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Reduction of the therapeutic dose of silencing RNA via its integration into the backbone of a pre-microRNA highly enriched in exosome-like vesicles

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Less than a few percent of the silencing RNA (siRNA) delivered via passive lipid nanoparticles and other delivery vehicles reaches the cytoplasm of cells. The high doses of siRNA and delivery vehicle that are thus required for achieving therapeutic outcomes can lead to toxicity. Here, we show that the integration of siRNA sequences into a Dicer-independent RNA stem-loop in pre-miR-451, a pre-microRNA that we found to be highly enriched in exosome-like vesicles secreted by many cell types, reduces the expression of siRNA-targeted genes in the liver, intestine and kidney glomeruli at siRNA doses that are at least 10-fold lower than the siRNA doses typically contained in lipid nanoparticles. Exosome-like vesicles that efficiently package siRNA can thus significantly reduce its therapeutic dose.

One-sentence editorial summary (to appear right below the article's title on the journal's website):
Integrating silencing RNA into the backbone of a pre-microRNA that is highly enriched in exosome-like vesicles reduces the therapeutic dose of the silencing RNA.

Canonically, pre-microRNA (miRNA) are 60-120 nt stem-loop structures which are cleaved by the RNAse III enzyme Dicer into short ~21 nt double-stranded (ds)RNAs[1]. This 21 nt dsRNA complex binds the microRNA effector Argonaute (Ago). After removal of one RNA strand this Ago-miRNA complex represses translation of target genes by translational repression and mRNA degradation[1]. Silencing RNAs (siRNA) are a therapeutic class which are designed to mimic Dicer-processed dsRNA that bind Ago. When siRNA are perfectly complementary to mRNA targets, Ago2 can enzymatically cleave target mRNA[2,3] providing remarkable potency and specificity to these drugs.

For therapeutic applications, delivery of siRNA into the cytoplasm of cells is a perennial challenge[4,5]. Quantitative analysis has shown that 300-2000 copies of siRNA that catalytically cleave mRNA are required in the cytoplasm of each cell to silence target mRNAs[6–8]. For example, 300 copies of siRNA per cell were required to silence target expression by 50% when siRNA was microinjected directly into the cytoplasm[8]. Alternatively 800 copies of siRNA loaded into Ago2 were required to silence a target mRNA effectively in the liver[7]. Measured in cells in culture or in liver of mice, current delivery vehicles like lipid nanoparticles enable only between 0.05% and 1% of siRNA to enter the cytoplasm of cells[6,7,9]. For this reason, high doses of siRNA and delivery vehicles are required to treat patients and this can lead to toxicity that has halted multiple clinical trials of silencing RNAs[10]. Lipid nanoparticles and siRNA accumulate most profoundly in the liver. Consequently, most siRNA therapeutics target liver expressed proteins. siRNA delivery to the liver with lipid nanoparticles or conjugated to GalNAc reduces target expression in patients by >80% for over 6 months with a single dose[2,3,11], and was recently approved for treatment of transthyretin amyloidosis. Delivery of siRNA to other organs remains a major challenge.

Extracellular vesicles called exosomes have been proposed as a delivery vehicle for siRNA. Exosomes are produced by budding of 60-120 nm vesicles into the lumen of late endosomes. These late endosomes or multivesicular bodies can subsequently fuse with the plasma membrane releasing their internal vesicles, called exosomes, into the extracellular space[12]. Studies showed that extracellular vesicles and exosome-like vesicles contain miRNA and can transfer miRNA activity into cells[13–18]. A hypothesis has emerged that exosome-like vesicles have an evolved ability to deliver small RNAs or other molecules between cells and this capacity could be captured for efficient drug delivery. Despite the attention devoted to them, how efficient exosome-like vesicles are in delivering their cargoes into the cytoplasm has not been directly evaluated. Indeed, in some cases target silencing was indistinguishable whether siRNA was electroporated into exosome-like vesicles or injected naked into mice[19]. In other cases where siRNA was electroporated with exosomes or coated onto the exosome surface, the siRNA doses required to knockdown target expression exceeded significantly that required by lipid nanoparticles[20,21]. This suggests that exosomes may actually have little inherent capacity to deliver RNAs.

To date, studies of siRNA delivery using exosomes may not have tested the actual delivery efficiency of exosomes. Topologically, siRNA on the exosome exterior will not enter the cytoplasm if exosome-like vesicles fuse with target cells, but rather it will remain trapped in endosomes or on the exterior of the cell, and hence inactive. As well, electroporating exosomes with siRNA may not actually introduce siRNA into exosomes, but rather precipitate siRNA which then co-purifies with exosomes[22]. Electroporation or otherwise altering the exosome surface may also interfere with the membrane architecture of exosomes required for

fusion with target cell membranes. For these reasons, the actual delivery efficiency of exosomes may still await testing.

MiRNAs are sparse in exosome-like vesicles with an absolute copy number ranging from 1 copy of a specific miRNA per 10^6 exosome-like vesicles to 1 miRNA copy per exosome-like vesicle[23]. This underscores that any attempt to deliver RNA therapeutics with exosome-like vesicles will likely require a means to package more RNA into exosome-like vesicles. However, exosome-like vesicles are loaded with a highly selective subset of RNAs from the cells which produce them[16,17,24]. For example, specific miRNAs which are similarly abundant in cells are found at a 10^6 range of relative quantity in exosome-like vesicles[23]. This suggests that simply over-expressing a miRNA may not enable its packaging into exosomes. Sequence motifs are likely not an adequate solution as they result in modest enrichment in exosomes[25,26] and are difficult to insert into a small siRNA without impairing its specificity.

Pre-miR-451 is an exception to the canonical pre-miRNA biogenesis cascade[27]. The Drosha-processed stem-loop of pre-miR-451 is only 42 nt and too short to be bound and cleaved by Dicer. Instead, pre-miR-451 binds directly to Ago2[28–30]. Ago2 cleaves the distal strand of the pre-miR-451 stem-loop. This generates a 34 nt product that is progressively trimmed by exonucleases to produce a ~21 nt miRNA[28–30]. Notably, if the structure of the pre-miR-451 stem-loop is maintained it can be reprogrammed with other miRNA sequences, that will be processed through the same Dicer-independent, Ago2-dependent pathway into mature effective miRNA[28–30].

Here, we demonstrate that integration of siRNA into an RNA backbone derived from pre-miR-451, but not a canonical pre-miRNA enables robust packaging into exosome-like vesicles. Exosome-like vesicles packaged with siRNA in this manner can efficiently reduce expression of target genes in primary cells in culture and in mice. Exosome-like vesicles packaged with siRNA can knockdown targets with at least 10-fold less siRNA than lipid nanoparticles, suggesting that exosomes are highly efficient delivery vehicles.

Pre-miR-451 products are selectively enriched in exosomes

Exosome-like vesicles were enriched by differential centrifugation from the media of mouse embryonic fibroblasts (MEF), MDA-MB-231, and motor-neuron-like cell line NSC-34 grown in exosome-depleted media. These preparations were enriched in exosome markers Tsg101 and Flotillin2 and contained small to moderate amounts of Ago2. As expected, Tubulin and a mitochondrial protein (Tomm20) were undetectable in these preparations (Fig.1a, Supp. Fig.1a). Particles in exosome preparations had a median size (100-120 nm) and size distribution consistent with exosome-like vesicles (Fig.1b, Supp. Fig.1b).

Following recent reports of siRNA delivery by exosomes in mice[19,20] we attempted to electroporate siRNA into exosomes. Utilizing a protocol and instrument identical to that previously published[19,20] we electroporated exosomes and siRNA vs. SOD1. Whether siRNA was electroporated in the presence or absence of exosomes, siRNA was similarly pelleted by a standard protocol to isolate exosomes by ultracentrifugation (Fig.1c). This suggests that electroporation may cause siRNA to aggregate or precipitate. In accord, while free siRNA was degraded by RNAseA, electroporated siRNA was resistant to digestion by RNAseA whether exosomes were present or not in the electroporation cuvette (Fig.1c). In agreement with a

previous publication[22], this suggests that siRNA is not introduced into exosomes by electroporation, but precipitates outside exosomes and co-purifies with them upon ultracentrifugation. We sought an alternative method to package siRNA into exosomes by harnessing cellular mechanisms of packaging RNAs into exosomes.

We evaluated the absolute copy number of endogenous miRNA per exosome-like vesicle. Nanoparticle tracking allowed counting of 100 nm polystyrene particles of known concentration with high accuracy and reproducibility across a range that coincided closely with that recommended by the instrument's software (Supp.Fig.1c). Exosome-like vesicles were diluted into this range for quantification in all experiments. To estimate the number of miRNA per exosome, standard curves of qPCR products were used correcting for RT-qPCR efficiency (Supp.Fig.1d). An independent method, digital droplet PCR, demonstrated that the standard curve method quantified miRNA with an error of <20% over a wide range of concentrations (Supp.Fig.1e). Some miRNAs were present at 1 to 10 copies per exosome (Fig.1d), in agreement with publications which quantified the most abundant miRNAs in exosome-like vesicles[31]. In contrast, many miRNAs like miR-16, miR-451 and miR-134 were present at extremely low numbers in exosome-like vesicles (1 copy per 10,000 exosome-like vesicles to 1 copy per 10 exosome-like vesicles, Fig.1d) in line with previous publications[23]. The relative abundance in exosome-like vesicles of several of the miRNAs tested did not greatly differ from their abundance in exosome-producing cells (Fig.1e), suggesting they are randomly packaged into exosome-like vesicles. Intriguingly, despite its low copy number in exosome-like vesicles (Fig.1d) miR-451 was 500 to 10,000-fold enriched in exosome-like vesicles compared to its levels in respective cells of diverse types (Fig.1e,f). miR-451 was similarly enriched in exosome-like vesicles normalized to U6 RNA and produced in serum-free Ultraculture (Lonza) media (Supp.Fig.1f).

miR-451 is abundant in fetal bovine serum (FBS) and trace amounts of miR-451 and exosomes may persist even in exosome-depleted FBS[32,33]. These sources of miR-451 could attach to or be internalized by cells to be maintained across cell culture passages. This suggests the possibility that the observed enrichment of miR-451 in exosomes could be due to contamination with residual miR-451 from FBS. While miR-451 was abundant in FBS, over 99.99% of this was eliminated by ultracentrifugation at 18 h and total amounts of miR-451 in exosomes increased approximately 32-fold when exosome-depleted media was incubated with cells (Supp. Fig. 2a). In addition, culture of three cell types in serum-free media continuously for 6 weeks, or dilution of cells by 8-fold into serum-free media did not deplete miR-451 in cells or exosomes, or impact its enrichment in exosomes (Supp. Fig. 2b-m). This demonstrates that miR-451 is being continuously expressed by many cell types and is strongly enriched in exosomes released by these cells.

miR-451 was similarly enriched in exosome-like vesicles when normalized to miR-16 (Fig.1e,f) which exhibits similar abundance to miR-451 in the exosome-like vesicles tested (Fig.1d), or to U6 RNA (Supp.Fig.1f). Finally, miR-451 was present inside exosome-like vesicles as it was only sensitive to RNAse treatment after pre-treatment with detergent (Fig.1g). Similarly, when exosomes were isolated by sucrose density gradient, miR-451 abundance peaked with exosome-containing fractions at a density of 1.10-1.18 (Fig.1h). miR-451 in these fractions was only sensitive to RNAse degradation after permeabilization of exosomes with detergent (Fig.1i). Furthermore, miR-451 was not digested with RNAse even when exosomes were pre-treated with Proteinase K to release any RNA from proteins or protein aggregates co-purifying with exosomes, while a

spiked in siRNA was degraded even without detergent (Supp.Fig.3a,b). This demonstrates that miR-451 is selectively packaged inside exosome-like vesicles compared to other cellular miRNAs.

