LETTERS

RNAi-mediated gene silencing in non-human primates

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The opportunity to harness the RNA interference (RNAi) pathway to silence disease-causing genes holds great promise for the development of therapeutics directed against targets that are otherwise not addressable with current medicines[1,2]. Although there are numerous examples of *in vivo* silencing of target genes after local delivery of small interfering RNAs (siRNAs)[3–5], there remain only a few reports of RNAi-mediated silencing in response to systemic delivery of siRNA[6–8], and there are no reports of systemic efficacy in non-rodent species. Here we show that siRNAs, when delivered systemically in a liposomal formulation, can silence the disease target apolipoprotein B (ApoB) in non-human primates. *APOB*-specific siRNAs were encapsulated in stable nucleic acid lipid particles (SNALP) and administered by intravenous injection to cynomolgus monkeys at doses of 1 or 2.5 mg kg^{-1}. A single siRNA injection resulted in dose-dependent silencing of *APOB* messenger RNA expression in the liver 48 h after administration, with maximal silencing of >90%. This silencing effect occurred as a result of *APOB* mRNA cleavage at precisely the site predicted for the RNAi mechanism. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and lasted for 11 days at the highest siRNA dose, thus demonstrating an immediate, potent and lasting biological effect of siRNA treatment. Our findings show clinically relevant RNAi-mediated gene silencing in non-human primates, supporting RNAi therapeutics as a potential new class of drugs.

ApoB is expressed predominantly in the liver and jejunum, and is an essential protein for the assembly and secretion of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), which are required for the transport and metabolism of cholesterol[9]. As a large, lipid-associated protein, ApoB is not accessible to targeting with conventional therapies, but it is a highly relevant and validated disease target. Elevated ApoB and LDL levels are correlated with increased risk of coronary artery disease, and inadequate control of LDL–cholesterol after acute coronary syndromes results in increased risk of recurrent cardiac events or death[10,11]. Approaches targeting ApoB with second-generation antisense oligonucleotides have progressed to pre-clinical and clinical studies[12]. Despite progress in the management of hypercholesterolaemia using HMG-CoA reductase inhibitors and other drugs that affect dietary cholesterol, there remains a significant need for new therapeutic approaches.

We have previously demonstrated silencing of *Apob* in rodents using cholesterol-conjugated siRNAs[6]. In the current study, we used a liposomal formulation of SNALP to evaluate systemic delivery of siRNA directed towards *APOB*. Preliminary evaluations were conducted in mice. Whereas administration of the *Apob*-specific siRNA siApoB-1, without formulation or chemical conjugation, at doses higher than 50 mg kg^{-1} was previously shown to have no

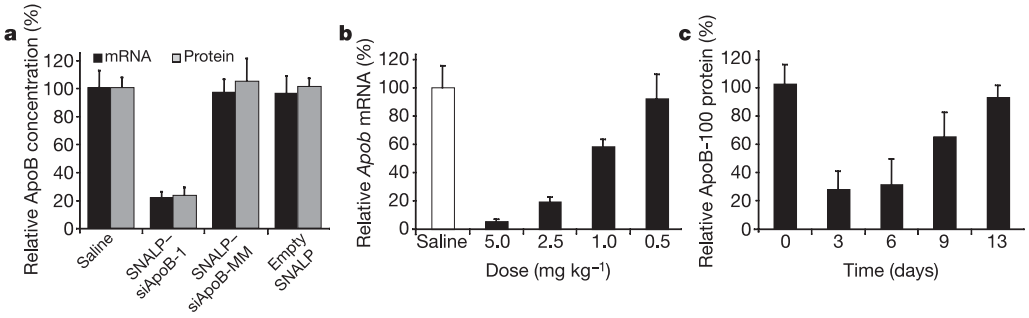

Figure 1 | SNALP–siRNA-mediated silencing of murine *Apob* is potent, specific, dose-dependent and long-lasting. a, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA and serum ApoB-100 protein levels measured two days after single i.v. injections of saline, SNALP–siApoB-1 (1 mg kg^{-1}), mismatched SNALP–siApoB-MM (1 mg kg^{-1}) or empty SNALP vesicles (25 mg kg^{-1}) ($n = 5$ per group). **b**, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA, assessed three days after i.v. administration of saline or 5, 2.5, 1 or 0.5 mg kg^{-1} SNALP–siApoB-2 ($n = 4$ per group). **c**, Serum ApoB-100 levels after i.v. administration of either saline or 2.5 mg kg^{-1} SNALP–siApoB-2 ($n = 6$ per group). Serum ApoB-100 levels for SNALP–siApoB-2-treated animals are relative to the saline-treated group for the same time point. Data show mean ± s.d.

