Published in final edited form as:

Nat Genet. ; 43(4): 371–378. doi:10.1038/ng.786.

Silencing of microRNA families by seed-targeting tiny LNAs

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Abstract

The challenge of understanding the widespread biological roles of animal microRNAs (miRNAs) has prompted the development of genetic and functional genomics technologies for miRNA loss-of-function studies. However, tools for exploring the functions of entire miRNA families are still limited. We developed a method that enables antagonism of miRNA function using seed-targeting 8-mer locked nucleic acid (LNA) oligonucleotides, termed tiny LNAs. Transfection of tiny LNAs into cells resulted in simultaneous inhibition of miRNAs within families sharing the same seed with concomitant upregulation of direct targets. In addition, systemically delivered, unconjugated tiny LNAs showed uptake in many normal tissues and in breast tumors in mice, coinciding with long-term miRNA silencing. Transcriptional and proteomic profiling suggested that tiny LNAs have negligible off-target effects, not significantly altering the output from mRNAs with perfect tiny LNA complementary sites. Considered together, these data support the utility of tiny LNAs in elucidating the functions of miRNA families *in vivo*.

miRNAs are ~22-nucleotide non-coding RNAs that regulate gene expression post-transcriptionally by mediating translational repression or promoting degradation of their target mRNAs^{1,2}. Animal miRNAs play critical roles in the control of diverse biological processes, including apoptosis, differentiation, cell proliferation and metabolism³. Furthermore, miRNA perturbations are prevalent in and are strongly associated with the development of human diseases, such as viral infections, cancer, cardiovascular and muscle diseases^{4–6}. Most animal miRNAs bind to partially complementary sites located predominantly in the 3' untranslated regions (UTRs) through Watson-Crick pairing of the miRNA seed region to the seed match site in the target mRNA². Prediction of targets by

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Accession codes. The microarray data are deposited in the public data repository ArrayExpress under the following accession codes: E-MEXP-2801 (antilet-7 mediated silencing of let-7 in the mouse liver), E-MEXP-2802 (miR-122 silencing in the mouse liver), E-MEXP-2803, (miR-21 inhibition in HeLa cells) and E-MEXP-2804 (let-7 inhibition in PC3 cells).

Note: Supplementary information is available on the Nature Genetics website.

AUTHOR CONTRIBUTIONS

S.O., C.O.d.S., A.P., M.H., O.B., C.R., C.F. and E.M.S. performed experiments and contributed data. H.F.H. performed synthesis of oligonucleotides. S.O., C.O.d.S., A.P., M.H., M.L., J.S., T.K., D.P., G.J.H. and S.K. designed experiments and discussed the data. S.K. supervised the study and wrote the manuscript together with S.O. and with input from other authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturegenetics/>.

identifying conserved seed sites in mammalian 3' UTRs or by analysis of transcriptional profiles and proteomic data supports the notion that miRNAs may regulate a large fraction of all protein-coding genes^{7–9}.

The challenges of deciphering the widespread roles of animal miRNAs demand the development of robust technologies that enable the determination of the biological functions of individual miRNAs and miRNA families *in vivo*. The expanding inventory of loss-of-function studies using miRNA gene knockouts has begun to shed light on the functions of animal miRNAs^{10–16}. However, generation of genetic knockouts to study miRNA families is either difficult or nearly insurmountable, depending upon the complexity of the family. The use of highly expressed transgenes with multiple miRNA binding sites, termed miRNA sponges, offers an alternative approach, allowing sequestration of entire miRNA families in cultured cells¹⁷. Although miRNA sponges were recently applied to deplete individual miRNAs in transgenic flies¹⁸ and in transplanted hematopoietic cells in mice¹⁹, the utility of this tool to explore miRNA function *in vivo* in higher organisms has not yet been assessed.

An alternative approach to miRNA gene knockouts and sponges is to use chemically modified antisense oligonucleotides, termed antimiRs. These act as competitive inhibitors of miRNAs, negating their ability to interact with and repress cellular target mRNAs. A variety of nucleotide modifications, including LNA, 2'-O-methyl (2'-O-Me) and 2'-O-methoxyethyl (2'-MOE) have been used to enhance the binding affinity of antimiRs to their cognate miRNAs. Additionally, phosphorothioate backbone modifications provide high biostability and facilitate delivery of antimiRs to the liver^{20–22}. The use of 3' cholesterol-conjugated, 2'-O-Me oligonucleotides with a partial phosphorothioate backbone represents another approach to silence miRNAs *in vivo*²³. Successful inhibition of individual miRNAs using antimiR oligonucleotides has been described *in vitro* and in *Caenorhabditis elegans*, *Drosophila*, mice, African green monkeys and hepatitis C virus-infected chimpanzees^{20–29}. However, no study thus far has addressed the functions of co-expressed miRNA family members, which requires a strategy able to simultaneously inhibit the activity of entire miRNA families.

Here we describe an approach that enables miRNA antagonism using fully LNA-modified phosphorothioate oligonucleotides, termed tiny LNAs, complementary to the miRNA seed region. We demonstrate the utility of unconjugated tiny LNAs in inhibiting single miRNAs and entire miRNA families in cultured cells in several tissues of adult mice and in a mouse breast tumor model *in vivo*. Our study validates the use of tiny LNA-based knockdown in exploring miRNA function, with important implications for the development of therapeutics targeting disease-associated miRNAs.

ONLINE METHODS

Oligonucleotides

The LNA oligonucleotides were synthesized with a phosphorothioate backbone, and their duplex melting temperatures were determined as described²¹ (Table 1 and Supplementary Table 1).

