Targeted Delivery of RNAi Therapeutics With Endogenous and Exogenous Ligand-Based Mechanisms

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Lipid nanoparticles (LNPs) have proven to be highly efficient carriers of short-interfering RNAs (siRNAs) to hepatocytes *in vivo*; however, the precise mechanism by which this efficient delivery occurs has yet to be elucidated. We found that apolipoprotein E (apoE), which plays a major role in the clearance and hepatocellular uptake of physiological lipoproteins, also acts as an endogenous targeting ligand for ionizable LNPs (iLNPs), but not cationic LNPs (cLNPs). The role of apoE was investigated using both *in vitro* studies employing recombinant apoE and *in vivo* studies in wild-type and *apoE*$^{-/-}$ mice. Receptor dependence was explored *in vitro* and *in vivo* using low-density lipoprotein receptor (*LDLR*$^{-/-}$)–deficient mice. As an alternative to endogenous apoE-based targeting, we developed a targeting approach using an exogenous ligand containing a multivalent *N*-acetylgalactosamine (GalNAc)-cluster, which binds with high affinity to the asialoglycoprotein receptor (ASGPR) expressed on hepatocytes. Both apoE-based endogenous and GalNAc-based exogenous targeting appear to be highly effective strategies for the delivery of iLNPs to liver.

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INTRODUCTION

RNA interference (RNAi) therapeutics are an emerging class of innovative medicines that offer the potential to potently and specifically reduce the expression of disease-causing genes, including those which are currently considered "undruggable" with small molecule or monoclonal antibody modalities. The key to fulfilling this potential is the safe and efficacious delivery of short-interfering RNAs (siRNAs), the molecules that mediate RNAi, to specific cell types and tissues. In recent years, significant progress has been made to overcome some of the obstacles associated with *in vivo* delivery of siRNA, and lipid nanoparticles (LNPs) represent one of the most advanced technological platforms.[1] Earlier work has established that siRNAs formulated in LNPs can successfully silence therapeutically relevant genes in nonhuman primates,[2,3] and a class of LNPs, termed "stable nucleic acid lipid particles", is being utilized in multiple liver-targeted RNAi programs in human clinical trials. More recently, we reported the discovery of novel ionizable[4] and cationic[5] lipids with significantly improved activity for next generation LNP-siRNA delivery systems. The discovery of both ionizable and cationic classes of lipids that mediate efficient LNP delivery of siRNA warrants the distinction of ionizable LNPs (iLNPs) and cationic LNPs (cLNPs).

The relative success of LNPs for liver delivery of siRNA molecules can, at least in part, be attributed to the favorable physiology of the liver, a well-perfused organ with a fenestrated endothelium. On the other hand, LNPs are known to interact with serum proteins, exchanging components and acquiring proteins in circulation that can potentially direct LNPs to specific cell types.[6,7] Apolipoproteins, for instance, were found to be generally adsorbed onto neutral liposomes, but only apolipoprotein E (apoE) was capable of enhancing uptake into hepatoma cells and primary hepatocytes.[8] More recently, Yan *et al.* have described the role of apoE in the clearance of neutral liposomes by hepatocytes *in vivo*.[9] ApoE is found on chylomicrons, very-low-density lipoproteins, and high-density lipoproteins and plays a major role in the clearance of very-low-density lipoproteins and chylomicron remnants by hepatocytes.[10,11] Multiple receptors have been associated with ApoE-mediated uptake, most notably low-density lipoprotein (LDL) receptor (LDLR), but also numerous other LDLR family members and scavenger receptor BI, which are also expressed on the surface of hepatocytes.[11–14]

Many of the most advanced LNP systems under development for delivery of siRNA, including stable nucleic acid lipid particle, contain ionizable cationic lipids. The weak basicity of the lipid headgroups affects the surface charge of the particles in a pH-dependent manner, rendering them positively charged at acidic pH but close to charge-neutral at physiologic pH. Hence, they have the potential to behave as neutral liposomes in circulation,

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acquiring apoE[9] and delivering siRNA to hepatocytes in a targeted manner (referred to as "endogenous targeting"). Alternatively, targeting may be achieved by the specific incorporation of a targeting ligand in the LNP (referred to as "exogenous targeting"). Exogenous targeting approaches have been widely used in the drug delivery field, both to improve drug potency and specificity. Specifically in the RNAi field, small molecules,[15,16] peptides,[17,18] proteins,[19] and antibodies[20] have been used to target LNPs to immune cells, tumor cells, and hepatocytes *in vivo*. In the case of hepatocyte targeting, one of the most studied and best characterized receptors is the asialoglycoprotein receptor (ASGPR), which was first identified by Ashwell and Morell[21,22] and is known to recognize terminal galactose or lactose residues.

In this study, we explored both endogenous and exogenous targeting strategies for the delivery of siRNA to liver by LNP delivery systems. The role of apoE in the activity of LNP delivery systems was studied to determine whether apoE is indeed responsible for the specific and potent delivery of siRNA to hepatocytes by iLNPs. The establishment of ligand-based targeting as being responsible for the potent liver delivery observed with iLNPs opens up two possibilities. First, it may be possible to use exogenously targeted iLNPs to deliver siRNA to liver through a potentially more favorable ligand-receptor interaction. Second, it may be possible to extend the clinical utility of iLNPs outside of liver applications through the use of exogenous targeting ligands specific for nonhepatic cell types. As proof-of-concept and as an alternative to an apoE-dependent approach, we employed a novel high-affinity *N*-acetylgalactosamine (GalNAc)–PEG–lipid for exogenous targeting *via* the ASGPR. iLNPs incorporating this lipid were developed and evaluated for their ability to deliver siRNA to hepatocytes in a ligand-dependent fashion. Accordingly, both endogenous and exogenous targeting mechanisms have been delineated for delivery of RNAi therapeutics, and represent novel strategies for the advancement of this promising approach to patients.

