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(72) Inventeurs/Inventors:
MACDONALD, MARCIA, CA;
LEE, AMY C. H., CA;
MACLACHLAN, IAN, CA

(73) Propriétaire/Owner:
PROTIVA BIOTHERAPEUTICS, INC., CA

(74) Agent: FETHERSTONHAUGH & CO.

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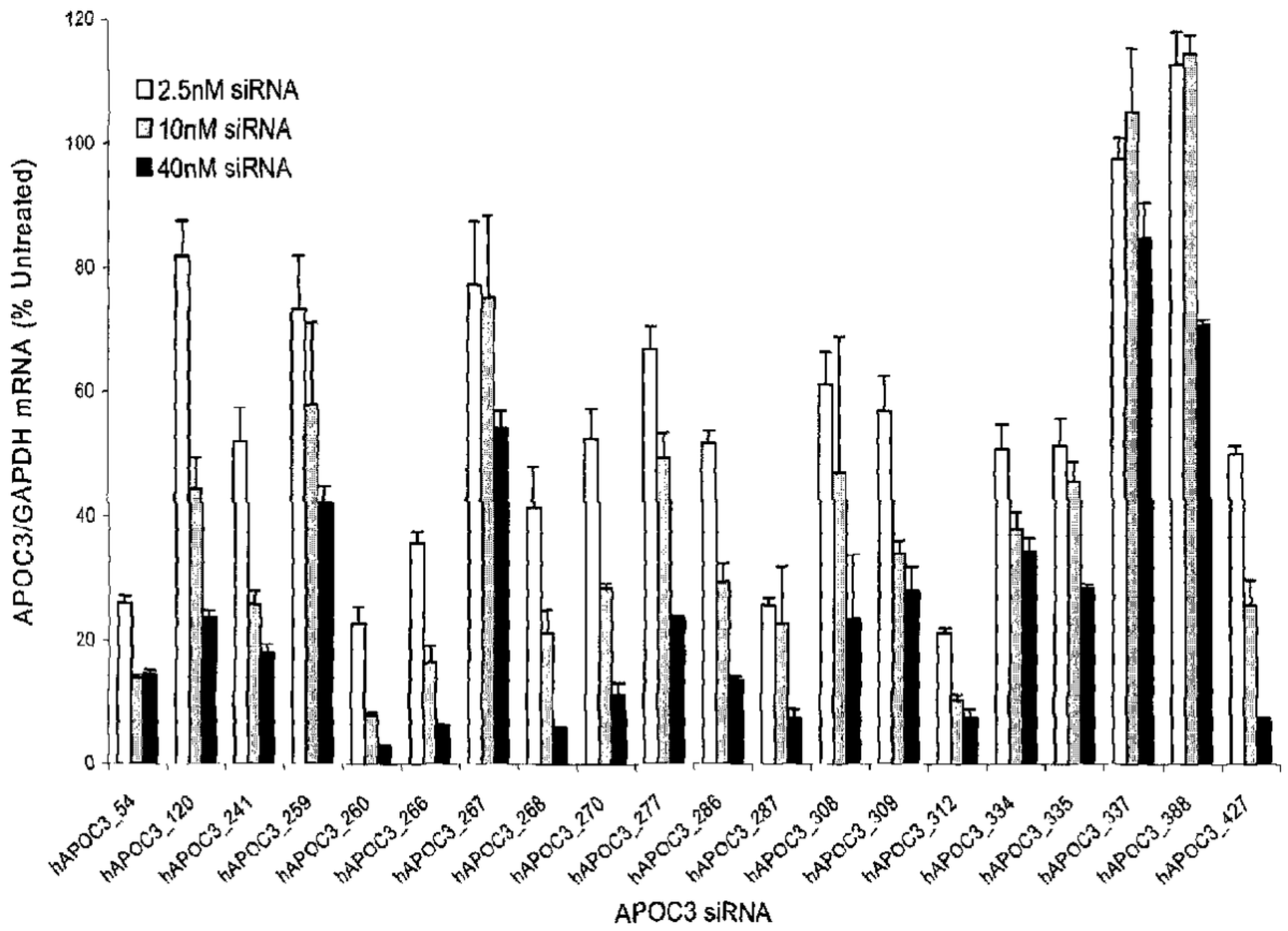

(57) Abrégé/Abstract:
The present invention provides compositions comprising therapeutic nucleic acids such as interfering RNA that target apolipoprotein C-III (APOC3) gene expression, lipid particles comprising one or more (e.g., a cocktail) of the therapeutic nucleic acids, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (e.g., for the treatment of lipid diseases or disorders such as atherosclerosis or a dyslipidemia such as hypertriglyceridemia or hypercholesterolemia).

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(71) Applicant *(for all designated States except US)*: **PROTIVA BIOTHERAPEUTICS, INC.** [CA/CA]; 100-8900 Glenlyon Parkway, Burnaby, British Columbia V5J 5J8 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MACDONALD, Marcia** [CA/CA]; 202-1556 5th Avenue E., Vancouver, British Columbia V5N 1L7 (CA). **LEE, Amy C.H.** [CA/CA]; 3315 Beaverbrook Drive, Burnaby, British Columbia V3J 7Y2 (CA). **MACLACHLAN, Ian** [CA/CA]; 8040 Aves Terrace, Mission, British Columbia V4S 1E5 (CA).

(74) Agent: **KINGWELL, Brian G.**; Smart & Biggar, 2200-650 West Georgia Street, Vancouver, British Columbia V6B 4N8 (CA).

