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LETTERS

LNA-mediated microRNA silencing in non-human primates

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microRNAs (miRNAs) are small regulatory RNAs that are important in development and disease[1–3] and therefore represent a potential new class of targets for therapeutic intervention[4]. Despite recent progress in silencing of miRNAs in rodents[5,6], the development of effective and safe approaches for sequence-specific antagonism of miRNAs *in vivo* remains a significant scientific and therapeutic challenge. Moreover, there are no reports of miRNA antagonism in primates. Here we show that the simple systemic delivery of a unconjugated, PBS-formulated locked-nucleic-acid-modified oligonucleotide (LNA-antimiR) effectively antagonizes the liver-expressed miR-122 in non-human primates. Acute administration by intravenous injections of 3 or 10 mg kg^{-1} LNA-antimiR to African green monkeys resulted in uptake of the LNA-antimiR in the cytoplasm of primate hepatocytes and formation of stable heteroduplexes between the LNA-antimiR and miR-122. This was accompanied by depletion of mature miR-122 and dose-dependent lowering of plasma cholesterol. Efficient silencing of miR-122 was achieved in primates by three doses of 10 mg kg^{-1} LNA-antimiR, leading to a long-lasting and reversible decrease in total plasma cholesterol without any evidence for LNA-associated toxicities or histopathological changes in the study animals. Our findings demonstrate the utility of systemically administered LNA-antimiRs in exploring miRNA function in rodents and primates, and support the potential of these compounds as a new class of therapeutics for disease-associated miRNAs.

miRNAs are small endogenous non-coding RNAs that post-transcriptionally repress the expression of protein-coding genes by base-pairing with the 3′ untranslated regions (UTRs) of the target messenger RNAs[1,2,7]. Emerging evidence suggests that animal miRNAs are important in the control of many biological processes[1–3,8]. In addition, miRNAs have been implicated in viral infections, cardiovascular disease and neurological and muscular disorders, as well as in the onset and progression of cancers[9–19]. miR-122 is a liver-expressed miRNA implicated in cholesterol and lipid metabolism[5,6] and in hepatitis C virus (HCV) replication[14,20], underscoring miR-122 as a potential therapeutic target for the treatment of hypercholesterolemia and hepatitis C infection. To develop an efficient approach for miR-122 targeting *in vivo*, we first evaluated the potency of different LNA-modified DNA oligonucleotides (LNA-antimiRs) in cultured Huh-7 cells with the use of a luciferase reporter assay for miR-122. Our screen implied that inhibition of miR-122 function was affinity dependent and identified a high-affinity LNA-antimiR (more than 50% LNA, melting temperature 80 °C), which mediated efficient derepression of the luciferase reporter at 5 nM concentration (Supplementary Fig. 1a). This oligonucleotide showed improved potency compared with a 2′-*O*-methyl oligonucleotide and two LNA-antimiRs of lower affinity in inhibiting HCV replication in Huh-7 cells harbouring the HCV-N replicon NNeo/C-5B (Supplementary Fig. 2). Moreover, when adapted to silencing of three additional miRNAs in HeLa cells, our LNA-antimiR design showed high potency for all targeted miRNAs (Supplementary Fig. 1b).

Next, we examined whether combining the high-affinity LNA-antimiR with phosphorothioate modifications could enable the delivery and silencing of miR-122 *in vivo* without additional conjugation chemistries. As shown in Fig. 1a, uptake of unconjugated LNA-antimiR in the murine liver was achieved by three intraperitoneal injections of saline-formulated LNA-antimiR with a complete phosphorothioate backbone. This coincided with the detection of a shifted band on the northern blot (Fig. 1a), indicating that the mature miR-122 is sequestered in a heteroduplex with LNA-antimiR. However, this mechanism of action does not necessarily apply to other types of miRNA antagonist. LNA-mediated silencing of miR-122 function led to a threefold derepression of the direct miR-122 target mRNA, *Aldoa*, which encodes aldolase A (ref. 6) (Fig. 1b). Single intraperitoneal injections at doses ranging from 1 to 200 mg kg^{-1} LNA-antimiR resulted in dose-dependent and sustained decrease in total plasma cholesterol with a median effective dose (ED_{50}) of 10 mg kg^{-1} (Fig. 1c). Moreover, intraperitoneally delivered LNA-antimiR at doses ranging from three injections of 1–25 mg kg^{-1} showed markedly improved efficiency in antagonizing miR-122 in comparison with mice that were treated with either cholesterol-conjugated antagomir-122 (refs 6, 21) or a phosphorothiolated LNA-antimiR with only 30% LNA and lower affinity[22] (melting temperature 70 °C) using the same dosing regimen (Supplementary Fig. 3). This is consistent with a previous report in mice in which efficient miR-122 silencing by antagomir-122 required much higher doses: from three doses of 40 mg kg^{-1} to three doses of 80 mg kg^{-1} (ref. 21), whereas our findings show that LNA enables the design of highly substituted LNA-antimiR oligonucleotides that can mediate potent miR-122 antagonism *in vivo* at a considerably lower dose.