New siRNA in pre-miR-451 structure are enriched in exosomes

The stem-loop hairpin of pre-miR-451 binds directly to Ago2, which cleaves the distal strand of RNA in the stem region[28–30]. Exonucleases then trim pre-miR-451 to ~22 nt producing a mature miRNA (Fig.2a). Other miRNA sequences can be integrated into the pre-miR-451 hairpin and processed through the same Dicer-independent pathway[28–30] (Fig.2a). We hypothesized that integrating siRNA into the pre-miR-451 backbone would cause these siRNA to be enriched in exosome-like vesicles. We integrated a siRNA targeting GFP into the pre-miR-451 backbone. As expected, this was processed into a ~22 nt mature siRNA in cells (Fig.2b). Integration of siRNA targeting GFP, TetR or SOD1 into the pre-miR-451 backbone, caused these siRNA, like miR-451, to be enriched from 58 to >7,000-fold in exosome-like vesicles produced by multiple cell types (Fig.2c-e Supp.Fig.3c,d). This was independent of over-expression as integration of the same siRNA in pre-miR-16 did not result in comparable enrichment in exosome-like vesicles measured as relative enrichment, or absolute copy number (Fig.2c-e, Supp.Fig.3c,d). When produced from the pre-miR-451 hairpin, siRNA targeting GFP or SOD1 were enriched up to 1 copy per exosome on average (Fig.2g, Supp.Fig.3d), similar to the level of the most abundant miRNA in exosome-like vesicles (Fig.1d)[23]. Finally, miR-451 (Fig.1h,i) and GFP siRNA expressed from the pre-miR-451 hairpin separated with exosomes on sucrose density gradients (Fig.2h,i). Ago2, like proteins inside exosomes including Alix and Tsg101 was only sensitive to Proteinase K digestion when exosomes were treated with detergent (Supp. Fig.3e). Similarly, GFP siRNA in exosomes purified by sucrose density gradient resisted RNAse digestion when these exosomes were pre-treated with Proteinase K, but were rendered sensitive to RNAse digestion after treatment of exosomes with detergent (Supp. Fig.3f). This provides strong evidence that Ago2, miR-451, and siRNA expressed from the pre-miR-451 hairpin are enclosed inside exosomes and not in large quantity in protein aggregates (Supp.Fig.3a,b,e,f).

Ago2 Not Required to Package Pre-miR-451 Hairpins Into Exosomes

Evidence above shows that siRNA with unrelated sequences can be integrated in the pre-miR-451 structure and will then be strongly enriched in exosome-like vesicles (Fig.2b-g). This suggests that it is the structure of the pre-miR-451 hairpin and not a specific siRNA sequence that is required for packaging into exosomes. Low levels of Ago2 are present in exosomes, even when purified by sucrose density gradient[34] (Fig.1a,h, Supplementary Fig.1a,3e). This suggests that once packaged into exosomes ~42 nt pre-miR-451 hairpins may be processed there by Ago2 into a mature miRNA in the hours to days between generation of exosome vesicles in MVB, their release from cells and isolation[35]. In agreement, in exosome-like vesicles these RNAs are highly abundant at sizes corresponding to pre-miR-451 precursors (~42 nt), intermediate processing products (24-34 nt) and mature siRNA (~22 nt, Fig.2h), while in cells the mature ~22 nt GFP siRNA was almost exclusively detected (Fig.2b). This confirms independent of RT-qPCR the enrichment in exosomes of siRNAs built into the pre-miR-451 structur and suggests that precursor forms of pre-miR-451 structural mimics rather than mature forms are preferentially packaged into exosomes.

To test directly, whether the precursor form of miR-451 is packaged into exosomes, *Ago2*$^{-/-}$ cells were employed which arrest processing of pre-miR-451 or its structural mimics at its 42 nt hairpin stage[28–30]. Confirming this, only the 42 nt form of GFP siRNA integrated in the pre-miR-451 hairpin was detected by

northern blot in *Ago2*$^{-/-}$ cells and exosomes, while a matured ~21 nt form was detected in cells rescued with wild-type Ago2 (Fig. 2j, Supp.Fig.3g). This means that in *Ago2*$^{-/-}$ cells only the ~42 nt precursor of pre-miR-451 structural mimics is available for packaging into exosomes. To reinforce the relative length of pre-miR-451 structural mimics in exosomes with an independent method we utilized an RT-qPCR kit (miScript, Qiagen) that preferentially amplifies either the ~22 nt mature miRNA/siRNA (Specific buffer) or longer precursors (Flexible buffer). Validating this assay with synthetic RNAs, 42 nt pre-miR-451 structural mimics were amplified more quickly with the Flexible buffer, while the corresponding 21 nt mature siRNA was amplified more quickly with the Specific buffer (Supp. Fig.3h). This enables the relative enrichment of pre-miR-451 precursors vs. mature forms to be expressed as a ratio of RNA abundance measured by Flexible vs. Specific buffers. In exosomes produced by wild-type cells, SOD1 siRNA produced from a pre-miR-451 structural mimic was amplified at similar cycle numbers with either the Flexible or Specific buffers (Supp. Fig. 3i), suggesting that the SOD1 siRNA exists in wild-type exosomes as a mixture of mature siRNA and longer pre-miR-451 structural mimics, as demonstrated by northern blot data (Fig.2h, Supp. Fig.3g). In contrast, exosomes produced by *Ago2*$^{-/-}$ cells were strongly enriched in long forms of pre-miR-451 structural mimics (Supp. Fig.3i), closely resembling amplification of the 42 nt synthetic version of pre-miR-451 structural mimics (Supp. Fig.3h). This reinforces northern blot data that *Ago2*$^{-/-}$ cells (Fig.2j) only contain 42 nt versions of pre-miR-451 structural mimics for packaging into exosomes.

Remarkably, the relative enrichment in exosome-like vesicles of pre-miR-451 or a pre-miR-451 structural mimic containing SOD1 siRNA were not significantly changed in the absence of Ago2 despite these cells only containing 42 nt precursors of these (Fig.2j-l). Similarly, over-expression of Ago2 in wild-type cells did not significantly change the enrichment of pre-miR-451 derivatives in exosome-like vesicles (Fig.2m). Ago2 expression appears to slightly reduce packaging of pre-miR-451 derivatives into exosomes (Fig.2j-m), although these effects were not statistically significant. This suggests that binding of pre-miR-451 derivatives to Ago2 may retain them in cells and pre-miR-451 hairpins are robustly packaged into exosome-like vesicles independent of Ago2. Once inside exosomes, low levels of Ago2 there (Fig.1a,h, Supplementary Fig.1a,3e) may process pre-miR-451 structural mimics into shorter versions. Cumulatively, this suggests that the 42 nt pre-miR-451 structural mimics can account for all selective packaging into exosome-like vesicles rather than a mature version of the miRNAs or siRNAs produced from it. At the same time, the data cannot exclude that ~20-24 nt forms of miR-451 or siRNA derived from pre-miR-451 structural mimics are also packaged into exosomes in wild-type cells.

Hairpins in RNAs inhibit processivity of reverse transcriptase, compete for primer binding and can impede PCR. Our data confirm this as, equimolar amounts of synthetic mature siRNA (21 nt) were amplified much earlier in RT-qPCR reactions than a similar amount of the same siRNA embedded in a 42 nt pre-miR-451 structural mimic that contains a hairpin (Supp. Fig.3h). This suggests that the presented RT-qPCR analyses may underestimate the enrichment of pre-miR-451 structural mimics in exosomes (where longer forms with hairpin structures are abundant) compared to their levels in cells where mature ~21 nt versions of these RNAs predominate.

Select Exosomes Deliver siRNA to Primary Motor Neurons

We sought to test whether exosome-like vesicles loaded with siRNA reprogrammed into the pre-miR-451 backbone could efficiently deliver these siRNA. Exosome-like vesicles from NSC-34 cells that were packaged with either siRNA targeting GFP or a control siRNA expressed from the pre-miR-451 backbone were incubated with primary mixed motor neuron cultures from GFP transgenic mice. siRNA detected by FISH accumulated in motor neurons and co-localized with target GFP mRNA and a marker of P-bodies (DDX6/Rck) (Fig.3a,b). This suggests the siRNA packaged in exosomes has reached the cytoplasm and engaged its target in RNA silencing. GFP knockdown in mixed motor neuron cultures measured by RT-qPCR peaked at a ratio of 1000 exosome-like vesicles per cell (Fig.3c). We used Fluorescence *In Situ* Hybridization (FISH) capable of detecting single mRNA molecules as an orthogonal method to quantify GFP mRNA. Similar to RT-qPCR results, both FISH for GFP mRNA and GFP protein fluorescence exhibited peak knockdown at ratios of 1000 exosome-like vesicles per cell (Fig.3d). Free siRNA administered to mixed motor neurons at the same dose had no effect on GFP expression (Fig.3e). Knockdown of GFP in primary mixed motor neurons did not require exosome-producing cells to express Ago2, demonstrating that siRNA in the pre-miR-451 hairpin can be packaged into exosomes equivalently in the absence of Ago2, and delivered into target cells in the precursor form (Fig.3f). Exosome-like vesicles produced by a distinct neuronal cell line, Neuro2a, and loaded with GFP siRNA also reduced GFP mRNA expression (Fig.3g,h, Supp.Fig.4a), while vesicles produced by BV2 microglia cells did not (Fig.3i,j, Supp.Fig.4b) despite both being packaged with GFP siRNA to the same level as exosome-like vesicles produced by NSC-34 cells (1 copy per exosome-like vesicle, Fig.2g). This demonstrates that the cell source of exosome-like vesicles can dictate whether effective siRNA delivery occurs. This suggests that exosome-like vesicles deliver siRNA to targeted, specific cell types.

Multiple previous studies have together established that between 300 and 2000 copies of a siRNA are required to elicit target knockdown in cells[6–8]. If exosome-like vesicles at a dose of 1000 siRNA per cell elicit target knockdown (Fig.3c-e) then exosomes would appear to deliver a large proportion of their siRNA contents into the cytoplasm, compared to 0.05-1% of siRNA for lipid nanoparticles[6,9]. However, as previously reported[36,37], recovery of spiked-in RNA of 21 nt was <10% when low amounts of RNA were used as in exosome preparations (Supplementary Fig.4c). The poor recovery of small RNAs with limited amounts of RNA input has not been accounted for in previous studies of miRNA copy number in exosomes. This suggests that estimates of siRNA and miRNA copy number in exosomes here (Fig.1-3) and in the literature may be underestimated by 10-30-fold, and the delivery efficiency of exosomes may be correspondingly lower.