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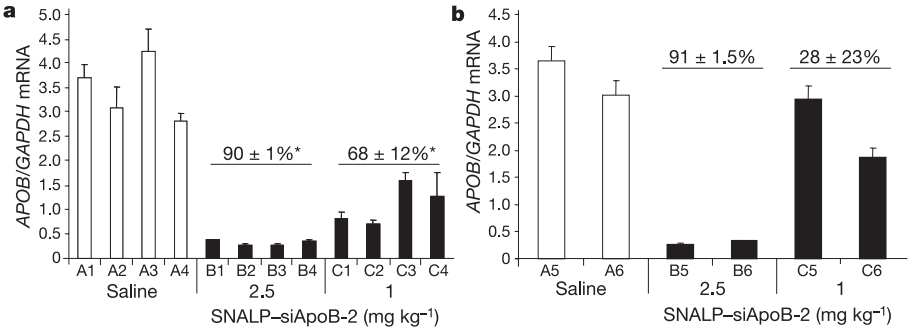

Figure 2 | Systemic silencing of *APOB* mRNA in non-human primates. **a**, **b**, Liver *APOB* mRNA levels for 12 biopsies (three isolated from each of four liver lobes) were quantified relative to *GAPDH* mRNA either 48 h (**a**, $n = 4$ animals per group) or 11 days (**b**, $n = 2$) after treatment with SNALP–siApoB-2. Data shown are mean *APOB*/*GAPDH* mRNA levels ± s.d. for each animal. Mean values (±s.d.) of the per cent *APOB* mRNA reduction relative to the saline treatment group are shown above each group. Asterisks indicate statistical significance compared with the saline-treated group ($P < 0.005$; ANOVA).

in vivo silencing activity[6], ~80% silencing of liver *Apob* mRNA and ApoB-100 protein was achieved with a single 1 mg kg^{-1} dose of SNALP-formulated siApoB-1 (Fig. 1a). In contrast, no detectable reduction was observed with a SNALP-formulated mismatched siRNA (siApoB-MM) or empty SNALP vesicles, indicating that silencing is specific to the siRNA and is not caused by the liposomal carrier. This silencing effect of SNALP-formulated siRNA represents more than a 100-fold improvement in potency compared with systemic administration of cholesterol-conjugated siApoB-1 (chol–siApoB-1) (Supplementary Fig. 1). Moreover, liposomal formulation of siRNA seems to be a general strategy for silencing hepatocyte targets, as demonstrated in mice for coagulation factor VII, green fluorescent protein and cyclophilin B (A.A., R. Constien and M.N.F., unpublished results).

As siApoB-1 was originally designed to be cross-reactive to both mouse and human ApoB genes, and we planned to conduct RNAi studies in non-human primates, a second ApoB-specific siRNA, siApoB-2, was designed to be cross-reactive with mouse, human and cynomolgus monkey ApoB genes. siApoB-2 was also selected on the basis of *in vitro* gene silencing activity and the absence of immunostimulatory activity (data not shown). Murine studies showed that encapsulated siApoB-2 showed a dose-dependent reduction in *Apob* mRNA, with >90% silencing achieved at the highest (5 mg kg^{-1}) dose (Fig. 1b). After a single 2.5 mg kg^{-1} dose of SNALP–siApoB-2, 80% silencing of liver *Apob* mRNA was associated with a 72% reduction in serum ApoB-100 protein. The silencing effect was detected for up to nine days, and was followed by recovery to normal protein levels by day 13 after treatment (Fig. 1c).

To address the therapeutic potential of this systemic RNAi approach, we evaluated the pharmacokinetics, efficacy and safety of SNALP-formulated siApoB-2 in cynomolgus monkeys. We first determined the circulating half-life of SNALP–siApoB-2 in plasma samples collected from cynomolgus monkeys ($n = 2$) receiving a single 2.5 mg kg^{-1} intravenous (i.v.) injection of the siRNA. An elimination half-life of 72 min was measured for the siRNA (Supplementary Fig. 2), compared with a 38-min half-life in mice (Supplementary Fig. 3a).