To validate this conclusion, we antagonized miR-122 in a diet-induced obesity mouse model by using two weekly intraperitoneal doses of 5 mg kg^{-1} LNA-antimiR for six weeks, which resulted in the efficient sequestration of mature miR-122 and a sustained decrease in total cholesterol by 30% without any elevations in hepatotoxicity markers in the serum or in lipid accumulation in the liver (Fig. 1d,

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e, and Supplementary Fig. 4). In contrast, treatment with either saline or LNA mismatch control did not affect the cholesterol levels, concurring with detection of the mature miR-122 by northern blots in both groups (Fig. 1d, e). The marked derepression of the miR-122 targets, *Aldoa* (Fig. 1f) and *Bckdk*[22] (data not shown), in LNA-antimiR-treated mice, but not in LNA-mismatch-treated mice, implies that antagonism of miR-122 *in vivo* by LNA-antimiR is specific. Consistent with this notion, clustering of the liver gene expression data revealed that all the LNA-antimiR-treated animals ($n = 5$ per group) had highly similar expression profiles as shown by a uniform cluster on the same main dendrogram branch, which was divergent from the saline and LNA mismatch control groups (Fig. 1g). Antagonism or ectopic expression of a miRNA has previously been shown to result in an increase or decrease in mRNAs, which show enrichment of miRNA seed matches in the 3′ UTRs[6,7,22,23]. Indeed, correlation of the presence of miR-122 seed matches with expression changes confirmed that messages with seed matches to miR-122 tended to be derepressed in the LNA antimiR-treated animals compared with those in control mice (Fig. 1h; Kolmogorov–Smirnov test, $P = 2.4 \times 10^{-14}$ for 8-mer seed). This demonstrates that the liver mRNA changes in the LNA-antimiR-treated mice are due mainly to the silencing of miR-122.

To examine whether our LNA-antimiR approach could be used for miR-122 antagonism in non-human primates, we undertook an efficacy study in African green monkeys (*Chlorocebus aethiops*). Systemic administration of PBS-formulated LNA-antimiR in drug-naive female African green monkeys by three intravenous injections of doses ranging from 1 to 10 mg kg^{-1} ($n = 5$ per group) resulted in a dose-dependent and sustained decrease in total plasma cholesterol in primates (Fig. 2a and Supplementary Information), which is consistent with the cholesterol lowering observed in miR-122-antagonized mice. Animals that received the high-dose LNA-antimiR (three doses of 10 mg kg^{-1}) showed a maximum decrease in cholesterol of 40% at 23 days after treatment ($P = 0.001$), whereas the middle-dose group (three doses of 3 mg kg^{-1} LNA-antimiR) showed a 20% lowering of cholesterol ($P = 0.02$) at the same time point (Fig. 2a). Despite the observed fluctuations in total cholesterol levels over time, the effect on cholesterol lowering was clearly dose dependent, as shown by the cholesterol trend plots of each treatment group normalized to control monkeys (Fig. 2b). Northern blot analyses of RNA samples extracted from LNA-antimiR-treated monkey liver biopsies, performed 24 h after the last dose, confirmed miR-122 silencing as demonstrated by dose-dependent accumulation of the shifted LNA-antimiR•miR-122 heteroduplex and the depletion of mature miR-122 compared with PBS-treated control monkey samples (Fig. 2c). In addition, *in situ* hybridization in frozen monkey-liver biopsies showed accumulation of the LNA-antimiR in the liver sections of treated monkeys but not in PBS controls (Fig. 2d–g), whereas high-resolution *in situ* hybridization showed that the LNA-antimiR was localized primarily in the cytoplasm of primate hepatocytes (Fig. 2h).