Cell culture and luciferase reporter assays

Cells were cultured as described in the Supplementary Note. The miRNA reporters were generated by cloning annealed 5' phosphorylated oligonucleotides corresponding to single perfect-match target sites for mouse miR-155 and human miR-21, miR-17, miR-18, miR-19, miR-221 and miR-222 into the 3' UTR of the *Renilla* luciferase gene in the dual-luciferase psiCHECK2 plasmid (Promega) (*XhoI/NotI* sites) (Supplementary Table 4). The 3' UTRs of AldoA and HMGA2 genes were cloned into the 3' UTR of the *Renilla* luciferase gene

(*XhoI/NotI* sites) in the dual-luciferase psiCHECK2 plasmid (Promega) using the PCR primers described in Supplementary Table 5. Transfections and luciferase activity measurements were carried out according to the manufacturer's instructions (Invitrogen Lipofectamine-2000 and Promega Dual-luciferase kits). The pre-miRs were from Ambion. For *in vivo* studies, a 1.2-kb PCR fragment corresponding to the Firefly luciferase gene was amplified from pGL3 vector (Promega) and subsequently cloned into *BglIII* and *XhoI* sites of a modified MSCV vector (Clontech), where the hygromycin resistance gene was replaced by the RFP gene. For luciferase control primers and miR-21 luciferase reporter primers, see Supplementary Table 5. The 4T1 cells were infected with virus supernatant and RFP-positive clones were sorted by flow cytometry.

Immunoprecipitation of Ago2 and detection of anti-miR-21 in the Ago2 complex

HeLa cells were transfected with 50 nM anti-miR-21 and grown for 48 h. The Ago2 protein complex was immunoprecipitated as described⁴⁷. The Ago2-bound RNA was isolated, electrophoresed in 10% denaturing polyacrylamide gel, transferred to Hybond NX membrane (Amersham) at 20 V for 45 min and cross-linked with EDC reagent at 50–60 °C for 2 h. The LNA-modified oligonucleotide probe (10 pmol) complementary to the 8-mer anti-miR-21 was end-labeled at 37 °C for 60 min using T4 polynucleotide kinase (NEB) and γ [³²P]ATP (Amersham) and purified on a Quick Spin Column (Roche) according to the manufacturer's instructions. The cross-linked membrane was pre-hybridized for 30 min at 35 °C in hybridization buffer followed by addition of pre-heated probe (1 min at 95 °C) and hybridization for 2 h at 50 °C. Membranes were washed twice in 2×SSC, 0.1% SDS at 50 °C for 10 min and scanned using a Storm 860 scanner (Molecular Dynamics).

Subcellular localization of anti-miR-21 in HEK293 cells

HEK293 cells stably expressing the FLAG-tagged Ago2 protein were incubated with 5 μ M FAM-labeled anti-miR-21 for 72 h followed by staining as described⁴⁸ using the following antibodies: FLAG antibody (M2, Sigma-Aldrich) or IgG antibody of the same isotype at 1:500 and Alexa 594-labeled goat mouse antibody (Molecular Probes) at 1:500. The cells were mounted using VECTASHIELD containing DAPI and analyzed using a 60× oil immersion objective on an Olympus IX81 confocal microscope with the merge image compiled using the ImageJ program.

RNA isolation and real-time quantitative RT-PCR

RNA was isolated from cells and mouse tissues using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). mRNA quantification was done using TaqMan assays and an Applied Biosystems 7500 real-time PCR instrument (Applied Biosystems).

RNA blot analysis

Total RNAs (15–20 μ g per sample) were electrophoresed in 20% TBE acrylamide gels (Invitrogen) using Hi-Density TBE sample buffer (Invitrogen) without pre-heating, transferred to GeneScreen Plus Membranes (PerkinElmer) and hybridized at 45 °C with dual FAM-labeled LNA probes (Exiqon) as described²¹. Synthetic miR-21 or miR-122 oligonucleotides and preannealed miR-21– and miR122–anti-miR heteroduplexes were used as controls. The blots were scanned using a Storm 860 scanner (Molecular Dynamics).

Protein blot analysis

Proteins were extracted from cultured cells and mouse tissues and subjected to protein blot analysis as described²⁵. The primary antibodies (Supplementary Note) were used according to the manufacturer's instructions.

Soft agar assays

PC3 (2.5×10^3) or HepG2 cells (2.0×10^3) were seeded on 0.35% soft agar plates 24 h after transfection with anti-miR-21, incubated at 37 °C for 12 days and stained with 0.005% crystal violet, followed by colony counting.

In vivo experiments

Female NMRI mice (Taconic) were dosed intravenously for three consecutive days with saline-formulated (0.9% NaCl) anti-miR-21, anti-let-7 or LNA scramble control compound, receiving daily doses of 10 mg/kg and were then killed 48 h after the last dose. For the anti-miR-21 time-course study, NMRI mice were dosed with a single intravenous injection of 25 mg/kg and killed at different time points. For miR-122 antagonism, female C57BL/6 mice (Taconic) received three intravenous doses of 5 mg/kg or 20 mg/kg of saline-formulated tiny or 15-mer anti-miR-122 over 7 days and were killed at day 7.

The 4T1 cells (5×10^4) expressing either Luc control or Luc-21 were injected into the fourth and/or ninth mammary gland of 8–10-week-old Balb/C female mice under isoflurane anaesthesia. Tumor-bearing mice were treated with three daily doses of 10 mg/kg anti-miR-21 or LNA scramble control starting at day 10 following transplantation. For *in vivo* imaging of luciferase activity, mice were injected intraperitoneally with 150 mg/kg D-Luciferin (Caliper LifeSciences), anaesthetized with isoflurane and imaged 10 min post luciferin injection. *In vivo* luciferase activity was measured using an IVIS100 imaging system (Caliper LifeSciences).

Biodistribution

The anti-miR-21 oligonucleotide was ^{35}S -labeled as described⁴⁹. Nine female C57BL/6 mice were given a single intravenous dose of 10 mg/kg of saline-formulated ^{35}S -anti-miR-21 (specific activity 75.7 $\mu\text{Ci}/\text{mg}$). Individual mice were killed at different time points ranging from 5 min to 21 days after administration, immediately frozen at -80°C and sectioned sagittally for quantitative whole-body autoradiography⁴⁹.