Exosomes Deliver siRNA to Multiple Organs in Mice

We tested whether exosome-like vesicles could deliver siRNA in mice. Exosome-like vesicles labeled with the far-red dye DiR and injected intravenously (IV) distributed broadly to many tissues including liver, kidneys, spleen, intestines and lungs (Fig.4a). In contrast, DiR alone subjected to the identical labeling method but without exosomes exhibited negligible signals in mice (Fig.4a). Exosome-like vesicles packaged with GFP siRNA at approximately 1-30 copies per exosome (Fig.2g, Supplementary Fig.4c) or control siRNA (1×10^{10} exosome-like vesicles) were injected IV into mice constitutively expressing GFP from a ubiquitous promoter. Despite the accumulation of exosome-like vesicles in heart, lungs and brown fat (Fig.4a), no knockdown of targets was detected in these organs at an estimated siRNA dose of 10-300 ng/kg (Fig.4b). In

contrast, in the small intestine and liver, exosomes packaged with GFP siRNA decreased GFP expression by approximately 50% (Fig.4c). Similar knockdown in liver and small intestine was observed if exosomes packaged with SOD1 siRNA by reprogramming the pre-miR-451 hairpin were injected IV into mice transgenic for human SOD1 G93A (Fig.4d). Increasing by ten-fold the dose of exosomes (10^{11}) and siRNA (estimated 100-3000 ng/kg) increased knockdown in liver to 50-60% and small intestine to 70-75% (Fig.4c,d). This demonstrates that exosomes can deliver siRNA to specific organs and knockdown targets in a dose-dependent manner.

The half-life of miRNA or siRNA in non-dividing cells is estimated at between 1 and 4 weeks[38,39]. This suggests that target knockdown induced by unmodified siRNA delivered by exosomes may similarly last for multiple weeks[38,39]. In agreement, knockdown of GFP mRNA after injection of exosomes packaged with GFP siRNA persisted for 12 days in liver where cell turnover is slow[40] (Fig.5a). In contrast, epithelium and other cells in the intestine turnover in less than a week[40]. In agreement, knockdown of GFP mRNA in the small intestine had dissipated by 12 days (Fig.5b). This suggests that the effects of unmodified siRNA delivered by exosomes can persist for extended periods of time in tissues with slow rates of cellular turnover.

Enhanced Delivery Efficiency Compared to Other Vehicles

Testing exosome delivery of siRNA on primary motor neurons suggested that exosomes may be highly efficient delivery vehicles. To test this *in vivo,* we compared exosomes packaged with siRNA using the pre-miR-451 backbone to lipid nanoparticles. As expected, InVivoFectamine lipid nanoparticles (ThermoFisher) at a high dose (1 mg/kg) of mature standard siRNA of the same sequence decreased target mRNA expression in the liver by >80% and by approximately 55% in small intestine (Fig.5c). Exosomes packaged with approximately 10-300 ng/kg siRNA elicited knockdown in liver and small intestine, while InVivoFectamine lipid nanoparticles with the same dose of siRNA had no effect on target mRNA levels (Fig.5c). We compared to advanced C12-200 lipid nanoparticles capable of silencing targets in liver at doses of 0.003 mg/kg and 0.03 mg/kg of siRNA[41]. At 0.03 mg/kg dose C12-200 lipid nanoparticles elicited 60% silencing of GFP in liver at 3 days, while exosomes at a siRNA dose 10-300-fold lower elicited similar silencing in liver (Fig.5d). This reinforces the notion from experiments on primary mixed motor neuron cultures in culture that exosomes deliver siRNA at least 10-fold more efficiently than lipid nanoparticles.

Others have proposed electroporation as a strategy to load siRNA into exosomes. In agreement with previous literature[22], our data suggests that siRNA is not packaged inside exosomes by electroporation, but is precipitated and co-purifies with them (Fig.1c). Nonetheless, we used the electroporation instrument and settings as published[19,20] to test the ability of exosomes electroporated with siRNA to deliver cargoes in mice. Mice were injected with exosomes electroporated with 10 ng/kg of siRNA. At this dose intact exosomes packaged with the pre-miR-451 backbone elicit 35-50% knockdown in liver (Fig.4c,5a), but exosomes electroporated with siRNA failed to decrease target expression (Fig.5e).

Electroporated cells often die due to excessive membrane permeabilization. Electroporation of exosomes rendered miR-106a and *let-7a* which are endogenously present inside exosomes sensitive to degradation by RNAseA nearly to the same extent as exosome permeabilization with detergents (Fig.5f). Furthermore, analysis of electroporated exosomes by Nanoparticle Tracking demonstrated a significant loss of exosome-

sized particles and an accumulation of smaller sized particles, consistent with exosome fragments, after electroporation (Fig.5g), even if median particle size was unchanged (Supplementary Fig.4d). This suggests that exosome membrane integrity is disrupted by electroporation and this could compromise the ability of exosomes to deliver siRNA. Confirming this, when the exosome membrane was disrupted by pre-treating exosomes with detergent their ability to deliver GFP siRNA into mixed motor neurons was abrogated (Fig.5h). Similarly, electroporation of exosomes abolished their ability to deliver siRNA in mice. Exosomes packaged with SOD1 siRNA using the pre-miR-451 hairpin reduced target expression in the liver and small intestine, but the same exosomes that had undergone electroporation did not (Fig.5i,j). This suggests that electroporation disrupts exosome membrane integrity and the capacity of exosomes to efficiently deliver siRNA.

Tissue and Cell-Specific Delivery Dictated by Exosome Source

Not all cell types produced exosomes capable of delivering siRNA to primary motor neurons (Fig.3). Exosomes may deliver cargoes to specific cell types. To investigate siRNA delivery and target knockdown in specific tissue regions or cell-types we utilized a Fluorescence *in Situ* Hybridization (FISH) method capable of detecting single mRNAs with a linear quantification method. We validated that this FISH method closely replicated target mRNA knockdown assessed by RT-qPCR in liver and small intestine (Fig.6). We then employed FISH to quantify target knockdown in specific regions and cell types of these organs. In small intestine, when total GFP mRNA was decreased by 55% by GFP siRNA delivered by exosomes (Fig.6c,d), loss of GFP mRNA and GFP fluorescence was 50% in villi and 80-85% in submucosa (Fig.7a-c, Supp.Fig.5a,b). In liver, when total tissue GFP fluorescence and mRNA was decreased by 35-50% (Fig.6g,h,7d,e), GFP mRNA and GFP fluorescence in hepatocytes, Kuppfer cells/macrophages (F4/80+), and stellate cells (GFAP+) was reduced by 60-70%, 20% and 0% respectively (Fig. 7d,f-i, Supp.Fig.5c-f). We also assessed siRNA target knockdown in specific regions of the kidney by FISH, where at the whole tissue level only marginal target knockdown was observed (Fig.7j,k). Interestingly, in Nephrin+ glomeruli GFP mRNA and GFP fluorescence was reduced by approximately 50%, whereas knockdown outside these cells was not noticeable (Fig.7j,l, Supp.Fig.5g). This demonstrates that exosomes exhibit delivery of siRNA to specific cell types and tissue regions in mice.

Interestingly, in many cell types, like Kuppfer cells, GFP siRNA delivered by exosomes was abundant, however no knockdown of GFP mRNA was observed. This suggests that in many cell types exosomes and their cargoes are internalized but rarely fuse with the target cell membrane to deliver their cargoes.

Reduction of Transthyretin Expression with Low siRNA Doses

To evaluate the therapeutic potential of siRNA delivery by exosomes we selected a validated target expressed in a cell-type to which delivery had been detected above. ONPATTRO was recently approved to treat patients with Transthyretin Amyloidosis by delivering siRNA targeting *Transthyretin* expressed predominantly in liver hepatocytes. SiRNA targeting *Transthyretin* was integrated into the pre-miR-451 backbone for packaging into exosomes. It would be preferable to treat patients with exosomes derived from human cells. Exosomes produced by primary human fibroblasts or by the human 293T cell line and packaged with siRNA using the pre-miR-451 backbone elicited knockdown of mRNA targets in liver by >80% at an estimated siRNA dose of 10-300 ng/kg (Fig.8a,b, Supplementary Fig.6a,b). Exosomes (10^{10}) from

human fibroblasts containing an estimated dose of 10-300 ng/kg siRNA targeting *Transthyretin* or a non-silencing siRNA were injected IV. This reduced levels of Transthyretin protein in blood in a time course reaching knock down of over >85% at 7 days (Fig.8c,d).

Collectively, this data demonstrates that pre-miR-451 is selectively packaged into exosomes and integrating siRNA into the hairpin structure of pre-miR-451 can induce their selective packaging into exosomes. By leaving exosomes intact, this strategy allows exosomes to deliver their siRNA cargoes and knockdown targets in specific cell types and tissues in mice with siRNA doses that are magnitudes lower than lipid nanoparticles or another exosome technology.

DISCUSSION

Together, the presented data uses four independent methods to demonstrate that pre-miR-451 and RNAs with unrelated sequences but the same secondary structure are robustly packaged into exosome-like vesicles (Fig.1-2). Cleavage and maturation of pre-miR-451 is arrested in *Ago2*$^{-/-}$ cells[28–30] at the stage of the 42 nt precursor as confirmed by northern blot and RT-qPCR (Fig.2j, Supplementary Fig. 3h,i). Despite this, In *Ago2*$^{-/-}$ cells pre-miR-451 or a pre-miR-451 structure containing SOD1 siRNA was still similarly enriched in exosomes (Fig.2k-m). In addition, exosomes produced by wild-type and *Ago2*$^{-/-}$ cells knocked down GFP siRNA targets in primary motor neurons similarly (Fig.3f). These two independent methods demonstrate the 42 nt hairpin that structurally resembles pre-miR-451 can account entirely for enrichment in exosomes of the studied miR-451 or siRNAs expressed from the pre-miR-451 hairpin.

The sequence of the pre-miR-451 structural mimic is completely changed when a siRNA against a new target is built into it (Fig.2a,f GFP, SOD1, TetR). Nonetheless, these RNAs are strongly enriched in exosomes much like pre-miR-451 derivatives (Fig.2). This also strongly suggests that the packaging of these RNAs into exosomes requires the shared structure of the pre-miR-451 hairpin and not mature ~21 nt siRNAs each with distinct sequences (Fig.1-2).

Mature ~20-24 nt lengths of GFP siRNA expressed from the pre-miR-451 backbone are the predominant species found in cells (Fig.2b), whereas precursors with lengths of ~42 nt and ~34 nt are abundant in exosomes along with the mature GFP siRNA (Fig.2h). This also suggests that the ~42 nt hairpins are selectively packaged into exosomes. Why matured ~21-34 nt forms of these RNAs are found in exosomes is less clear. It is possible that these matured forms are sorted into exosomes in an independent process. Alternatively, exosome-like vesicles harvested from cell media may have been present in MVB prior to release for significant periods, and exosome-like vesicles may remain in cell culture media for hours before collection. During this time 42 nt hairpins structurally resembling pre-miR-451 may undergo maturation by low levels of Ago2 packaged inside exosomes (Fig.1a,h, Supp.Fig.3e)[34,35]. This would account for the ability of only the 42 nt pre-miR-451 structural mimics to drive packaging into exosomes in *Ago2*$^{-/-}$ cells and the relative abundance of matured forms of pre-miR-451 structural mimics in exosomes.

