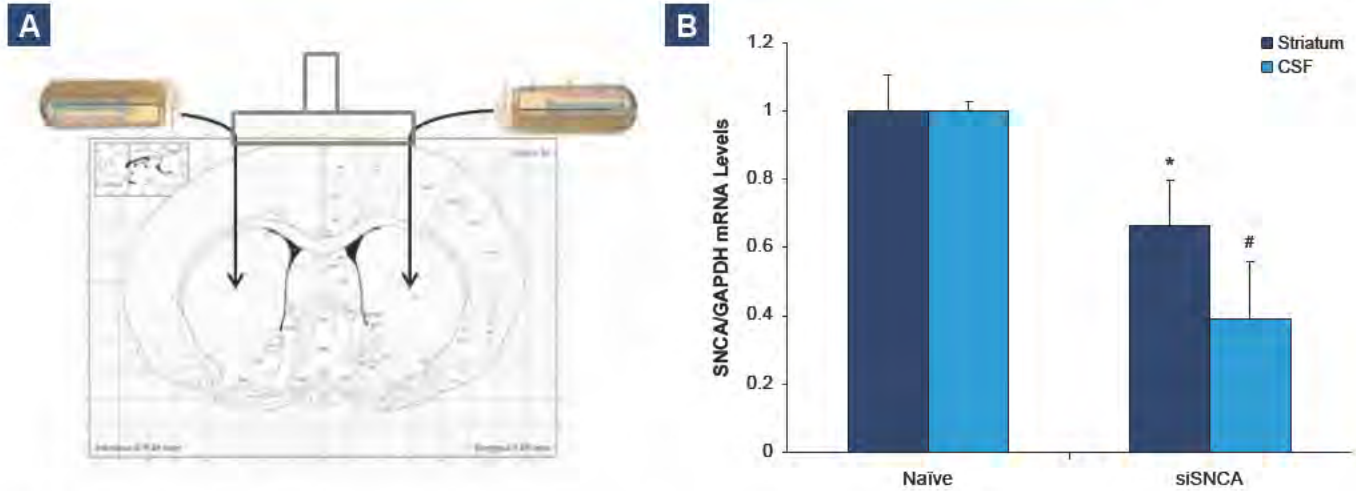
Dose-dependent silencing of cynomolgus monkey (*Macaca fascicularis*) liver and serum *TTR* 48 hours after intravenous administration of indicated doses (mg kg<sup>-1</sup>) of LNP-formulated *TTR* siRNA (*TTR*). Levels of *TTR* were normalized to *GAPDH* (liver) or *SERPINA1* (serum) levels. Group averages, relative to a 3 mg kg<sup>-1</sup> LNP-Luc siRNA treated control group (Luc), are shown. Error bars represent standard deviations (n=3 per group). Significance relative to the Luc siRNA treated controls was determined by Student's t-test (a,b) or ANOVA (c,d) (\*, p<0.001; #, p<0.005, †, p<0.05).