Human serum albumin binding assay

Human serum albumin (Sigma) was dissolved in PBS (Sigma), filtered on YM-30 columns (Microcon) and incubated at 37 °C for 30 min with 5' FAM-labeled anti-miR-122 oligonucleotides, synthesized with a complete phosphorothioate or with an unmodified phosphodiester backbone. The samples were centrifuged at 1,500g in YM-30 columns, diluted with distilled water and dispensed for fluorescence measurements using a FLUOstar Optima Microplate Reader.

Cholesterol and transaminase measurements

Total cholesterol, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured as described²⁴.

Microarray expression profiling, proteomics and miRNA target site analyses

Total RNAs were labeled using Whole Transcript Sense Target Labeling Assay (Affymetrix) and hybridized to MoGene 1.0 ST or HuGene 1.0 ST arrays according to the manufacturer's instructions. Pre-processing of the microarray data was done using the Variance Stabilization and Normalization (VSN) method for background correction, between-array normalization and transformation, and summarization was done using the Robust Multi-array Average (RMA) method. The low-level analysis was done using Chip Definition Files where probes were reorganized into Ensembl gene-specific probe set⁵⁰. In each experiment, we included a filtering step to remove genes that had low absolute

expression values and were therefore not likely to be expressed in the tissue under study. The analysis was limited to probe sets exceeding the overall median intensity for at least half of the replicates in a single group.

PC3 cells (6×10^6) were seeded, transfected with 25 nM antileet-7 or LNA control and harvested 48 h post transfection. Washed cell pellets were lysed in 8 M urea, 50 mM Triethylammonium bicarbonate (TEAB, pH 8.5) 0.05% w/v ProteaseMax containing phosphatase inhibitor and protease inhibitor cocktails, pelleted at 14,000g and the soluble protein concentrations were determined. Proteins were extracted from mouse liver samples as described²⁵. Aliquots of 100 µg total protein from each sample were reduced, alkylated and precipitated⁵¹. Following re-suspension, samples were digested with Trypsin (1:50 w/w) overnight at 37 °C. The solution volumes were reduced to a final volume of ~20 µl, and labeling with iTRAQ was performed essentially as described⁵¹. After labeling, the solutions were combined and desalted with C18 Sep-Pak cartridges. Bound peptides were eluted, reconstituted in OFFGEL sampling buffer and subjected to high resolution fractionation using a reduced glycerol protocol⁵². Each of the 24 fractions was then analyzed by capillary liquid chromatography-tandem mass spectrometry using a protocol modified from reference 53. Mass spectrometry acquisition parameters were adapted from reference 54, with the top four most intense ions selected for consecutive collision induced dissociation (CID) and high energy collisional dissociation (HCD).

Protein identification and quantification was carried out with Mascot 2.3 (Matrix Science) against the human IPI database (87,040 sequences). Methylthiolation of cysteine, N-terminal and lysine iTRAQ modifications were set as fixed modifications, methionine oxidation as variable and one missed cleavage was allowed. iTRAQ ratios were calculated as intensity weighted, using only peptides with expectation values <0.05. Global ratio normalization was performed using intensity summation with no outlier rejection. A more detailed description of the proteomics protocols is given in the Supplementary Note.

Sequence information was downloaded from Ensembl (build 56) and in the case of one-to-many mapping between Ensembl genes and sequence information, the longest sequence was chosen. MicroRNA targets were predicted by searching for canonical seed match sites in the 3' UTR sequences. Sylamer analysis was carried out as described in reference 40 using spliced complementary DNA sequence information from Ensembl version 59.

RESULTS

Design of miRNA seed-targeting tiny LNAs

Our goal was to develop an antimiR approach that would enable inhibition of miRNA families by targeting their shared seed region (Fig. 1). We have previously reported efficient silencing of the liver-specific miR-122 in rodents and primates using a high-affinity 15-mer LNA-modified antimiR^{24,26}. Here we hypothesized that 8-mer antimiRs complementary to the miRNA seed could have adequate affinity to attain efficient inhibition when synthesized as fully LNA-substituted phosphorothioate oligonucleotides. Indeed, 8-mer tiny LNAs targeting the seed regions of miR-21, miR-122, miR-155 and the miR-17/18/19 and let-7 families all showed high duplex melting temperatures (T_m) ranging from 60 °C to 78 °C (Table 1 and Supplementary Table 1).

Inhibition of miR-21 function by an 8-mer tiny LNA

To test our knockdown strategy in cultured cells, we first chose to target miR-21, a potentially oncogenic miRNA that is upregulated and abundant in many solid tumors and in most cancer cell lines³⁰. Thus, a miR-21 luciferase reporter harboring a perfect-match miR-21 target site in its 3' UTR was repressed in HeLa cells as compared to luciferase

controls (Fig. 2a and Supplementary Fig. 1a). Transfection of the tiny LNA complementary to nucleotides 2–9 in the mature miR-21 sequence resulted in potent antagonism of miR-21, as shown by specific de-repression of the miR-21 reporter with a half-maximal inhibitory concentration (IC_{50}) of 0.9 nM (Fig. 2a,b and Supplementary Fig. 1b). This coincided with sequestration of miR-21 in a slower-migrating heteroduplex with anti-miR-21, de-repression of HeLa mRNAs with canonical miR-21 seed match sites and upregulation of the *Pdcd4* target protein³¹ (Fig. 2c–e). Moreover, tiny LNA-transfected PC3 and HepG2 cells formed significantly fewer colonies on soft agar compared to LNA control treated cells (Fig. 2f and Supplementary Fig. 1c), as reported previously using other anti-miR approaches³². Of note, an 8-mer seed-targeting 2'-*O*-Me-modified oligonucleotide showed no effect on the miR-21 reporter, consistent with its markedly lower T_m of 37 °C (Fig. 2a and Table 1).