RESULTS

ApoE enhances uptake of and gene silencing by iLNPs *in vitro*

We conducted a series of uptake experiments to study whether iLNPs may behave similar to neutral LNPs in their ability to acquire apoE, resulting in enhanced apoE-directed cellular uptake. HeLa cells were used in these studies as they express receptors for apoE, but do not express and secrete apoE into the media, allowing for clear interpretation of the results. Fluorescently labeled siRNA was formulated in either iLNPs[4] or as control, cLNPs[23] and incubated with HeLa cells under different media conditions: serum-free media, media, and media+apoE. Visualization was performed using automated confocal microscopy. Consistent with prior literature on neutral liposomes,[8,9] apoE dramatically enhanced the cellular uptake of siRNA formulated in the iLNP, but had no

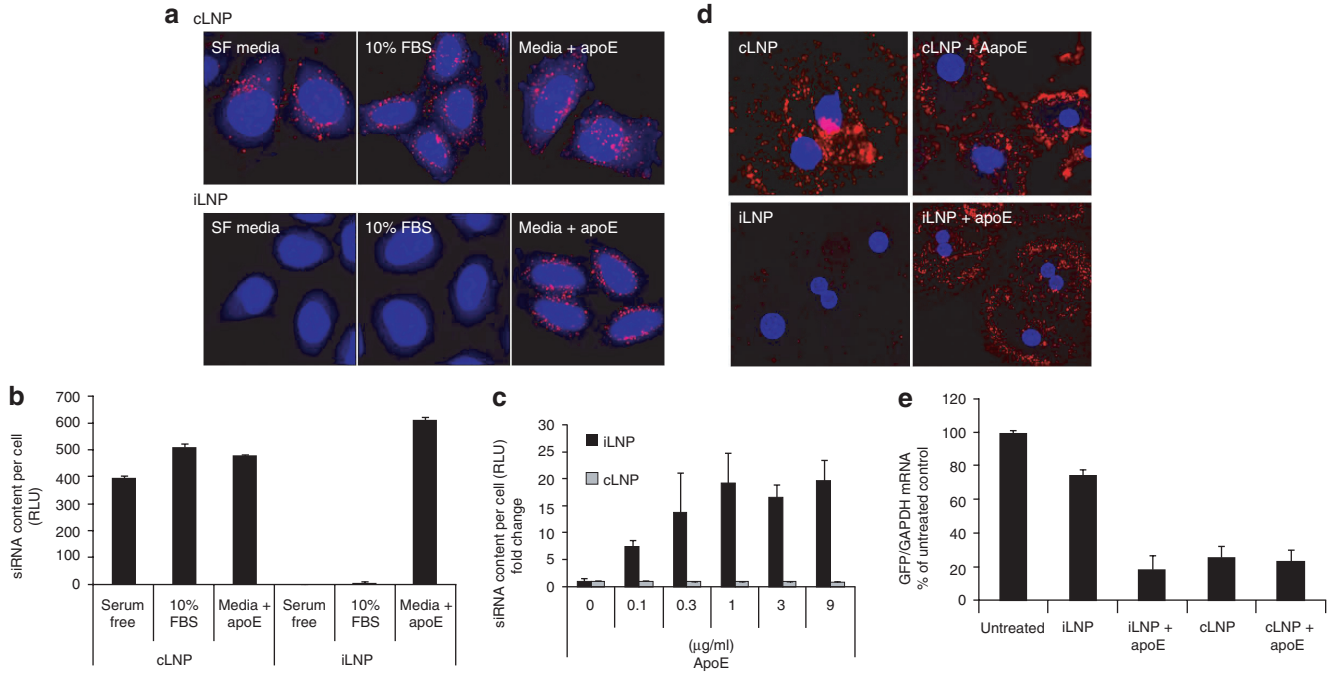

Figure 1 iLNPs but not cLNPs are dependent on apoE for cellular uptake and silencing *in vitro*. (**a**) Alexa-Fluor 647–labeled siRNA formulated in iLNPs or cLNPs were added to HeLa cells at 20 nmol/l and incubated for 4 hours at 37 °C under different media conditions: serum-free media, media with 10% FBS, or full media supplemented with 1 μg/ml of apoE. Cells were washed, fixed, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) then viewed by automated confocal microscopy. (**b**) Quantitation of cellular uptake. (**c**) Enhancement in cellular uptake achieved by preassociating increasing amounts of apoE with iLNPs or cLNPs. Data are expressed as fold increase over LNP without apoE. (**d**) cLNPs or iLNPs formulated with an Alexa-Fluor 647–labeled siRNA were incubated with primary hepatoctyes in the absence or presence of apoE for 4 hours. Cells were washed, fixed, and counterstained with DAPI, then viewed by automated confocal microscopy. (**e**) Silencing of GFP in HeLa-GFP cells following treatment of cells with iLNP and cLNP in the absence or presence of apoE. ApoE, apolipoprotein E; cLNP, cationic LNP; iLNP, ionizable LNP; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; LNP, lipid nanoparticle; RLU, relative light units; siRNA, short-interfering RNA.

effect on the uptake of cLNP (**Figure 1a,b**). Because apoE3 is the dominant isoform in humans, recombinant human apoE3 was used in these studies. However, the E2 and E4 isoforms were also tested and found to have similar activity (**Supplementary Materials and Methods** and **Supplementary Figure S1**). Complementary uptake studies were performed with fluorescently labeled lipid instead of siRNA and identical results were obtained, indicating that apoE mediates the uptake of the intact iLNP (**Supplementary Materials and Methods** and **Supplementary Figure S2**). The observed enhancement of cellular uptake of the iLNPs mediated by apoE was found to be dose dependent. Cellular uptake relative to that in the absence of apoE was increased ~6- and 14-fold at 0.1 and 0.3 µg/ml apoE, saturating to ~20-fold at 1 µg/ml apoE (**Figure 1c**). Consistent with earlier results, the level of uptake of the cLNP was unchanged with increasing apoE in the media.