LNA-mediated antagonism of miR-122 in primates was effective and long-lasting, as shown by a decrease in total plasma cholesterol for seven weeks ($P < 0.05$, two-sided *t*-test) in the LNA-antimiR high-dose group and for five weeks ($P < 0.05$) in the middle-dose group (Fig. 2b and Supplementary Information). The cholesterol

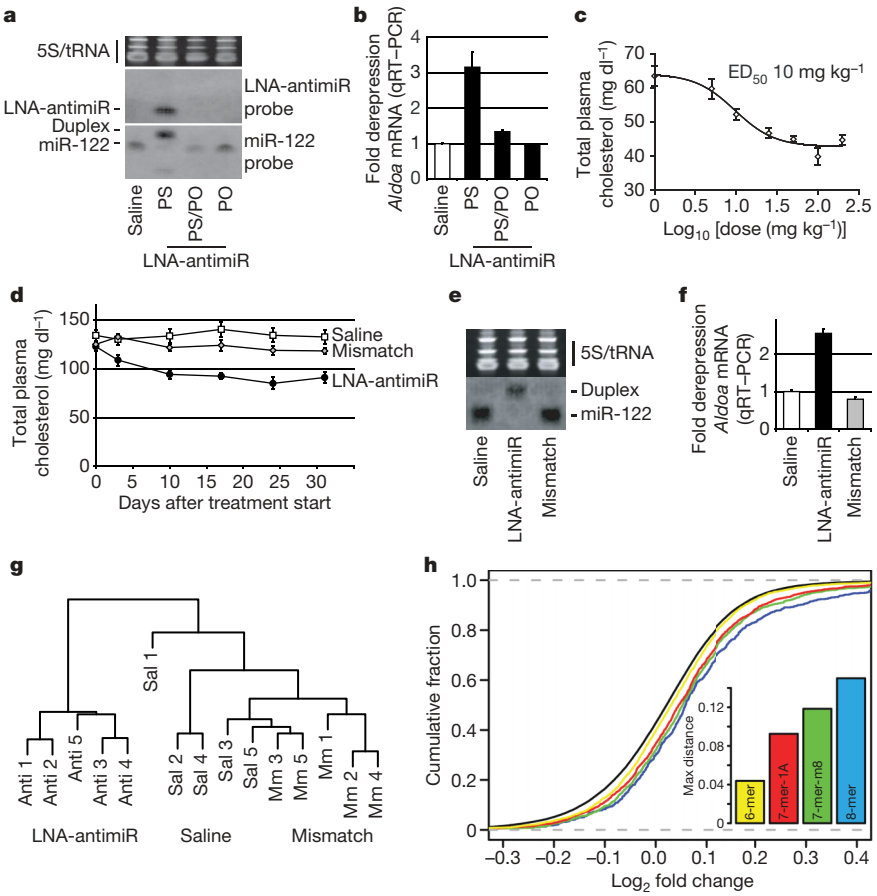

Figure 1 | Silencing of miR-122 function in normal and hypercholesterolaemic mice by LNA-antimiR. **a**, Northern blot of liver RNA from mice treated with LNA-antimiR with a complete phosphorothioate backbone (PS), a mixed phosphorothioate/phosphodiester backbone (PS/PO) or an unmodified phosphodiester (PO) backbone. The northern blot was probed for LNA-antimiR and re-probed for miR-122. **b**, Derepression of the direct miR-122 target *AldoA* (same samples as in **a**, normalized to glyceraldehyde-3-phosphate dehydrogenase; means and s.e.m., $n = 5$). **c**, Total plasma cholesterol in mice after treatment with single intraperitoneal doses of LNA-antimiR ranging from 1 to 200 mg kg^{-1} (means ± s.e.m., $n = 5$ except for 1 mg kg^{-1} LNA-antimiR ($n = 4$)). **d**, Total plasma cholesterol levels in hypercholesterolaemic mice treated with saline, LNA-antimiR or LNA mismatch control, respectively, at a dose of 5 mg kg^{-1} intraperitoneally twice weekly over a six-week period (means ± s.e.m., $n = 10$ except for saline ($n = 9$)). **e**, miR-122 northern blot (same mice as in **d**). **f**, Quantification of *AldoA* mRNA (same samples as in **e**; means and s.e.m., $n = 10$ except for saline ($n = 9$)). **g**, Unsupervised clustering of liver mRNA expression profiles (same mice as in **d**,). **h**, Expression changes of liver mRNAs in LNA-antimiR-treated mice relative to controls. mRNAs are grouped by the presence or absence of different types of canonical miR-122 seed matches in the 3′ UTR. Separation from mRNAs without seed matches (maximum distance from the black trace) is shown in the inset; this was significant for all types of site ($P = 1.4 \times 10^{-6}$, $P = 2.2 \times 10^{-13}$, $P < 10^{-15}$ and $P = 2.4 \times 10^{-14}$ (Kolmogorov–Smirnov test) for 6-mer, 7-mer-1A (6-mer site with an A across from nucleotide position 1 of miR-122), 7-mer-m8 (an exact match to nucleotide positions 2–8 of miR-122) and 8-mer sites, respectively).