To determine whether 8-mer anti-miRs must interact with the seed region to inhibit miRNA function, we designed three additional complementary 8-mer LNAs tiled across the mature miR-21 sequence (Fig. 2g). In contrast to the seed-targeting anti-miR-21, transfection of tiny LNAs binding adjacent to the seed, to the central region or to the 3'-end of miR-21 had no effect on the miR-21 reporter. We also assessed the specificity and the optimal length of seed-targeting LNAs by introducing one or two adjacent mismatches at all possible nucleotide positions in the anti-miR-21 sequence and by shortening the 8-mer LNA to either 7 or 6 nucleotides. The mismatched LNAs, as well as the shorter species, showed substantially reduced potency, consistent with their lower duplex melting temperatures (Supplementary Fig. 2a,b and Supplementary Table 1). Similarly, we observed lower potencies for extended 9- to 12-mer LNAs, suggesting that fully LNA-modified anti-miRs longer than 8 nucleotides might be structurally too rigid to allow effective binding to the miRNA in competition with the target mRNAs.

Next, we investigated whether the tiny anti-miR-21 is associated with Argonaute2 (Ago2) protein complexes. To this end, we carried out Ago2 immunoprecipitations in anti-miR-21-transfected HeLa cell lysates (Fig. 2h) followed by RNA blot analysis of the Ago2-bound RNAs. Hybridization of the RNA blot with a complementary LNA probe showed presence of the anti-miR-21 in the Ago2 complex, but not in the IgG control (Fig. 2i). To determine the subcellular localization, we delivered a FAM-labeled anti-miR-21 by unassisted uptake³³ to HEK293 cells expressing FLAG-tagged Ago2. Immunofluorescence microscopy of fixed HEK293 cells revealed a punctuated distribution for the FAM-labeled anti-miR-21 and for the Ago2 protein, predominantly in the cytoplasm (Fig. 2j and Supplementary Fig. 3), where some of the fluorescent anti-miR-21 foci co-localized with Ago2 (Fig. 2j). Together, these findings support the notion that tiny LNAs target Ago2-bound mature miRNAs.

Silencing of miRNA families in cultured cells by tiny LNAs

To further explore knockdown of individual miRNAs, we designed 8-mer anti-miRs complementary to the seed regions of miR-122 and miR-155 (Fig. 3a,b and Table 1). We assessed the potency of the tiny anti-miR-122 by cloning the 3' UTR of the *Aldoa*²¹ target downstream of the *Renilla* luciferase gene. The activity of this reporter was repressed by a pre-miR-122 in HeLa cells, whereas co-transfection of the anti-miR-122 resulted in concentration-dependent de-repression of the *Aldoa* 3' UTR reporter and upregulation of aldolase A as compared to the anti-miR-155 and LNA scramble controls (Fig. 3a and Supplementary Fig. 4). To test the potency of anti-miR-155, we constructed a reporter harboring a perfect-match miR-155 target site in the 3' UTR of *Renilla* luciferase. This reporter was effectively repressed in lipopolysaccharide (LPS)-stimulated mouse RAW264.7 macrophages, which express elevated levels of miR-155 (ref. 25). Co-transfection of anti-miR-155 into RAW264.7 cells de-repressed the miR-155 reporter in a concentration-dependent manner, whereas tiny anti-miR-122 and LNA control showed no effect (Fig. 3b

and Supplementary Fig. 4). This antimiR was also able to block repression of the miR-155 target PU.1 (ref. 25) by transfected pre-miR-155 (Fig. 3b).

We next tested whether the tiny LNA approach could be used to inhibit simultaneously the activity of miRNAs within the same family. The miR-221/222 family consists of two members that negatively regulate the cell-cycle inhibitor p27^{Kip1} through two target sites in its 3' UTR^{34,35}. We synthesized a 7-nucleotide LNA complementary to the miR-221/222 seed region and constructed two reporters with perfect-match target sites for miR-221 and miR-222 in the 3' UTR of *Renilla* luciferase. Both reporters were effectively repressed in PC3 cells (Fig. 3c) in accordance with the high expression of miR-221 and miR-222 in this cancer cell line³⁵. In contrast, co-transfection of the tiny antimiR into PC3 cells led to concentration-dependent de-repression of both reporters when assayed either individually or in combination, implying that this tiny LNA is able to inhibit both members of the miR-221/222 family. This is supported by a coincident, concentration-dependent upregulation of p27^{Kip1} (Fig. 3d). Similarly, transfection of tiny LNAs targeting the seed region of the miR-17, miR-18 and miR-19 families resulted in de-repression of their respective luciferase reporters in HeLa cells (Supplementary Fig. 5).

The let-7 family was originally identified in *C. elegans*³⁶ and comprises nine members in humans³⁷. Expression of the let-7 family is downregulated in a number of tumor types, including non-small-cell lung cancer. *In vitro* studies have shown that the let-7 miRNAs can restrain cellular proliferation³⁷, which can, in part, be explained by their target mRNAs, including the well-characterized oncogenes *RAS* (*HRAS*, *KRAS* and *NRAS*)³⁷ and *HMGA2* (ref. 38). To assess inhibition of individual let-7 family members by a seed-targeting antilet-7, we obtained pre-miRs representing all nine let-7 members, let-7a, -7b, -7c, -7d, -7e, -7f, -7g, -7i and miR-98, and assayed a cell line panel to identify a context with minimum levels of endogenous let-7. In a screen of 14 different cell lines, the hepatocellular cancer cell line Huh-7 had the lowest expression of let-7 (data not shown). All nine pre-miRs effectively repressed the HMGA2 3' UTR reporter in Huh-7 cells (Fig. 3e). By comparison, co-transfection of antilet-7 alongside each pre-let-7 resulted in concentration-dependent de-repression of the HMGA2 reporter, suggesting that this tiny LNA is capable of inhibiting the entire let-7 family in Huh-7 cells. To test whether anti-let-7 could also inhibit endogenous let-7, we co-transfected it alongside the HMGA2 3' UTR reporter into HeLa or PC3 cells that express most let-7 family members. This resulted in robust de-repression of the HMGA2 reporter in both cell lines (Fig. 3f and Supplementary Fig. 6a) and upregulation of the *RAS* and *HMGA2* targets at the protein level (Fig. 3g and Supplementary Fig. 6b). Furthermore, transfection of the tiny antilet-7 into PC3 cells resulted in significant de-repression of predicted let-7 target mRNAs with canonical 3' UTR seed sites (Supplementary Fig. 6c). We thus conclude that tiny seed-targeting LNAs can be used to inhibit the function of individual miRNAs and entire miRNA families in cultured cells.