In order to verify these findings in liver cells, similar uptake studies were conducted using primary hepatocytes. Although primary hepatocytes are known to express and secrete apoE into culture medium, apoE supplementation was found to enhance the cellular uptake of iLNPs, but not cLNPs (**Figure 1d**). These results validated the findings in HeLa cells and suggested that apoE-based targeting of siRNA-containing iLNPs to hepatocytes was possible. Finally, we determined the extent to which enhanced cellular uptake mediated by apoE translated into enhanced gene silencing. iLNPs and cLNPs containing formulated GFP-targeting siRNA were incubated with GFP-expressing HeLa cells in the presence or absence of apoE. GFP silencing was found to be independent of apoE for cLNP (~75% silencing), whereas it was strongly enhanced by apoE (~80% versus 20%) for iLNP (**Figure 1e**). These results indicate that apoE is a potent enhancer of the uptake and silencing activity of iLNPs in cultured cells *in vitro*.

In vivo activity of iLNP is ApoE dependent

We next tested whether the *in vivo* activity of iLNPs determined *in vitro* was similarly dependent on the presence of apoE *in vivo*. To study this, we took advantage of the mouse factor VII gene-silencing model.[24] Factor VII–targeting siRNA (siFVII) was formulated in iLNP and administered to wild-type and $apoE^{-/-}$ mice at 0.022, 0.067, and 0.2 mg/kg *via* tail-vein injection. As a control, siFVII was also formulated in cLNP and administered at 5 mg/kg in both wild-type and $apoE^{-/-}$ mice. The lower intrinsic *in vivo* potency of the cLNP used in this experiment required this higher dose to obtain comparable silencing activity as with the highly potent iLNP formulation. The iLNP mediated a potent, dose-dependent silencing of factor VII in the wild-type mice with >50% silencing at the lowest dose level tested (**Figure 2a**). However, at the same dose levels, the activity of this formulation was essentially abolished in the $apoE^{-/-}$ mice. In contrast, the cLNP preparation demonstrated identical levels of RNAi activity in both wild-type and $apoE^{-/-}$ mice. Earlier studies utilizing nontargeting control siRNAs have established that the *in vivo* gene silencing observed with the iLNP and cLNP formulations is siRNA sequence specific.[4,23,24]

To establish that the loss of activity observed for iLNP in the $apoE^{-/-}$ mice was due to the absence of apoE as an endogenous targeting ligand rather than other differences between the wild-type and $apoE^{-/-}$ strains, we tested whether the activity

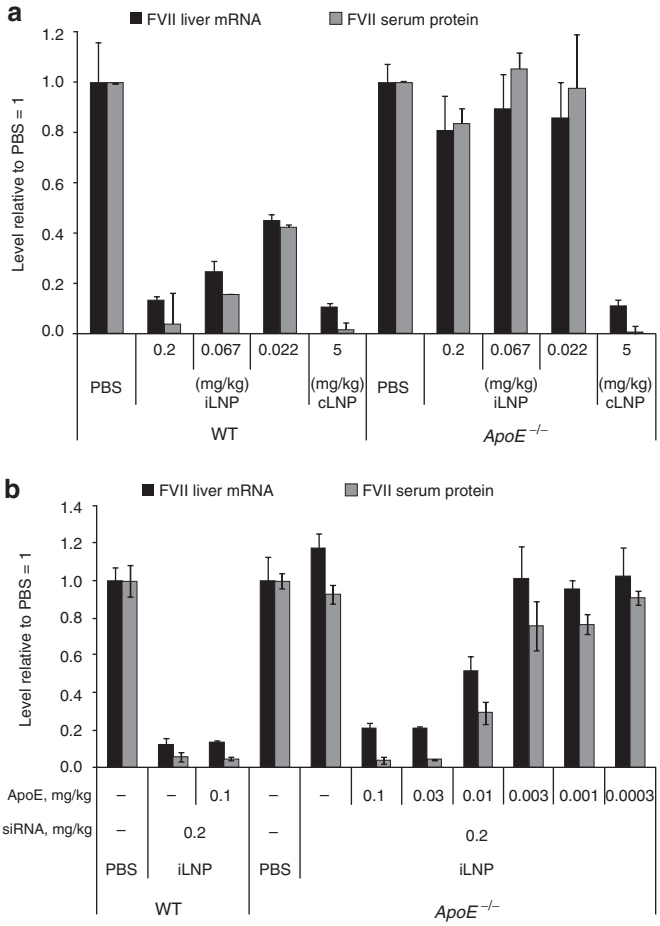

Figure 2 iLNP activity *in vivo* is dependent on apoE. (**a**) *In vivo* factor VII gene-silencing activity of iLNPs and cLNPs in wild-type and $apoE^{-/-}$ mice. FVII siRNA formulated in iLNPs or cLNPs were administered via bolus tail-vein injection to wild-type or $apoE^{-/-}$ mice. Animals were killed at 48 hours postadministration and serum and livers were collected. Serum factor VII protein and liver factor VII mRNA levels were determined. Data points are expressed as a percentage of PBS control animals and represent group mean (n = 5) ± SD. (**b**) Rescue of iLNP activity in $apoE^{-/-}$ mice using exogenous apoE. iLNPs containing FVII siRNA were premixed with increased amounts of recombinant human apoE protein and incubated overnight at 4 °C. The next day, iLNPs were administered to $apoE^{-/-}$ mice at a constant siRNA dose of 0.2 mg/kg. Control animals received PBS or iLNPs without apoE protein. For comparison, wild-type mice received either PBS, iLNP, or iLNP associated with the highest concentration of apoE protein. Data points are expressed as a percentage of PBS control animals and represent group mean (n = 5) ± SD. ApoE, apolipoprotein E; cLNP, cationic LNP; FVII, factor VII; iLNP, ionizable LNP; LNP, lipid nanoparticle; PBS, phosphate-buffered saline; siRNA, short-interfering RNA; WT, wild type.

of the iLNP in the $apoE^{-/-}$ mice could be rescued by premixing recombinant human apoE with the formulation before administration. In these experiments, $apoE^{-/-}$ mice received 0.2 mg/kg factor VII siRNA formulated in iLNP, which was first premixed with increasing amounts of recombinant human apoE, ranging from 0.0003 to 0.1 mg/kg. Silencing activity of the iLNP was found to be efficiently rescued in the $apoE^{-/-}$ mice in an apoE dose-dependent manner, with maximal activity achieved at 0.03 mg/kg apoE (**Figure 2b**). In this study, apoE was incubated with the LNPs overnight at 4 °C before administration.

