Reduction of the therapeutic dose of silencing RNA by packaging it in extracellular vesicles via a pre-microRNA backbone

Ryan Reshke1,2,3,7, James A. Taylor1,2, Alexandre Savard1,2,7, Huishan Guo1,2, Luke H. Rhym4, Piotr S. Kowalski5, My Tran Trung1,2, Charles Campbell1,2, Wheaton Little5, Daniel G. Anderson4 and Derrick Gibbings1,2,3,6*

A small percentage of the short interfering 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 to achieve therapeutic outcomes can lead to toxicity. Here, we show that the integration of siRNA sequences into a Dicer-independent RNA stem–loop based on pre-miR-451 microRNA—which is highly enriched in small extracellular vesicles secreted by many cell types—reduces the expression of the genes targeted by the siRNA in the liver, intestine and kidney glomeruli of mice at siRNA doses that are at least tenfold lower than the siRNA doses typically delivered via lipid nanoparticles. Small extracellular vesicles that efficiently package siRNA can significantly reduce its therapeutic dose.

Canonically, pre-microRNAs (pre-miRNAs) are 60–120 nucleotide (nt) stem–loop structures that are cleaved by the ribonuclease (RNase) III enzyme Dicer into short, approximately 21 nt double-stranded (ds) RNAs. This 21 nt dsRNA complex binds the microRNA effector Argonaute (Ago). After removal of one RNA strand, the Ago–miRNA complex represses translation of target genes by translational repression and mRNA degradation. siRNAs are designed to mimic Dicer-processed dsRNA that bind Ago and are used therapeutically. When siRNAs are perfectly complementary to mRNA targets, Ago2 can enzymatically cleave target mRNA,1–4 conferring remarkable potency and specificity to these drugs.

For therapeutic applications, delivery of siRNA into the cytoplasm of cells is a perennial challenge.5,6 Quantitative analysis has shown that 300–2,000 copies of siRNA that can catalytically cleave mRNA are required in the cytoplasm of each cell to silence target mRNAs13–15. For example, 300 copies of siRNA per cell were required to silence target expression by 50% when siRNA was microinjected directly into the cytoplasm. Alternatively, 800 copies of siRNA loaded into Ago2 were required to silence a target mRNA effectively in the liver.7 Measured in cultured cells or in the liver of mice, current delivery vehicles such as lipid nanoparticles enable only between 0.05% and 1% of siRNA to enter the cytoplasm of cells.8,9,16 For this reason, high doses of siRNA and their delivery vehicles are required to reach a therapeutic dose in patients; this can lead to toxicity, which has halted multiple clinical trials of silencing RNAs10. Lipid nanoparticles and siRNA accumulate predominantly in the liver. Consequently, most siRNA therapeutics target proteins expressed in the liver. siRNA delivery to the liver with lipid nanoparticles reduces target expression in patients by more than 80% for over 6 months with a single dose17,18, and was recently approved for treatment of transthyretin amyloidosis. Delivery of siRNA to other organs remains a major challenge.

Extraacellular vesicles, and in particular small extracellular vesicles, have been proposed as a delivery vehicle for siRNA. Small extracellular vesicles (sEVs) can be produced by budding from the plasma membrane or by budding of 60–120 nm vesicles into the lumen of late endosomes. These late endosomes can subsequently fuse with the plasma membrane, releasing the sEVs inside them into the extracellular space.11 Studies showed that extracellular vesicles and sEVs contain miRNA and can transfer miRNA activity into other cells.12,13 A hypothesis has emerged that sEVs have an evolved ability to deliver small RNAs or other molecules between cells and that this capacity could be captured for efficient drug delivery. Despite the attention on sEVs, the efficiency with which they deliver their cargoes into the cytoplasm has not been directly evaluated. Indeed, in some cases, target silencing was indistinguishable whether siRNA was electroporated into sEVs or injected directly into mice14. In other cases, where siRNA was electroporated with sEVs or coated onto the sEV surface, the siRNA doses required to knockdown target expression markedly exceeded that required for siRNA in lipid nanoparticles.15,16 This suggests that sEVs may actually have little inherent capacity to deliver RNAs.

Studies of siRNA delivery using sEVs to date may not, however, test the actual delivery efficiency of sEVs. Topologically, siRNA on the sEV exterior will not enter the cytoplasm if sEVs fuse with target cells, but will rather remain trapped in endosomes or on the exterior of the cell, and thus inactive. Also, electroporating sEVs with siRNA may not actually introduce siRNA into sEVs, but rather precipitate siRNA, which then co-purifies with sEVs.17 Electroporation or otherwise altering the sEV surface may also interfere with the membrane architecture of sEVs required for fusion with target cell...
membranes. For this reason, the actual delivery efficiency of sEVs still requires testing.

miRNAs are sparse in sEVs, with an absolute copy number ranging from one copy of a specific miRNA per 10° sEVs to one copy per sEV. This underscores that any attempt to deliver RNA therapeutics using sEVs will probably require a means of packaging more RNA into sEVs. sEVs are loaded with a highly selective subset of RNAs from the cells that produce them. For example, specific miRNAs that are similarly abundant in cells are found with a 10°-wide range of relative quantity in sEVs. This suggests that simply overexpressing a miRNA may not enable its packaging into sEVs. Sequence motifs are probably not an adequate solution, as they result in modest enrichment in sEVs and are difficult to insert into a small siRNA.

Pre-miR-451 is an exception to the canonical pre-miRNA biogenesis cascade. The stem–loop of pre-miR-451, after processing by Drosha, is only 42 nt long and too short to be bound and cleaved by Dicer. Instead, pre-miR-451 binds directly to Ago2. 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 an approximately 21 nt miRNA. Notably, if the structure of the pre-miR-451 stem–loop is maintained, it can be reprogrammed with other miRNA sequences, which will be processed through the same Dicer-independent, Ago2-dependent pathway into mature effective miRNA.

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 sEVs. sEVs packaged with siRNA in this manner can efficiently reduce expression of target genes in primary cells in culture and also in mice. sEVs packaged with siRNA can knockdown targets with at least tenfold less siRNA than lipid nanoparticles, suggesting that sEVs are highly efficient delivery vehicles.

Pre-miR-451 products are selectively enriched in sEVs

sEVs were enriched by differential centrifugation from supernatants of mouse embryonic fibroblasts (MEFs), MDA-MB-231 human breast cancer cell line and NSC-34 motor neuron-like cell line grown in sEV-depleted media. These preparations were enriched in sEV markers Tsg101 and Rottlin 2 and contained small to moderate amounts of Ago2. As expected, tubulin and a mitochondrial protein (Tommm20) were undetectable in these preparations (Fig. 1a and Supplementary Fig. 1a). Particles in sEV preparations had a median size (100–120 nm) and size distribution consistent with sEVs (Fig. 1b and Supplementary Fig. 1b).

Following recent reports of siRNA delivery by sEVs in mice, we attempted to electroporate siRNA into sEVs. Using a protocol and instrument identical to the one described, we electroporated sEVs and siRNA targeted against superoxide dismutase (SOD1). Whether siRNA was electroporated in the presence or absence of sEVs, siRNA was similarly pelleted by a standard protocol to isolate sEVs by ultracentrifugation (Fig. 1c). This suggests that electroporation may cause siRNA to aggregate or precipitate. Consistent with this suggestion, whereas free siRNA was degraded by RNase A, electroporated siRNA was resistant to digestion by RNase A whether sEVs were present or not in the electroporation cuvette (Fig. 1c). In agreement with ref. 2, this suggests that siRNA is not introduced into sEVs by electroporation but precipitates outside sEVs and copurifies with them on ultracentrifugation. We sought an alternative method to package siRNA into sEVs by harnessing cellular mechanisms of packaging RNAs into sEVs.

We evaluated the absolute copy number of endogenous miRNA per sEV. Nanoparticle tracking enabled counting of 100 nm poly-styrene particles of known concentration with high accuracy and reproducibility across a range that coincided closely with that recommended by the instrument’s software (Supplementary Fig. 1c). sEVs were diluted into this range for quantification in all experiments. To estimate the number of miRNA per sEVs, standard curves of quantitative PCR (qPCR) products were used correcting for efficiency of qPCR with reverse transcription (RT-qPCR) (Supplementary Fig. 1d). An independent method, digital droplet PCR, demonstrated that the standard curve method quantified miRNA with an error of less than 20% over a wide range of concentrations (Supplementary Fig. 1e). Some miRNAs were present at 1 to 10 copies per sEV (Fig. 1d), in agreement with studies which quantified the most abundant miRNAs in sEVs. By contrast, many miRNAs, such as miR-16, miR-451 and miR-134, were present at extremely low numbers in sEVs (1 copy per 10,000 sEVs to 1 copy per 10 sEVs; Fig. 1d), in line with previous studies. The relative abundance in sEVs of several of the miRNAs tested did not differ greatly from their abundance in sEV-producing cells (Fig. 1e), suggesting that they are randomly packaged into sEVs. Notably, despite its low copy number in sEVs (Fig. 1d), miR-451 was 500 to 10,000-fold enriched in sEVs compared with its levels in the respective cells (Fig. 1e,f). miR-451 was similarly enriched in sEVs normalized to U6 RNA and produced in serum-free Ultraceulture medium (Lonzza) (Supplementary Fig. 1f).