Delivery and efficacy of tiny LNAs *in vivo*

To determine whether the high binding affinity of tiny LNAs combined with a complete phosphorothioate backbone could enable delivery and silencing of miRNAs *in vivo* without additional conjugation or formulation chemistries, we first determined the biodistribution of a radiolabeled tiny LNA after systemic delivery into mice. To this end, we injected nine female mice with single intravenous doses of 10 mg/kg ³⁵S-labeled antimiR-21. Individual mice were killed at different time points ranging from 5 min to 21 days after injection, which was followed by sagittal sectioning for whole body autoradiography (Fig. 4a). All animals showed a broad biodistribution with high levels of radioactivity in the kidney cortex, liver, lymph nodes, bone marrow and spleen, whereas we detected no radioactivity in the brain (Fig. 4a and Supplementary Fig. 7). We observed a rapid decline of radioactivity in heart blood with terminal elimination half-lives of 8–10 h. We detected radioactivity in the

urinary tract and in bile in all mice, suggesting that both were routes of elimination. By comparison, most tissues showed a slow decline of radioactivity with half-lives of 100–600 h (Supplementary Fig. 7 and data not shown).

We next assessed *in vivo* silencing of miR-21 by injecting mice intravenously at a dose of 10 mg/kg of saline-formulated antimiR-21 on three consecutive days. RNA blot analysis of kidney, liver and lung RNAs showed sequestration of miR-21 in a heteroduplex with the antimiR-21 (Fig. 4b), coinciding with upregulation of the miR-21 target BTG2 (ref. 32) in the same tissues (Fig. 4c). Moreover, sequestration of miR-21 by antimiR-21 was already observed after 1 h and was still effective after 21 days in the kidney and liver of mice injected with a single dose of 25 mg/kg (Fig. 4d). Together, these data imply that systemically delivered, unconjugated tiny LNAs are taken up by a broad range of tissues which, in turn, leads to long-lasting silencing of miRNA function in these tissues *in vivo*.

Targeting of miR-21 in a mouse breast tumor model

To determine whether tiny LNAs could be used to target miRNAs in solid tumors *in vivo*, we tested delivery of the antimiR-21 compound in a mouse orthotopic breast tumor model. Young Balb/c female mice were injected with 4T1 cells, a mouse mammary tumor cell line used to model stage IV human adenocarcinoma³⁹. We injected 4T1 cells expressing a miR-21 luciferase reporter into the fourth gland and cells expressing a control luciferase reporter into the ninth gland. Tumor-bearing mice were injected intravenously at a dose of 10 mg/kg antimiR-21 or LNA control on three consecutive days starting at day 10 after tumor initiation. We obtained *in vivo* images before treatment and at 3, 7 and 9 days after the last dose. There was significant de-repression of the miR-21 luciferase reporter in tumors of antimiR-21-treated mice, indicating successful delivery to the tumor site (Fig. 4e, upper panel, right-side tumors and Fig. 4f). In contrast, we detected minimal or no alteration of the miR-21 reporter activity in the LNA-control-treated mice (Fig. 4e, lower panel, right-side tumors). We observed no obvious changes in the activity of the luciferase control tumors or of tumor size in either treatment group. The effect of antimiR-21 mediated de-repression of the miR-21 reporter was sustained for 9 days after last injection (D9 in Fig. 4f), with the reporter still being de-repressed in most tumors at day 11 (Supplementary Fig. 8a) followed by a return to basal luciferase activity at day 14 (Supplementary Fig. 8b,c).

To assess the effect of antimiR-21 treatment on tumor growth, we injected Balb/c mice with 4T1 cells as above and then treated them with six injections of 30 mg/kg of antimiR-21 or LNA control after tumor initiation. We evaluated tumor size twice weekly, starting one week after the initial treatment. Mice from the antimiR-21-treated group ($n = 10$, two tumors per mouse) showed no difference in tumor growth as compared to the LNA controls ($n = 8$, two tumors per mouse). Thus, in the context of 4T1 cells, an invasive breast tumor cell line, knockdown of miR-21 was not sufficient to impair tumor development. Nevertheless, our results show that the antimiR-21 compound can be delivered to solid tumors, suggesting that tiny LNAs targeting cancer-associated miRNAs could be useful for the development of new therapeutic strategies.

Silencing of miR-122 in the mouse liver by tiny LNA

We have previously shown that silencing of the liver-specific miR-122 using a 15-mer LNA-antimiR leads to long-lasting decrease of serum cholesterol in rodents and primates²⁴. Because miR-122 was functionally inhibited by seed-targeting antimiR-122 in cultured cells, we decided to compare the 8-mer and 15-mer antimiRs side-by-side *in vivo*. We injected mice intravenously with three doses of 5 mg/kg or 20 mg/kg and killed them on day 7. RNA blot analysis showed sequestration of miR-122 in a slower-migrating hetero-duplex with both antimiRs. Concomitantly, we observed a dose-dependent lowering of total cholesterol

without any hepatotoxicity (Fig. 5a,b and Supplementary Fig. 9) and significant de-repression of the miR-122 targets *AldoA* and *Bckdk*²¹ in the anti-miR-122-treated mice (Fig. 5c). In contrast, treatment with an 8-mer anti-miR-122 synthesized with a phosphodiester backbone had no effect on miR-122, *AldoA*, *Bckdk* or serum cholesterol levels, coinciding with its markedly reduced binding to human serum albumin (Supplementary Fig. 10). These findings highlight the importance of phosphorothioate backbone modifications in facilitating delivery of tiny LNAs *in vivo*. Unsupervised hierarchical clustering of liver mRNA profiles from the treated mice clustered into two distinct branches (Fig. 5d). All controls grouped into a highly compact cluster, suggesting that the 8-mer LNA control has a minimal effect on liver gene expression. By comparison, the expression profiles of the anti-miR-treated animals formed a separate uniform cluster, indicating that the two anti-miRs have similar effects on the liver transcriptome (Fig. 5d).