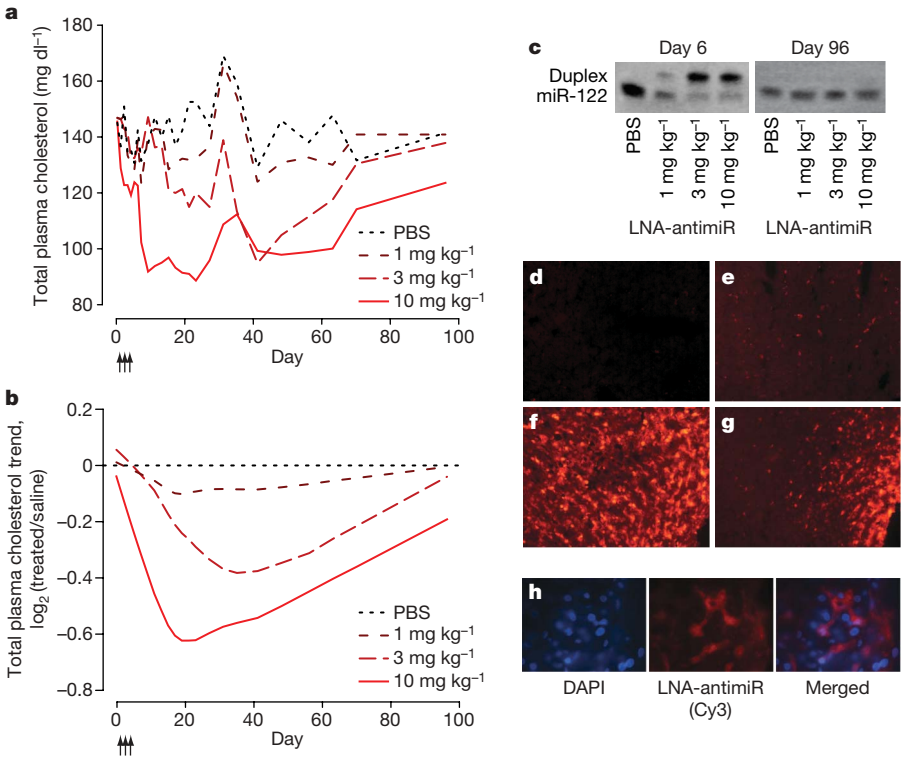

Figure 2 | Silencing of miR-122 in non-human primates by LNA-antimiR. **a**, Total plasma cholesterol levels in African green monkeys treated with LNA-antimiR or PBS by three i.v injections over five days (arrows) ($n = 5$ per group). **b**, Trend plots of the cholesterol data in **a**, normalized to the PBS control group, Lowess-smoothened and $\log_2$-transformed. **c**, Northern blot analysis of monkey liver RNA samples from liver biopsies performed on day 6 and 96. **d**–**g**, *In situ* detection of LNA-antimiR in liver biopsies on day 6 (same animals as in **c**): **d**, PBS; **e**, 1 mg kg^{-1} LNA-antimiR; **f**, 3 mg kg^{-1} LNA-antimiR; **g**, 10 mg kg^{-1} LNA-antimiR. **h**, Cytoplasmic localization of LNA-antimiR in hepatocytes (day 6, 10 mg kg^{-1} LNA-antimiR).

levels gradually returned towards baseline over a period of three months after treatment with LNA-antimiR, which is consistent with normalization of mature miR-122 levels and clearance of the LNA-antimiR compound from the liver as detected by northern blots and *in situ* hybridization, respectively, in a second set of monkey liver biopsies performed 96 days after initiation of LNA-antimiR treatment (Fig. 2c, and data not shown). Decreases in both high-density lipoprotein (HDL) and its major apolipoprotein, ApoA-I, as well as in low-density lipoprotein (LDL) and its principal apolipoprotein, ApoB, were detected in LNA-antimiR-treated monkeys (Supplementary Information), which concur with previous findings in miR-122-antagonized mice[5]. Although differences in the ApoA-I/ApoB ratios between the high-dose LNA-antimiR-treated and control animals (Supplementary Information) did not achieve statistical significance ($P > 0.05$, two-sided *t*-test on each day), the ratio seemed to be slightly lower in the high-dose group, suggesting a more pronounced effect on ApoA-I and HDL.