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

miR-451 was similarly enriched in sEVs when normalized to miR-16 (Fig. 1e,f) which exhibits similar abundance to miR-451 in the sEVs tested (Fig. 1d), or to U6 RNA (Supplementary Fig. 1f). Finally, miR-451 was present inside sEVs as it was only sensitive to RNase treatment after pre-treatment with detergent (Fig. 1g). Similarly, when sEVs were isolated by sucrose density gradient centrifugation, miR-451 abundance peaked with sEV-containing fractions at a density of 1.10–1.18 g ml⁻¹ (Fig. 1h). miR-451 in these fractions was only sensitive to RNase degradation after permeabilization of sEVs with detergent (Fig. 1i). Furthermore, miR-451 was not digested with RNase even when sEVs were pre-treated with proteinase K to release any RNA from proteins or protein aggregates co-purifying with sEVs, whereas a spiked-in siRNA was degraded even without detergent (Supplementary Fig. 3a,b). This demonstrates that miR-451 is selectively packaged inside sEVs compared with other cellular miRNAs.

New siRNAs in pre-miR-451 structures are enriched in sEVs

The stem–loop hairpin of pre-miR-451 binds directly to Ago2, which cleaves the distal strand of RNA in the stem region. Exonucleases then trim pre-miR-451 to around 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. We hypothesized that integrating siRNA into the pre-miR-451 backbone would cause these siRNA to be enriched in sEVs. We integrated a siRNA targeting GFP into the pre-miR-451 backbone. As expected, this was processed into a mature siRNA of about 22 nt in cells (Fig. 2b). Integration of siRNA targeting GFP, tetracycline repressor (TetR) or SOD1 into the pre-miR-451 backbone caused these siRNA, like miR-451, to be
enriched from 58-fold to more than 7,000-fold in sEVs produced by multiple cell types (Fig. 2c–e and Supplementary 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 sEVs measured as relative enrichment or absolute copy number (Fig. 2c–e and Supplementary Fig. 3c,d). When produced from the pre-miR-451 hairpin, siRNA targeting GFP or SOD1 were enriched from 58-fold to more than 7,000-fold in sEVs produced by NSC-34 cells. Top: densities of fractions measured by refractometry (black line) and quantity of miR-451 (red dots, RT-qPCR, n = 2) and NSC-34 (n = 6) (mesenchymal stem cell (MSC) and macrophage) and n = 3 (all others). g, RT-qPCR to measure relative quantity of miR-451 in sEVs (NSC-34) left untreated, treated with RNase or treated with detergent and RNase. n = 2. h, Sucrose density gradient fractionation of sEVs produced by NSC-34 cells. Top: densities of fractions measured by refractometry (black line) and quantity of miR-451 (red dots, RT-qPCR, 2–16 without normalization) in these fractions. Western blot for Ago2 was performed on a separate membrane with the same fractions as western blots for Alix and flotillin 2. i, RT-qPCR analysis of combined fractions 6 and 7 from h either left untreated, treated with RNase A and RNase T1, or RNase A/T1 and 0.5% Triton X-100. Repeated measurements one-way analysis of variance (ANOVA) with Holm-Sidak correction; n = 3. d, Absolute copy numbers of miRNAs in sEVs measured by standard curve RT-qPCR analysis in sEVs produced by three cell types (MDA-MB-231 (n = 3), MEF (n = 2) and NSC-34 (n = 3)). e, Enrichment of miRNAs in sEVs, expressed as the level of the respective miRNA in total sEV RNA relative to its level in total cell RNA as measured by RT-qPCR, normalized to miR-16. MEF (n = 2) and NSC-34 (n = 3). f, Enrichment of miR-451 in sEVs versus its level in cells in the listed cell types. n = 6 (mesenchymal stem cell (MSC) and macrophage) and n = 3 (all others). g, RT-qPCR to measure relative quantity of miR-451 in sEVs (NSC-34) left untreated, treated with RNase or treated with detergent and RNase. n = 2. h, Sucrose density gradient fractionation of sEVs produced by NSC-34 cells. Top: densities of fractions measured by refractometry (black line) and quantity of miR-451 (red dots, RT-qPCR, 2–16 without normalization) in these fractions. Western blot for Ago2 was performed on a separate membrane with the same fractions as western blots for Alix and flotillin 2. i, RT-qPCR analysis of combined fractions 6 and 7 from h either left untreated, treated with RNase A and RNase T1, or RNase A/T1 and 0.5% Triton X-100. Repeated measurements one-way analysis of variance (ANOVA) with Holm-Sidak correction; n = 3. d, Absolute copy numbers of miRNAs in sEVs measured by standard curve RT-qPCR analysis in sEVs produced by three cell types (MDA-MB-231 (n = 3), MEF (n = 2) and NSC-34 (n = 3)). e, Enrichment of miRNAs in sEVs, expressed as the level of the respective miRNA in total sEV RNA relative to its level in total cell RNA as measured by RT-qPCR, normalized to miR-16. MEF (n = 2) and NSC-34 (n = 3). f, Enrichment of miR-451 in sEVs versus its level in cells in the listed cell types. n = 6 (mesenchymal stem cell (MSC) and macrophage) and n = 3 (all others). g, RT-qPCR to measure relative quantity of miR-451 in sEVs (NSC-34) left untreated, treated with RNase or treated with detergent and RNase. n = 2. h, Sucrose density gradient fractionation of sEVs produced by NSC-34 cells. Top: densities of fractions measured by refractometry (black line) and quantity of miR-451 (red dots, RT-qPCR, 2–16 without normalization) in these fractions. Western blot for Ago2 was performed on a separate membrane with the same fractions as western blots for Alix and flotillin 2. i, RT-qPCR analysis of combined fractions 6 and 7 from h either left untreated, treated with RNase A and RNase T1, or RNase A/T1 and 0.5% Triton X-100. Repeated measurements one-way analysis of variance (ANOVA) with Holm-Sidak correction; n = 3. d, Absolute copy numbers of miRNAs in sEVs measured by standard curve RT-qPCR analysis in sEVs produced by three cell types (MDA-MB-231 (n = 3), MEF (n = 2) and NSC-34 (n = 3)). e, Enrichment of miRNAs in sEVs, expressed as the level of the respective miRNA in total sEV RNA relative to its level in total cell RNA as measured by RT-qPCR, normalized to miR-16. MEF (n = 2) and NSC-34 (n = 3). f, Enrichment of miR-451 in sEVs versus its level in cells in the listed cell types. n = 6 (mesenchymal stem cell (MSC) and macrophage) and n = 3 (all others). g, RT-qPCR to measure relative quantity of miR-451 in sEVs (NSC-34) left untreated, treated with RNase or treated with detergent and RNase. n = 2. h, Sucrose density gradient fractionation of sEVs produced by NSC-34 cells. Top: densities of fractions measured by refractometry (black line) and quantity of miR-451 (red dots, RT-qPCR, 2–16 without normalization) in these fractions. Western blot for Ago2 was performed on a separate membrane with the same fractions as western blots for Alix and flotillin 2. i, RT-qPCR analysis of combined fractions 6 and 7 from h either left untreated, treated with RNase A and RNase T1, or RNase A/T1 and 0.5% Triton X-100. Repeated measurements one-way analysis of variance (ANOVA) with Holm-Sidak correction; n = 3. d, Absolute copy numbers of miRNAs in sEVs measured by standard curve RT-qPCR analysis in sEVs produced by three cell types (MDA-MB-231 (n = 3), MEF (n = 2) and NSC-34 (n = 3)). e, Enrichment of miRNAs in sEVs, expressed as the level of the respective miRNA in total sEV RNA relative to its level in total cell RNA as measured by RT-qPCR, normalized to miR-16. MEF (n = 2) and NSC-34 (n = 3). f, Enrichment of miR-451 in sEVs versus its level in cells in the listed cell types. n = 6 (mesenchymal stem cell (MSC) and macrophage) and n = 3 (all others). g, RT-qPCR to measure relative quantity of miR-451 in sEVs (NSC-34) left untreated, treated with RNase or treated with detergent and RNase. n = 2. h, Sucrose density gradient fractionation of sEVs produced by NSC-34 cells. Top: densities of fractions measured by refractometry (black line) and quantity of miR-451 (red dots, RT-qPCR, 2–16 without normalization) in these fractions. Western blot for Ago2 was performed on a separate membrane with the same fractions as western blots for Alix and flotillin 2. i, RT-qPCR analysis of combined fractions 6 and 7 from h either left untreated, treated with RNase A and RNase T1, or RNase A/T1 and 0.5% Triton X-100. Repeated measurements one-way analysis of variance (ANOVA) with Holm-Sidak correction; n = 3.
Fig. 2 | Reprogramming the pre-miR-451 backbone with siRNAs causes their enrichment in sEVs. a, Schematic 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, RT–qPCR measuring enrichment of TetR (c) or GFP (d) siRNA in sEVs versus 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. Two-tailed unpaired t-test; n = 3.

b, RT–qPCR measuring enrichment of SOD1 siRNA in sEVs versus the level in cells when SOD1 siRNA was integrated in either the pre-miR-451 backbone or the pre-miR-16 backbone (control) and transiently expressed in MEF. Two-tailed unpaired t-test; n = 3.

e, RT–qPCR measuring enrichment of SOD1 siRNA in sEVs versus 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. Two-tailed unpaired t-test; n = 3.