Sequence motifs can also influence packaging of RNAs into exosomes. For example, sequence motifs that bind hnRNPA2B1, Ybx1 and HuR have all been shown to promote inclusion of specific small RNAs into exosomes[24–26]. While the driving force behind packaging of RNA with the structure of pre-miR-451 into

exosomes is the hairpin secondary structure, sequence motifs may also influence packaging of these hairpins into exosomes.

For years the major physiological role of exosome-like vesicles was hypothesized to be in expelling proteins from cells or activating receptors on target cells. These effects did not assume the ability of exosome-like vesicles to deliver their cargoes into the cytoplasm of target cells. Cells internalize many inert and biological particles from lipid nanoparticles, to apoptotic cells and pathogens and traffic these to lysosomes for degradation. Therefore, it is important to distinguish exosome internalization from a more complex and possibly rare event in which exosomes fuse with target cells and deliver their luminal contents into the cytoplasm. We observed that in some circumstances cells internalized exosomes and accumulated siRNA but exhibited no signs of siRNA delivery measured as target knockdown (stellate cells in liver, or exosomes produced by BV2 cells on primary motor neurons, not shown). This highlights that exosome internalization by cells does not always lead to delivery of exosome contents. Cells expend considerable resources to establish and maintain differentiated states and these are critical for tissue specialization in multicellular organisms. Exosomes, by transferring RNA contents between cells have the capacity to disturb or overthrow the identity and function of target cells. Our evidence emphasizes that delivery by exosomes is targeted to specific cell types. Biologically, this may help prevent widespread loss of cell identity and function.

Various papers have now shown that exosome-like vesicles contain RNA and target cells exhibited signs of using siRNA, miRNA or mRNA from exosome-like vesicles[13,15–17]. This led to the emergence of the model that exosomes escape from endolysosomal degradation to fuse with target cell membranes and deliver their cargoes into the cytoplasm of cells. However, even naked RNAs incubated with cells at high enough doses will enter the cytoplasm in sufficient quantities to be active, and this can be substantially improved to the extent that liposomes and lipid nanoparticles can delivery up to 1 in every 100 to 1000 siRNA they contain[5,6,9]. In this context, where exosomes were used in prior studies to deliver siRNA, the siRNA doses required were much higher than lipid nanoparticles[5,19–21] suggesting that exosomes loaded in these manners were relatively inefficient delivery vehicles[7,9]. This leaves open the possibility that exosome-like vesicles may have no natural propensity to fuse with target cells and deliver RNA. Therefore, whether exosomes are actually efficient at delivering RNA into target cells has not been rigorously tested to our knowledge and many experimental effects observed with exosomes could be due to supraphysiological doses of exosome-like vesicles or excessive amounts of siRNA loaded onto exosomes.

Using absolute quantification of siRNA and exosome-like vesicles we have tested the efficiency of exosome-like vesicles in delivering siRNA into target cells both *in vitro* and *in vivo*. Previous quantitative analyses has shown that 300-2000 copies of siRNA that catalytically cleave mRNA are required in the cytoplasm of each cell to silence target mRNAs[6–8]. Our results demonstrate that exosome-like vesicles containing 1000 to 30,000 copies of siRNA per cell are sufficient to silence target mRNAs in recipient cells. This suggests that between 3-30% of siRNA inside intact exosome-like vesicles is delivered into the cytoplasm. Supporting this claim, exosomes knockdown targets in liver and intestine at siRNA doses at least 10-fold lower than lipid nanoparticles or other best-in-class engineered delivery vehicles that only deliver about 1 in every 100 to 1000 siRNA they contain into cells[6,7,9]. This suggests that exosome-like vesicles are likely between 10-300-fold better at delivering siRNA than contemporary engineered vehicles. This provides evidence that

exosomes are highly efficient delivery vehicles and may have evolved this efficiency to fulfill biological roles in targeted intercellular communication. At the same time, this suggests that the majority of exosomes (70-97%) do not deliver their contents into target cells and are likely degraded in lysosomes. An increasing number of studies have described subpopulations of exosomes differentiated by surface markers and size[42–44]. It is possible that one subpopulation of exosome-like vesicles is exceptionally efficient in delivering contents including RNA into the cytoplasm while other subpopulations of vesicles are internalized and degraded by cells.

Lipid nanoparticles contain much more siRNA per particle than most miRNA or siRNA in exosomes. Consequently, to inject similar amounts of siRNA requires injecting substantially larger numbers of exosomes than lipid nanoparticles. It is possible that this excess of exosomes (compared to lipid nanoparticles) saturates non-productive pathways of particle entrapment or uptake. Saturating such “sinks” for particles with an excess of exosomes could theoretically contribute to the enhanced delivery efficacy of exosomes.

We found that electroporating exosomes ablated their ability to deliver siRNA and knockdown targets in mouse liver and small intestine. Similarly, according to literature and our data, exosomes electroporated with siRNA or coated with siRNA required much higher doses of siRNA (ranging from 0.04 mg/kg[20] to 6 mg/kg[19]) to elicit target knockdown in mice compared to siRNA packaged inside exosomes using the pre-miR-451 hairpin (10-300 ng/kg) or lipid nanoparticles[5]. Adhering to standard cell biology models of lipid membrane fusion, this suggests that exosomes must remain intact to fuse with target cells and deliver their cargoes.

As previously reported[23], results here demonstrate that miRNA are present at low copy number in exosome-like vesicles. Accounting for RNA recovery from standard exosome samples (Supp.Fig.4c), miRNAs, or siRNA packaged into exosomes with the pre-miR-451 backbone may be present in exosomes at up to 30-300 copies per exosome (Fig.1d, 2g, Supp.Fig.3d). Many other miRNA are detectable in exosomes but occur at 100 to 10,000-fold lower copies per exosome. miRNA have more subtle effects on mRNA degradation and translational inhibition than siRNA used here which enzymatically cleave their targets. Consequently, our results suggest that only the most abundant miRNAs in exosome-like vesicles could feasibly have biological effects *in vivo*. Expression or inhibition of miRNAs in exosome-producing cells will have a wide spectrum of effects on lipids, proteins and small molecules that are released in exosomes with miRNA, and in some cases may account for effects observed in recipient cells independent of the delivery of miRNA by exosome-like vesicles. Measuring absolute quantities of miRNAs in exosomes rather than relative levels can establish the plausibility that observed effects are due to miRNA in exosomes.

The current results demonstrate that exosome-like vesicles are highly efficient delivery vehicles, providing evidence to support previous speculation. This suggests that harnessing exosome-like vesicles to deliver siRNA may allow siRNA doses to be reduced by orders of magnitude in patients. To bring this possibility to practical application will require a method to robustly package siRNA inside exosome-like vesicles and manufacture them at a feasible scale. The current results show that integration in the pre-miR-451 backbone can enrich an siRNA of choice to the level of the most abundant miRNA in exosome-like vesicles and thereby reduce volumes required to manufacture exosome delivery vehicles by 100 to 10,000-fold. This

suggests that the pre-miR-451 backbone may enable a method for the manufacture and delivery of therapeutic siRNA.

METHODS

Exosome Isolation

All cells were grown to 70-80% confluence to produce exosomes, except human fibroblasts, which reached 90-100% confluence prior to collection. Exosomes were collected from cells grown in DMEM (Wisent Bioproducts; catalog # 319-015-CL; 4.5 g/L glucose) with 10% exosome-depleted heat-inactivated FBS (Wisent Bioproducts; # 080-150) prepared as described[45], or alternatively cultured for 18-24 hours in serum-free UltraCULTURE medium (Catalogue No. 12-725F, Lonza). Exosomes were produced using two methods: differential ultracentrifugation, or tangential flow filtration and ultracentrifugation.

For differential ultracentrifugation exosome containing media was spun at 300 g for 10 minutes and supernatants were subsequently centrifuged at 2,000 g for 10 minutes, 10,000 g for 30 minutes (SW-32Ti rotor, Beckman-Coulter Life Sciences; polycarbonate tubes # 355631, Beckman-Coulter Life Sciences) and 100,000 g for two hours (SW-32Ti rotor). Exosome pellets were washed by resuspending in 1 mL 1x PBS (Wisent Bioproducts; catalogue # 311-010-CL) and centrifuged at 100,000 g for 30 minutes (TLA-100.3, Beckman-Coulter Life Sciences; Polypropylene microfuge tubes # 357448, Beckman-Coulter Life Sciences).

HEK293T and human fibroblast exosomes were isolated using Tangential Flow Filtration (TFF) and ultracentrifugation. Exosome conditioned media was spun at 300 g for 10 minutes and supernatants were subsequently spun at 2,000 g for 10 minutes. Supernatants were passed through a 0.22 μm filter (Thermo-Scientific, catalogue # 09-741-04) and concentrated to a final volume of 15 mL using the KR2i TFF system (Spectrum Labs) with a 75 cm^2 modified polyethersulfone hollow fiber column with 500 kDa cut off (Spectrum, D02-E500-10-S) at a flow rate of 140 mL/min and transmembrane pressure of 2.5 psi to achieve a shear rate of 2000 sec^{-1}. The concentrated media underwent 10x buffer exchange in 1x PBS before being pelleted at 100,000 g for 30 minutes (TLA-100.3 rotor). All exosomes were resuspended in 50 µL 1x PBS and were quantified using Nanoparticle Tracking Analysis (NTA, ZetaView, Particle Metrix).

Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) was performed on a ZetaView PMX-110 (ParticleMetrix). The ZetaView Nanoparticle Tracking instrument (ParticleMetrix) was calibrated for experiments following every instrument start-up. Focusing and alignment are performed automatically using 102 nm polystyrene beads (Microtrac, catalogue # 900383). Exosome samples were diluted in PBS into the range determined by the instrument to be accurate for measurement (typically 1:10,000 – 1:500,000). One mL of sample was injected into the machine and allowed to equilibrate until it reached an acceptable level according to the built-in particle drift sensor. Once the sample fell within the acceptable reading concentration range, video acquisition and analysis were performed using the parameters outlined in Table 1 (Supplementary Information). Undiluted concentration and median-size are reported. The acceptable measurement range was determined using serial dilutions of 110 nm polystyrene beads of known ($5.0x10^{12}$ particles/mL) concentration (Microtrac, catalogue # 400168).

Measurement of enrichment of miRNA and siRNA in exosomes relative to cells

RNA was prepared from cells or their exosomes using Trizol reagent (Thermo-Fisher Scientific, Catalogue # 15596018,) as described by the manufacturer and using 5 µg of glycogen (Thermo-Fisher Scientific,

Catalogue # R0551) as a carrier in the alcohol precipitation step. RNA was quantified by spectrophotometry and 250 ng was added to a reverse transcription reaction using the miScript II microRNA reverse transcription kit using the Specific buffer (Qiagen, Catalogue # 218161). qPCR was performed using the miScript qPCR kit (Qiagen, Catalogue # 218076) on a BioRad CFX-384 or BioRad CFX-96 instrument using 12.5 µl or 25 µl reaction volumes respectively. Primer sequences are included in Table 2 (Supplementary Information). Optimization of the reaction melting temperatures (T_A) was performed such that reaction efficiency was between 95-105%. For all subsequent reactions T_A was 55 °C except GFP siRNA reactions where T_A was 58 °C. Fold enrichment was measured using the ΔΔCt method comparing query miRNA levels in exosomes vs. cells to mir-16 or U6 (reference) levels in exosomes vs. cells.