## Figure 7. Knock-down Measurements Extended to miRNA



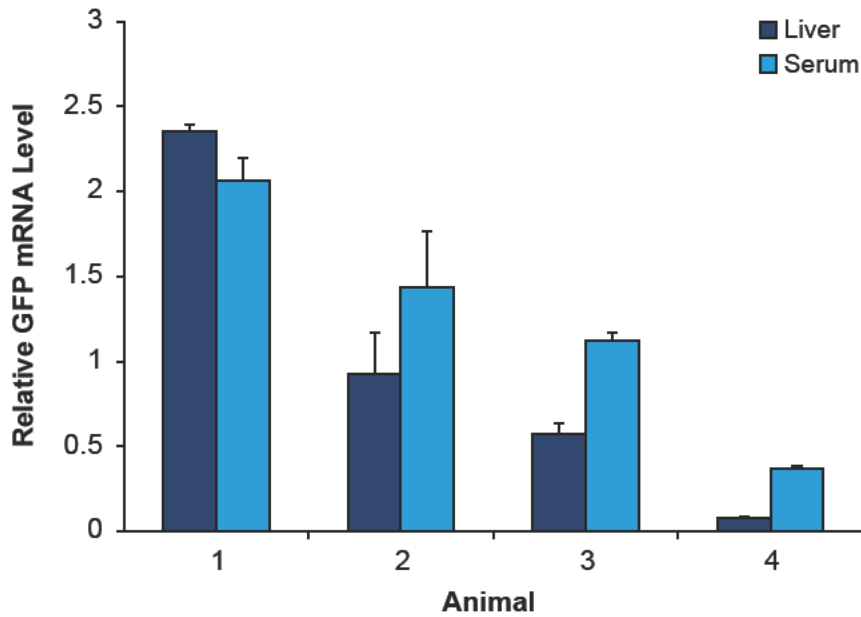
**Figure 7. Targeted reduction of liver *miR-122* leads to a comparable reduction in circulating *miR-122* levels.** **A.** Relative levels of *miR-122* detected by qPCR in equal amounts of total RNA isolated from rat liver or from filtered, centrifuged rat serum obtained 3 days following intravenous administration of 1 mg kg<sup>-1</sup> LNP-formulated oligonucleotide targeting *miR-122* (anti-miR). Group averages compared to a control group receiving 1 mg kg<sup>-1</sup> LNP-formulated mismatch control oligonucleotide (MM) are shown. Error bars represent standard deviations (n=5 per group). Significance was determined by Student's t-test (\*, p<0.05). **B.** *miR-122* target aldolase was upregulated in liver but this upregulation could not be detected in the serum, probably because aldolase is ubiquitously expressed.

## Figure 8. $\alpha$ -Synuclein Silencing Monitored in Rat CSF



**Figure 8. Silencing of *Snca* in the Rat Brain Is Reflected in *Snca* mRNA Levels Measured in Cerebrospinal Fluid.** **A** Cartoon depicting the intra-striatal bi-lateral brain surgery (Anterior-posterior 0.5 mm, Medial-lateral 3 mm, Dorsal-ventral 5.1 mm). siRNA targeting *Snca* mRNA (4 mg/mL) was infused for 7 days at 1  $\mu$ L/hour rate. CSF and striatum were collected from all animals at Day 7. **B.** 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. Error bars represent standard deviations. Significance was determined by Student's t-test (\*,  $p < 0.001$ , #,  $p < 0.05$ ).

## Figure 9. Exogenous Gene Expression Detected in Serum



**Figure 9. Comparable levels of GFP mRNA are measured in rat liver and serum following administration of adenoviral-GFP vector.** Relative levels of GFP mRNA measured in total RNA isolated from liver and filtered, centrifuged serum of four individual rats four days following intravenous injection of  $10^{11}$  pfu Adeno-GFP. Values presented are the means of two technical replicates for serum and two separate pieces of liver. Error bars represent standard deviations.

# Conclusions

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- Liver-specific RNAs detected in mouse, rat, monkey and human serum
  - Detected RNAs represent full transcript (shown using multiple QPCR probes, data not shown)
- Serum RNA is associated with exosomes
  - Protected from RNase digestion
  - Presence of vesicles: EM, DLS, sucrose density gradient, protein markers
- RNA silencing in liver can be detected in serum
  - Comparable mRNA silencing measured in liver and serum
    - Similar dose-response, kinetics
    - Multiple genes
  - Results extended to non-human primates
  - Liver RNAi can be confirmed by detection of specific 5'-RACE product in serum
- Tissue RNAi detection extended to CSF
  - SNCA silencing observed similar to striatum
- Exogenous gene expression in liver can be monitored
  - Ad-GFP expressed GFP mRNA observed in serum
- This new method, circulating extracellular RNA detection (cERD), allows quantitative measurement of tissue-specific mRNA silencing from biological fluids including blood and CSF"

# References

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2. Valadi, H et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* **9**, 654-659 (2007).
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