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 sEVs after stable expression from the pre-miR-451 backbone in the indicated cell types. n = 3.

h, Northern blot of GFP siRNA or U6 (loading control) in WT, Ago2−/− or Ago2+/+ cells stably rescued with Ago2. Intermed., intermediate.

i, RT–qPCR analysis of GFP siRNA processed integrated in the pre-miR-451 backbone in fractions of density gradient in Fig. 1k using the 2−ΔΔCt method without normalization.

j, Western blot of Ago2 and tubulin (loading control) in WT, 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. Intermed., intermediate.

k, RT–qPCR of miR-16 and miR-451 (k) or SOD1 siRNA integrated in the pre-miR-451 hairpin structure (l) in sEVs versus cells, using Ago2−/− or Ago2+/+ cells stably rescued with Ago2. Enrichment in sEVs versus cells normalized to U6 (k) or miR-16 (l) RNA. n = 3.

m, RT–qPCR to measure fold change in enrichment in sEVs versus cells of miR-451 after transiently expressing GFP (control) or Ago2. Full blot images from b, g and k are presented in the Supplementary Information. One-way ANOVA with Holm–Sidak multiple comparison, unless otherwise mentioned. Data are mean ± s.e.m.
Fig. 3 | sEVs 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 sEVs produced by NSC-34 cells and loaded with the indicated siRNA integrated in the pre-miR-451 backbone. Scale bars, 100 μm. White arrows highlight co-localization of siRNA and target mRNA. b, Confocal microscopy of DDX6 (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 sEVs loaded with the indicated siRNA integrated in the pre-miR-451 backbone. Scale bars, 10 μm. White arrows highlight co-localization of siRNA and target mRNA in P bodies. c, RT–qPCR for GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with the indicated doses of sEVs produced by NSC-34 cells. n = 4 at 1,000 sEVs 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 sEVs 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 1,000 sEVs per cell. n = 3. f, RT–qPCR for GFP mRNA in primary mixed motor neuron cultures from GFP transgenic mice after incubation with sEVs produced by MEF cells produced by Ago2−/− or Ago2−/− cells stably rescued with Ago2. n = 3. g, h, i, RT–qPCR for GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with the indicated doses of sEVs produced by Neuro2a cells (g) or BV2 cells (i). Reference mRNAs were averaged GAPDH, GusB and TfrR. n = 2. h, i, Quantification of GFP fluorescence and GFP mRNA in primary mixed motor neuron cultures of GFP transgenic mice after incubation with the indicated doses of sEVs produced by Neuro2a cells (h) or BV2 cells (i). One-way ANOVA with Holm–Sidak multiple comparison. For microscopy analysis, fields of view containing approximately 100 cells were quantified (4 per slide, n = 4 slides, ~1,600 cells in total). Data are mean ± s.e.m.
the structure of the pre-miR-451 hairpin and not a specific siRNA sequence that is required for packaging into sEVs. Low levels of Ago2 are present in sEVs, even when purified by sucrose density gradient44 (Fig. 1a,b and Supplementary Figs. 1a,3e). This suggests that once packaged into sEVs, pre-miR-451 hairpins approximately 42 nt in length may be processed there by Ago2 into mature miRNAs in the hours to days between generation of sEVs vesicles in late endosomes, their release from cells and isolation42. In agreement, whereas in cells, mature forms of GFP-targeted siRNA of about 21 nt integrated into pre-miR-451 dominate (Fig. 2b), in sEVs, these RNAs are highly abundant at sizes corresponding to pre-miR-451 precursors (approximately 42 nt), intermediate processing products (24–34 nt) and mature siRNA (about 22 nt; Fig. 2h). This confirms, independently of RT–qPCR analysis, the enrichment in sEVs of pre-miR-451 derivatives in sEVs (Fig. 2m). Ago2 are present in sEVs, even when purified by sucrose density gradient34 (Fig. 1a,h and Supplementary Figs. 1a,3e). This suggests that precursor forms of pre-miR-451 structural mimics rather than siRNAs built into the pre-miR-451 structural mimic and suggests that precursor forms of pre-miR-451 structural mimics rather than mature forms are preferentially packaged into sEVs.

To test directly whether the precursor form of miR-451 is packaged into sEVs, we used Ago2−/− cells, which arrest processing of pre-miR-451 or its structural mimics at the 42 nt-hairpin stage24–30. Confirming this arrest, only the 42 nt form of GFP-targeting siRNA integrated in the pre-miR-451 hairpin was detected by northern blot in Ago2−/− cells and sEVs, whereas a mature approximately 21 nt form was detected in cells rescued with wild-type (WT) Ago2 (Fig. 2j and Supplementary Fig. 2g). This means that in Ago2−/− cells, only the approximately 42 nt precursor of pre-miR-451 structural mimics is available for packaging into sEVs. To determine the relative length of pre-miR-451 structural mimics in sEVs by an independent method, we used an RT–qPCR kit (miScript, Qiagen) that preferentially amplifies either the ~ 22 nt mature miRNA or 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, whereas the corresponding 21 nt mature siRNA was amplified more quickly with the specific buffer (Supplementary Fig. 3h). This enables the relative enrichment of pre-miR-451 precursors versus mature forms to be expressed as a ratio of RNA abundance measured by flexible versus specific buffers. In sEVs produced by WT cells, SOD1 siRNA produced from a pre-miR-451 structural mimic was amplified at similar cycle numbers with either the flexible or specific buffers (Supplementary Fig. 3i), suggesting that the SOD1 siRNA exists in WT sEVs as a mixture of mature siRNA and longer pre-miR-451 structural mimics, as demonstrated by northern blot data (Fig. 2h and Supplementary Fig. 3g). By contrast, sEVs produced by Ago2−/− cells were strongly enriched in long forms of pre-miR-451 structural mimics (Supplementary Fig. 3i), closely resembling amplification of the 42 nt synthetic version of pre-miR-451 structural mimics (Supplementary Fig. 3h). This reinforces northern blot data showing that Ago2−/− cells (Fig. 2j) only contain 42 nt versions of pre-miR-451 structural mimics for packaging into sEVs.

Of note, the relative enrichment in sEVs 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 WT cells did not significantly change the enrichment of pre-miR-451 derivatives in sEVs (Fig. 2m). Ago2 expression appears to slightly reduce packaging of pre-miR-451 derivatives into sEVs (Fig. 2j–m), although these effects were not
Fig. 5 | sEVs loaded with siRNA integrated in the pre-miR-451 backbone knock down target expression with lower doses than lipid nanoparticles or electroporated sEVs. a, b, RT-qPCR of GFP mRNA in liver (a) and small intestine (b) of GFP transgenic mice 3 d or 12 d after injection of 10^10 sEVs packaged with GFP siRNA or control siRNA. n = 4 mice per group at 3 d, n = 3 mice per group at 12 d. c, RT-qPCR of SOD1 mRNA in liver and small intestine of SOD1(G93A) mice 3 d after injection of InvivoFectamine lipid nanoparticles packaged with 1 mg kg\(^{-1}\) siRNA, 10 ng kg\(^{-1}\) siRNA or 10^10 sEVs packaged with 10–300 ng kg\(^{-1}\) of the identical SOD1 siRNA. n = 4 mice per group. LNP, lipid nanoparticles. d, e, RT-qPCR of GFP mRNA in liver 3 d after intravenous injection of C12–200 lipid nanoparticles or sEVs packaged with GFP siRNA at the indicated doses. n = 3 mice for control siRNA, n = 4 mice per group of sEVs or lipid nanoparticle treatments. e, RT-qPCR of GFP mRNA in liver of GFP transgenic mice 3 d after injection of sEVs electroporated with GFP siRNA using previously published conditions\(^{20}\). f, RT-qPCR of SOD1 mRNA in liver and small intestine of mice treated with sEVs packaged with control siRNA or sEVs left untreated, electroporated as described\(^{20}\), or treated with detergent. n = 3 or n = 2 experiments as shown. g, Size distribution of sEVs after electroporation measured by nanoparticle tracking analysis. n = 6 sEVs preparations. h, RT-qPCR of GFP mRNA in mixed motor neurons after incubation for 2 d with sEVs (10,000 per cell) left intact or disrupted with detergent. n = 5 untreated, n = 3 for intact sEVs and n = 2 for detergent-treated sEVs. i, j, RT-qPCR of SOD1 mRNA in liver (i) and small intestine (j) after treatment of mice with sEVs packaged with control siRNA or sEVs that were left untreated or electroporated after packaging with SOD1 siRNA using the pre-miR-451 hairpin. n = 4 mice per group. All sEVs were derived from NSC-34 cells. One-way ANOVA with Holm–Sidak multiple comparison. Data are mean ± s.e.m.