To validate this conclusion, we identified all differentially expressed liver mRNAs by comparing the two anti-miR-122 treatment groups to the LNA control and to each other. Cluster analysis showed a high level of concordance between the 15-mer and 8-mer anti-miR-122-treated animals, underscored by only 32 differentially expressed transcripts out of the 1,003 mRNAs identified (Fig. 5e). Furthermore, silencing of miR-122 in mice by the two LNAs resulted in significant and highly similar de-repression of predicted miR-122 target mRNAs with canonical 3' UTR seed match sites (Fig. 5f). Notably, of the liver mRNAs upregulated by both anti-miRs, 63 had 8-mer seed sites, whereas 134 mRNAs had 7-mer seed sites in the 3' UTRs (Fig. 5g). Gene Ontology (GO) enrichment analysis of the downregulated mRNAs in the anti-miR-treated mice showed an overrepresentation of GO terms related to lipid and steroid metabolic processes, including cholesterol biosynthesis (data not shown), consistent with lowering of serum cholesterol, as has also been reported previously in rodents and primates^{20,21,23,24,26}. Taken together, these data show that both 15-mer and tiny LNA-anti-miRs lead to similar physiological responses in treated animals and to highly comparable effects on miR-122 targets in the mouse liver.

Assessment of off-target effects

Short, 8-mer oligonucleotides have many predicted perfect-match recognition sequences in cellular mRNAs and, thus, we asked whether potential binding of tiny LNAs to such sites could lead to off-target effects at the mRNA or protein level. Sylamer⁴⁰ analysis of microarray data from cell culture and *in vivo* experiments with tiny anti-miR-21, anti-let-7 or anti-miR-122 showed significant enrichment of seed-match-containing mRNAs in the most upregulated mRNA pools, consistent with the notion that functional miRNA inhibition leads to de-repression of direct targets (Supplementary Figs. 11,12 and Supplementary Tables 2,3). By comparison, we observed no effect of the tiny LNAs on mRNAs with 6-, 7- or 8-nucleotide perfect-match binding sites, suggesting that mRNAs with such sites are not affected. Next, we assessed the impact of tiny LNAs on protein production by analyzing liver protein samples from mice treated with anti-miR-122 and protein extracts from anti-let-7-transfected PC3 cells employing iTRAQ (isobaric tags for relative and absolute quantitation) labeling of peptides combined with liquid chromatography and tandem mass spectrometry. This resulted in quantification of 3,772 and 2,402 proteins from the mouse liver and PC3 cells, respectively, of which 3,184 and 1,908 could be mapped uniquely to Ensembl gene IDs. In both data sets, the effect of tiny LNA-mediated miRNA silencing could be detected on the protein output, as shown by significant de-repression of proteins derived from mRNAs with canonical seed-match sites (Fig. 6a,b and Supplementary Fig. 13). In contrast, none of the tiny LNAs showed a significant shift in levels of proteins encoded by mRNAs with tiny LNA binding sites (Fig. 6a,b and Supplementary Fig. 13).

To further investigate the effect of tiny LNAs on protein levels using a highly sensitive readout, we designed sixteen 8-mer LNAs encompassing the entire *Renilla* luciferase

reporter gene. As expected, co-transfection of small interfering RNAs (siRNAs) or RNase H-recruiting LNA gapmers targeting *Renilla* resulted in potent knockdown of the *Renilla* luciferase reporter (Fig. 6c,d). In contrast, tiny LNAs targeting 8-mer recognition sequences within the siRNA or LNA gapmer target sites showed negligible effects on luciferase activity (Fig. 6c). Similarly, ten additional tiny LNAs binding at perfect-match sites in the 5' UTR, open reading frame (ORF) or 3' UTR of *Renilla* luciferase showed a modest effect at best on luciferase activity (Fig. 6c). In summary, our data suggest that 8-mer tiny LNAs do not significantly affect the protein output from mRNAs with perfect-match binding sites.

DISCUSSION

We developed a method that exploits the high affinity of 7- to 8-mer, fully modified LNA oligonucleotides toward complementary RNA molecules to antagonize miRNA function. When designed to target the miRNA seed, tiny LNAs enable specific and concentration-dependent inhibition of entire miRNA families in cultured cells. Our data highlight the importance of targeting the miRNA seed and suggest that this region is more accessible for inhibition, as additional tiny LNAs tiled across mature miR-21 (Fig. 2g), miR-155 and let-7 sequences (Supplementary Fig. 14) had no or limited effect on miRNA activity. This is consistent with the model in which the miRNA seed region is exposed and preorganized in the Argonaute complex to favor efficient pairing to the seed-match site in the target mRNA^{2,41,42}. In addition, structural studies have shown that the fixed *N*-type conformation of the LNA nucleoside preorganizes LNA oligonucleotides in a high-affinity conformation for binding complementary RNA sequences, thereby adopting an A-form helix geometry typical for RNA-RNA duplexes⁴³. Thus, the remarkable thermodynamic stability of tiny LNAs described here facilitates their effective binding to miRNA seeds in competition with cellular target mRNAs leading to sequestration of the miRNA. The importance of a high binding affinity of tiny LNAs is further highlighted by the fact that an isosequential 8-mer 2'-*O*-Me-modified anti-miR-21 oligonucleotide with a low T_m of 37 °C showed no inhibition of miR-21 activity.