iLNP activity is reduced in *LDLR*$^{-/-}$ primary hepatocytes and mice

Having demonstrated the importance of the endogenous ligand apoE for iLNP activity, we turned our attention to the role of hepatic receptors in the apoE-mediated uptake of iLNPs. Among the hepatic receptors for apoE, the most widely studied is the LDLR. Confocal fluorescence microscopy studies were conducted to examine differences in uptake of LNPs in primary hepatocytes from both wild-type and *LDLR*$^{-/-}$ mice. Relative to wild-type primary hepatocytes, the level of uptake for iLNPs was diminished in *LDLR*$^{-/-}$ primary hepatocytes, and a substantial fraction of labeled iLNPs was found to concentrate on the plasma membrane of the *LDLR*$^{-/-}$ cells, suggesting that internalization of the iLNPs was significantly inhibited (**Figure 3a**). Next, the dependence of iLNPs on the LDLR *in vivo* was studied by comparing silencing activity in both wild-type and *LDLR*$^{-/-}$ mice. Animals received either saline or factor VII siRNA formulated in iLNPs at 0.01, 0.03, and 0.1 mg/kg. Consistent with *in vitro* results demonstrating impaired uptake in *LDLR*$^{-/-}$ hepatocytes, *in vivo* silencing activity of the iLNP was greatly diminished in the *LDLR*$^{-/-}$ mice (**Figure 3b**).

An exogenous targeting ligand, GalNAc, can also rescue activity in *apoE*$^{-/-}$ mice

As exogenous apoE addition was able to rescue activity of the iLNP in *apoE*$^{-/-}$ mice, we hypothesized that the specific incorporation of other hepatocyte-targeting ligands into the iLNP formulation could serve a similar role. Hence, a trivalent GalNAc ligand was designed, synthesized, and incorporated into the iLNP formulation as a PEG–lipid (**Figure 4a**). The GalNAc moiety was conjugated to the distal end of a 2,000 MW polyethylene glycol (PEG) utilizing a distearyl (C18) lipid (GalNAc–PEG–DSG) providing a stable hydrophobic anchor for the targeted PEG–lipid to the iLNP. The unmodified PEG–lipid typically used for the formulation of LNPs contains shorter dimyristyl (C14) chains (PEG–DMG) to ensure rapid removal of the PEG shield during circulation.

The binding activity of GalNAc–iLNPs, prepared with GalNAc–PEG–DSG incorporation levels ranging from 0.005 to 0.5 mol%, was investigated using a cell-free receptor competition assay. The commercially available dendritic cell ASGPR (CD301) was used in the assay. In all cases, the total PEG–lipid in the formulation was kept constant at 1.5 mol%. When plotted as a function of siRNA dose, formulations with higher levels of GalNAc–PEG–DSG were found to be more potent competitors (**Figure 4b**). However, because GalNAc is the binding partner for ASGPR, when the data are plotted as a function of GalNAc–PEG–DSG concentration, all GalNAc–iLNP data fit a single binding competition curve (**Figure 4c**). Importantly, binding was found to be GalNAc-dependent, with no appreciable binding detected for the nontargeted control iLNP.

Next, factor VII siRNA was formulated in the same GalNAc–iLNP compositions described above. These formulations, along with the untargeted control, were administered to *apoE*$^{-/-}$ mice at an siRNA dose level of 0.2 mg/kg. Similar to the result obtained with apoE premixing, a GalNAc dose-dependent increase in silencing activity was observed, with near maximal effect occurring at 0.15 mol% GalNAc–PEG–DSG (**Figure 5a**). The potency of the 0.5 mol% GalNAc–iLNP was next examined by dosing

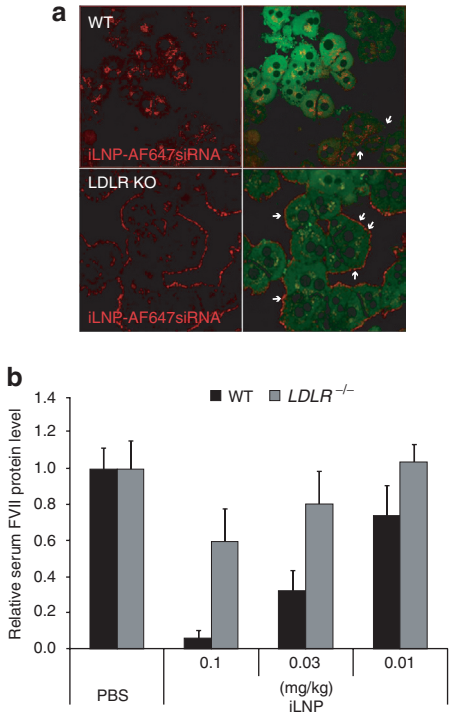

Figure 3 Loss of LDLR impairs iLNP activity *in vitro* and *in vivo*. (**a**) Primary hepatocytes were isolated from wild-type or *LDLR*$^{-/-}$ mice. Cells were incubated with Alexa-Fluor 647–labeled siRNA formulated in iLNPs in the presence of apoE for 4 hours. Arrows indicate iLNPs containing labeled siRNA which appear to accumulate at the plasma membrane and fail to enter cells efficiently in *LDLR*$^{-/-}$ hepatocytes. (**b**) Factor VII gene-silencing activity of iLNPs in wild-type and *LDLR*$^{-/-}$ mice. FVII siRNA formulated in iLNPs were administered *via* bolus tail-vein injection. No exogenous apoE was added to the formulations. Animals were killed at 48 hours postadministration and serum samples were collected and analyzed for serum factor VII protein levels. Data points are expressed as a percentage of PBS control animals and represent group mean (n = 5) ± SD. FVII, factor VII; iLNP, ionizable LNP; LDLR, low-density lipoprotein receptor; LNP, lipid nanoparticle; PBS, phosphate-buffered saline; siRNA, short-interfering RNA; WT, wild type.