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 sEVs independent of Ago2. Once inside sEVs, low levels of Ago2 there (Fig. 1a,h and Supplementary Figs. 1a,3e) may process pre-miR-451 structural mimics into shorter versions. Cumulatively, this suggests that the 42nt pre-miR-451
Fig. 6 Quantitative FISH accurately measures mRNA in mouse tissues. a, e, Confocal microscopy of SOD1 siRNA (FISH) and SOD1 mRNA (FISH) versus GAPDH mRNA (normalization control) in sections of mouse liver and small intestine 3 d after injection of sEVs loaded with control siRNA or SOD1 siRNA integrated in the pre-miR-451 backbone. Scale bars, 50 μm. b, f, Quantification of SOD1 siRNA by FISH in liver and small intestine sections 3 d after intravenous injection of sEVs 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 intravenous injection of sEVs 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 3 d after intravenous injection of sEVs loaded with control siRNA or GFP siRNA integrated in the pre-miR-451 backbone. Two-tailed unpaired t-tests with Welch’s correction; n = 6 animals per experimental group. All sEVs were derived from NSC-34 cells. Two pieces of tissue per slide (duplicates) were analysed in two randomly selected areas (four in total per animal per tissue). n = 4 mice per group for RT–qPCR data. Data are mean ± s.e.m.
Fig. 7 | sEVs packaged with siRNA knock down target genes in specific regions and cell types of liver, small intestine and kidney. a–l, Experiments were performed on tissues of GFP transgenic mice 3 d after injection of $10^8$ sEVs (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 (a, scale bars, 50 μm); liver stained for GFAP, a marker for stellate cells (d, scale bars, 10 μm); macrophages (g, scale bars, 50 μm); or kidney stained for nephrin (white arrows), a podocyte marker (j, scale bars, 10 μm for control siRNA, 50 μm for GFP siRNA). b,c,e,f,h,i 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 3 d after injection of sEVs packaged with GFP siRNA or control siRNA using the pre-miR-451 backbone. n = 4. Two-tailed unpaired t-test with Welch’s correction; n = 6 animals per experimental group. Two pieces of tissue per slide (duplicates) were analysed in two randomly selected areas (four 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. All sEVs were derived from NSC-34 cells. Data for whole-liver and whole-small intestine knockdown contain results from three mice also used in Fig. 6, in addition to data from three more mice included only in Fig. 7. Data are mean ± s.e.m.

Structural mimics can account for all selective packaging into sEVs rather than a mature version of the miRNAs or siRNAs produced from it. At the same time, the data cannot exclude that approximately 20–24 nt forms of miR-451 or siRNA derived from pre-miR-451 structural mimics are also packaged into sEVs in WT 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 (Supplementary Fig. 3h). This suggests that the presented RT–qPCR analyses may underestimate the enrichment of pre-miR-451 structural mimics in sEVs (where longer forms with secondary structure are abundant) compared with their levels in cells where mature approximately 21 nt versions of these RNAs predominate. Select sEVs deliver siRNA to primary motor neurons

We sought to test whether sEVs loaded with siRNA reprogrammed into the pre-miR-451 backbone could efficiently deliver these siRNA.
sEVs 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 GFPTg transgenic mice. siRNA detected by fluorescence in situ hybridization (FISH) accumulated in motor neurons and co-localized with target GFP mRNA and a marker of P-bodies (DDX6, also known as RCK) (Fig. 3a,b). This suggests the siRNA packaged in sEVs 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 1,000 sEVs per cell (Fig. 3c). We used 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 1,000 sEVs 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 sEV-producing cells to express Ago2, demonstrating that siRNA in the pre-miR-451 hairpin can be packaged into sEVs equivalently in the absence of Ago2, and delivered into target cells in the precursor form (Fig. 3f). sEVs produced by a distinct neuronal cell line, Neuro2a, and loaded with GFP siRNA also reduced GFP mRNA expression (Fig. 3g,h, and Supplementary Fig. 4a), whereas vesicles produced by BV2 microglia cells did not (Fig. 3i,j and Supplementary Fig. 4b), despite both being packaged with GFP siRNA to the same level as sEVs produced by NSC-34 cells (1 copy per sEV-like vesicle; Fig. 2g). This demonstrates that the cell source of sEVs can determine whether siRNA delivery is effective. This suggests that sEVs deliver siRNA to targeted, specific cell types.

Multiple previous studies have together established that between 300 and 2,000 copies of a siRNA are required to elicit target knockdown in cells6–8. If sEVs at a dose of 1,000 siRNAs per cell cause target knockdown (Fig. 3c–e), then sEVs would appear to deliver a large proportion of their siRNA contents into the cytoplasm, compared with 0.05–1% of siRNA for lipid nanoparticles6,9. However, as previously reported36,37, recovery of spiked-in RNA of 21 nt was lower than 10% when small amounts of RNA were used, as in sEVs 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 sEVs. This suggests that estimates of siRNA and miRNA copy number in sEVs here (Figs. 1–3) and in the literature may be underestimated by 10- to 30-fold, and the delivery efficiency of sEVs may be correspondingly lower.

**SEVs deliver siRNA to multiple organs in mice**

We tested whether sEVs could deliver siRNA in mice. sEVs labelled with the far-red dye DiR and injected intravenously distributed broadly to many tissues including liver, kidneys, spleen, intestines and lungs (Fig. 4a). By contrast, DiR alone, subjected to the identical labelling method but without sEVs, resulted in negligible signals in mice (Fig. 4a). sEVs packaged with GFP-targeted siRNA at approximately 1–30 copies per sEV (Fig. 2g and Supplementary Fig. 4c) or control siRNA (10^9 sEVs) were injected intravenously into mice constitutively expressing GFP from a ubiquitous promoter. Despite the accumulation of sEVs 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^-1 (Fig. 4b). By contrast, in the small
Articles

**Nature Biomedical Engineering**

while sEVs at a siRNA dose 10- to 300-fold lower elicited similar knockdown induced by unmodified siRNA delivered by sEVs may persist for extended periods of time in tissues with slow rates of cellular turnover.

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

**Enhanced delivery efficiency compared with other vehicles**

Testing sEV delivery of siRNA on primary motor neurons suggested that sEVs may be highly efficient delivery vehicles. To test this in vivo, we compared sEVs packaged with siRNA using the pre-miR-451 backbone to lipid nanoparticles. As expected, In VivoFectamine lipid nanoparticles (ThermoFisher) at a high dose (1 mg kg\(^{-1}\)) of mature standard siRNA of the same sequence as in sEVs decreased target mRNA expression in the liver by >80% and by approximately 55% in small intestine (Fig. 5c). sEVs packaged with approximately 10–300 ng kg\(^{-1}\) siRNA elicited knockdown in liver and small intestine, whereas In VivoFectamine lipid nanoparticles with the same dose of siRNA had no effect on target mRNA levels (Fig. 5c). We compared sEVs with advanced C12–200 lipid nanoparticles capable of silencing targets in liver at doses of 0.003 mg kg\(^{-1}\) and 0.03 mg kg\(^{-1}\) (Fig. 5c). At 0.3 mg kg\(^{-1}\), C12–200 lipid nanoparticles elicited 60% silencing of GFP in liver at 3 d, while sEVs at a siRNA dose 10- to 300-fold lower elicited similar silencing in liver (Fig. 5d). This reinforces the notion from experiments on primary mixed motor neuron cultures that sEVs deliver siRNA at least tenfold more efficiently than lipid nanoparticles.

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

Electroporated cells often die due to excessive membrane permeabilization. Electroporation of sEVs rendered mir-106a and let-7a endogenously present inside sEVs sensitive to degradation by RNase A nearly to the same extent as sEVs permeabilized with detergents (Fig. 5f). Furthermore, analysis of electroporated sEVs by nanoparticle tracking demonstrated a significant loss of sEV-sized particles and an accumulation of smaller sized particles, consistent with sEV fragments, after electroporation (Fig. 5g), even if median particle size was unchanged (Supplementary Fig. 4d). This suggests that sEV membrane integrity is disrupted by electroporation and this could compromise the ability of sEVs to deliver siRNA. Confirming this, when the sEV membrane was disrupted by pre-treating sEVs with detergent their ability to deliver GFP siRNA into mixed motor neurons was abrogated (Fig. 5h). Similarly, electroporation of sEVs abolished their ability to deliver siRNA in mice. sEVs packaged with SOD1 siRNA using the pre-miR-451 hairpin reduced target expression in the liver and small intestine, but the same sEVs that had undergone electroporation did not (Fig. 5i).