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(54) Title: COMPOSITIONS AND METHODS FOR SILENCING APOLIPOPROTEIN C-III EXPRESSION

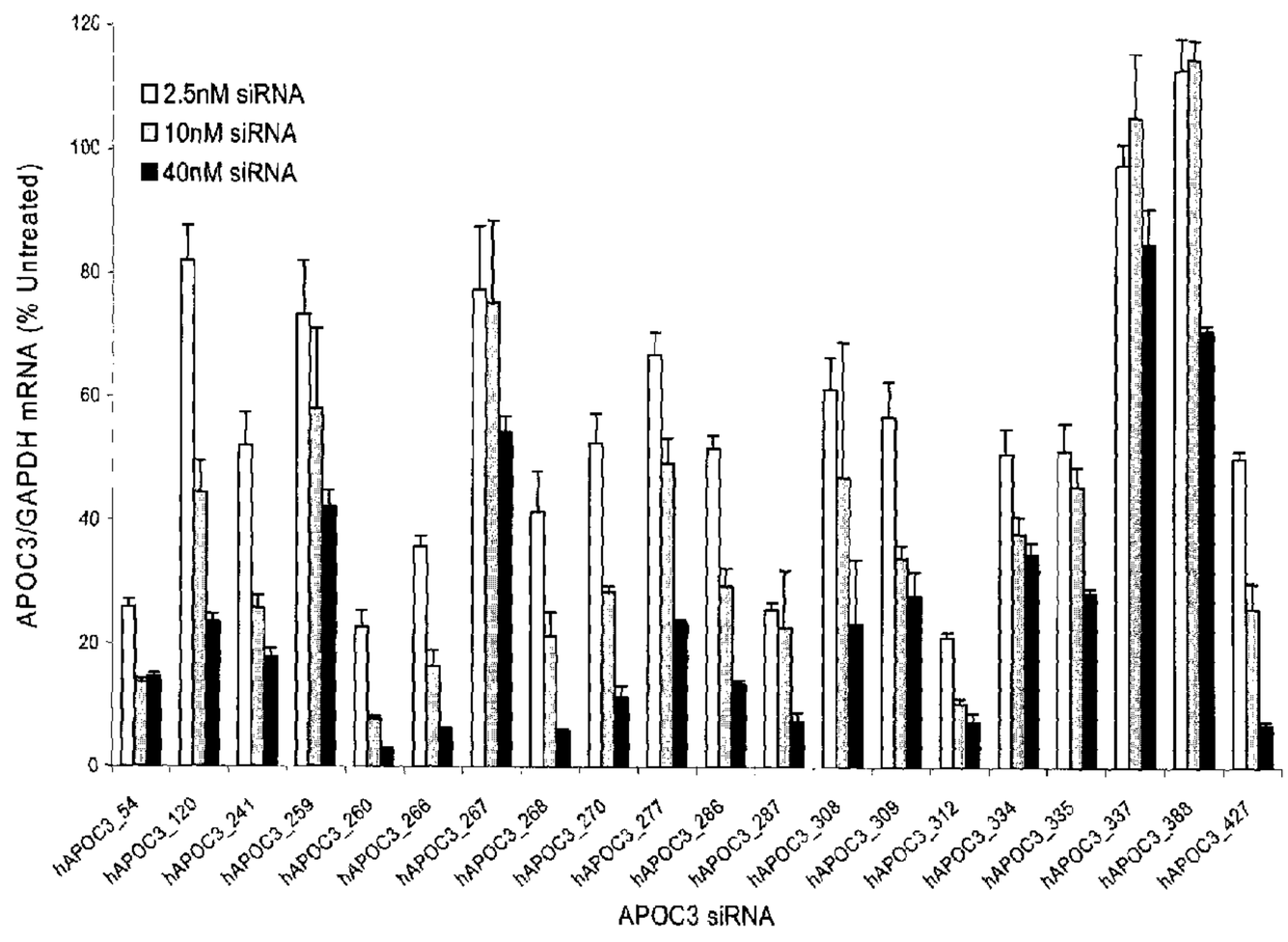

FIG. 8

(57) Abstract: The present invention provides compositions comprising therapeutic nucleic acids such as interfering RNA that target apolipoprotein C-III (APOC3) gene expression, lipid particles comprising one or more (e.g., a cocktail) of the therapeutic nucleic acids, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (e.g., for the treatment of lipid diseases or disorders such as atherosclerosis or a dyslipidemia such as hypertriglyceridemia or hypercholesterolemia).

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COMPOSITIONS AND METHODS FOR SILENCING APOLIPOPROTEIN C-III EXPRESSION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is related to U.S. Provisional Application Nos. 61/147,235, filed January 26, 2009, and 61/293,452, filed January 8, 2010.

BACKGROUND

[0002] Lipoproteins are globular, micelle-like particles that consist of a non-polar core of acylglycerols and cholesteryl esters surrounded by an amphiphilic coating of protein, phospholipid, and cholesterol. Lipoproteins have been classified into five broad categories on the basis of their functional and physical properties: chylomicrons, which transport dietary lipids from intestine to tissues; very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL), all of which transport triacylglycerols and cholesterol from the liver to tissues; and high density lipoproteins (HDL), which transport endogenous cholesterol from tissues to the liver.

[0003] Lipoprotein particles undergo continuous metabolic processing and have variable properties and compositions. Lipoprotein densities increase without decreasing particle diameter because the density of their outer coatings is less than that of the inner core. The protein components of lipoproteins are known as apolipoproteins. At least nine apolipoproteins are distributed in significant amounts among the various human lipoproteins.