Measurement of copy number of miRNAs per exosome

RNA was prepared from known numbers of exosomes (quantified by Nanoparticle tracking) and reverse transcribed using the miScript system (Qiagen) as above. qPCR was performed on exosome samples, simultaneously with qPCR reactions using purified products of known concentration as templates in order to generate a standard curve and calculate absolute copy number of miRNA or siRNA in exosomes.

Mice

All experiments performed using mice were approved by the Animal Care Committee of the University of Ottawa under protocol CMM-2273 and performed according to guidelines of the Canadian Council on Animal Care and the International Guiding Principles for Biomedical Research Involving Animals. Mice transgenic for eGFP under the expression of the ubiquitous Chicken B-actin enhancer and cytomegalovirus promoter[46] (C57BL/6-Tg(CAG-EGFP)1Osb/J, The Jackson Laboratory, Catalogue # 003291)) were bred and females used between the ages of 15-18 weeks. Mice expressing a low copy number of human SOD1 G93A[47] (C57/B6.Cg-Tg(SOD1*G93A)dl1Gur/J, The Jackson Laboratory, Catalogue # 002299) were also bred and males between the ages of 15-18 weeks (25-30 g) were used. Groups of mice were matched for age and litter-mates for IV injections. For IV injections mice were warmed with a heat lamp, restrained in a commercial device (Braintree Scientific restrainer, Catalogue # NC0690443) and injected into the lateral tail vein (U-100 insulin syringe 28G ½" needle, Beckton-Dickinson, Catalogue # 329424) with a 5 mL/kg (100-150 µL) suspension of exosomes ($10E^{10}$ – $10E^{11}$ particles as specified) in PlasmaLyte A, (Baxter, Catalogue # JB2544) after a 10x diafiltration from the Tangential Flow Filtration system with PlasmaLyte A. Mice were euthanized with intraperitoneal sodium pentobarbital (120 mg/kg, Bimeda-MTC, Catalogue # 8015E) and perfused with PBS (10 mL) before tissue collection. People performing injections and tissue harvest were blinded to the treatment.

Cell lines

The following cell lines were used: NSC-34 (CLU140, Cedarlane), MDA-MB-231 (ATCC), C8S (CRL-2535, ATCC), C8D1A (CRL-2541, ATCC), MN-1 (gift of J. Cote, University of Ottawa), Neuro2a (CCL-131, ATCC), 293T (CRL-3216, ATCC), MSC-TERT (Cedarlane), and Human neonatal dermal fibroblasts (Lonza, CC-2509). BV2 cells were a gift of Dr. David Park (University of Ottawa). Bone marrow-derived macrophages were generated from femurs of wild-type C57/B6 mice selected by adherence and matured in GM-CSF for 1 week. Cell lines were not authenticated. Cell lines were verified to be free of mycoplasma contamination by

assessing for cytoplasmic DAPI staining. All cell culture was performed at 37 °C in 5% CO_2 in humidified incubators.

Mixed motor neuron extraction and culture

Mice embryos were collected from pregnant mice between day E13.5 and E14.5. Spinal cords were dissected using a Zeiss Stereo Discovery V20 microscope (Carl Zeiss, Oberkochen, Germany). The clean spinal cords were placed in dissection buffer (sucrose 40 g/L, dextrose 1g/L and HEPES 2.4g/L in 1x PBS), minced with scissors and incubated with trypsin (Sigma-Aldrich) for 30 min at 37°C. The cells were separated using a 1 mL pipette and placed in NFeed neuronal culture media (MEM/HBSS, Hyclone, Catalogue # SH3002402), Insulin 10 µg/mL (Sigma-Aldrich, Catalogue # I-6634), transferrin 200 µg/mL (USBiologicals, Catalogue # T8205-47-1G), BSA 10 µg/mL (Sigma, Catalogue # A9418), putrescine 32 µg/mL (Sigma, Catalogue # P-5780), selenium 26 ng/mL (Sigma-Aldrich, Catalogue # S-1382), T3 20 ng/mL (Sigma-Aldrich, Catalogue # T-6397), hydrocortisone 9.1 ng/mL (Sigma-Aldrich, Catalogue # H-0888), progesterone 13 ng/mL (Sigma-Aldrich, Catalogue # P-8783) and 2.5S NGF 5 ng/mL (EMD Millipore, Catalogue # 01-125), Horse serum 1.3% (Invitrogen Life Technologies, Catalogue # 16050-015) and ABAM 1% (Gibco, Catalogue # 15240062). Cells were counted and seeded at 300 000 cells/well in 12 well plates pre-treated with poly-D-Lysine 1 mg/mL (Sigma-Aldrich, Catalogue # P7280,) and Matrigel 0.5% (Corning, VWR, Catalogue # 354234,). After 4-7 days, mitotic cells were killed using Arabinofuranosylcytosine 1.4 µg/mL (EMD Millipore, Catalogue # 251010).

Fluorescent *In* Situ Hybridization (FISH)

Tissues were collected from mice and placed in 4% PFA in 1x PBS for 24h. PFA was replaced by 1x PBS with 30% sucrose until the tissues sank to the bottom of tubes. Tissues were then placed in Optimal Cutting Temperature solution (OCT, 23-730-571, Fisher) and frozen on dry ice. Tissue sections of 5 µm were collected on slides and placed at -80°C. Slides were heated to room temperature (RT) before staining. Slides were placed in 4% PFA in PBS for 10 min at ambient temperature. They were washed with 1x PBS and placed at 37°C for 20 min in permeabilization buffer (10 µg/mL proteinase K, 0.2% Triton X-100 in PBS). Slides were returned to room temperature, washed in PBS and blocked 1h with 1% BSA, 100 µg/mL salmon sperm DNA and 250 µg/mL yeast extract RNA in PBS. Slides were washed with 1x PBS and treated for autofluorescence reduction with $NaBH_4$ 0.1% in water for 1h. Slides were washed with Stellaris wash A buffer (LGC Biosearch Technologies, Catalogue # SMF-WA1-60) and incubated with Stellaris fluorescent mRNA probes (LGC Biosearch Technology, SOD1 [Custom assay], GFP [VSMF-1014-5], GAPDH [SMF-3002-1]) and DIG-coupled siRNA probes (SOD1 siRNA, GFP siRNA, negative control siRNA, Integrated DNA Technologies) in hybridization buffer (90% Stellaris Hybridization buffer, Catalogue # SMF-HB1-10, 10% formamide). Slides were incubated with the probes in the dark at 37°C overnight. Slides were returned to room temperature and washed with wash A buffer and incubated with a sheep anti-DIG antibody (Enzo Life Sciences, Catalogue # ENZ-ABS266-0100) diluted 1:100 in blocking solution for 1h. Slides were washed with wash A buffer and incubated with a donkey anti-sheep AlexaFluor488 or 647 antibody (Life Technologies, Catalogue # A-21448) diluted 1:500 in blocking solution for 1h. Slides were washed with wash A buffer and incubated with DAPI (Life Technologies) 1:10 000 in PBS 5 min. A final wash was performed with Stellaris wash B buffer and slides were mounted with Citifluor AF3 antifade solution (Electron Microscopy Sciences, Catalogue # AF3-25) and sealed with nail polish. Where specific cell types were

stained antibodies used were: macrophages (anti-EMR1 [F4/80] Santa-Cruz, Catalogue # sc-365340) and Stellate cells (anti-GFAP, DAKO, Catalogue # Z0334).

Production of stable cell lines:

We adopted published siRNA sequences in order to reprogram pre-mir-451 to target GFP[48], Sod1[49], TetR[50] and Ttr (US patent US9399775). Where published siRNA sequences contained less complementary sequence than the length of the 22 nucleotide mature mir-451, we added bases complementary to the targets at the 3' end of the antisense strand. The mature antisense strands were designed as follows: Gfp 5' - atgaacttcagggtcagcttgc; Sod1 5' - ttcagtcagtcctttaatgctt; TetR 5' - tcttgatcttccaatacgcaac; Ttr 5' - ttatagagcaagaacactgttt. Pre-mir-451 reprogrammed to express GFP siRNA or TetR siRNA were generated as a G-block (IDT DNA) with the following sequences:

GFP siRNA

agatcttactgactgccagggcacttgggaatggcaaggatgaacttcagggtcagcttgcgttgaccctgaagttcattcttgctatacccagaaaacgt gccttttggtaccaagctt

TetR siRNA

agatcttactgactgccagggcacttgggaatggcaaggtcttgatcttccaatacgcaaccgtgttggaagatcaagctcttgctatacccagaaaacgt gccttttggtaccaagctt

Ttr siRNA

ggatcctactgactgccagggcacttgggaatggcaaggttatagagcaagaacactgtttagtgttcttgctttatattcttgctatacccagaaaacgtgc cttttggtaccaagctt

These were cloned between the BamHI and HindIII sites of pSilencer 2.1-U6(Puro) (Invitrogen, Catalogue # AM5762M) as a BglII-HindIII fragment with the exception of the Ttr siRNA construct which was cloned as a BamHI-HindIII fragment.

Further constructs were generated from these plasmids by inverse PCR using the following primer pairs: SOD1 siRNA agcattaaaggactgactgaaccttgccattcccaagtgc and ttttaaaggactgactgactcttgctatacccagaaaac.

Reprogrammed pre-mir-16 constructs were generated by inverse PCR using the human pre-mir-16 sequence cloned as a BamHI-HindIII fragment in pSilencer 2.1-U6(Puro) (as template with the following primer pairs: GFP siRNA gaatcttaagcaagctgaccctgaagttcatggatcccgcgtcctttcc
and taaaattatgtaagctgtcctctgaagttcatttttttggaaaagcttggcac
; TetR siRNA gaatcttaagttgcgtattggaagatcaagaggatcccgcgtcctttcc
and taaaattatgttgcgtaatgtgaagatcaagatttttttggaaaagcttggcac
; SOD1 siRNA gaatcttaaaagcattaaaggactgactgaaggatcccgcgtcctttcc and
Taaaattataagcattaaagtgactgactgaatttttttggaaaagcttggcac.

Constructs encoding reprogrammed pre-miR-451 in pSilencer were moved into a vector allowing production of lentiviral particles. Briefly, the following DNA fragments were amplified by PCR using Herculase II DNA polymerase (Agilent, Catalogue # 600677) as recommended by the manufacturer and combined into a single vector by Gibson assembly (NEB, Catalogue # E2611S): The region coding reprogrammed mir-451, amplified using oligonucleotide primers 5' – tacagaatcgttgcctgcacTTTCTTGGGTAGTTTGCAGTTT and 5' – atgcaacaagacacgagacgGTTTTCCCAGTCACGACGTT; a fragment of pGIPZ (Dharmacon) encoding all

components of a lentiviral transfer vector, amplified using oligonucleotide primers 5' – cgtctcgtgtcttgttgcat and 5' – ggtggcagatcctctagtag; a fragment of pXPAC-luc (Systems Biosciences) encoding the luciferase gene, amplified using oligonucleotide primers 5' – ctactagaggatctgccaccgtgggccagtcctctgatag and 5' – gcggccgctacttgtacacttgaaggcgtgctggtact; a fragment of pGIPZ encoding an IRES site and puromycin resistance gene, amplified using oligonucleotide primers 5' – tgtacaagtagcggccgc and 5' – gtgcaggcaacgattctgta. The resulting vector allowed transduction of cells in cell culture to produce stable cell lines expressing luciferase, puromycin resistance and the reprogrammed mir-451 construct from a CAG enhanced CMV promoter. Lentiviral particles were produced using standard methods from transfected HEK293T cells and were concentrated by ultracentrifugation. Dilutions of these lentiviral particle stocks were added to cell growth media, and after 3 days cells were spilt into media containing puromycin to select for transduced cells. Transduction was confirmed by measurement of luciferase activity within the cells.