This suggests that electroporation disrupts sEV membrane integrity and the capacity of sEVs to efficiently deliver siRNA.

**sEV source-dependent tissue- and cell-specific delivery**

Not all cell types produced sEVs capable of delivering siRNA to primary motor neurons (Fig. 3). sEVs may deliver cargos to specific cell types. To investigate siRNA delivery and target knockdown in specific tissue regions or cell types we utilized a 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 used 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 sEVs (Fig. 6c,d), loss of GFP mRNA and GFP fluorescence was 50% in villi and 80–85% in submucosa (Fig. 7a–c and Supplementary Fig. 5a,b). In liver, when total tissue GFP fluorescence and mRNA was decreased by 35–50% (Figs. 6g,h, 7d,e), GFP mRNA and GFP fluorescence in hepatocytes, Kupffer cells and macrophages (F4/80\(^{+}\)), and stellate cells (glial fibrillary acidic protein (GFAP\(^{+}\))) was reduced by 60–70%, 20% and 0%, respectively (Fig. 7d,f–i and Supplementary Fig. 5c–f). We also assessed siRNA target knockdown in specific regions of the kidney by FISH; at the whole-tissue level, only marginal target knockdown was observed (Fig. 7j,k). Notably, in Nephrin\(^{+}\) glomeruli, GFP mRNA and GFP fluorescence were reduced by approximately 50%, whereas knockdown outside these cells was not noticeable (Fig. 7j,k and Supplementary Fig. 5g). This demonstrates that sEVs exhibit delivery of siRNA to specific cell types and tissue regions in mice.

Of note, in many cell types, such as Kupffer cells, GFP siRNA delivered by sEVs was abundant; however no knockdown of GFP mRNA was observed. This suggests that in many cell types, sEVs and their cargos 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 sEVs, we selected a validated target expressed in a cell type to which delivery had been detected above. Patisiran (trade name Onpattro) was recently approved to treat patients with transthyretin amyloidosis by delivering siRNA targeting the transthyretin (TTR) gene, which is expressed predominantly in liver hepatocytes. siRNA targeting transthyretin was integrated into the pre-miR-451 backbone for packaging into sEVs. It would be preferable to treat patients with sEVs derived from human cells. sEVs 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\(^{-1}\) (Fig. 8a,b and Supplementary Fig. 6a,b). sEVs (10\(^{10}\)) from human fibroblasts containing an estimated dose of 10–300 ng kg\(^{-1}\) siRNA targeting transthyretin or a non-silencing siRNA were injected intravenously. This reduced levels of transthyretin protein in blood in a time course, reaching knockdown of more than 85% at 7 d (Fig. 8a,d).

Collectively, these data demonstrate that pre-miR-451 is selectively packaged into sEVs, and integrating siRNA into the hairpin structure of pre-miR-451 can induce their selective packaging into sEVs. By leaving sEVs intact, this strategy enables sEVs to deliver their siRNA cargos and knock down targets in specific cell types and tissues in mice with siRNA doses that are magnitudes lower than with lipid nanoparticles or other sEVs technologies.
Discussion

The data presented in this study uses four independent methods to demonstrate that pre-miR-451 and RNAs with unrelated sequences but the same secondary structure are robustly packaged into sEVs (Figs. 1, 2). Cleavage and maturation of pre-miR-451 is arrested in Ago2−/− cells28–30 at the stage of the 42 nt precursor as confirmed by northern blot and RT–qPCR (Fig. 2) and 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 sEVs (Fig. 2k–m). In addition, sEVs produced by WT and Ago2−/− cells similarly knocked down GFP siRNA targets in primary motor neurons (Fig. 3f). These two independent methods demonstrate that the 42 nt hairpin that structurally resembles pre-miR-451 can account entirely for enrichment in sEVs 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; GFP, SOD1 or TetR). Nonetheless, these RNAs are highly enriched in sEVs much like pre-miR-451 derivatives (Fig. 2). This also strongly suggests that the packaging of these RNAs into sEVs requires the shared structure of the pre-miR-451 hairpin and not mature siRNAs of approximately 21 nt, each with distinct sequences (Figs. 1 and 2).

Mature, approximately 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 about 42 nt and about 34 nt are abundant in sEVs along with the mature GFP siRNA (Fig. 2h). This also suggests that the approximately 42 nt hairpins are selectively packaged into sEVs. Why matured 21–34 nt forms of these RNAs are found in sEVs is less clear. It is possible that these matured forms are also sorted into sEVs in an independent process. An alternative explanation is also possible: sEVs collected from cell matured forms are also sorted into sEVs in an independent process.

Types of sequence motifs may also influence packaging of these RNA hairpins with the structure of pre-miR-451 into sEVs is the secondary hairpin structure, these sEVs differentiated by surface markers and size42–44. It is possible an increasing number of studies have described subpopulations of sEVs packaged inside them into recipient cells than contemporary engineered vehicles. This provides evidence that sEVs are highly efficient delivery contents including RNA into the cytoplasm whereas other subpopulations of vesicles are internalized and degraded by cells.

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

We found that electroporating sEVs ablated their ability to deliver siRNA and knockdown targets in mouse liver and small intestine. Similarly, according to previous studies and our data, sEVs electro- porated with siRNA or coated with siRNA required much higher doses of siRNA (ranging from 0.04 mg kg−1 (ref. 20) to 6 mg kg−1 (ref. 19)) to elicit target knockdown in mice compared with siRNA packaged inside sEVs endogenously using the pre-miR-451 hairpin (10–300 ng kg−1) or lipid nanoparticles. Adhering to standard models of sEVs, 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 sEVs is targeted to specific cell types. Biologically, this may help prevent widespread loss of cell identity and function.

Several studies have shown that sEVs contain RNA and that target cells exhibited signs of using siRNA, miRNA or mRNA from sEVs13,15–17. This has led to the emergence of the model that sEVs 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 deliver up to 1 in every 100 to 1,000 siRNAs that they contain45–47. In this context, when sEVs were used in previous studies to deliver siRNA, the siRNA doses required were much higher than that of lipid nanoparticles46–48–50, suggesting that sEVs loaded in these manners were relatively inefficient delivery vehicles49. This leaves open the possibility that sEVs may have no natural propensity to fuse with target cells and deliver RNA. Therefore, whether sEVs are actually efficient at delivering RNA into target cells has not been rigorously tested to our knowledge and many experimental effects observed with sEVs could be due to supraphysiological doses of sEVs or excessive amounts of siRNA loaded onto sEVs.

Using absolute quantification of siRNA and sEVs, we have tested the efficiency of sEVs in delivering siRNA into target cells both in vitro and in vivo. Previous quantitative analyses has shown that 300–2,000 copies of siRNA that catalytically cleave mRNA are required in the cytoplasm of each cell to silence target mRNAs45. Our results demonstrate that sEVs containing 1,000 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 sEVs is delivered into the cytoplasm. Supporting this claim, sEVs knock down targets in liver and intestine at siRNA doses at least tenfold lower than lipid nanoparticles or other best-in-class engineered delivery vehicles that only deliver about 1 in every 100 to 1,000 siRNA they contain into cells42–45. This suggests that sEVs are probably between 10- to 300-fold better at delivering siRNA that is packaged inside them into recipient cells than contemporary engineered vehicles.

This provides evidence that sEVs are exceptionally efficient in delivering contents including RNA into the cytoplasm whereas other subpopulations of vesicles are internalized and degraded by cells. Lipid nanoparticles contain much more siRNA per particle than most miRNA or siRNA in sEVs. Consequently, to inject similar amounts of siRNA requires injecting substantially larger numbers of sEVs than lipid nanoparticles. It is possible that this excess of sEVs (compared to lipid nanoparticles) saturates non-productive pathways of particle entrapment or uptake. Saturating such ‘sinks’ for particles with an excess of sEVs could theoretically contribute to their enhanced delivery efficacy.
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. Undiluted concentration and more dilute concentration based on concentration are reported. The acceptable measurement range was determined using serial dilutions of 110 nm polymerose beads of known concentration (5.0 x 10^3 particles per ml) (Microtrac, catalogue no. 400168). Supplementary Table 1 shows settings used for nanoparticle tracking analysis.