[0004] Apolipoprotein C-III is a constituent of HDL and triglyceride-rich lipoproteins and has a role in hypertriglyceridemia, a risk factor for coronary artery disease. Apolipoprotein C-III slows the clearance of triglyceride-rich lipoproteins by inhibiting lipolysis, both through inhibition of lipoprotein lipase and by interfering with lipoprotein binding to the cell-surface glycosaminoglycan matrix (*see,* Shachter, *Curr. Opin. Lipidol.*, 12:297-304 (2001)).

[0005] The gene encoding human apolipoprotein C-III (also called *APOC3* and apoC-III) was cloned in 1984 (*see,* Levy-Wilson *et al.*, *DNA*, 3:359-364 (1984); Protter *et al.*, *DNA*, 3:449-456 (1984); Sharpe *et al.*, *Nucleic Acids Res.*, 12:3917-3932 (1984)). The coding sequence is interrupted by three introns (*see,* Protter *et al.*, *supra*). The human *APOC3* gene is located approximately 2.6 kilobases to the 3' direction of the apolipoprotein A-1 gene and these two genes are convergently transcribed (*see,* Karathanasis, *Proc. Natl. Acad. Sci. U.S.A.*, 82:6374-6378 (1985)). Also cloned was a variant of the human *APOC3* gene resulting in a Thr74 to Ala74 mutation from a patient with unusually high levels of serum apoC-III protein. As the Thr74 is O-glycosylated, the Ala74 mutant therefore resulted in

increased levels of serum apoC-III protein lacking the carbohydrate moiety (*see,* Maeda *et al.*, *J. Lipid Res.*, 28:1405-1409 (1987)).

[0006] Five polymorphisms have been identified in the promoter region of the *APOC3* gene: C(-641) to A; G(-630) to A; T(-625) to deletion; C(-482) to T; and T(-455) to C. All of these polymorphisms are in linkage disequilibrium with the SstI polymorphism in the 3' untranslated region. The SstI site distinguishes the S1 and S2 alleles and the S2 allele has been associated with elevated plasma triglyceride levels (*see,* Dammerman et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90:4562-4566 (1993)). The *APOC3* promoter is downregulated by insulin and this polymorphic site abolishes insulin regulation. Thus, the potential overexpression of apoC-III resulting from the loss of insulin regulation may be a contributing factor to the development of hypertriglyceridemia associated with the S2 allele (*see,* Li *et al.*, *J. Clin. Invest.*, 96:2601-2605 (1995)). The T(-455) to C polymorphism has been associated with an increased risk of coronary artery disease (*see,* Olivieri *et al.*, *J. Lipid Res.*, 43:1450-1457 2002)).

[0007] In addition to insulin, other regulators of *APOC3* gene expression have been identified. A response element for the nuclear orphan receptor rev-erb alpha has been located at positions -23/-18 in the *APOC3* promoter region and rev-erb alpha decreases *APOC3* promoter activity (*see,* Raspe *et al.*, *J. Lipid Res.*, 43:2172-2179 (2002)). The *APOC3* promoter region -86 to -74 is recognized by two nuclear factors, CIIIb1 and CIIIB2 (*see,* Ogami *et al.*, *J. Biol. Chem.*, 266:9640-9646 (1991)). *APOC3* expression is also upregulated by retinoids acting via the retinoid X receptor, and alterations in retinoid X receptor abundance affects *APOC3* transcription (*see,* Vu-Dac *et al.*, *J. Clin. Invest.*, 102:625-632 (1998)). Specificity protein 1 (Sp1) and hepatocyte nuclear factor-4 (HNF-4) have been shown to work synergistically to transactivate the *APOC3* promoter via the HNF-4 binding site (*see,* Kardassis *et al.*, *Biochemistry*, 41:1217-1228 (2002)). HNF-4 also works in conjunction with SMAD3-SMAD4 to transactivate the *APOC3* promoter (*see,* Kardassis *et al.*, *J. Biol. Chem.*, 275:41405-41414 (2000)).

[0008] Transgenic and knockout mice have further defined the role of apoC-III in lipolysis. Overexpression of *APOC3* in transgenic mice leads to hypertriglyceridemia and impaired clearance of VLDL-triglycerides (*see,* de Silva *et al.*, *J. Biol. Chem.*, 269:2324-2335 (1994); Ito *et al.*, *Science*, 249:790-793 (1990)). Knockout mice with a total absence of apoC-III protein exhibited significantly reduced plasma cholesterol and triglyceride levels compared with wild-type mice and were protected from postprandial hypertriglyceridemia (*see,* Maeda *et al.*, *J. Biol. Chem.*, 269:23610-23616 (1994)).

[0009] Recently, it was discovered that about 5% of the Lancaster Amish are heterozygous carriers of a null mutation in exon 3 of the *APOC3* gene consisting of a C to T transition at nucleotide 55, resulting in an Arg19 to Ter (R19X) substitution (*see,* Pollin *et al.*, *Science*, 322:1702-1705 (2008)). As the

mutation occurs in the signal peptide of the protein, a complete lack of production of apoC-III from alleles carrying the mutation was predicted. Carriers of the R19X null mutation expressed half the amount of apoC-III present in noncarriers. Mutation carriers compared with noncarriers had lower fasting and postprandial serum triglycerides, higher levels of HDL cholesterol, and lower levels of LDL cholesterol. Subclinical atherosclerosis, as measured by coronary artery calcification, was less common in carriers than noncarriers, which suggested that lifelong deficiency of apoC-III protein has a cardioprotective effect.

[0010] In view of the foregoing, there is a need for therapeutic agents capable of effectively inhibiting *APOC3* function and methods for their *in vivo* delivery to target tissues such as the liver. The present disclosure addresses these and other needs.