apoE$^{-/-}$ mice at 0.006, 0.02, 0.06, and 0.2 mg/kg. Indeed, the formulation was found to have an ED_{50} ~0.02 mg/kg, which was remarkably comparable to the potency of both the native iLNP in wild-type mice and the native iLNP preassociated with apoE in the *apoE*$^{-/-}$ mice (**Figures 5b** and **2a**). Similarly, to verify a reduced dependence on the receptors for apoE, the potency of the 0.5 mol% GalNAc–iLNP was tested in *LDLR*$^{-/-}$ mice. Both wild-type and *LDLR*$^{-/-}$ mice were administered siFVII formulated in GalNAc–iLNP at a dose of 0.01, 0.03, and 0.1 mg/kg. In contrast to the native iLNP, the potency of GalNAc–iLNP was unchanged in the *LDLR*$^{-/-}$ mice (**Figures 5c** and **3b**).

Inactivation of endogenous ApoE-mediated targeting mechanism and development of GalNAc-specific iLNP

Although the GalNAc-targeted iLNPs described above provided significantly improved activity in the *apoE*$^{-/-}$ mice, they showed no improvement in activity in wild-type mice (**Figures 5c** and **3b**). This is presumably due to the fact that apoE plasma levels are not limiting and that an endogenous apoE-based targeting mechanism is sufficiently functional in wild-type animals. In order to

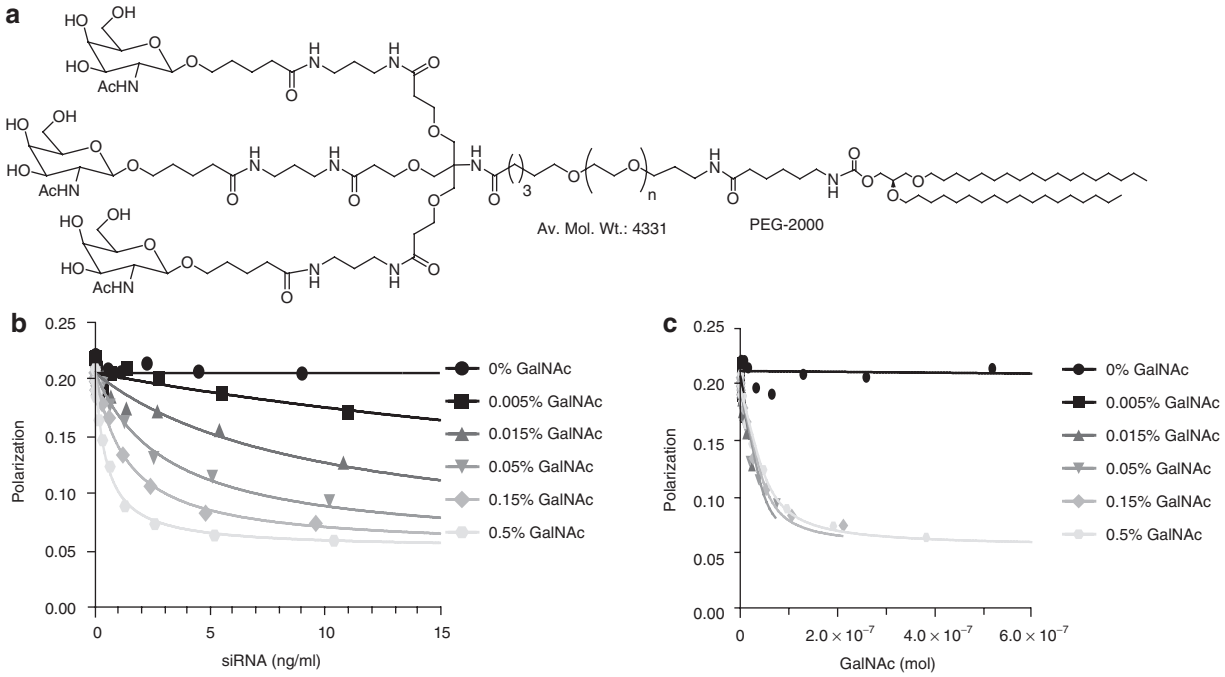

Figure 4 Receptor binding activity of GalNAc–iLNPs. (**a**) Structure of GalNAc–PEG–DSG. (**b**) Cell-free receptor competition-binding activity of iLNPs containing 0–0.5 mol% GalNAc–PEG–DSG. Data plotted as a function of siRNA concentration. (**c**) Competition-binding data replotted as a function of GalNAc concentration. DSG, distearyl (C18) lipid; GalNAc, *N*-acetylgalactosamine; iLNP, ionizable LNP; PEG, polyethylene glycol; siRNA, short-interfering RNA.

prove the concept of exogenous GalNAc-specific iLNP targeting in wild-type mice, it was necessary to first inactivate the endogenous apoE-based targeting mechanism for iLNPs. We hypothesized that this might be possible by increasing both the density and stability of the PEG shielding on the base iLNP formulation. Indeed, so-called "stealth" liposomes[25,26] have up to 10 mol% PEG–lipid in the formulation and the PEG is typically stably associated with the liposome *via* a distearyl lipid anchor. The dense, stable PEG shell on the surface of stealth liposomes minimizes interactions with serum proteins and cell surfaces, allowing for longer circulation half-life. Based on this approach, we used PEG–DSG instead of PEG–DMG and increased the total level of PEG–lipid to 10 mol% in the formulation.