Fluorescent labelling of exosomes

For biodistribution studies, exosomes were labelled with XenoLight DiR Fluorescent Dye (PerkinElmer, Catalogue # 125964). Exosomes were incubated with 41.5 µg/µL DiR in a total volume of 1 mL 1x PBS at room temperature on a rotator for 30 minutes. Exosomes were then centrifuged at 100,000 g for 30 min (TLA-100.3 rotor) and resuspended in an appropriate volume for injection. 1x PBS was treated with the same concentration of dye and centrifuged and resuspended identically to exosomes to produce an exosome-free "dye only" control.

Mixed Motor Neuron transfer

siRNA-containing exosomes were quantified by NTA (Zetaview) and added to mixed motor neuron cultures at a concentration of 1 000 exosomes per cell in 12 well plates containing 300,000 cells/well on removable cover slips. After 72 hours, coverslips were removed from the wells for FISH analysis. The remaining cells in the well were lysed using Trizol (Life Technologies, Catalogue # 15596026) for qPCR analysis.

In other experiments, siRNA-containing exosomes from Ago2$^{-/-}$ MEFs and wild-type rescued MEFs were quantified by NTA (Zetaview) and added to mixed motor neuron cultures at a concentration of 10,000 exosomes per cell in 24 well plates. Untreated wells were used as naïve controls. After 72 hours, cells were lysed using Trizol for qPCR analysis.

qPCR analysis of mixed motor neurons and mouse organs

Cells or mouse tissues were homogenized in Trizol (Sigma-Aldrich) and RNA was extracted following the manufacturer's instructions. Reverse transcription was conducted with the M-MuLV enzyme (NEB, Catalogue # M0253S) and 18 nt oligoDT primer (IDT, custom synthesis). Taqman qPCR was performed using probes from ThermoFisher for GFP (Mr04097229_mr), human SOD1 (Hs00533490_m1) and mouse housekeeping genes GusB (Mm01197698_m1), Pgk1 (Mm00435617_m1), Gapdh (Mm99999915_g1) and Tfrc (Mm00441941_m1) (ThermoFisher) on a CFX-384 instrument (Bio-Rad).

Image Analysis.

ZEN 2.3 analysis software (Carl Zeiss, Oberkochen, Germany) was used for FISH image analysis. Briefly, images were acquired by confocal microscopy (Zeiss LSM800 AxioObserverZ1, Carl Zeiss, Oberkochen,

Germany) with a 63X Plan-Apochromat 1.4 Oil lens. Colors were added after acquisition. Analyses were done blindly. Mean intensity of the whole image or specific cell types (GFAP, Iba1) were measured with the software. Mean intensity average, SEM and statistical analysis were performed using Graphpad Prism (GraphPad Software, Inc. La Jolla, CA, USA).

For experiments on mixed motor neuron cultures fields of view with approximately 100 cells were analyzed. Four randomly selected fields of view were analyzed per slide and two slides from two independent wells were analyzed in each of two independent experiments (~1600 cell total).

RNAse treatment of exosomes

Where described, exosomes suspended in PBS + 0.25 M sucrose were treated with 10 µg RNase A (ThermoFisher-Scientific, Catalogue #EN0531) or the same amount of RNase A + 0.5% Triton X-100.

DNase I/ Proteinase K/ RNase A treatment

Reactions of 10^{10} exosomes purified on sucrose density gradients and containing GFP siRNA were suspended in 0.25M sucrose in PBS. These were spiked with 1 µg plasmid DNA and 100 pg single guide strand SOD1 siRNA (IDT, custom synthesis) to control for DNAse and RNAse activity respectively. Where required reactions were first treated with 10 µg DNase I (10 min, 37°C, Roche, Catalogue # 10104159001) then 0.5% SDS/Triton X-100 and proteinase K (100 µg/ml, 10 min, 37 ˚C, Sigma-Aldrich, Catalog # P2308). All reactions were then treated with PMSF at a final concentration of 5 mM for 10 minutes at room temperature. Reactions that received DNase I/Proteinase K/RNase A treatments were then finally treated with 10 µg RNase A for 10 minutes at 37 C. All reactions were then split into three equal parts for Western blot, RT-qPCR or DNA analysis. RNA was extracted with 0.5 ml Trizol while DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol for analysis on an ethidium-bromide stained agarose gel. Identical methods were used for analysis of ultracentrifuged exosomes by RT-qPCR. For western blot analysis of ultracentrifuged exosomes in Supplementary Fig.3f a Proteinase K concentration of 10 µg/mL was used.

Northern blots

RNA preparations were separated on 17.5 % polyacrylamide gels in 0.5X TBE (Wisent Bioproducts, Catalogue # 880-545-CL) and electroblotted (TurboBlot, BioRad) onto positively charged nylon membranes (GE Healthcare, Catalogue # 1417240) also in 0.5 X TBE. RNAs were crosslinked to membranes by UV using a Stratalinker delivering 240 mJ to each side of the membrane. Membranes were prehybridized for 1 h in 10 ml PerfectHyb buffer (Sigma-Aldrich, Catalogue # H7033) at 40 °C and hybridized with labeled probe overnight under the same conditions. Membranes were washed twice in 5 X SSC + 0.1 % SDS and once in 1X SSC + 0.1% SDS before exposure to storage phosphor screens (GE Healthcare). Screens were imaged using a Typhoon Trio machine (GE Healthcare). Custom probe oligonucleotides (IDT) were labeled as follows: 18 pmol of oligonucleotide was labeled with 30 µCi of [gamma-32P]-ATP (PerkinElmer, Catalogue # BLU002A500UC) using T4 PNK (NEB, Catalogue # M0201S) as recommended by the manufacturer. Labeled probes were separated from unincorporated nucleotides by ethanol precipitation using 10 µg glycogen (ThermoFisher, Catalogue # R0551) as a carrier. Oligonucleotide probe sequences were as follows: GFP siRNA probe 5'-gcaagctgaccctgaagttcat; U6 probe 5'-aggggccatgctaatcttct.

Fluorescent Western Blots (Transthyretin)

Blood (20-50 uL) was collected from the tail vein at different time points in heparinized tubes (Greiner Bio-one, 450477). Whole blood was separated by centrifugation at 3500 g for 15 minutes. Equal amount of serum (5 μL) was collected and diluted in water and Laemmli sample buffer 4x (Bio-Rad, 161-0747) with 10% 2-Mercaptoethanol (Bio-Rad, 1610710) and boiled for 5 min at 99 °C. Samples were loaded on an SDS-PAGE gel (10% acrylamide) and transferred to a positively charged nylon membrane (Roche, Catalogue # 11209299001). Membranes were washed with 1x TBST (3x5 min) and blocked with 1x TBST containing 5% milk for 1 h. Membranes were incubated at 4 °C overnight with the primary antibody (rabbit anti-mouse TTR, Invitrogen, PA5-80197). Membranes were washed with TBST (3x5 min) and incubated 1h with secondary antibody (Li-Cor Odyssey, goat anti-rabbit IRdye 800 (TTR), 926-32211, donkey anti-mouse IRDye 680 (IgG, loading control), 925-68072). Membranes were washed with 1x TBST (3x5 min) and imaged with Li-Cor Odyssey Fc imaging system (Li-Cor Biotechnology, Lincoln, USA). Images were analyzed using Image Studio software (Li-Cor Biotechnology, Lincoln, USA). Please refer to Table 3 in Supplementary Information for details on antibodies used.

Electroporation of Exosomes

siRNA, exosomes or a mixture of both were electroporated exactly as described[20]. Briefly, 10^9 exosomes isolated as described above and/or 1 µg siRNA (custom synthesized *in vivo* grade siRNA, Dharmacon) were resuspended in 400 µl electroporation buffer (1.15 mM potassium phosphate pH 7.2, 25 mM potassium chloride, 21% Optiprep), transferred to a 4 mm electroporation cuvette (VWR) and pulsed in a Gene Pulser Xcell (Bio-Rad). The following electroporation settings were used: 400 V, 125 µF and ∞ ohms. After pulsing the cuvette was immediately transferred to ice. The electroporated mixture was recovered from the cuvette, centrifuged at 100,000 g for 30 minutes in a TLA-100.3 rotor (Beckman), resuspended in 1 ml PBS and recentrifuged as above before final resuspension in 20 µl 1x PBS.

Digital Droplet PCR

Digital droplet PCR (ddPCR) was performed using the Bio-Rad QX200 system (QX200 droplet generator; C1000 thermotouch thermal cycler; QX200 droplet reader) following the manufacturer's recommendations, using the QX200 ddPCR EvaGreen supermix (Bio-Rad) and the QX200 droplet generation oil for Evagreen (Bio-Rad, Catalogue # 186-4034). PCR was performed using the SOD1 siRNA primer and the miScript Universal primer from the miScript kit described above (Qiagen) both present at 0.5 µM in the reaction. Optimal primer annealing temperatures (57 °C) and template dilutions were determined empirically as previously described[51]. PCR activation was performed as recommend for the QX200 ddPCR EvaGreen supermix. PCR cycling conditions were otherwise those recommended for the miScript qPCR system (Qiagen).

Quantification of miR-451 in exosomes after Long-Term Culture in Serum-Free Media

NSC-34, HEK293 and human neonatal fibroblast cells were cultured in DMEM + 10% FBS or in Serum-free media (Lonza UltraCULTURE + 1% Penecillin-Streptomycin + 1% L-glutamine) for six weeks. Cells were passaged at a sub-cultivation ratio of 1:10 approximately every 5 days. Cells were centrifuged at 300 g for 10

minutes and resuspended in media at each passage. Following six weeks of culture, cells at 80% confluence were washed with 1x PBS and media (either DMEM + 10% FBS or Lonza Ultraculture serum-free media) was replaced with serum-free media for exosome collection. Exosomes were isolated by differential ultracentrifugation. miR-451 levels in exosomes were quantified by RT-qPCR.

Quantification of miR-451 in Exosome-Depleted Media With and Without Exposure to Cells

Exosome-depleted DMEM prepared by ultracentrifugation as above was used to collect exosomes from 80% confluent NSC-34 cells (exosome-conditioned media). Exosomes were isolated by differential ultracentrifugation from equal volumes of standard DMEM + 10% FBS, exosome-depleted DMEM, and exosome-conditioned media. Pellets obtained from the final 100,000xg spin were used to quantify miR-451 by RT-qPCR.