BRIEF SUMMARY

[0011] The present disclosure relates to compositions comprising therapeutic nucleic acids such as interfering RNA that target apolipoprotein C-III (*APOC3*) gene expression, lipid particles comprising one or more (*e.g.,* a cocktail) of the therapeutic nucleic acids, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.,* for the treatment of lipid diseases or disorders such as atherosclerosis or a dyslipidemia such as hypertriglyceridemia or hypercholesterolemia).

[0012] More particularly, the disclosure relates to compositions comprising unmodified and chemically modified interfering RNA (*e.g.,* siRNA) molecules which silence *APOC3* gene expression. The present disclosure also relates to serum-stable nucleic acid-lipid particles (*e.g.,* SNALP) and formulations thereof comprising one or more (*e.g.,* a cocktail) of the interfering RNA (*e.g.,* siRNA) described herein, a cationic lipid, and a non-cationic lipid, which can further comprise a conjugated lipid that inhibits aggregation of particles.

[0013] In one aspect, the present disclosure relates to an siRNA that targets *APOC3* gene expression, wherein the siRNA comprises a sense strand and a complementary antisense strand, and wherein the siRNA comprises a double-stranded region of about 15 to about 60 nucleotides in length. In certain embodiments, the present disclosure compositions comprising a combination (*e.g.,* a cocktail) of siRNAs that target *APOC3* and at least 1, 2, 3, 4, 5, 6, 7, or 8 additional genes associated with metabolic diseases and disorders. The siRNA molecules of the present disclosure are capable of silencing *APOC3* gene expression, reducing triglyceride levels, and/or reducing cholesterol levels *in vivo*.

[0014] Human *APOC3* sequences are set forth in Genbank Accession No. NG_008949 REGION: 5001..8164 (SEQ ID NO:1), which corresponds to the human *APOC3* genomic sequence, and Genbank Accession No. NM_000040.1 (SEQ ID NO:2), which corresponds to the human *APOC3* mRNA

sequence. Mouse *Apoc3* sequences are set forth in Genbank Accession No. NC_000075 REGION: complement(46041134..46043380), which corresponds to the mouse *Apoc3* genomic sequence, and Genbank Accession No. NM_023114.3, which corresponds to the mouse *Apoc3* mRNA sequence.

[0015] Each of the siRNA sequences present in the compositions of the disclosure may independently comprise at least one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides such as 2'OMe nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. Preferably, uridine and/or guanosine nucleotides are modified with 2'OMe nucleotides. In particular embodiments, each of the siRNA sequences present in the compositions comprises at least one 2'OMe-uridine nucleotide and at least one 2'OMe-guanosine nucleotide in the sense and/or antisense strands.

[0016] In some embodiments, each of the siRNA sequences present in the compositions may independently comprise a 3' overhang of 1, 2, 3, or 4 nucleotides in one or both strands of the siRNA or may comprise at least one blunt end. In certain instances, the 3' overhangs in one or both strands of the siRNA each independently comprise 1, 2, 3, or 4 of any combination of modified and unmodified deoxythymidine (dT) nucleotides, 1, 2, 3, or 4 of any combination of modified (*e.g.,* 2'OMe) and unmodified uridine (U) ribonucleotides, or 1, 2, 3, or 4 of any combination of modified (*e.g.,* 2'OMe) and unmodified ribonucleotides having complementarity to the target sequence (3' overhang in the antisense strand) or the complementary strand thereof (3' overhang in the sense strand).

[0017] In further embodiments, the present disclosure relates to a composition comprising at least one or a cocktail (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) of the unmodified and/or modified siRNA sequences set forth in Tables 1-10. In particular embodiments, the disclosure relates to a composition comprising at least one or a cocktail of the siRNA sequences set forth in Table 7. In these embodiments, each siRNA sequence set forth in Table 7 may comprise a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides in one or both strands of the siRNA. In other particular embodiments, the composition comprises at least one or a cocktail of the siRNA sequences set forth in Table 10, and each siRNA sequence present in the composition comprises nucleotides 1-19 of one of the sense and/or antisense strand sequences set forth in Table 10. In certain embodiments, the composition comprises at least one or a cocktail of the siRNA sequences set forth in Table 10, and each siRNA sequence present in the composition consists of one of the sense and/or antisense strand sequences set forth in Table 10. In preferred embodiments, the present invention provides a composition comprising at least one or a cocktail of the modified siRNA sequences set forth in Tables 1-6. In these embodiments, each sequence set forth in Tables 1-6 may comprise a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other preferred

embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more (*e.g.,* all) of the siRNA sequences present in the composition comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more modified nucleotides such as 2'OMe nucleotides, *e.g.,* in the double-stranded region.

[0018] The present disclosure also relates to a pharmaceutical composition comprising one or a cocktail of interfering RNA (*e.g.,* siRNA) molecules that target *APOC3* gene expression and a pharmaceutically acceptable carrier.

[0019] In another aspect, the present disclosure relates to a nucleic acid-lipid particle that targets *APOC3* gene expression. The nucleic acid-lipid particle typically comprises one or more unmodified and/or modified siRNA that silence *APOC3* gene expression, a cationic lipid, and a non-cationic lipid. In certain instances, the nucleic acid-lipid particle further comprises a conjugated lipid that inhibits aggregation of particles. Preferably, the nucleic acid-lipid particle comprises one or more unmodified and/or modified siRNA that silence *APOC3* gene expression, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles.