We observed no acute or subchronic toxicities in the LNA-antimiR-treated primates as shown by the clinical chemistries, which remained within normal limits for all measurements throughout the study in the treatment groups (Supplementary Information), with the exception of transient, liver-biopsy-associated spikes in creatine phosphokinase, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin. There were no changes in blood coagulation profiles associated with treatment with LNA-antimiR (Fig. 3a and Supplementary Information). Moreover, histopathology investigations of the liver biopsies revealed no treatment-correlated abnormalities in the LNA-antimiR-treated primates (Fig. 3b–e and Supplementary Information). To dissociate the effects of any liver-biopsy-associated toxicities in the safety evaluation of LNA-antimiR treatment, two additional non-biopsy groups ($n = 5$) treated either with the high dose of LNA-antimiR (three doses of 10 mg kg^{-1}) or PBS, respectively, were included in the primate study. We did not observe any hepatotoxicity or renal toxicity in these animals as demonstrated by the absence of increases in the levels of the plasma transaminases ALT and AST or of bilirubin, creatine phosphokinase or creatinine after treatment with three doses of 10 mg kg^{-1} LNA-antimiR in comparison with PBS controls (Fig. 3a

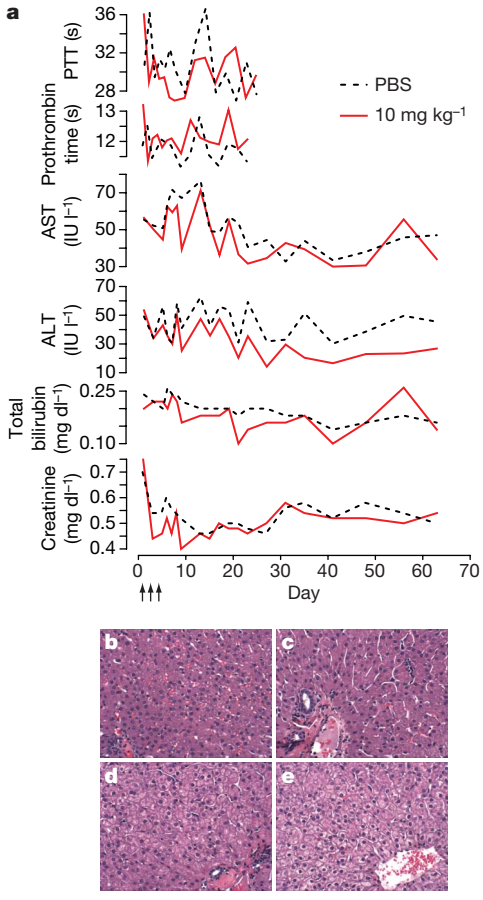

Figure 3 | LNA-mediated miR-122 silencing is safe in non-human primates. **a**, Partial thromboplastin time (PTT), prothrombin time, AST, ALT, total bilirubin and creatinine levels assessed in African green monkeys after treatment with PBS or three doses of 10 mg kg^{-1} LNA-antimiR. **b**–**e**, Photomicrographs of haematoxylin/eosin-stained sections from liver biopsies on day 6: **b**, PBS; **c**, 1 mg kg^{-1} LNA-antimiR; **d**, 3 mg kg^{-1} LNA-antimiR; **e**, 10 mg kg^{-1} LNA-antimiR.

and Supplementary Information). All study animals tolerated both the LNA-antimiR compound and sample collection procedures well, and all were in good health for at least ten months after treatment with LNA-antimiR.

Taken together, our results demonstrate potent antagonism of miR-122 by a simple delivery of a unconjugated high-affinity LNA-antimiR oligonucleotide in mice and non-human primates. The therapeutic value of antagonizing miR-122 was inferred in two species, in which treatment of hypercholesterolaemic mice with two weekly intraperitoneal injections of 5 mg kg^{-1} LNA-antimiR and treatment of African green monkeys with three intravenous injections of 3 or 10 mg kg^{-1} LNA-antimiR resulted in an effective and long-lasting decrease in plasma cholesterol without any evidence for LNA-associated toxicities. Although additional studies will be needed to optimize the dosing regimen and to assess the biodistribution and safety of LNA-antimiR compounds after long-term treatment, our findings highlight the utility of LNA-mediated silencing in studying miRNA function in rodents and primates as well as in the future development of miRNA-based therapeutics.