MiR-451 Quantification in Cells and Exosomes after Serial Dilution of Cells

Cell lines were grown in DMEM + 10% FBS + Pen/strep and were washed extensively with PBS prior to harvesting with Trypsin. Indicated dilutions of cells were then seeded in serum-free Ultraculture (Lonza) in 10 cm plates, with the most concentrated plates receiving 2 x 10^6 cells. Media was replaced with fresh Ultraculture daily. Once each dilution reached approximately 80 % confluence, media that had been on the plates for 20 h was collected for exosome collection and quantification of miRNA levels by RT-qPCR.

Preferential Detection of short and long forms of pre-miR-451.

RNA from exosomes from *Ago2*$^{-/-}$ and *Ago2*$^{-/-}$ cells rescued with Ago2 was isolated and subjected to reverse transcription using the miScript RT kit (Qiagen) with either the Specific or Flexible buffers. Specific buffer is designed to preferentially produce short amplicons corresponding to mature miRNA, while Flexible buffer produces both short and long amplicons. To verify the preferences of these buffers, synthetic RNAs mimicking the mature 22 nt strand or Drosha-processed 42 nt strand were included in separate reactions (sequences 5'-UUCAGUCAGUCCUUUAAUGCUU or 5'-UUCAGUCAGUCCUUUAAUGCUUAUUAAAGGACUGACUGAUUC respectively; IDT). qPCR using the miScript system was then used to assess the fold-change in product levels of Flexible to Specific buffer.

Measurement of RNA Recovery from Exosome Samples

To determine the efficiency with which small ~22 nt single-stranded RNAs are recovered in Trizol preparations, a synthetic RNA mimicking the fully processed SOD1 siRNA in exosomes was used. Final cell RNA preparations contained approximately 50 μg or 1 μg RNA, while exosomes preparations contained approximately 1 μg RNA (measured by spectrophotometry). Briefly, cell and exosome pellets in 1 ml of Trizol were spiked with 5 μl of 2 nM ssRNA and RNA was prepared according to the manufacturer's instructions, including the addition of 10 μg glycogen (RNA grade, Thermo-Fisher Scientific, Catalogue # R0551) to the isolated aqueous phase to aid RNA precipitation. After alcohol precipitation RNA was resuspended in 50 μL RNase free water. Five μL of the 2 nM ssRNA was similarly diluted to serve as the Input sample. Recovery of the siRNA vs. SOD1 was quantified using absolute copy numbers using a standard curve as above.

Sucrose density gradient:

Exosomes produced from NSC-34 cells transduced with pre-miR-451 hairpin vectors with GFP siRNA integrated were isolated by differential ultracentrifugation. Exosome pellets were resuspended in 1.85 mL of 0.95 M sucrose solution. The 0.95 M sucrose solution containing exosomes was inserted in a sucrose step-gradient composed of six 1.85 mL layers of sucrose concentrations of 2.0 M, 1.65 M, 1.30 M, 0.95 M, 0.6 M and 0.25 M. The sucrose step-gradient was centrifuged at 200,000 g for 20 hours at 4°C. Twelve fractions of 0.925 mL were collected and their density was measured with a refractometer (No.16046 ERMA). About 250-500 μL of fractions was diluted to 30 mL in cold 1x PBS and centrifuged at 100,000 g at 4 °C for 70 minutes. The pellets were resuspended in 25 μL of cold 1x PBS and used for Western blotting.

Fraction RT-qPCR and RNase A/T1 treatment:

Trizol LS (ThermoFisher, Catalogue # 10296028) was used to isolate RNA from 250 μL of each fraction and RT-qPCR was performed with the miSCRIPT kit (Qiagen). For RNase A/T1 treatments equal volumes of Fraction 6 and 7 that contained >80% of exosome markers (Flotillin2, Tsg101) were combined. Fifty μL of these combined fractions was used for each reaction in a total volume of 75 μL. Fractions were treated with RNase A/T1 Mix (1.5 μL at 2 mg/mL of RNAse A and 5000 U/mL RNase T1, ThermoFisher, Catalogue # EN0551,) at 37°C for 10 minutes with or without 0.5% Triton X-100 in 1x PBS.

Lipid Nanoparticles

In some experiments, lipid nanoparticles were produced using InvivoFectamine 3.0 (ThermoFisher, Catalogue # IVF3001) loaded with synthetic siRNA vs. SOD1 (IDT) without chemical modifications according to the manufacturer's instructions. The SOD1 siRNA represents a canonical double-stranded siRNA with 3' overhangs and the targeting sequence is identical in sequence to that produced by the SOD1 siRNA integrated into the pre-miR-451 hairpin above. In other experiments C12-200 lipid nanoparticles were prepared and loaded with siRNA targeting GFP (Qiagen, GFP-22 siRNA positive control). The targeting sequence of GFP siRNA is identical to that produced by the GFP siRNA integrated into the pre-miR-451 hairpin elsewhere. C12-200 lipid nanoparticles were produced as previously described[41].

Statistics

In all cases where sample size is mentioned, measures were taken from distinct samples. Statistics were calculated in GraphPad Prism.

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Reporting Summary

Data Availability

The main data supporting the results in this study are available within the paper and its Supplementary Information. The raw and analysed datasets generated during the study are available for research purposes from the corresponding authors on reasonable request.

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Author Contributions

JT performed cloning, lentivirus production, northern blots, analysis of RNA enrichment in exosome-like vesicles and absolute quantification of RNA in exosome-like vesicles. AS maintained mouse colonies, performed ICV and IV injections and tissue harvests helped analyze exosome distribution, performed western blots of exosome-like vesicles, generated cultures of primary mixed motor neurons, performed some RT-qPCR and performed and analyzed microscopy. RR produced exosome-like vesicles, performed Nanoparticle tracking analysis, performed tissue harvests, labeled exosome-like vesicles and analyzed their distribution and analyzed RNA enrichment in exosome-like vesicles and mRNA target knockdown by RT-qPCR. MTT maintained mouse colonies, generated mouse protocols, genotypes mice and performed ICV and IT injections and tissue harvests. CC helped establish protocol for primary mixed motor neuron culture and performed some analyses of miRNA levels in exosome-like vesicles. HG performed western blots of exosome-like vesicles, density gradient analyses of exosomes. LR and PK helped design lipid nanoparticle experiments and produced C12-200 lipid nanoparticles. DA helped design lipid nanoparticle experiments and supervised LR and PK. WL helped design experiments. JT, AS and RR analyzed experiments and helped design experiments. DG conceived the project, designed experiments and wrote the manuscript.

Competing Financial Interests

JT and DG are inventors on a patent application which claims the use of the pre-miR-451 backbone for enrichment of small RNAs in exosome-like vesicles.

Additional Information

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Figures and Figure Captions

Fig. 1 | Exosome-like vesicles contain few miRNAs but among these miR-451 is highly abundant compared to its level in cells. a, Western blot of equal amounts of proteins from MDA-MB-231 cells and the exosome-like vesicles they produce for markers of exosome-like vesicles (Tsg101, Flotillin2), proteins excluded from exosome-like vesicles (mitochondrial Tomm20) and Ago2. **b,** Nanoparticle tracking analysis of exosome-like vesicles. Representative plot of particle number (y-axis) vs. diameter (x-axis, nm) of exosome preparations produced by NSC-34 cells. **c,** Left, RT-qPCR of SOD1 siRNA recovered by ultracentrifugation after electroporation with or without exosomes. Statistics: Unpaired 2-tailed t-test. N=3 for all groups. Right, RT-qPCR of SOD1 siRNA recovered by ultracentrifugation and then treated with RNAseA/T1 after electroporation with or without exosomes. Statistics are derived from a repeated measurements one way ANOVA with Holmes-Sidack correction. N=3. **d,** Absolute copy numbers of miRNAs in exosome-like vesicles produced by three cell types (MDA-MB-231 [N=3], MEF [N=2] and NSC-34 [N=3]) measured by standard curve RT-qPCR analysis. **e,** Relative enrichment of miRNAs in exosome-like vesicles expressed as the level of the respective miRNA in total exosome RNA / its level in total cell RNA as measured by RT-qPCR normalizing to miR-16. (MEF [N=2] and NSC-34 [N=3]). **f,** Enrichment of miR-451 in exosome-like vesicles vs. its level in the listed cell types. N=6 MSC, macrophage, N=3 all others. **g,** RT-qPCR to measure relative quantity of miR-451 in exosome-like vesicles left untreated, treated with RNAse or treated with detergent and RNAse. N=2. **h,** Sucrose density gradient fractionation of exosomes produced by NSC-34 cells. Upper graph represents densities of fractions measured by refractometry (black line) and quantity of miR-451 (red dots, RT-qPCR 2^{-Ct} without normalization) in these fractions. Bottom, Western blot for Ago2 was performed on a separate membrane with the same fractions as western blots for Alix and Flotillin2. **i,** RT-qPCR analysis of combined fraction 6 and 7 from (**h**) that was left untreated, treated with RNAse A/T1 or RNAse A/T1 and 0.5% Triton X-100. Full blot images from (**a**) and (**h**) are available in Supplementary Information. Where error bars are shown, the central value is the mean and the bars show the standard error in the mean.

Fig. 2 | Reprogramming the pre-miR-451 backbone with siRNAs causes their enrichment in exosome-like vesicles. a, A diagram of the predicted pre-miR-451 secondary structure detailing its cleavage by Drosha, Ago2 and subsequent trimming by exonucleases. **b,** Northern blot of GFP siRNA produced from the pre-miR-451 backbone in HeLa and NSC-34 cells. **c,d** RT-qPCR measuring enrichment of TetR (**c**) or GFP (**d**) siRNA in exosome-like vesicles vs. their levels in cells when these were integrated in either the pre-miR-451 backbone or the pre-miR-16 backbone (control) and transiently expressed in MEF. Statistics: two-tailed unpaired t-test. N=3. **e,** RT-qPCR measuring enrichment of SOD1 siRNA in exosome-like vesicles vs. their levels in cells when SOD1 siRNA was integrated in either the pre-miR-451 backbone or the pre-miR-16 backbone (control) and transfected into the indicated cell types. Statistics: 1 way ANOVA with Holmes-Sidack correction was used. N=4, 3, 2, 6 for BV2, C8D1A, C8S and NSC-34 pre-mir-16 control transfected respectively and 4, 3, 3, 5 for pre-miR-451 transfected. **f**, Schematic portraying the reprogramming of the pre-miR-451 hairpin structure with siRNA targeting SOD1 or GFP siRNA. **g,** Copy number of GFP or SOD1 siRNA in exosome-like vesicles after stable expression from the pre-miR-451 backbone in the indicated cell types. N=3. **h,** Northern blot of GFP siRNA or U6 (control) in equal amounts of RNA from cells or exosome-like vesicles of MEF. **i,** RT-qPCR analysis of GFP siRNA integrated in the pre-miR-451 backbone in fractions of density gradient in Fig.1h using 2^{-Ct} method without normalization. **j,** Left, Western blot of Ago2 and

Tubulin (loading control) in wild-type, *Ago2*$^{-/-}$ or *Ago2*$^{-/-}$ cells stably rescued with *Ago2*. Right, Northern blot of GFP siRNA programmed into the pre-miR-451 backbone or U6 (loading control) in cellular RNA of *Ago2*$^{-/-}$ cells or *Ago2*$^{-/-}$ cells stably rescued with *Ago2*. **k,l** RT-qPCR of miR-16 and miR-451 (**k**) or SOD1 siRNA integrated in the pre-miR-451 hairpin structure (**l**) in exosome-like vesicles vs. cells using *Ago2*$^{-/-}$ or *Ago2*$^{-/-}$ cells stably rescued with *Ago2*. Enrichment in exosome-like vesicles vs. cells normalized to U6 (**k**) or miR-16 (**l**) RNA is depicted. N=3. **m,** RT-qPCR to measure fold-change in enrichment in exosome-like vesicles vs. cells of miR-451 after transiently expressing GFP (control) or Ago2. Full blot images from (**b**), (**h**) and (**j**) are available in Supplementary Information. For statistics, unless otherwise mentioned a one way ANOVA with Holmes-Sidack multiple comparison was used. Where error bars are shown, the center value is the mean and the bars show the standard error in the mean.