[0020] In some embodiments, the nucleic acid-lipid particle comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more of the unmodified or modified sequences set forth in Tables 1-10. In particular embodiments, the nucleic acid-lipid particle comprises one or a cocktail (*e.g.,* at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) of the siRNA sequences set forth in Table 7. In these embodiments, each siRNA sequence present in the nucleic acid-lipid particle composition may comprise a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides in one or both strands of the siRNA. In other particular embodiments, the nucleic acid-lipid particle comprises one or a cocktail (*e.g.,* at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) of the siRNA sequences set forth in Table 10, and each siRNA sequence present in the nucleic acid-lipid particle composition comprises nucleotides 1-19 of one of the sense and/or antisense strand sequences set forth in Table 10. In certain embodiments, the nucleic acid-lipid particle comprises one or a cocktail (*e.g.,* at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) of the siRNA sequences set forth in Table 10, and each siRNA sequence present in the nucleic acid-lipid particle composition consists of one of the sense and/or antisense strand sequences set forth in Table 10. In preferred embodiments, the nucleic acid-lipid particle comprises at least one or a cocktail of the modified siRNA sequences set forth in Tables 1-6. In these embodiments, each sequence present in the nucleic acid-lipid particle composition may comprise a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other preferred embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more (*e.g.,* all) of the siRNA sequences present in the nucleic acid-lipid particle formulation comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more modified nucleotides such as 2'OMe nucleotides, *e.g.,* in the double-stranded region.

[0021] In other embodiments, the siRNA molecules are fully encapsulated in the nucleic acid-lipid particle (*e.g.,* SNALP). With respect to formulations comprising an siRNA cocktail, the different types of siRNAs may be co-encapsulated in the same nucleic acid-lipid particle, or each type of siRNA species present in the cocktail may be encapsulated in its own nucleic acid-lipid particle.

[0022] The present disclosure also relates to pharmaceutical compositions comprising a nucleic acid-lipid particle and a pharmaceutically acceptable carrier.

[0023] Nucleic acid-lipid particles of the disclosure are useful for the prophylactic or therapeutic delivery of interfering RNA (*e.g.,* siRNA) molecules that silence *APOC3* gene expression. In some embodiments, one or more of the siRNA molecules described herein are formulated into nucleic acid-lipid particles, and the particles are administered to a mammal (*e.g.,* a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) requiring such treatment. In certain instances, a therapeutically effective amount of the nucleic acid-lipid particle can be administered to the mammal, *e.g.,* for reducing apoC-III protein levels to prevent morbidity and/or mortality associated with cardiac-related disorders. The nucleic acid-lipid particles of the invention are particularly useful for reducing plasma and/or serum levels of triglycerides, cholesterol, and/or glucose and find utility in preventing, treating, or reducing susceptibility to a lipid disorder such as atherosclerosis or a dyslipidemia such as hypertriglyceridemia or hypercholesterolemia. The nucleic acid-lipid particles of the invention (*e.g.,* SNALP) find utility in targeting cells, tissues, and/or organs associated with metabolic diseases and disorders, such as hepatocytes as well as other cell types of the liver. Administration of the nucleic acid-lipid particle can be by any route known in the art, such as, *e.g.,* oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, or intradermal. In particular embodiments, the nucleic acid-lipid particle is administered systemically, *e.g.,* via enteral or parenteral routes of administration.

[0024] In some embodiments, downregulation of *APOC3* gene expression is determined by detecting *APOC3* mRNA or apoC-III protein levels in a biological sample from a mammal after nucleic acid-lipid particle administration. In other embodiments, downregulation of *APOC3* gene expression is determined by measuring triglyceride, cholesterol, and/or glucose levels in a biological sample from a mammal after nucleic acid-lipid particle administration.

[0025] In certain embodiments, the present disclosure relates to a method for treating a mammal having hyperlipidemia comprising administering to a mammal suffering from hyperlipidemia an siRNA that silences *APOC3* expression (*e.g.,* encapsulated in a nucleic acid-lipid particle such as SNALP), thereby reducing hyperlipidemia in the mammal. In certain other embodiments, the present disclosure relates to a method for delaying the onset of hyperlipidemia in a mammal comprising administering to a

mammal at risk for developing hyperlipidemia an siRNA that silences *APOC3* expression (*e.g.*, encapsulated in a nucleic acid-lipid particle such as SNALP), thereby delaying the onset of hyperlipidemia. In further embodiments, the present disclosure relates to a method for lowering triglyceride levels in a mammal comprising administering to a mammal in need of a reduction in triglyceride levels an siRNA that silences *APOC3* expression (*e.g.*, encapsulated in a nucleic acid-lipid particle such as SNALP), wherein the administering results in reduced triglyceride levels in the mammal. In other embodiments, the present disclosure relates to provides a method for lowering cholesterol levels in a mammal comprising administering to a mammal in need of a reduction in cholesterol levels an siRNA that silences *APOC3* expression (*e.g.*, encapsulated in a nucleic acid-lipid particle such as SNALP), wherein the administering results in reduced cholesterol levels in the mammal.

[0026] In a further aspect, the present disclosure relates to compositions comprising at least one siRNA that silences *APOC3* expression and at least one siRNA that silences *APOB* expression. In certain instances, the siRNA targeting *APOC3* and the siRNA targeting *APOB* are formulated in the same nucleic acid-lipid particle (*e.g.*, SNALP). As a non-limiting example, the cocktail of *APOC3* and *APOB* siRNA molecules may be co-encapsulated in the same nucleic acid-lipid particle. In certain other instances, the *APOC3* and *APOB* siRNA molecules are formulated in separate nucleic acid-lipid particles. In these instances, one formulation may be administered before, during, or after the administration of the other formulation to a mammal in need thereof. Exemplary siRNA sequences targeting *APOB* that are suitable for use in the present invention are described in, *e.g.*, U.S. Patent Publication Nos. 20060134189 and 20070135372.