METHODS SUMMARY

The LNA-modified oligonucleotides were synthesized as unconjugated LNA/DNA mixmers with a complete phosphorothioate backbone. The 2′-*O*-methyl oligonucleotides for HCV replication assays and the antagomir-122 were synthesized as described[6,14]. Saline-formulated compounds were administered into normal and hypercholesterolaemic C57BL/6J mice by intraperitoneal injections, and blood samples were collected for measurements of cholesterol and serum transaminases. Liver samples were prepared for RNA extraction, miRNA quantification and liver histopathology. Microarray expression profiling of mouse liver RNAs was performed in accordance with standard Affymetrix protocols and the data were submitted to the ArrayExpress database. For data analysis, standard Bioconductor[24] packages were used. Transcript 3′ UTRs were searched for the presence of miR-122 seed matches[25] by using in-house Perl scripts. mRNA quantification was conducted with standard TaqMan assays (Applied Biosystems). Thirty female drug-naive young adult African green monkeys were assigned to six groups ($n = 5$ per group) and dosed once daily on days 1, 3 and 5 by intravenous infusions over about 10 min at a rate of 24 ml kg^{-1} h^{-1}. Four treatment groups received PBS or 1, 3 or 10 mg kg^{-1} PBS-formulated LNA-antimiR, all of which received liver biopsies, whereas two groups received PBS or 10 mg kg^{-1} PBS-formulated LNA-antimiR without liver biopsies. Blood samples were collected for clinical chemistry and haematology measurements. Total cholesterol was determined enzymatically in microtitre plates. Lipoprotein cholesterol distributions were determined by fast protein liquid chromatography and the apolipoprotein levels by enzyme-linked immunosorbent assay. *In situ* detection of LNA-antimiR was performed on frozen liver sections of LNA-antimiR-treated and control monkeys by using a FAM-labelled LNA probe and horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-fluorescein isothiocyanate antibodies (Dako) combined with Cyanine 3-Plus tyramide (Perkin-Elmer). Northern blot analyses of liver RNAs were performed with a 5′ FAM-labelled LNA-modified miR-122 probe and an anti-fluorescein–HRP antibody (NEF710; Perkin-Elmer) combined with the enhanced chemiluminescence advanced kit for detection (GE Healthcare Life Sciences).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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1. Ambros, V. The functions of animal microRNAs. *Nature* **431,** 350–355 (2004).
2. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116,** 281–297 (2004).
3. Kloosterman, W. P. & Plasterk, R. H. The diverse functions of microRNAs in animal development and disease. *Dev. Cell* **11,** 441–450 (2006).
4. Soifer, H. S., Rossi, J. J. & Saetrom, P. MicroRNAs in disease and potential therapeutic applications. *Mol. Ther.* **12,** 2070–2079 (2007).
5. Esau, C. *et al.* miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab.* **3,** 87–98 (2006).
6. Krutzfeldt, J. *et al.* Silencing of microRNAs *in vivo* with 'antagomirs'. *Nature* **438,** 685–689 (2005).
7. Grimson, A. *et al.* MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell* **27,** 91–105 (2007).
8. Alvarez-Garcia, I. & Miska, E. A. MicroRNA functions in animal development and human disease. *Development* **132,** 4653–4662 (2005).
9. Abelson, J. F. *et al.* Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science* **310,** 317–320 (2005).
10. Calin, G. A. & Croce, C. M. MicroRNA signatures in human cancers. *Nature Rev. Cancer* **6,** 857–866 (2006).
11. Eisenberg, I. *et al.* Distinctive patterns of microRNA expression in primary muscular disorders. *Proc. Natl Acad. Sci. USA* **104,** 17016–17021 (2007).
12. Esquela-Kerscher, A. & Slack, F. J. Oncomirs—microRNAs with a role in cancer. *Nature Rev. Cancer* **6,** 259–269 (2006).
13. He, L. *et al.* A microRNA polycistron as a potential human oncogene. *Nature* **435,** 828–833 (2005).
14. Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. & Sarnow, P. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* **309,** 1577–1581 (2005).
15. Lu, J. *et al.* MicroRNA expression profiles classify human cancers. *Nature* **435,** 834–838 (2005).
16. Pedersen, I. M. *et al.* Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* **449,** 919–922 (2007).
17. Triboulet, R. *et al.* Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315,** 1579–1582 (2007).
18. van Rooij, E. *et al.* Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316,** 575–579 (2007).
19. Yang, B. *et al.* The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. *Nature Med.* **13,** 486–491 (2007).
20. Randall, G. *et al.* Cellular cofactors affecting hepatitis C virus infection and replication. *Proc. Natl Acad. Sci. USA* **104,** 12884–12889 (2007).
21. Krutzfeldt, J. *et al.* Specificity, duplex degradation and subcellular localization of antagomirs. *Nucleic Acids Res.* **35,** 2885–2892 (2007).
22. Elmen, J. *et al.* Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res.* doi:10.1093/nar/gkm1113 (2007).
23. Lim, L. P. *et al.* Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433,** 769–773 (2005).
24. Gentleman, R. C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5,** R80.1–R80.16 (2004).
25. Lewis, B. P., Burge, C. B. & Bartel, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120,** 15–20 (2005).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.E. and M. Lindow contributed equally to this study. J.E., M. Lindow, M. Lawrence, U.B., S.O., M.H., A.P., S.S. and E.M.S. performed experiments and contributed data. H.F.H. performed the synthesis of all oligonucleotides. J.E., M. Lindow, M. Lawrence, M. Lindholm, H.F.H., P.K., S.G., P.S., E.M.S. and S.K. designed experiments and discussed the data. S.K. supervised the study and wrote the paper together with J.E. and M. Lindow.