A key feature of our approach is that it enables simple systemic delivery of unconjugated, saline-formulated tiny LNAs *in vivo* when combined with a phosphorothioate backbone. As shown here, intravenously injected tiny LNAs are taken up by a range of mouse tissues, which leads to long-lasting silencing of miRNA function. This has important ramifications for functional studies of animal miRNAs *in vivo*. First, unlike other chemically modified anti-miR oligonucleotides, tiny LNAs enable simultaneous knockdown of co-expressed miRNA family members that may have redundant biological functions. Although genetic approaches have been used successfully to generate triple and double knockouts for functional studies of the *C. elegans* let-7 (ref. 10) and mouse miR-208 families¹⁶, respectively, silencing of miRNA seed families using tiny LNAs is less time consuming and less labor intensive than this approach. Furthermore, by using different dosing regimens, tiny LNAs can be used to study the physiological consequences of acute and partial inhibition of miRNA families *in vivo*, which is technically challenging using genetic knockouts. Second, we found that silencing of the liver-expressed miR-122 in mice by a tiny 8-mer LNA in comparison with a 15-mer anti-miR led to highly similar effects *in vivo*. Notably, of the liver mRNAs de-repressed by both anti-miRs, about 200 messages had canonical 8-mer or 7-mer seed matches in the 3' UTRs. These results suggest that the tiny LNA approach has the potential to pinpoint physiologically relevant miRNA targets *in vivo*, thereby complementing bioinformatic, proteomic and Ago HITS-CLIP (high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation) approaches for uncovering miRNA-target interactions on a genomic scale^{8,9,44,45}. Moreover, tiny LNAs are also applicable to miRNA-target interaction studies in cultured cells and should be especially useful for identifying common targets shared by co-expressed miRNA family

members. Third, apart from efficient miRNA silencing in several mouse tissues, systemically delivered tiny LNAs showed uptake and miR-21 knockdown in a mouse model of breast cancer, thus underscoring the potential of this technology in functional studies of disease-implicated miRNAs *in vivo*. Given the emerging roles of miRNA seed families in disease, such as the let-7 family in lung cancer^{37,46}, the miR-19 family in Myc-driven B-cell lymphomas¹⁵ and the miR-208 family in cardiac remodeling¹⁶, we envision tiny LNAs as important tools to study the functional overlap within miRNA seed families in animal disease models. Finally, our data demonstrate *in vivo* efficacy and the lack of acute toxicities in mice, which imply that tiny LNAs may be well suited to the development of therapeutic strategies aimed at pharmacological inhibition of disease-associated miRNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank A. Koustrup, H. Brostrøm, A. Konge, J.J. Jørgensen, M. R. Møller, L. Bang and M. Meldgaard for excellent technical assistance, and A. Lund and T. Bou-Kheir for reagents. This study was supported by grants from the Danish National Advanced Technology Foundation and the Danish Medical Research Council to S.K.

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Figure 1.

Schematic overview of the miRNA silencing approach using seed-targeting tiny LNAs. **(a)** miRNAs bind to partially complementary target sites in the 3' UTRs of target mRNAs and thereby mediate translational repression or mRNA degradation. **(b)** Tiny LNAs are designed as fully LNA-modified phosphorothioate oligonucleotides complementary to the seed region. **(c)** The high binding affinity of tiny LNAs enables functional inhibition of co-expressed members of miRNA seed families, which leads to de-repression of target mRNAs.

Figure 2.

Inhibition of miR-21 function by tiny anti-miR-21. (a) Relative luciferase activity of the miR-21 reporter co-transfected into HeLa cells with tiny anti-miR-21, 2'-O-Me anti-miR-21, LNA scramble (scr) or mismatch (mm) controls. Error bars, s.e.m. (b) We determined the concentration of anti-miR-21 required for half-maximal inhibition (IC₅₀) of miR-21 in HeLa cells using the miR-21 reporter. Error bars, s.e.m. (c) RNA blot analysis of miR-21 in HeLa cells transfected with 5 nM anti-miR-21 or LNA scramble control. U6 is shown as a control. (d) De-repression of HeLa mRNAs with canonical miR-21 seed sites after transfection with 5 nM anti-miR-21. Cumulative distributions of mRNA changes between anti-miR-21-treated and mock-treated cells are shown for each seed site (one-sided Kolmogorov-Smirnov test). (e) Protein blot analysis of Pcd4 expression in HeLa cells (same cells as in c). Tubulin is shown as a control. (f) Colony formation of PC3 cells transfected with anti-miR-21 or LNA scramble control. * $P < 0.05$; $n = 3$; error bars, s.e.m. (g) Relative luciferase activity of the miR-21 reporter in HeLa cells co-transfected with tiny LNAs targeting the mature miR-21 sequence. Error bars, s.e.m. (h) Protein blot analysis of Ago2 immunoprecipitates from anti-miR-21-transfected HeLa cells. IgG was used as a control. (i) RNA blot analysis of

RNAs from immunoprecipitated Ago2 samples probed for antimiR-21. The antimiR-21 oligonucleotide (14 pg and 140 pg per sample) was used as a control. **(j)** Subcellular localization of antimiR-21 in HEK293 cells using immunofluorescence microscopy (red, FLAG-tagged Ago2; green, FAM-labeled antimiR-21; blue, nuclei co-stained with DAPI; scale bar, 10 μ m).

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Figure 3.

Silencing of miRNA families by seed-targeting tiny LNAs in cultured cells. **(a)** Relative luciferase activity of the AldoA 3' UTR reporter co-transfected into HeLa cells with pre-miR-122 and tiny antimiR-122 or LNA scramble control. Error bars, s.e.m. Protein blot analysis of AldoA expression in HeLa cells transfected with 5 nM antimiR-122 or LNA scramble control. Tubulin is shown as a loading control. **(b)** Relative luciferase activity of the miR-155 reporter co-transfected into LPS-treated RAW264.7 cells with tiny antimiR-155 or LNA scramble control. Error bars, s.e.m. Protein blot analysis of PU.1 expression in RAW264.7 cells co-transfected with pre-miR-155 and 5 nM tiny antimiR-155 or LNA scramble control. Tubulin is shown as a loading control. **(c)** Relative luciferase activity of the miR-221 and miR-222 reporters co-transfected into PC3 cells with tiny antimiR or LNA scramble control. Error bars, s.e.m. **(d)** Protein blot analysis of p27 expression in PC3 cells transfected with tiny antimiR or LNA scramble control. Tubulin is shown as a loading control. **(e)** Relative luciferase activity of the HMGA2 3' UTR reporter co-transfected into Huh-7 cells with 10 nM pre-let-7a, -7b, -7c, -7d, -7e, -7f, -7g, -7i or miR-98 and tiny antilelet-7 or LNA scramble control. Error bars, s.e.m. **(f)** Relative luciferase activity of the HMGA2 3' UTR reporter co-transfected into HeLa cells with tiny antilelet-7. Error bars, s.e.m. **(g)** Protein blot analysis of HMGA2 and RAS expression in HeLa cells transfected with tiny antilelet-7 or LNA scramble control. Tubulin is shown as a loading control.