[0027] In a related aspect, the present disclosure relates to compositions comprising at least one siRNA that silences *APOC3* expression (*e.g.*, encapsulated in a nucleic acid-lipid particle such as SNALP) and at least one lipid-lowering agent which decreases apoC-III levels but does not mediate RNA interference. Such lipid-lowering agents include, but are not limited to, statins, fibrates, thiazolidinediones, ezetimibe, niacin, beta-blockers, nitroglycerin, calcium antagonists, and fish oil. One skilled in the art will appreciate that one or more *APOC3* siRNA molecules (*e.g.*, encapsulated in a nucleic acid-lipid particle such as SNALP) may be administered before, during, or after the administration of one or more lipid-lowering agents to a mammal in need thereof.

[0027a] Various embodiments of the claimed invention relate to a small-interfering RNA (siRNA) that silences apolipoprotein C-III (APOC3) gene expression, wherein the siRNA comprises a sense strand and a complementary antisense strand, and wherein the siRNA comprises a double stranded region of about 19 to about 25 nucleotides in length, and wherein the antisense strand comprises a sequence set

forth in SEQ ID NO:770 and/or wherein the sense strand comprises a sequence set forth in SEQ ID NO:769.

[0027b] Various embodiments of the claimed invention relate to a nucleic acid-lipid particle comprising: (a) an siRNA as claimed; (b) a cationic lipid; and (c) a non-cationic lipid.

[0027c] Various embodiments of the claimed nucleic acid-lipid particles may be useful for treating and/or ameliorating one or more symptoms associated with atherosclerosis or dyslipidemia in a mammal.for treating, reducing susceptibility to atherosclerosis or dyslipidemia in a mammal, preventing or delaying the onset of atherosclerosis or dyslipidemia in a mammal, lowering triglyceride levels in a mammal or lowering cholesterol levels in a mammal.

[0028] Other objects, features, and advantages of the present disclosure will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] Figure 1 illustrates data demonstrating that *Apoc3* siRNAs display dose-dependent activity *in vitro*. A panel of siRNAs targeting mouse *Apoc3* mRNA and a firefly luciferase (Luc) control siRNA were transfected into mouse primary hepatocytes and silencing activity was assessed by QuantiGene Assay 24 h post-treatment. Cells were treated with SNALP-formulated *Apoc3* siRNA at 2 nM (black bars) and 20 nM (gray bars). Sequence numbers represent the nucleotide position of mouse *Apoc3* mRNA (Genbank Accession No. NM_023114.3) that is complementary to the 3' end of the antisense strand of the siRNA.

[0030] Figure 2 illustrates data demonstrating the *in vitro* activity of unmodified versus 2'OMe-modified *Apoc3* siRNA. Unmodified siRNA duplexes 465, 467, and 492 and 2'OMe-modified duplexes 465.1, 465.2, 467.1, 467.2, 492.1, and 492.2 were transfected into mouse primary hepatocytes and silencing activity was assessed by QuantiGene Assay 24 h post-treatment. Cells were treated with SNALP-formulated *Apoc3* siRNA at 1.25 nM (black bars), 5 nM (gray bar), and 20 nM (white bars).

[0031] Figure 3 illustrates data demonstrating that SNALP-mediated apoCIII silencing is potent and long-lasting. Target mRNA silencing in liver following a single dose of SNALP-formulated siRNA is shown. (A) 48 hours after siRNA administration or after initiation of 100 mg/kg/d fenofibrate delivered by oral gavage. (B) Comparison of silencing activity at various time points after administration of 0.5 mg/kg SNALP-formulated siRNA targeting apoCIII and apoB.

[0032] Figure 4 illustrates data demonstrating that 2′OMe-modified *Apoc3* siRNAs induce no measurable interferon response in mice. Hepatic levels of *Ifit1* mRNA, a sensitive measure of low-grade immunostimulatory activity, 4 hours after IV administration of SNALP-formulated 2′OMe-modified *Apoc3* siRNA and unmodified luciferase control siRNA (Unmod Luc) to C57BL/6 mice, are shown.

[0033] Figure 5 illustrates data demonstrating that SNALP-mediated apoCIII silencing does not increase liver TG. Hepatic triglyceride levels, 48 hours after IV administration of SNALP-formulated *Apoc3* siRNA and Apob siRNA to C57BL/6 mice, are shown.

[0034] Figure 6 illustrates data demonstrating that siRNA-based silencing of apoCIII improves plasma lipids in LDLR-deficient mice. Hepatic *Apoc3* mRNA levels (A), plasma triglycerides (B), and plasma cholesterol (C) following a single IV administration of SNALP-formulated *Apoc3* siRNA to LDLR-deficient mice fed a Western diet for 12 days prior to injection are shown.

[0035] Figure 7 is a schematic depicting the amelioration of dyslipidemia and the reduction in susceptibility to atherosclerotic cardiovascular disease associated with SNALP-mediated silencing of apoCIII.

[0036] Figure 8 illustrates data demonstrating an *in vitro* activity screen of *APOC3* siRNA sequences. Native human *APOC3* siRNA sequences targeting *APOC3* mRNA were reverse transfected into HepG2 cells and silencing activity was assessed by QuantiGene Assay 48 h post-treatment. Cells were treated with SNALP formulated-*APOC3* siRNA at 2.5nM (white bar), 10nM (grey bar), and 40nM (black bar). Sequence numbers represent the nucleotide position of *APOC3* mRNA (Genbank Accession No. NM_000040.1) that is complementary to the 3' end of the antisense strand of the siRNA.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0037] Coronary artery disease (CAD) or atherosclerotic cardiovascular disease (CVD) is the leading cause of illness and death worldwide. The risk of developing CAD is closely associated with alterations in blood lipids (*i.e.,* dyslipidemias), particularly elevated plasma cholesterol (*i.e.,* hypercholesterolemia). While the symptoms and signs of CAD are noted in the advanced state of disease, most individuals with CAD show no evidence of disease for decades as the disease progresses before the first onset of symptoms, often a "sudden" heart attack, finally arises. After decades of progression, some of the atheromatous plaques that develop may rupture and (along with the activation of the blood clotting system) start limiting