Author Information The microarray data have been submitted to the ArrayExpress database under accession number E-MEXP-1406. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.K. (sk@santaris.com).

METHODS

Oligonucleotides. Unconjugated LNA-modified DNA oligonucleotides were synthesized with a complete phosphorothioate backbone, except for uptake studies where additional LNA oligonucleotides with a 50% phosphorothioate or a phosphodiester backbone were used. The sequence of the high-affinity LNA-antimiR was 5′-CcAttGTcaCaCtCC-3′, the LNA mismatch oligonucleotide used in mouse studies was 5′-CcAttCTcaCaCtGC-3′, and the LNA control used in the HCV replication assays was 5′-CcAttCTgaCcCtAC-3′ (LNA in capitals, DNA in lower case, capital C denotes LNA methylcytosine). The 2′-*O*-methyl oligonucleotides for HCV replication assays and the antagomir-122 were synthesized as described[6,14].

***In vivo* experiments.** C57BL/6J female mice were administered once on alternate days over a five-day period with saline or saline-formulated LNA-antimiR, antagomir-122 or LNA mismatch control, allowing the mice to receive an injection volume of 10 ml kg^{-1} with daily intraperitoneal doses ranging from 1 to 25 mg kg^{-1}. The mice were killed 48 h after treatment. Before killing, retro-orbital sinus blood was collected in EDTA-coated tubes followed by isolation of the plasma fraction and measurement of total cholesterol with ABX Pentra Cholesterol CP (Horiba ABX Diagnostics). In the mouse dose–response study, single intraperitoneal injections ranging from 1 to 200 mg kg^{-1} LNA-antimiR were administered and plasma cholesterol was measured six days after treatment. Diet-induced obesity mouse model was generated by feeding C57BL/6J female mice on a high-fat diet (D12492; Research Diets) for 13 weeks. Hypercholesterolaemic mice were treated with two weekly intraperitoneal doses of 5 mg kg^{-1} LNA-antimiR or LNA control for six weeks. Serum ALT and AST levels were determined with enzymatic assays (Horiba ABX Diagnostics).

Thirty female drug-naive young adult African green monkeys were assigned to six treatment groups ($n = 5$ per group) and dosed once daily on days 1, 3 and 5 by intravenous infusion over about 10 min at a rate of 24 ml kg^{-1} h^{-1} by means of a catheter inserted into the saphenous vein. Four groups, all of which received liver biopsies, were treated with PBS or 1, 3 or 10 mg kg^{-1} PBS-formulated LNA-antimiR, and two other groups received PBS or 10 mg kg^{-1} PBS-formulated LNA-antimiR without liver biopsies. The animals were sedated with ketamine (7.5 mg kg^{-1}) and xylazine (1.5 mg kg^{-1}) before and during dosing and at biopsy and phlebotomy time points. Percutaneous liver biopsies were performed 1 and 90 days after treatment to obtain two core biopsies from the right and left lobes. Half of each biopsy was immediately immersed in RNAlater (Qiagen), and the remaining biopsy was divided into samples for fixation in paraformaldehyde for haematoxylin/eosin staining and into cryopreservation for *in situ* analysis. Blood samples were obtained for the biopsied animals before and 24 h after treatment by means of superficial venipuncture, and additional blood samples were collected for all treatment groups throughout the study. Samplings were performed before feeding after a period of 12 h without access to food, to minimize dietary effects on cholesterol measurements.