The heavily PEG-shielded iLNP, in the absence of GalNAc-targeting ligand, was found to lack significant gene-silencing activity at doses as high as 3 mg/kg, presumably due to inhibition of apoE binding to the iLNP (**Figure 6**). In contrast, the targeted iLNP, in which 0.5 mol% PEG–DSG was replaced with GalNAc–PEG–DSG, mediated a clear, dose-responsive factor VII–silencing effect, with an ED_{50} <1 mg/kg. To demonstrate that the activity of the GalNAc-containing iLNP was in fact due to receptor targeting, a factor VII–silencing study was conducted in *ASGR2*$^{-/-}$ mice. Although these mice still express *ASGR1*, as both ASGR1 and ASGR2 subunits are required for high-affinity ligand binding, the *ASGR2*$^{-/-}$ mice effectively serve as an ASGPR knockout model in which to confirm the activity of the GalNAc-targeted iLNPs.[27] Indeed, the activity of the GalNAc-targeted formulation was nearly abolished in the *ASGR2*$^{-/-}$ mice, indicating that the GalNAc–ASGPR interaction is responsible for the *in vivo* activity of the GalNAc-targeted iLNP (**Figure 6**).

DISCUSSION

The data presented in this study suggest that apoE acts as an endogenous targeting ligand and plays a major role in the plasma clearance and hepatic uptake of iLNPs (LNPs with near neutral surface charge at physiological pH), but not cLNPs (LNPs with positive surface charge at physiological pH). *In vitro* studies demonstrated that both uptake and subsequent gene-silencing activity of iLNPs were dramatically enhanced by the presence of apoE in the culture medium. Conversely, apoE had no effect on the uptake or gene-silencing activity of a cLNP control. These findings were corroborated by *in vivo* studies in wild-type and *apoE*$^{-/-}$ mice. The potent *in vivo* gene-silencing activity of the iLNP observed in wild-type mice was nearly completely abolished in *apoE*$^{-/-}$ mice, whereas that of the cLNP control was unchanged. Although the cLNP control helped to establish the general RNAi competency of the *apoE*$^{-/-}$ mice, other factors unrelated to the loss of apoE as an endogenous targeting ligand (*e.g.*, alterations in gene expression profile or serum lipoprotein composition) could potentially have accounted for the loss in iLNP activity observed in this strain. To rule these factors out, experiments were conducted whereby exogenous apoE protein was premixed with iLNPs before administration to *apoE*$^{-/-}$ mice. Exogenous apoE was found to fully rescue the activity of iLNPs in a dose-dependent manner, confirming that the loss of iLNP activity in *apoE*$^{-/-}$ mice is indeed due to the absence of apoE as an endogenous targeting ligand.

To complement studies establishing apoE as an endogenous targeting ligand for iLNPs, we sought to study the receptor dependence of iLNP activity. Unfortunately, such a study is complicated by the fact that there are numerous potential receptors for apoE, whose activities may be redundant and for which the loss of one

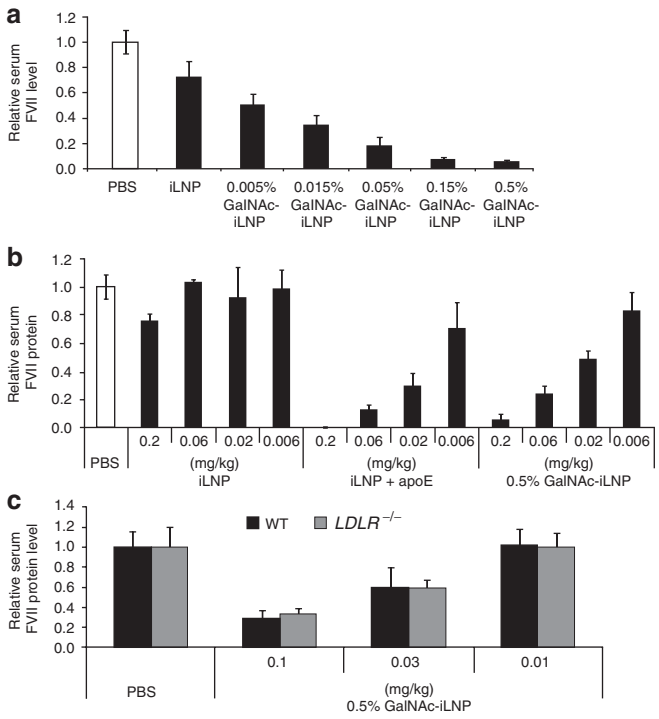

Figure 5 Exogenous targeting with GalNAc is able to rescue activity of iLNPs in *apoE*$^{-/-}$ mice. (**a**) siFVII was formulated in GalNAc–iLNPs containing 0.005–0.5 mol% GalNAc–PEG–DSG and administered to *apoE*$^{-/-}$ mice at a dose of 0.2 mg/kg. Control animals received PBS or FVII siRNA formulated in iLNP at 0.2 mg/kg. Serum factor VII levels were determined in animals 48 hours postadministration. Data points are expressed as a percentage of PBS control animals and represent group mean ($n = 3$) ± SD. (**b**) Comparative potency of 0.5% GalNAc–iLNP (containing 0.5 mol% of GalNAc–PEG–DSG) to iLNP preassociated with apoE (at a ratio of 0.5 mg apoE/mg siRNA) in *apoE*$^{-/-}$ mice. Control animals received PBS or iLNP. Data points are expressed as a percentage of PBS control animals and represent group mean ($n = 3$) ± SD. (**c**) Factor VII gene-silencing activity of 0.5% GalNAc–iLNP in wild-type and *LDLR*$^{-/-}$ mice. Serum factor VII levels were determined in animals 48 hours postadministration. Data points are expressed as a percentage of PBS control animals and represent group mean ($n = 5$) ± SD. ApoE, apolipoprotein E; DSG, distearyl (C18) lipid; GalNAc, *N*-acetylgalactosamine; iLNP, ionizable LNP; PBS, phosphate-buffered saline; PEG, polyethylene glycol; siFVII, factor VII–targeting siRNA; siRNA, short-interfering RNA.