Figure 4.

Silencing of miR-21 *in vivo* by tiny anti-miR-21. **(a)** Uptake of ³⁵S-labeled anti-miR-21 in mice over time after treatment with single intravenous doses of 10 mg/kg. Individual mice were killed at different time points and sectioned sagittally for whole-body autoradiography. Uptake of ³⁵S-labeled anti-miR-21 is shown for selected tissues. **(b)** RNA blot analysis of liver, kidney and lung RNA from mice treated with three intravenous doses of 10 mg/kg tiny anti-miR-21 or LNA control or with saline. The RNA blot was probed for miR-21 and U6. **(c)** Protein blot analysis of BTG2 expression in kidney, liver and lung of mice treated with three intravenous doses of 10 mg/kg tiny anti-miR-21 or LNA scramble control or with saline. **(d)** RNA blot analysis of liver and kidney RNA from mice killed at different time points after treatment with single intravenous doses of 25 mg/kg anti-miR-21 or LNA scramble control. The RNA blot was probed for miR-21 and U6. **(e)** Representative images of miR-21 luciferase reporter de-repression in breast tumor (right side tumors) after treatment with anti-miR-21 (upper panel) or LNA control (lower panel). Left side tumors express luciferase alone. We carried out imaging before first injection (D0) and at 3, 7 and 9 days (D3, D7 and D9) after the last injection. **(f)** *In vivo* image analysis quantification of two independent experiments. Luciferase activity was normalized to tumor size. **P* < 0.05, *n* = 7 for anti-miR-treated group and *n* = 5 for LNA control; ***P* < 0.01, *n* = 5 for both groups. Error bars, s.e.m.

Figure 5.

Silencing of miR-122 in the mouse liver by seed-targeting tiny LNA. **(a)** RNA blot analysis of liver RNAs from mice after treatment with three intravenous doses of 20 mg/kg tiny anti-miR-122, 15-mer anti-miR-122 or LNA scramble control or with saline. The RNA blot was probed for miR-122 and U6. **(b)** Total plasma cholesterol levels in mice treated with three intravenous injections of tiny anti-miR-122, 15-mer anti-miR-122, LNA scramble control or with saline (error bars, s.e.m.; $n = 5$; *** $P < 0.001$, one-way ANOVA). **(c)** Quantification of the AldoA and Bckdk mRNAs (same samples as in a, normalized to GAPDH; error bars, s.e.m.; $n = 5$; *** $P < 0.001$, one-way ANOVA). **(d)** Hierarchical clustering of samples using filtered microarray expression data from mice treated with tiny anti-miR-122, 15-mer anti-miR-122, LNA scramble control or with saline (same samples as in a; $n = 5$, except for 15-mer anti-miR and LNA scramble control, where $n = 4$). **(e)** Hierarchical clustering of genes showing differential expression at a false discovery rate of 1% when comparing anti-miR-122 profiles to controls or when comparing the two

antimiR-122 profiles directly. Predicted miR-122 targets (7-mer and 8-mer seed match sites) are indicated with black lines between the heatmap and the dendrogram. **(f)** De-repression of liver mRNAs with canonical miR-122 seed match sites (same samples as in **e**). We determined the significance of the differences from mRNAs with no sites using one-sided Kolmogorov-Smirnov tests. **(g)** Venn diagrams showing the number of liver mRNAs upregulated by antimiRs both with 8-mer or 7-mer seed matches in the 3' UTRs.

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Figure 6.

Off-target analysis. **(a)** Schematic representation of the different seed match sites in miR-122 target mRNAs and the putative perfect-match binding sites of the tiny anti-miR-122 and the LNA scramble control, respectively. **(b)** De-repression of proteins encoded by mRNAs with canonical miR-122 seed match sites after treatment with 3 × 20 mg/kg tiny anti-miR-122. The cumulative fraction plots show the distribution of log₂ fold changes between the anti-miR-122- and LNA-control-treated mice for each seed match type. Proteins encoded by mRNAs with perfect-match binding sites to anti-miR-122 or LNA scramble control did not show a significant shift relative to saline control. **(c)** Relative luciferase activity of a *Renilla* luciferase reporter co-transfected into HeLa cells with three siRNAs, three LNA gapmers targeting the *Renilla* ORF (red and orange), three tiny LNAs with binding sites within the siRNA or LNA gapmer target sequences (gray), tiny LNAs binding to the 5' UTR (black), ORF (blue) or 3' UTR (green) of *Renilla* luciferase, respectively. Error bars, s.e.m. **(d)** Schematic representation of the mechanism of action of siRNAs and LNA gapmers and binding of tiny LNAs to a perfect-match mRNA site.

Table 1

AntimiR oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')	T_m (°C)
antimiR-21	GATAAGCT	65
antimiR-122	CACACTCC	78
antimiR-155	TAGCATTA	61
antimiR-221/222	ATGTAGC	56
antilet-7	ACTACCTC	75
2'-O-Me antimiR-21	gauaagcu	37
LNA mismatch	GGTAA <u>ACT</u>	39
LNA scramble	TCATACTA	<30

LNA nucleotides are shown in uppercase and 2'-O-methyl-modified nucleotides are shown in lowercase. The LNA mismatch control had two mismatches (underlined) to the miR-21 seed region. We measured the T_m values of the LNA control oligonucleotides for the miR-21-LNA mismatch and the miR-21-LNA scramble duplexes.