**Measurement of enrichment of miRNA and siRNA in sEVs relative to cells.** RNA was prepared from cells or their sEVs using Trizol reagent (ThermoFisher Scientific, catalogue no. 15596018) as described by the manufacturer, and using 5 μg of glycogen (ThermoFisher Scientific, catalogue no. R0551) as a carrier in the alcohol precipitation step. RNA was quantified by spectrophotometry and 250 ng were added to a reverse transcription reaction using the miScript II microRNA reverse transcription kit using the Flex buffer option (Qiagen, catalogue no. 218016). Reverse transcription was performed as described by the manufacturer using the miScript SYBR Green PCR kit (catalogue no. 218075, Qiagen). qPCR was performed using the miScript qPCR kit (Qiagen, catalogue no. 218076) on a Bio-Rad CFX-384 or Bio-Rad CFX-96 instrument using 12.5 μl or 25 μl reaction volumes, respectively. Primer sequences are included in the table below. Optimization of the reaction method (5 μl miRNA template) was performed such that reaction efficiency was between 95–105%. For all subsequent reactions Tm was 55°C except GFP siRNA reactions, where Tm was 58°C. Fold enrichment was measured using the ΔΔCt method comparing query miRNA levels in sEVs versus cells or miR-16 or U6 (reference) levels in sEVs versus cells. Please refer to Table 2 in Supplementary Information for primer sequences used for RT-qPCR.

**Measurement of copy number of miRNAs per sEV.** RNA was prepared from known numbers of sEVs (quantified by nanoparticle tracking) and reverse transcribed using the miScript system (Qiagen) as described above. qPCR was performed on sEV samples, simultaneously with qPCR reactions using positive product of known concentration as templates to generate a standard curve. Correlation of sample Ct values with standard curve Ct values enabled measurement of the number of molecules in the reverse transcription reaction. As the number of sEVs in the original sample was known, this allowed calculation of the number of molecules of specific miRNAs per sEV.

**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 β-actin enhancer and cytomegalovirus promoter46 (C57BL/6-Tg(CAG-EGFP)1Osb/J, The Jackson Laboratory, catalogue no. 007674) were also bred and males between the ages of 15 and 18 weeks (25–30 g) were used. Groups of mice were matched for littermates for intravenous injections. For intravenous injections mice were restrained (Braintree Scientific restrainer, catalogue no. NC069443) and injected in the lateral tail vein (U-100 insulin syringe 28G half inch needle, Beckton-Dickinson, catalogue no. 329424) with a 5 μl/kg (100–150 μl) suspension of sEVs (10^6–10^7 particles as specified) in PlasmaLyte A (Baxter, catalogue no. JR2544) after a 10x dilution in the PlasmaLyte A. Mice were euthanized with intraperitoneal sodium pentobarbital (120 mg/kg, Bimed-MTC, catalogue no. 8015E) and perfused with PBS (10 ml) before tissue collection. Those perfusions and tissue collection were blinded to the treatment.

**Cell lines.** The following cell lines were used: NSC-34 (CLU140, Cedara), MDA-MB-231 (ATCC), C83 (CRL-2353, ATCC), C8D1A (CRL-2541, ATCC), MN-1 (gift from J. Cote, University of Ottawa), Neuro2a (CCL-131, ATCC), 293T (CRL-3216, ATCC), MSC-TERT (Cedara), and Human neonatal dermal fibroblasts (Lonza, CC-2509). BV2 cells were a gift from D. Park (University of Ottawa). Bone marrow-derived macrophages were generated from femurs of WT C57/6 mice selected by adherence and matured in GM-CSF for one week. Cell lines were not authenticated. Cell lines were verified to be free of mycoplasma contamination by assessing for cytoplasmic 4,6-diamidino-2-phenylindole (DAPI) staining. All cell culture was performed at 37°C in 5% CO2 in humidified incubators.

**Mixed motor neurons extraction and culture.** Mice embryos were collected from pregnant mice between embryonic day (E)13.5 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 1 g/l and HEPES 2.4 g/l in 1x PBS), minced with scissors and incubated with trypsin (Sigma-Aldrich) for 30 min at 37°C. The tissue was minced using a 1 ml pipette and placed in N2feral neuron culture medium (MEM/HBSS, Hyclone, catalogue no. SH3002402), insulin 10 μg/ml (Sigma-Aldrich, 5493T) and human fibroblasts, which reached 90–100% confluence before collection. Measurement of copy number of miRNAs per sEV. RNA was prepared from Known numbers of sEVs (quantified by Nanoparticle tracking) and reverse transcribed using the miScript system (Qiagen) as described above. qPCR was performed on sEV samples, simultaneously with qPCR reactions using positive product of known concentration as templates to generate a standard curve. Correlation of sample Cc values with standard curve Cc values enabled measurement of the number of molecules in the reverse transcription reaction. As the number of sEVs in the original sample was known, this allowed calculation of the number of molecules of specific miRNAs per sEV.

**Methods**

**sEVs isolation.** All cells were grown to 70–80% confluence to produce sEVs, except human fibroblasts, which reached 90–100% confluence before collection. sEVs were collected from cells grown in DMEM (Wisent Bioproducts; catalogue no. 319–015-CL; 4.5 g/l glucose) with 10% sEV-depleted heat-inactivated FBS (Wisent Bioproducts, catalogue no. 080–150) prepared as described48, or alternatively cultured for 18–24 h in serum-free UltraCULTURE medium (catalogue no. 12–725F, Lonza). sEVs were produced using two methods: differential ultracentrifugation, or tangential flow filtration and ultracentrifugation.

For differential ultracentrifugation, sEVs containing media was spun at 300 g for 10 min and supernatants were subsequently centrifuged at 2000 g for 10 min, 10,000 g for 30 min (SW-32Ti rotor, Beckman-Couler Life Sciences; polycarbonate tubes, catalogue no. 355631, Beckman-Couler Life Sciences) and 200,000 g for 2 h (SW-32Ti rotor), sEV pellets were washed by resuspending in 1 ml 1x PBS (Wisent Bioproducts; catalogue no. 311–010-CL) and centrifuged at 100,000 g for 30 min (SW-32Ti rotor, Beckman-Coulter Life Sciences; polycarbonate tubes, catalogue no. 357448, Beckman-Couler Life Sciences).

HEK293T and human fibroblast sEVs were isolated using tangential flow filtration and ultracentrifugation. sEVs-conditioned media was spun at 300 g for 10 min and supernatants were subsequently spun at 2,000 g for 10 min. Supernatants were passed through a 0.22 μm filter (Thermo-Scientific, catalogue no. 09–741–04) and concentrated to a final volume of 15 ml using the CR2 tangential flow filtration system (Spectrum Labs) with a 75 cm² modified polyethersulfone hollow fibre column with 500 kDa cut-off (Spectrum, D02–ES00-10-5) at a flow rate of 140 ml/min and transmembrane pressure 2.5 psi to achieve a shear rate of 2,000 s⁻¹. The concentrated media underwent 10x buffer exchange in 1x PBS before being pelleted at 100,000 g for 30 min (TLA-100.3 rotor). All sEVs were resuspended in 50 μl 1x PBS and were quantified using nanoparticle tracking analysis on the ZetaView (ParticleMetrix).

**Nanoparticle tracking analysis.** Nanoparticle tracking analysis 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 no. 900383). sEVs samples were loaded in PBS into the range determined by the instrument to be accurate for measurement (typically 1:10,000–1:500,000). One millilitre of sample was injected into the machine and allowed to equilibrate until it reached an acceptable

of lipid membrane fusion in cell biology, this suggests that sEVs must remain intact to fuse with target cells and deliver their cargoes. As previously reported41, results here demonstrate that miRNA are present at low copy number in sEVs. Accounting for RNA recovery from standard sEV samples (Supplementary Fig. 4c), miRNAs, or siRNA packaged into sEVs with the pre-miR-451 backbone may be present in sEVs at up to 30–300 copies per sEVs (Fig. 1d and Supplementary Fig. 3g). Many other miRNAs are detectable in sEVs, but they occur at 100–10,000-fold lower copy number per sEV. 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 sEVs could feasibly have biological effects in vivo. Expression or inhibition of miRNAs in sEV-producing cells will have a wide spectrum of effects on lipids, proteins and small molecules that are released in sEVs with miRNA, and in some cases may account for effects observed in recipient cells independent of the delivery of miRNA by sEVs. Measuring absolute quantities of miRNAs in sEVs rather than relative levels can establish the plausibility that observed effects are due to miRNA in sEVs.