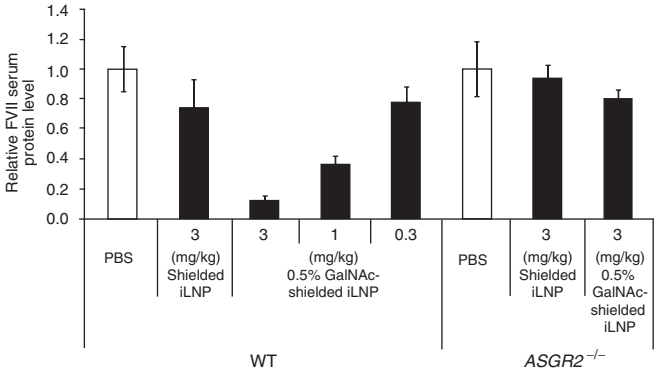

Figure 6 GalNAc-targeted, "shielded" iLNPs demonstrate GalNAc-specific gene-silencing activity *in vivo*. FVII siRNA was formulated in a PEG-shielded GalNAc–iLNP containing ionizable lipid/DSPC/cholesterol/PEG–DSG/GalNAc–PEG–DSG in a 50/10/30/9.5/0.5 molar ratio. Wild-type or *ASGR2*$^{-/-}$ mice were administered PBS, nontargeted shielded-iLNPs, or 0.5% GalNAc-shielded iLNPs at the doses indicated. Serum factor VII levels were determined in animals 48 hours postadministration. Data points are expressed as a percentage of PBS control animals and represent group mean ($n = 5$) ± SD. ASGR, asialoglycoprotein-binding receptor; DSG, distearyl (C18) lipid; GalNAc, *N*-acetylgalactosamine; iLNP, ionizable LNP; PBS, phosphate-buffered saline; PEG, polyethylene glycol; siFVII, factor VII–targeting siRNA; siRNA, short-interfering RNA.

receptor pathway may be compensated for by other mechanisms. Nonetheless, the dependence of iLNPs on LDLR was studied *in vitro* and *in vivo*. Relative to uptake in wild-type primary hepatocytes, uptake of iLNPs was considerably diminished in *LDLR*$^{-/-}$ primary hepatocytes. Consistent with the *in vitro* observations, *in vivo* gene-silencing activity of iLNPs was found to be substantially reduced, although not abolished, in *LDLR*$^{-/-}$ mice. As mentioned above, the observation of only a partial loss in activity is likely attributable to the fact that apoE-targeted iLNPs are likely to be internalized by multiple hepatic apoE receptors, where the absence of one receptor alone may be compensated for by the others. As with the *apoE*$^{-/-}$ mice, there are multiple differences between the wild-type and *LDLR*$^{-/-}$ mice, which could potentially complicate the interpretation of the observed loss in activity. However, the fact that the GalNAc–iLNPs had indistinguishable activity in wild-type and *LDLR*$^{-/-}$ animals serves as an additional control. Taken together the data establish LDLR as an important receptor for the hepatic uptake of apoE-dependent iLNPs.

As an alternative to endogenous apoE-based targeting, an exogenous targeting strategy utilizing a trivalent GalNAc ligand with high affinity to the ASGPR, was explored. A trivalent GalNAc cluster was synthesized as a conjugate to a PEG–lipid and was subsequently incorporated into an iLNP. Earlier work has established that particles up to 70 nm in size can be effectively recognized and processed by the ASGPR, whereas the binding of 90 nm particles to the ASGPR is significantly impaired.[28,29] Consistent with this upper size limit for ASGPR binding, the resultant GalNAc–iLNPs used in this work had mean particle diameters in the range 40–70 nm (**Supplementary Table S1**). Indeed, cell-free binding assays established that the GalNAc–iLNPs could bind a soluble GalNAc receptor in a GalNAc-dependent manner. To establish whether the exogenous ligand GalNAc could direct iLNPs to hepatocytes *in vivo*, the activity of GalNAc–iLNPs was studied in *apoE*$^{-/-}$ mice. Similar to exogenously premixed apoE, GalNAc was also able to "rescue" activity of iLNPs in the *apoE*$^{-/-}$ mice, in a GalNAc-dependent manner. As little as 0.15 mol% GalNAc ligand was sufficient to give near maximal activity. Further, the potency of a GalNAc–iLNP was found to be comparable to an exogenous apoE-associated iLNP in *apoE*$^{-/-}$ mice, suggesting that there is nothing unique to apoE *per se* in mediating efficient iLNP-based gene silencing. Rather, the findings indicate that both apoE and GalNAc simply serve as effective ligands to allow uptake of iLNPs by hepatocytes *via* receptor-mediated endocytosis. In addition, the activity of GalNAc–iLNPs in wild-type and *LDLR*$^{-/-}$ mice was remarkably indistinguishable, consistent with the fact that GalNAc–iLNPs are not dependent on the apoE/LDLR interaction for hepatocyte uptake.

Although the incorporation of GalNAc was able to rescue activity of iLNP in *apoE*$^{-/-}$ mice, GalNAc targeting provided no

enhancement of activity in wild-type mice. This is presumably due to the fact that the apoE mechanism is sufficiently functional in wild-type mice to effectively direct nonexogenously targeted iLNPs to liver. To prove the concept of an exogenously targeted iLNP in wild-type mice, we first inactivated the endogenous apoE-targeting mechanism by increasing the PEG shielding on the iLNP. The "inactivated", highly PEG-shielded iLNP showed little activity in wild-type mice, even at doses as high as 3 mg/kg. However, significantly enhanced activity (ED_{50} <1 mg/kg) was observed when GalNAc was incorporated into the shielded iLNP. The reduced potency of the shielded GalNAc–iLNP relative to the original GalNAc–iLNP is likely due to the presence of the stable PEG shield, which may act to inhibit LNP uptake and/or endosomal release. The formulation may need to be optimized further in order to improve potency. Nevertheless, as a general strategy, shielded iLNPs may be useful for inactivating the endogenous apoE-targeting system, particularly for targeting iLNPs to nonhepatic tissues. Finally, the receptor dependence of the shielded GalNAc–iLNP was studied in mice lacking the ASGR2-subunit of the ASGPR; no silencing activity was observed in the *ASGR2*$^{-/-}$ mice, confirming the role of ASGPR in the uptake of GalNAc-targeted iLNPs.