Fig. 3 | Exosome-like vesicles loaded with siRNA integrated in the pre-miR-451 backbone efficiently deliver siRNA to primary motor neurons. a, Confocal microscopy of GFP fluorescence, GFP mRNA (FISH) and siRNA targeting GFP (FISH) in primary mouse motor neurons from GFP transgenic mice after incubation with exosome-like vesicles produced by NSC-34 cells and loaded with the indicated siRNA integrated in the pre-miR-451 backbone. Scale bar = 100 μm. **b,** Confocal microscopy of DDX6/Rck (P-body marker), SOD1 mRNA (FISH) and siRNA targeting SOD1 (FISH) in primary mouse motor neurons from human SOD1 G93A transgenic mice after incubation with exosome-like vesicles loaded with SOD1 siRNA integrated in the pre-miR-451 backbone. Scale bar = 10 μm. **c,** RT-qPCR for GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with the indicated doses of exosome-like vesicles produced by NSC-34 cells. N=4 at 1,000 exosomes per cell, N=2 all others. **d,** Quantification of GFP fluorescence and GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with the indicated doses of exosome-like vesicles produced by NSC-34 cells. **e,** RT-qPCR for GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with amounts of siRNA alone equivalent to that in 1000 exosome-like vesicles / per cell. N=3. **f,** RT-qPCR for GFP mRNA in primary mixed motor neuron cultures from GFP transgenic mice after incubation with exosome-like vesicles produced by *Ago2*$^{-/-}$ MEF cells or *Ago2*$^{-/-}$ MEF cells stably rescued with *Ago2*. N=3. **g, i,** RT-qPCR for GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with the indicated doses of exosome-like vesicles produced by Neuro2a cells **(g)** or BV2 cells **(i)**. Reference mRNAs were averaged GAPDH, GusB and TfrR. N=2. **h, j,** Quantification of GFP fluorescence and GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with the indicated doses of exosome-like vesicles produced by Neuro2a cells **(h)** or BV2 cells **(j)**. For statistics a 1 way ANOVA with Holmes-Sidack multiple comparison was used. For microscopy analysis, fields of view containing approximately 100 cells were quantified (x 4/slide, n=4 slides) (~1600 cell total). Where error bars are shown, the center value is the mean and the bars show the standard error in the mean.

Fig. 4 | Exosome-like vesicles loaded with siRNA integrated in the pre-miR-451 backbone knockdown target expression in mouse liver and small intestine after intravenous (IV) injection. a, IVIS pre-clinical imaging of organs harvested from mice injected with exosome-like vesicles labeled with DiR or DiR prepared similarly without exosome-like vesicles. Right, quantification of fluorescence in organs of IV injected mice. N=5 mice, box and whisker plot identifies median values with error bars spanning the maximum and minimum data points, statistics derived by two-tailed unpaired t-test with Welch's correction. **b,** RT-qPCR of

GFP mRNA in brown fat, heart and lungs three days after injection of 10^{10} exosomes (NSC-34) packaged with GFP siRNA or control siRNA. N=4 mice per group. Statistics derived from two-tailed unpaired t-test with Welch's correction. **c,** RT-qPCR of GFP mRNA in liver and small intestine of GFP transgenic mice three days after injection of 10^{10} or 10^{11} exosomes (NSC-34) packaged with GFP siRNA or control siRNA. N=4 mice per group for liver and control groups, N=6 for small intestine GFP siRNA. **d,** RT-qPCR of SOD1 mRNA in liver and small intestine of SOD1 G93A mice three days after injection of 10^{10} or 10^{11} exosomes (NSC-34) packaged with SOD1 siRNA or controls (equal numbers of untreated mice and mice treated with exosomes packaged with control siRNA). N=4 mice per group. For statistics, unless otherwise mentioned a 1 way ANOVA with Holmes-Sidack multiple comparison was used. Where error bars are shown, except in (**a**), the center value is the mean and the bars show the standard error in the mean.

Fig. 5 | Exosome-like vesicles loaded with siRNA integrated in the pre-miR-451 backbone knockdown target expression with lower doses than lipid nanoparticles or electroporated exosomes. a,b, RT-qPCR of GFP mRNA in liver **(a)** and small intestine **(b)** of GFP transgenic mice three or 12 days after injection of 10^{10} exosomes packaged with GFP siRNA or control siRNA. N=4 mice per group at 3 days, N=3 mice per group at 12 days. **c,** RT-qPCR of SOD1 mRNA in liver and small intestine of SOD1 G93A mice three days after injection of InvivoFectamine lipid nanoparticles packaged with 1 mg/kg siRNA, 10 ng/kg siRNA or 10^{10} exosomes packaged with 10-300 ng/kg of the identical SOD1 siRNA. N=4 mice per group. **d,** RT-qPCR of GFP mRNA in liver three days after IV injection of C12-200 lipid nanoparticles or exosomes packaged with GFP siRNA at the indicated doses. N=3 mice for control siRNA, N=4 mice per group of exosomes or lipid nanoparticle treatments. **e,** RT-qPCR of GFP mRNA in liver of GFP transgenic mice three days after injection of exosomes electroporated with GFP siRNA using conditions previously published[20]. N=3 mice per group. **f,** RT-qPCR of *let-7* and miR-106 after treatment of exosome pellets with RNAse A/T1 after exosomes were left untreated, electroporated as described[20], or treated with detergent N=3 or N=2 experiments as shown. **g,** Size distribution of exosome-like vesicles after electroporation measured by Nanoparticle tracking analysis. N=6 exosome preparations. **h,** RT-qPCR of GFP mRNA in mixed motor neurons after incubation for 2 days with exosomes (10,000/cell) left intact or disrupted with detergent. N=5 untreated, N=3 for intact exosomes, N=2 for detergent-treated exosomes. **i,j,** RT-qPCR of SOD1 mRNA in liver **(i)** and small intestine **(j)** after treatment of mice with exosomes packaged with control siRNA or exosomes that were left untreated or electroporated after packaging with SOD1 siRNA using the pre-miR-451 hairpin N=4 mice per group. For statistics a 1 way ANOVA with Holmes-Sidack multiple comparison was used. Where error bars are shown, the center value is the mean and the bars show the standard error in the mean.

Fig. 6 | Quantitative FISH for mRNA accurately knockdown measured by RT-qPCR in mouse. a,e, Confocal microscopy of SOD1 siRNA (FISH) and SOD1 mRNA (FISH) vs. GAPDH mRNA (normalization control) in sections of mouse liver and small intestine three days after injection of exosome-like vesicles loaded with control siRNA or SOD1 siRNA integrated in the pre-miR-451 backbone. Scale bar = 50 μm. **b,f,** Quantification of SOD1 siRNA by FISH in liver and small intestine sections three days after IV injection of exosome-like vesicles loaded with control siRNA or SOD1 siRNA integrated in the pre-mIR-451 backbone. **c,g,** RT-qPCR of SOD1 mRNA in liver and intestine 72 h after IV injection of exosome-like vesicles loaded with control siRNA or SOD1 siRNA integrated in the pre-mIR-451 backbone. N=5 mice per group **d,h,**

Quantification of SOD1 mRNA by FISH in liver and small intestine sections three days after IV injection of exosome-like vesicles loaded with control siRNA or GFP siRNA integrated in the pre-miR-451 backbone. For statistics two-tailed unpaired t-tests with Welch's correction were used. N=6 animals per experimental group. Two pieces of tissue per slide (duplicates) were analyzed in two randomly selected areas (4 total per animal per tissue). N=4 mice per group for RT-qPCR data. Where error bars are shown, the center value is the mean and the bars show the standard error in the mean.

Fig. 7 | Exosomes packaged with siRNA knockdown target genes in specific regions and cell-types of liver, small intestine and kidney. Experiments herein were performed on tissues of GFP transgenic mice three days after injection of 10^{10} exosomes (low dose) packaged with GFP siRNA or control siRNA using the pre-miR-451 backbone. **a,d,g,j,** Confocal microscopy of GFP fluorescence and GFP mRNA (FISH) in sections of small intestine Scale bar = 50 μm **(a)**, liver labeled with markers for stellate cells Scale bar = 10 μm (**d,** GFAP) or macrophages Scale bar = 50 μm (**g**, F4/80), or kidney labeled for podocytes Scale bar = 10 μm for control siRNA, 50 μm for GFP siRNA**.** (**j**, Nephrin). **b,c,e,f,h,i,l,** Quantification of GFP fluorescence in small intestine submucosa **(b)** and villi **(c)**, in total liver **(e)** hepatocytes **(f)** liver macrophages (F4/80) **(h)** liver stellate cells (GFAP) **(i),** and kidney podocytes (Nephrin) (**l**) **k,** RT-qPCR of GFP mRNA in total kidney three days after injection of exosomes packaged with GFP siRNA or control siRNA using the pre-miR-451 backbone n=4. For statistics a two-tailed unpaired t-test with Welch's correction was used. N=6 animals per experimental group. Two pieces of tissue per slide (duplicates) were analyzed in two randomly selected areas (4 total per animal per tissue). For cell-specific analysis 3-5 cells positive for the label were quantified per image in 2-3 images per tissue slice and 2 tissue slices per animal. Data for whole liver and whole small intestine knockdown contains results from 3 mice also used in Figure 6, in addition to data from 3 more mice only included in Figure 7. Where error bars are shown, the center value is the mean and the bars show the standard error in the mean

Fig. 8 | Exosomes packaged with siRNA targeting TTR reduce TTR levels in blood by >85%.
a,b RT-qPCR of GFP mRNA three days after injection of 10^{10} exosomes produced by 293T cells (**a**)**,** or human primary fetal fibroblasts (**b**)**,** and packaged with about 10-300 ng/kg GFP siRNA using the pre-miR-451 backbone compared to untreated mice. N=4 mice per group. Experiments in (**a**) and (**b**) were performed in parallel with the same control mice. **c,** Western blot of TTR from blood (vs. IgG loading control) seven days after injection of 10^{10} exosomes produced by human primary fetal fibroblasts and packaged with about 10-300 ng/kg control siRNA or TTR siRNA using the pre-miR-451 backbone. Right, quantification of TTR levels in blood at two days and seven days after exosome administration. N=4 mice in control group, N=3 in TTR siRNA group. For (**a**) and (**b**), statistics are derived from a two-tailed unpaired t-test with Welch's correction. For (**c**), one way ANOVA with Holmes-Sidack multiple comparison was used. Where error bars are shown, the center value is the mean and the bars show the standard error in the mean. Full blot images from (**c**) are available in Supplementary Information.