To evaluate efficacy, cynomolgus monkeys were treated with saline or SNALP-formulated siApoB-2 at doses of 1 or 2.5 mg kg^{-1} ($n = 6$ per group). siApoB-2 treatment was associated with a clear and statistically significant dose-dependent gene-silencing effect on cynomolgus liver *APOB* mRNA. Forty-eight hours after treatment, *APOB* mRNA was reduced by 68 ± 12% (mean ± s.d., $n = 4$, $P = 0.004$) and 90 ± 1% ($n = 4$, $P = 0.002$) for the 1 mg kg^{-1} and 2.5 mg kg^{-1} groups, respectively (Fig. 2a). Gene silencing was found to be consistent across the liver and correlated with detectable tissue levels of siApoB-2 (Supplementary Fig. 4). We also confirmed this *APOB* mRNA silencing to be mediated by RNAi, as demonstrated by 5′ rapid amplification of cDNA ends (RACE) analysis and identification of the predicted cleavage site, exactly ten nucleotides from the 5′ end of the antisense strand of siApoB-2 (Supplementary Fig. 5). Notably, *APOB* mRNA silencing was maintained for 11 days after the single 2.5 mg kg^{-1} treatment, with *APOB* mRNA levels still reduced by 91 ± 1.5% (Fig. 2b). Monkeys treated with the 1 mg kg^{-1} dose showed varying degrees of recovery from ApoB silencing at the day 11 time point. Although *APOB* mRNA was efficiently silenced in the liver, SNALP–siApoB-2 showed no silencing of *APOB* expressed in the jejunum (Supplementary Fig. 6), consistent with the absence of significant biodistribution of SNALP-formulated siRNAs to intestinal tissues in mice (Supplementary Fig. 3b).

The degree and persistence of RNAi-mediated silencing observed in cynomolgus monkeys far exceeds the results obtained with rodents. The lasting RNAi-mediated effects *in vivo* are consistent with observed long-lasting silencing by siRNAs in other studies[13,14], and the longer duration observed in primates may relate to species differences in the efficiency and stability of the RNA-induced

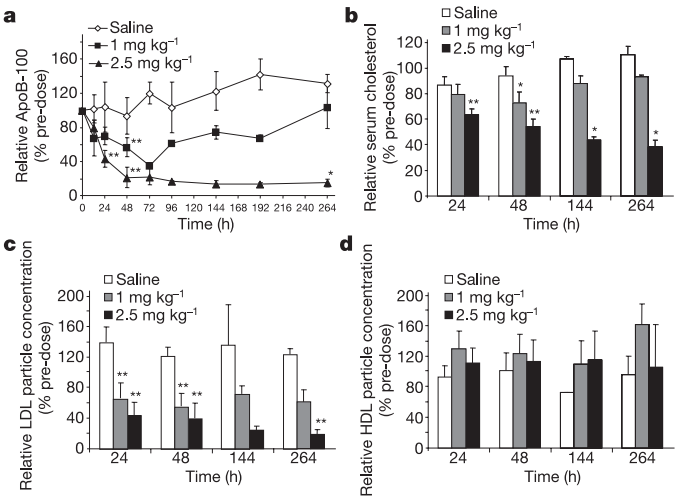

Figure 3 | Phenotypic effects of RNAi-mediated silencing of *APOB* mRNA in non-human primates. **a**–**d**, Serial plasma samples were obtained from cynomolgus monkeys treated with saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, and measured for ApoB-100 (**a**), total serum cholesterol (**b**), LDL (**c**) and HDL (**d**) levels. Data show levels as a percentage of pre-dose values and are expressed as mean ± s.d. Data sets collected at 0, 12, 24 and 48 h have a group size of six, and data sets collected at later time points have a group size of two. Data points marked with asterisks are statistically significant compared with saline-treated animals (*$P < 0.05$, **$P < 0.005$; ANOVA).

silencing complex (RISC), the mitotic state of hepatocytes and/or the tissue stability of the siRNA.

The expected biological effects resulting from *APOB* mRNA silencing include reduction in the blood levels of ApoB-100 protein, total cholesterol and LDL. To evaluate the kinetics of these downstream effects, we analysed plasma sampled serially from individual monkeys before and during the 11-day time course of the single-dose siApoB-2 study. Plasma ApoB-100 protein levels were reduced as early as 12 h after administration of 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, reaching nadirs of 35 ± 2% and 22 ± 9% of pre-treatment levels, respectively, 72 h after treatment (Fig. 3a). Animals that received the higher siRNA dose maintained a marked reduction in ApoB protein between 2 and 11 days after treatment, consistent with the lasting effect on mRNA silencing. Monkeys that received the lower siRNA dose showed an intermediate degree of ApoB protein reduction that returned to pre-dose levels by day 11, consistent with the observed recovery in *APOB* mRNA.