blood flow to the heart muscle. CAD is the most common cause of sudden death, and is also the most common reason for death of men and women over 20 years of age. According to present trends in the United States, half of healthy 40-year-old males will develop CAD in the future, and one in three healthy 40-year-old women. As the degree of CAD progresses, there may be near-complete obstruction of the lumen of the coronary artery, severely restricting the flow of oxygen-carrying blood to the myocardium. Individuals with this degree of CAD typically have suffered from one or more myocardial infarctions (heart attacks), and may have signs and symptoms of chronic coronary ischemia, including symptoms of angina at rest and flash pulmonary edema. It is therefore clear that CAD and other diseases associated with elevated blood cholesterol, triglyceride, and/or glucose levels represent a significant unmet medical need that requires the development of novel therapeutic agents for more effective treatment options.

[0038] Apolipoprotein C-III (*APOC3*) is an important regulator of lipoprotein metabolism that has been implicated in the progression of atherosclerosis through its association with hypertriglyceridemia and its direct induction of endothelial dysfunction. Example 2 below describes the preclinical development of chemically modified siRNA targeting *Apoc3* in mice. *Apoc3*-targeting siRNA formulated in stable nucleic acid-lipid particles (SNALP) were administered by intravenous injection to female C57BL/6 mice at doses of 0.5 and 5 mg/kg. Both doses demonstrated potent efficacy, reducing hepatic *Apoc3* mRNA by more than 90% and reducing plasma triglycerides by 35-45%, without an increase in hepatic triglycerides. No measurable immune response was induced with these formulations, minimizing the potential for nonspecific effects in models of chronic inflammatory disease, such as atherosclerosis. In addition, Example 3 below illustrates the identification of human *APOC3* siRNA sequences which demonstrated potent silencing activity. As such, these Examples demonstrate the clinically relevant effects and benefits of siRNA-based silencing of *APOC3* in mammals, *e.g.,* the utility of *Apoc3*-targeting SNALP in animal models of dyslipidemia and atherosclerosis, as well as the utility of SNALP-formulated siRNA targeting the human *APOC3* gene for treating, preventing, reducing the risk of developing, or delaying the onset of a lipid disorder such as atherosclerosis or a dyslipidemia, *e.g.,* a hyperlipidemia such as elevated triglyceride levels (hypertriglyceridemia) and/or elevated cholesterol levels (hypercholesterolemia).

II. Definitions

[0039] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0040] The term "interfering RNA" or "RNAi" or "interfering RNA sequence" as used herein includes single-stranded RNA (*e.g.,* mature miRNA, ssRNAi oligonucleotides, ssDNAi oligonucleotides) or double-stranded RNA (*i.e.*, duplex RNA such as siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.,* by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or sequence, or may comprise a region of mismatch (*i.e.,* a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof. Preferably, the interfering RNA molecules are chemically synthesized.

[0041] Interfering RNA includes "small-interfering RNA" or "siRNA," *e.g.,* interfering RNA of about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.,* each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-

complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule.

[0042] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see*, *e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes,* 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.,* 31:981-987 (2003); Knight *et al.*, *Science,* 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.,* 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0043] As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an interfering RNA (*e.g.,* siRNA) sequence that does not have 100% complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0044] The phrase "inhibiting expression of a target gene" refers to the ability of an interfering RNA (*e.g.,* siRNA) of the present invention to silence, reduce, or inhibit the expression of a target gene (*e.g.*, *APOC3* and/or other genes associated with metabolic diseases and disorders). To examine the extent of gene silencing, a test sample (*e.g.*, a biological sample from an organism of interest expressing the target gene or a sample of cells in culture expressing the target gene) is contacted with an interfering RNA (*e.g.,* siRNA) that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample is compared to expression of the target gene in a control sample (*e.g.*, a biological sample from an organism of interest expressing the target gene or a sample of cells in culture expressing the target gene) that is not contacted with the interfering RNA (*e.g.,* siRNA). Control samples (*e.g.*, samples expressing the target gene) may be assigned a value of 100%. In particular embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the value of the test sample relative to the control sample is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%,

20%, 10%, 5%, or 0%. Suitable assays include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, *e.g.,* dot blots, Northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0045] An "effective amount" or "therapeutically effective amount" of a therapeutic nucleic acid such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of an interfering RNA. In particular embodiments, inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring the expression of a target gene or target sequence include, but are not limited to, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, *e.g.,* dot blots, Northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0046] By "decrease," "decreasing," "reduce," or "reducing" of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.*, a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFNγ, IFNα, TNFα, IL-6, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0047] As used herein, the term "responder cell" refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth factors such as TNF-α, IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TGF, and combinations thereof. Detectable immune responses also include, *e.g.*, induction of interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) mRNA.

[0048] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0049] The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10^{o}C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0050] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42^{o}C, or, 5x SSC, 1% SDS, incubating at 65^{o}C, with wash in 0.2x SSC, and 0.1% SDS at 65^{o}C. For PCR, a temperature of about 36^{o}C is typical for low stringency amplification, although annealing temperatures may vary between about 32^{o}C and 48^{o}C depending on primer length. For high stringency PCR amplification, a temperature of about 62^{o}C is typical, although high stringency annealing temperatures can range from about 50^{o}C to about 65^{o}C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90^{o}C-95^{o}C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72^{o}C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.,* in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y. (1990).