Primate haematology, clinical chemistry and plasma lipid measurements and lipoprotein analysis. Haematology measurements were made by optical and mechanical methods and an automated cell counter; clinical chemistries were measured with a Hitachi 747 analysis system (Antech Diagnostics). Total plasma cholesterol was determined enzymatically in microtitre plates. Lipoprotein cholesterol distributions were determined by fast protein liquid chromatography and apolipoproteins with an enzyme-linked immunosorbent assay by M. Wilson.

***In situ* hybridization.** Detection of LNA-antimiR was performed on 10-μm primate liver cryosections. Slides were thawed, fixed in 4% paraformaldehyde for 10 min at room temperature (25 °C) and treated with acetic anhydride/triethanolamine followed by rinsing in PBS. Slides were pre-hybridized at 48 °C for 30 min in 50% formamide, 5 × SSC (standard saline citrate), 500 μg ml^{-1} yeast transfer RNA, 1 × Denhardt's solution. LNA-antimiR was detected with a complementary FAM-labelled LNA probe hybridized to liver sections for 30 min at 48 °C followed by three 10-min post-hybridization washes in 0.1 × SSC at 52 °C. After exposure for 10 min to 3% H_2O_2, slides were preincubated for 15 min with TN buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl) containing 1% blocking reagent (TNB buffer, TSA kit; Perkin-Elmer) and subsequently with polyclonal rabbit anti-fluorescein isothiocyanate antibodies conjugated to HRP (diluted 1:500 in TNB; Dako) for 30 min. Slides were rinsed with TN buffer containing 0.3% Triton X-100, and incubated with Cyanine 3-Plus tyramide (diluted 1:100 in amplification buffer; Perkin-Elmer). The slides were rinsed and mounted in Vectashield containing DAPI (Vector Laboratories) and analysed on a Leica epifluorescence microscope equipped with a charge-coupled device camera (Leica Microsystems) and NIS-Elements software.

Microarray expression profiling and miR-122 target site analysis. Liver RNAs from hypercholesterolaemic mice were labelled and hybridized to Affymetrix Mouse Genome 430 2.0 arrays in accordance with the manufacturer's instructions. The expresso function from the *affy*-package was used for low-level data analysis with rma-based background correction, quantile normalization and summarizing probe sets by Bioconductor's[24] implementation of the Li and Wong summary method. Expression profiles were subjected to hierarchical clustering with euclidean distance measure and Ward's agglomeration method. The array data were submitted to the ArrayExpress database. Affymetrix probe sets were mapped to Ensembl genes and transcripts by using Ensembl-Biomart. Transcript 3′ UTRs were searched for miR-122 seed matches[25] with the use of in-house Perl scripts. When genes had alternative 3′ UTRs, only the longest sequence was used.

Northern blot analysis and real-time RT–PCR. Trizol-extracted liver RNAs (10–15 μg per sample) were subjected to electrophoresis in 15% denaturing Novex TBE-urea polyacrylamide gels (Invitrogen), transferred to Zeta Probe plus membrane (Bio-Rad) and hybridized overnight with 5′ FAM-labelled LNA-modified miR-122 probe in ULTRAhyb-Oligo (Ambion) at 45 °C. The membranes were washed twice for 30 min in Low Stringency wash solution no. 1 (Ambion) at 45 °C, rinsed twice in PBST and blocked in ECL advanced blocking solution (GE Healthcare Life Sciences) for 1 h at room temperature, and then rinsed twice in PBST. An antifluorescein-HRP antibody (NEF710, diluted 1:1,000 in blocking solution; Perkin-Elmer) was incubated with the membrane for 1 h at room temperature, followed by rinsing twice in PBST, washing for 15 min and then three washes (5 min each) in PBST at 25 °C. The ECL advanced kit (GE Healthcare Life Sciences) was used for detection employing the VersaDoc imaging system (Bio-Rad). mRNA quantification was performed with TaqMan assays and a 7500 real-time PCR instrument (Applied Biosystems).