Serum cholesterol levels were similarly reduced, in a dose-dependent manner and with comparable kinetics (Fig. 3b). The maximum cholesterol reduction of 62 ± 5.5% ($n = 2$, $P = 0.006$) observed for the high dose siRNA group would be considered clinically significant for patients with hypercholesterolaemia, and exceeds levels of cholesterol reduction reported clinically for currently approved cholesterol-lowering drugs.

Administration of SNALP–siApoB-2 also resulted in dramatic and rapid dose-dependent reduction in the ApoB-containing lipoprotein particle LDL. Reduction in LDL relative to pre-dose levels was observed as early as 24 h after treatment for both doses of SNALP–siApoB-2 (Fig. 3c). In contrast, there were no significant changes in circulating levels of the non-ApoB-containing high-density lipoprotein particle (HDL, Fig. 3d). The reduction in LDL persisted over the 11-day study for both siApoB-2 treatment groups, with a maximum 82 ± 7% decrease compared to pre-treatment levels observed for the high-dose group at day 11 ($n = 2$, $P = 0.003$). The time required for the biological effects to return to pre-dose levels was not determined for the high-dose group because the endpoint for this study was defined using rodent data, which indicated a faster rate of recovery. The rapid onset and lasting effect on lipoprotein metabolism suggest that siRNAs targeting *APOB* may be a valuable therapeutic strategy for achieving plaque stabilization in acute coronary syndromes[10,11], as HMG-CoA reductase inhibitors can require up to 4–6 weeks to have the desired clinical effects[15].

An important consideration for the therapeutic application of siRNA relates to its general safety, as well as to the safety profile associated with specific delivery technologies. General tolerability as well as specific toxicities (such as activation of complement, coagulation and cytokines) were evaluated for all monkeys in this study. We observed no treatment-related effects on the appearance or behaviour of animals treated with SNALP–siApoB-2 compared with saline-treated animals. There was no evidence for complement activation, delayed coagulation, pro-inflammatory cytokine production (Supplementary Table 1) or changes in haematology parameters (data not shown), toxicities that have been observed previously with treatments using related approaches[16–19]. Across a systematic evaluation, the only detected change in primates treated with SNALP–siApoB-2 was a transient increase in liver enzymes in monkeys that received the high dose of SNALP–siApoB-2. The observed transaminosis peaked 48 h after treatment and was highly variable across individual animals. These effects, which were observed only at the highest dose of SNALP–siApoB-2, were completely reversible, with normalization by day 6 notwithstanding continued biological efficacy.

Our study highlights the potential for therapeutic gene silencing using systemic RNAi in non-human primates. A single, low dose of *APOB*-specific siRNA resulted in rapid and lasting RNAi-mediated gene silencing, with associated and profound phenotypic changes. The study was limited by the premature termination of the protocol after 11 days, which prevented full evaluation of the time course for RNAi-mediated effects. Although further optimization of treatment regimen and safety profile characterization may be required, our data suggest that systemic delivery of siRNAs for targeting hepatocyte-specific genes in a higher species is possible. Furthermore, the rapid and long-lasting silencing of *APOB* using RNAi may represent a new strategy for reducing LDL–cholesterol in several relevant clinical settings.

METHODS

Additional details of the methods used are provided in the Supplementary Information.

siRNA formulation. The SNALP formulation contained the lipids 3-*N*-[(ω-methoxypoly(ethylene glycol)$_{2000}$)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio.

***In vivo* experiments.** Saline and siRNA preparations were administered by tail vein injection under normal pressure and low volume (0.01 ml g^{-1}) for all rodent experiments. Cynomolgus monkeys ($n = 6$ per group) received either 2 ml kg^{-1} phosphate buffered saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2 at a dose volume of 1.25 ml kg^{-1} as bolus i.v. injections via the saphenous vein. For mRNA measurements, three liver biopsies per lobe were collected 48 h ($n = 4$) or 264 h ($n = 2$) after siRNA administration.

Bioanalytical methods. The QuantiGene assay (Genospectra) was used to quantify reduction in *APOB* mRNA levels relative to the housekeeping gene *GAPDH* in lysates prepared from mouse liver or cynomolgus monkey liver and jejunum as previously described[6] but with minor variations. Mouse[6] and cynomolgus monkey ApoB-100 protein levels were quantified by enzyme-linked immunosorbent assay (ELISA). LDL and HDL lipoprotein content were determined for plasma samples (250 μl) as described previously[6].

Statistical analysis. *P*-values were calculated for comparison of SNALP–siApoB-2-treated animals with saline-treated animals using analysis of variance (ANOVA, two-factor without replication) with an alpha value of 0.05. *P*-values less than 0.05 were considered significant.

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

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