In summary, the results presented in this work establish the role of apoE as an endogenous targeting ligand for iLNPs, but not cLNPs, and demonstrate an alternative targeting strategy for the hepatic delivery of siRNA using GalNAc as an exogenous ligand. Taken together, the findings on endogenous targeting and the strategies presented for exogenous, apoE-independent targeting provide fundamentally new mechanistic insights for LNP-mediated delivery of RNAi therapeutics.

MATERIALS AND METHODS

iLNP and cLNP formulations. iLNPs were prepared with the ionizable lipid DLin-KC2-DMA, disteroylphosphatidyl choline, cholesterol, and PEG–DMG using a spontaneous vesicle formation formulation procedure as previously described.[4] The iLNPs had a component molar ratio of ~50/10/38.5/1.5 (DLin-KC2-DMA/disteroylphosphatidyl choline/cholesterol/PEG–DMG). The final lipid:siRNA weight ratio was ~12:1. GalNAc–iLNPs were prepared in a similar manner by inclusion of trivalent GalNAc–PEG–DSG into the lipid mixture at the appropriate molar ratios. Synthesis of the trivalent GalNAc cluster has been previously described.[30,31] The conjugation of the GalNAc cluster to PEG–can be found in the PCT publication WO 2009/082607. Control cLNPs were prepared with the cationic lipidoid, $98N_{12}$-5(I), cholesterol, and PEG–DMG as previously described.[23] The particle size of LNPs was determined by dynamic light scattering (Zetasizer Nano ZS; Malvern, Malvern, UK) and the mean diameter was in the range 40–70 nm for all LNPs used in this work (**Supplementary Table S1**).

In vitro ***imaging and gene-silencing studies.*** HeLa cells were purchased from ATCC (Manassas, VA) and were used for labeled siRNA uptake experiments. The HeLa-GFP cell line was generated using a BAC expression system at the Max-Plank Institute for Molecular Cell Biology and Genomics and were used for GFP-silencing experiments. Primary hepatocytes were isolated using a standard collagenase perfusion method and cultured on collagen coated plates (BD Biosciences, Franklin Lakes, NJ). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with penicillin, streptomycin, and fetal bovine serum. For experiments, cells were seeded in 96-well plates (Greiner, Kremsmünster, Austria) overnight and then LNP formulations of Alexa-647 tagged GFP-targeting siRNA (AS-AAGUCGUGCUGCUUCAUGUdTdTAlexa647, S-AcAuGA AGcAGcACGACuUdTsdT; 2′-*O*-methyl-modified nucleotides are in lower case) (Alnylam, Cambridge, MA) were added at 20 nmol/l for the indicated time points. In apoE preassociation experiments, LNPs were preassociated with human recombinant apoE3 (Fitzgerald Industries, Acton, MA) for 5 minutes at 37 °C before adding to cells. Cells were fixed in 4% paraformaldehyde and counterstained with 4′,6-diamidino-2-phenylindole or in some experiments counterstained with Hoechst without fixation and imaged live. All images were acquired using an Opera automated spinning disc confocal system (Perkin Elmer, Wellesley, MA) and data analyzed using Acapella Software (Perkin Elmer). Quantification of uptake or GFP silencing was done by taking an average of 20 fields from three replicate wells.

In vivo ***screening of LNPs for FVII activity.*** Six- to eight-week-old, female C57Bl/6 mice were obtained from Charles River Laboratories (Willmington, MA). ApoE$^{-/-}$ (stock #002052), LDLR$^{-/-}$ (stock #002207), and ASGR2$^{-/-}$ (stock #002361) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were held in a pathogen-free environment and all procedures involving animals were performed in accordance with local, state, and federal regulations as applicable and approved by the Institutional Animal Care and Use Committee. LNP-siRNA systems containing siFVII[24] were diluted to the appropriate concentrations in sterile phosphate-buffered saline immediately before use and the formulations were administered intravenously *via* the lateral tail vein in a total volume of 10 ml/kg. At various time points, blood was collected from animals and processed to serum (Microtainer Serum Separator Tubes; Becton Dickinson). Serum was tested immediately or stored at −70 °C for later analysis for serum factor VII levels. Serum factor VII levels were determined using the colorimetric Biophen VII assay kit (Anaira, Mason, OH) as previously described.[23,24]

Cell-free ASGPR binding assays. The binding activity of GalNAc–iLNP to the dendritic cell ASGPR (CD301) (cat. no. 4888-CL; R&D Systems, Minneapolis, MN) was determined by the competition equilibrium method. The binding reaction was observed by measuring fluorescence polarization of a fluorescein-labeled trientennary GalNAc reporter probe [(GalNAc)3-fl; synthesized at Alnylam]. Polarization data were collected on a SpectraMax M5e (Molecular Devices, Sunnyvale, CA). Excitation, emission, and emission cutoff wavelengths of 485, 538, and 515 nm were used, respectively. The final buffer conditions were 0.2× phosphate-buffered saline, 8 mmol/l HEPES (7.2), 0.12 mol/l NaCl, 4 mmol/l $CaCl_2$, 1 mg/ml bovine serum albumin, T = 25 °C. First, direct binding of CD301 and (GalNAc)3-fl probe was initially characterized. Data fit well to a one-site-binding model, K_d = 50 nmol/l. Second, to determine GalNAc–iLNP/CD301-binding activity, varying amounts of each GalNAc–iLNP formulation were added to a fixed amount of (GalNAc)3-fl and CD301 (total (GalNAc)3-fl = 20 nmol/l; total CD301 = 40 nmol/l). The CD301/competitor reaction is indirectly observed by its effect on the CD301/(GalNAc)3-fl reaction. Polarization data from the competition experiments were fit to a single-site competition-binding model using the Prism5 software package (GraphPad Software, La Jolla, CA). In all fits employed, the SE was <10% of the mean.

SUPPLEMENTARY MATERIAL

Figure S1. Multiple apoE isoforms enhance iLNP-mediated silencing.
Figure S2. ApoE mediates the uptake of the entire iLNP particle.
Table S1. LNP physical characterization.
Materials and Methods.

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