The current results demonstrate that sEVs are highly efficient delivery vehicles, providing evidence to support previous speculation. This suggests that harnessing sEVs to deliver siRNA may enable siRNA doses in patients to be reduced by orders of magnitude. To bring this possibility to practical application will require a method to robustly package siRNA inside sEVs 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 sEVs and thereby reduce volumes required to manufacture sEV delivery vehicles by 100- to 1000-fold. This suggests that the pre-miR-451 backbone may be the foundation for a method for the manufacture and delivery of therapeutic siRNA.**
Articles

**FISH.** Tissues were collected from mice and placed in 4% PFA in 1× PBS for 24 h. PFA was replaced by 1× PBS with 30% sucrose until the tissues sank to the bottom of tubes. Tissues were then placed in optimal cutting temperature solution (OCT, 23-730-71, 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 before staining. Sections were washed with 1× PBS and incubated in 10% normal goat serum containing 1% Triton X-100 for 1 h. They were then washed with 1× PBS and placed at 37°C for 20 min in permeabilization buffer (10% methanol, 0.2% Triton X-100). Slides were returned to room temperature, washed in PBS and blocked for 1 h with 1% BSA, 100 µg/ml salmon sperm DNA and 250 µg/ml yeast extract RNA in PBS. Slides were washed with 1× PBS and incubated with the secondary antibody, Alexa Fluor 488, for 1 h. Slides were washed with Stellaris wash A buffer (LGC Biosearch Technologies, catalogue no. SMF-WA1-60) and incubated with Stellaris fluorescent miRNA probes (LGC Biosearch Technology, SOD1 (custom assay), GFP, (VSMF-1014-5), GAPDH (SMF-3002-1, β-actin) and DIG-coupled siRNA probes (SOD1 siRNA, GFP siRNA and negative control siRNA; Integrated DNA Technologies) in hybridization buffer (10% Stellaris hybridization buffer, SMF-HB1-10, 10% formamide). Slides were probed with the dots 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 no. ENZ-ABS266-0100) diluted 1:100 in blocking solution for 1 h. Slides were washed with wash B and blocks overnight. Staining was performed with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies, catalogue no. A-11034) diluted 1:500 in blocking solution for 1 h. Slides were washed with wash A buffer and mounted with Vectorshield (Vector Laboratories). Stained sections were imaged on a Zeiss Axiovert 200M microscope using a 63 Plan-Apochromat 1.4 Oil lens. Images were acquired by confocal microscopy (Zeiss LSM880). Images were then analysed per slide and two slides from two independent wells were analysed in each of two independent experiments (n=1,000 cells in total).

**RNA sequencing.** Total RNA was purified from pure motor neurons using an RNeasy Plus Mini Kit (Qiagen). Each sample was reverse-transcribed to cDNA using SuperScript III Reverse Transcriptase (Life Technologies). qPCR was performed to select a pool of optimal RNA samples. Two biological replicates were run for each sample and each qPCR assay was performed in technical triplicates. The expression of motor neuron genes was assessed after normalisation to the housekeeping genes (Actb, Gapdh and Tfb1m) and Zosterea nigricans (Znt1) via the comparative Ct method (GraphPad Prism (GraphPad Software)). The results showed comparable expression levels for all genes tested, with no genes showing a fold change greater than 0.5 or less than 2. The expression of motor neuron genes in the different groups was compared using a two-way ANOVA with Tukey’s multiple comparisons test.

**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 Tri-reagent isolation protocol. Reverse transcription was conducted with the M-MuLV enzyme (NEB, catalogue no. M0253S) and 18S oligotd primer (Integrated DNA Technologies) as described above.

**Image analysis.** ZEN 2.3 analysis software (Carl Zeiss, Oberkochen, Germany) was used for FISH image analysis. Briefly, images were acquired using confocal microscopy (Zeiss LSM880 AxioObserver Z1). Images were then analysed per slide and two slides from two independent wells were analysed in each of two independent experiments (n=1,000 cells in total).
proteinase K or RNase A treatments were first treated with 10 μg DNase I (Roche, catalogue no. 1010415901) for 10 min at 37 °C. Reactions that received detergent were then treated with 0.5% SDS or Triton X-100. Reactions that received DNase I, proteinase K and RNase A treatments were then treated with 10 μg RNase A for 10 min at 37 °C. All reactions were then treated with PMSF at a final concentration of 5 mM for 10 min at room temperature. Reactions that received DNase I, proteinase K and RNase A treatments were run on an agarose gel and stained with ethidium bromide to visualize the equal volume of phenol:chloroform:isoamyl alcohol and the aqueous portion was run on an agarose gel and stained with ethidium bromide to visualize the apical DNA. Identical methods were used for analysis of ultracentrifuged siRNAs by RT-qPCR. For western blot analysis of ultracentrifuged siRNAs in Supplementary Fig. 3f, 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 no. 880-545-CL) and electroblotted (Turboblot, Bio-Rad) onto positively charged nylon membranes (GE Healthcare, catalogue no. 1417240) also in 0.5x TBE. RNAs were crosslinked to membranes by UV using a Stratallinker delivering 240 mJ to each side of the membrane. Membranes were prehybridized for 1 h in 10x PerfectHyb buffer (Sigma-Aldrich, catalogue no. H7003) at 40 °C and hybridized with labelled probe overnight under the same conditions. Membranes were washed twice in 5x SSC + 0.1% SDS and once in 1x SSC + 0.1% SDS before exposure to X-ray film (GE Healthcare). Screens were imaged using a Typhoon Trio machine (GE Healthcare). Custom probe oligonucleotides (Integrated DNA Technologies) were labelled as follows: 18 pmol of oligonucleotide was labelled with 30 μCi of [γ-32P]-ATP (PerkinElmer, catalogue no. BLU002A500UC) using T4 PNK (NEW, catalogue no. M0201S) as recommended by the manufacturer. Labelled probes were denatured heating the matured nucleotides to 95 °C and cooled to 4 °C. Oligonucleotide probe sequences were as follows: GFP siRNA probe 5′-GCAAGGCTGACCCTGAAAGUCA and U6 probe 5′-AGGCCCCGATCATCTCTTCT.

**Western blot.** Blood (20–50 μl) was collected from the tail vein at different time points in heparinized tubes (Greiner Bio-One, 450477). Whole blood was separated by centrifugation at 3,000 g for 15 min. An equal amount of serum (5 μl) was collected and diluted in water and 4x Laemmli sample buffer (Bio-Rad, 161-0747) with 10% β-mercaptoethanol (Bio-Rad, 1610710) and boiled for 5 min at 99 °C. Samples were separated by SDS-PAGE (10% acrylamide). Membranes were transferred to nylon membrane (Roche, 1417240) for 1 h at 100 V. Membranes were washed with 1x TBS (3.5 min) and blocked with 5% milk in 1x TBS + 5% milk for 1h. Membranes were incubated overnight at 4 °C with primary antibody (rabbit anti-mouse TTR, Invitrogen, PA5-80197). Membranes were washed with TBS (3.5x5 min) and incubated for 1 h with secondary antibody (Li-Cor Odyssey, goat anti-rabbit IRDye 800 (TTR), 926-32211, donkey anti-mouse IRDye 680 (IgG, Santa Cruz, sc-2020) or Dredoham peroxidase (Dako, 1050), the latter was visualized with 100 μl of 2 nM ssRNA and RNA was prepared according to the manufacturer’s instructions, including the addition of 10 μg glycogen (RNA grade, ThermoFisher Scientific, catalogue no. R0551) to the isolated aqueous phase to aid RNA precipitation. After alcohol precipitation RNA was resuspended in 50 μl RNAse-free water. Five microlitres of the 2 μl ssRNA was similarly diluted to serve as the input sample. Recovery of the ssRNA versus SOD1 was quantified using absolute copy numbers with a standard curve as above.

**Sucrose density gradient.** sEVs produced from NSC-34 cells transduced with pre-miR-451 hairpin vectors were isolated by differential ultracentrifugation. sEVs pellets were resuspended in 1.85 ml of 0.95 μM sucrose solution. The 0.95 μM sucrose solution containing sEVs was inserted in a sucrose step gradient composed of six 1.85 ml layers with 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 200,000g for 20 h at 4 °C. Twelve fractions of 0.925 ml were collected and their density was measured with a refractometer (No. 10646 ARMA). About 250–500 μl of fractions was diluted to 30 ml in cold 1x PBS and centrifuged at 100,000g at 4 °C for 70 min. The pellets were resuspended in 25 μl of cold 1x PBS and used for western blotting.

**Lipid nanoparticles.** In some experiments, lipid nanoparticles were produced using InvivoFectamid 3.0 (ThermoFisher, catalogue no. IV3F001) loaded with synthetic siRNA versus SOD1 (Integrated DNA Technologies) 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 to that produced by the SOD1 siRNA integrated into the pre-miR-451 hairpin above. In other experiments C12–20 lipid
nanoparticles were prepared and loaded with siRNA targeting GFP (Qiagen, GFP-22 siRNA positive control). The targeting sequence of GFP-22 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 described1.

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

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The main data supporting the results of this study are available within the paper and its Supplementary Information. The raw and analysed datasets are too numerous to be readily shared publicly, but can be obtained for research purposes from the corresponding author on reasonable request.

Received: 22 January 2018; Accepted: 4 December 2019; Published online: 14 January 2020

References
1. Ha, M. & Kim, V. N. Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. 15, 509–524 (2014).
2. Fitzgerald, K. et al. A highly durable RNAi therapeutic inhibitor of PCSK9. N. Engl. J. Med. 376, 41–51 (2017).
3. Coelho, T. et al. Safety and efficacy of RNAi therapy for transthyretin amyloidosis. N. Engl. J. Med. 369, 819–829 (2013).
4. Whitehead, K., Langer, R. & Anderson, D. G. Knocking down barriers: Delivery materials for in vivo gene silencing. Nat. Rev. Mol. Cell Biol. 7, 58–66 (2006).
5. Kanasty, R., Dorkin, J. R., Vegas, A. & Anderson, D. Delivery materials for systemic RNA interference. Nat. Biotechnol. 25, 129–138 (2007).
6. Fitzgerald, K. et al. A highly durable RNAi therapeutic inhibitor of PCSK9. N. Engl. J. Med. 376, 41–51 (2017).
7. Kanasty, R., Dorkin, J. R., Vegas, A. & Anderson, D. Delivery materials for siRNA therapeutics. Nat. Mater. 12, 967–977 (2013).
8. Wittrup, A. et al. Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. Nat. Biotechnol. 33, 1–9 (2015).
9. Pei, Y. et al. Quantitative evaluation of siRNA delivery in vivo. RNA 16, 2533–2563 (2010).
10. Veldhoen, S., Laufer, S. D., Trampe, A. & Restle, T. Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect. Nucleic Acids Res. 34, 6561–6573 (2006).
11. Gilleron, J. et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. Nat. Biotechnol. 31, 638–646 (2013).
12. Garber, K. Amaryl terminates revusiran program, stock plunges. J. Virol. Methods 20, 332–343 (2018).
13. Colombo, M., Raposo, G. & Théry, C. Biogenesis, secretion, and intercellular transfer of exosomes. Nat. Rev. Mol. Cell Biol. 6, 153–164 (2005).
14. Bartlett, D. W. & Davis, M. E. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. Nucleic Acids Res. 34, 322–333 (2006).
15. Foust, K. D. et al. Therapeutic AAV9-mediated suppression of mutant SOD1 protein 1 is required to sort microRNAs into exosomes in cells and in a cell-free reaction. eLife 5, e19276 (2016).
16. Chevillet, J. R. et al. Reversible Holt–Howard microRNA binding controls extracellular export of miR-122 and augments stress response. EMBO Rep. 17, 1184–1203 (2016).
17. Villarroya-Beltri, C. et al. Sumoylated hnRNA/PAB1 controls the sorting of miRNAs into exosomes through binding to specific motifs. Nat. Commun. 4, 2323 (2013).
18. Yang, J. S. & Lai, E. C. Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. Mol. Cell 43, 892–903 (2011).
19. Clefentines, D. et al. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. Science 328, 1694–1698 (2010).
20. Shelke, G. V., Lasser, C., Gho, Y. S. & Lottl, D. Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. J. Extracell. Vesicles 3, 24735 (2014).
21. Valadi, H. et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of transfer from cell to cell. FEBS Lett. 503, 165 (2002).
22. Taylor, S. C., Carbonneau, J., Shelton, D. N. & Bolvin, G. Optimization of droplet digital PCR from RNA and DNA extracts with direct comparison to RT–qPCR: clinical implications for quantification of oseltamivir-resistant subpopulations. J. Virol. Methods 224, 58–66 (2015).
Acknowledgements
The authors acknowledge E. Lai (Memorial Sloan-Kettering Cancer Center) for providing mouse embryonic fibroblast cell lines (WT, Ago2−/− and Ago2−/− rescued with Ago2). R.R. was funded in part by a scholarship in translational research from the Centre for Neuromuscular Disease and the University of Ottawa Brain and Mind Research Institute. This research was funded by grants from the Canadian Institutes of Health Research (proof of principle grant, PPP-141720), the National Research and Engineering Council of Canada (discovery grant no. 436104), The Quebec Consortium for Drug Discovery (CQDM Explore grant) and the ALS Association Treat Program (grant no. 15-LGCA-290) awarded to D.G.

Author contributions
J.A.T. performed cloning, lentivirus production, northern blots, analysis of RNA enrichment in sEVs and absolute quantification of RNA in sEVs. A.S. maintained mouse colonies, performed injections and tissue collections, helped analyse sEVs distribution, performed western blots of sEVs, generated cultures of primary mixed motor neurons, performed some RT–qPCR and performed and analysed microscopy. R.R. produced sEVs, performed nanoparticle tracking analysis, performed tissue collections, labelled sEVs and analysed their distribution, and analysed RNA enrichment in sEVs and mRNA target knockdown by RT–qPCR. M.T.T. maintained mouse colonies, generated mouse protocols, genotyped mice and performed injections and tissue collections. C.C. helped establish protocol for primary mixed motor neuron culture and performed some analyses of miRNA levels in sEVs. H.G. performed western blots of sEVs and density gradient analyses of sEVs. L.H.R. and P.S.K. helped design lipid nanoparticle experiments and produced C12–200 lipid nanoparticles. D.G.A. helped design lipid nanoparticle experiments and supervised L.H.R. and P.S.K. W.L. helped design experiments. J.A.T., A.S. and R.R. analysed experiments and helped design experiments. D.G. conceived the project, designed experiments and wrote the manuscript.

Competing interests
J.A.T. and D.G. are inventors on a filed patent that claims the use of the pre-miR-451 backbone for enrichment of small RNAs in sEVs. The remaining authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41551-019-0502-4.

Correspondence and requests for materials should be addressed to D.G.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2020
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | RT-qPCR: Bio-Rad CFX Maestro 1.1 version 4.1.2433.1219; ddPCR: Bio-Rad Quantasoft version 1.7.4; Nanoparticle tracking analysis: ParticleMetrix Zetaveiw software version 8.02.31. |
|-----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

| Data analysis   | Statistics: GraphPad Prism 7 version 7.02; Microscopy: Zeiss Zen 2.3 software.                                                                                      |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The main data supporting the results of this study are available within the paper and its Supplementary Information. The raw and analysed datasets are too numerous to be readily shared publicly, yet can be obtained for research purposes from the corresponding author on reasonable request.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences  
- [ ] Behavioural & social sciences  
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

- **Sample size**  
  Sample size was determined on the basis of anticipated variability between measures.

- **Data exclusions**  
  Samples with failed RNA extractions (RNA quantity <20% of average) were excluded prior to RT-qPCR analyses.

- **Replication**  
  All attempts at data replication were successful. Experiments were replicated with independent samples, as indicated in the figure legends.

- **Randomization**  
  Mice of only one sex were used, and were randomly assigned among groups while ensuring balance in age and littermates.

- **Blinding**  
  In experiments with mice, the experimenters were blinded to the sample being injected until the quantifications were complete.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | Antibodies            |
| [ ] | Eukaryotic cell lines |
| [ ] | Palaeontology         |
| [ ] | Animals and other organisms |
| [ ] | Human research participants |
| [ ] | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | ChIP-seq              |
| [ ] | Flow cytometry        |
| [ ] | MRI-based neuroimaging |

**Antibodies**

| Antibodies used |
|-----------------|
| Alzheimer (clone) Company Catalog # |
| Alix (3A9) Cell Signaling Technology 21715 |
| Ago2 (C34C6) Cell Signaling Technology 28975 |
| B-Actin (AC-15) Sigma-Aldrich A5441 |
| Flotillin2 (C42A3) Cell Signaling Technology 34365 |
| GFP (GF28R) eBiosciences 14-6674-82 |
| Tomm20 (FL-145) Santa-Cruz Biotechnology sc-11415 |
| TSG101 (4A10) GeneTex GTX70255 |
| TTR (Polyclonal) ThermoFisher PA5-80197 |
| Tubulin (DM1A) Sigma-Aldrich T6199 |
| Anti-Mouse HRP Jackson ImmunoResearch 115-035-174 |
| Ant-Rabbit HRP Jackson ImmunoResearch 111-035-144 |

**Validation**

Antibodies were used with species recommended by the manufacturers. The specificity of antibodies was confirmed by the detection of bands at the appropriate mass, enrichment in exosomes vs. cells (Alix, Flotillin2, Tsg101) and blotting in cells/tissues lacking the antigen (Ago2, GFP).

**Eukaryotic cell lines**

**Policy information about** [cell lines](#)

**Cell line source(s)**

- NSC-34 (Cedarlane), MDA-MB-231 (ATCC), C8S (ATCC), C8D1A (ATCC), MN-1, Neuro2a (ATCC), MSC-TERT (Cedarlane). BV2 cells were a gift of Dr. David Park (University of Ottawa).
Authentication

None of the cell lines used were authenticated.

Mycoplasma contamination

The cell lines were tested for mycoplasma by staining with DAPI and via assessment for cytoplasmic DNA by microscopy. The cell lines used in this study were negative for mycoplasma contamination.

Commonly misidentified lines

(See ICLAC register)

No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

GFP transgenic mice (Jackson Labs, catalogue number 003291, CS7BL/6-Tg(CAG-EGFP)1Osb/J). All GFP mice used were female. SOD1 G93A transgenic mice (low-copy number, Jackson Labs, catalogue number 002299, B6.Cg-Tg(SOD1*G93A)dl1Gur/J). All SOD1 mice used were male.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.

Ethics oversight

Ethical approval for experimentation with mice was obtained from 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.

Note that full information on the approval of the study protocol must also be provided in the manuscript.