[0051] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This

occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous references, *e.g.*, Current Protocols in Molecular Biology, Ausubel *et al.*, eds.

[0052] The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (*i.e.,* at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0053] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0054] A "comparison window," as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.,* by the local homology algorithm of Smith and Waterman, *Adv. Appl.*

Math., 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.,* 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA,* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology*, Ausubel *et al*., eds. (1995 supplement)).

[0055] Non-limiting examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.,* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.*, 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. Another example is a global alignment algorithm for determining percent sequence identiy such as the Needleman-Wunsch algorithm for aligning protein or nucleotide (*e.g.,* mRNA) sequences.

[0056] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA,* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0057] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of small interfering RNA (siRNA), Dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring,

and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.,* degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.,* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.,* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes,* 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0058] The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0059] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0060] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0061] The term "lipid particle" includes a lipid formulation that can be used to deliver a therapeutic nucleic acid (*e.g.*, interfering RNA) to a target site of interest (*e.g.,* cell, tissue, organ, and the like). In preferred embodiments, the lipid particle of the invention is a nucleic acid-lipid particle, which is typically formed from a cationic lipid, a non-cationic lipid, and

optionally a conjugated lipid that prevents aggregation of the particle. In other preferred embodiments, the therapeutic nucleic acid (*e.g.*, interfering RNA) may be encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation.

[0062] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.*, a cationic lipid, a non-cationic lipid, and optionally a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.*, interfering RNA) is fully encapsulated within the lipid. In certain instances, SNALP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.*, sites physically separated from the administration site), and they can mediate silencing of target gene expression at these distal sites. The nucleic acid may be complexed with a condensing agent and encapsulated within a SNALP as set forth in PCT Publication No. WO 00/03683.

[0063] The lipid particles of the invention (*e.g.*, SNALP) typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 20040142025 and 20070042031.

[0064] As used herein, "lipid encapsulated" can refer to a lipid particle that provides a therapeutic nucleic acid such as an interfering RNA (*e.g.*, siRNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid (*e.g.*, interfering RNA) is fully encapsulated in the lipid particle (*e.g.*, to form a SNALP or other nucleic acid-lipid particle).

[0065] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polyamide oligomers

(*e.g.,* ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to dialkyloxypropyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, PEG conjugated to ceramides (*see, e.g.,* U.S. Patent No. 5,885,613), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.,* non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

[0066] The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0067] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

[0068] The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

[0069] The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0070] The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0071] The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0072] The terms "cationic lipid" and "amino lipid" are used interchangeably herein to include those lipids and salts thereof having one, two, three, or more fatty acid or fatty alkyl chains and a pH-titratable amino head group (*e.g.,* an alkylamino or dialkylamino head group). The cationic lipid is typically protonated (*i.e.,* positively charged) at a pH below the pK_a of the cationic lipid and is substantially neutral at a pH above the pK_a. The cationic lipids of the invention may also be termed titratable cationic lipids. In some embodiments, the cationic lipids comprise: a protonatable tertiary amine (*e.g.,* pH-titratable) head group; C_{18} alkyl chains, wherein each alkyl chain independently has 0 to 3 double bonds; and ether or ketal linkages between the head group and alkyl chains. Such lipids include, but are not limited to, DSDMA, DODMA, DLinDMA, DLenDMA, DLin-K-DMA, DLin-K-C2-DMA (also known as DLin-C2K-DMA, XTC2, and C2K), DLin-K-C3-DMA, and DLin-K-C4-DMA.

[0073] The term "salts" includes any anionic and cationic complex, such as the complex formed between a cationic lipid and one or more anions. Non-limiting examples of anions include inorganic and organic anions, *e.g.,* hydride, fluoride, chloride, bromide, iodide, oxalate (*e.g.,* hemioxalate), phosphate, phosphonate, hydrogen phosphate, dihydrogen phosphate, oxide, carbonate, bicarbonate, nitrate, nitrite, nitride, bisulfite, sulfide, sulfite, bisulfate, sulfate, thiosulfate, hydrogen sulfate, borate, formate, acetate, benzoate, citrate, tartrate, lactate, acrylate, polyacrylate, fumarate, maleate, itaconate, glycolate, gluconate, malate, mandelate, tiglate, ascorbate, salicylate, polymethacrylate, perchlorate, chlorate, chlorite, hypochlorite, bromate, hypobromite, iodate, an alkylsulfonate, an arylsulfonate,

arsenate, arsenite, chromate, dichromate, cyanide, cyanate, thiocyanate, hydroxide, peroxide, permanganate, and mixtures thereof. In particular embodiments, the salts of the cationic lipids disclosed herein are crystalline salts.

[0074] The term "alkyl" includes a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include, but are not limited to, methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and the like, while saturated branched alkyls include, without limitation, isopropyl, *sec*-butyl, isobutyl, *tert*-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like, while unsaturated cyclic alkyls include, without limitation, cyclopentenyl, cyclohexenyl, and the like.

[0075] The term "alkenyl" includes an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both *cis* and *trans* isomers. Representative straight chain and branched alkenyls include, but are not limited to, ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

[0076] The term "alkynyl" includes any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include, without limitation, acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, 3-methyl-1 butynyl, and the like.

[0077] The term "acyl" includes any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. The following are non-limiting examples of acyl groups: -C(=O)alkyl, -C(=O)alkenyl, and -C(=O)alkynyl.

[0078] The term "heterocycle" includes a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle may be attached via any heteroatom or carbon atom. Heterocycles include, but are not limited to, heteroaryls as defined below, as well as morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

[0079] The terms "optionally substituted alkyl", "optionally substituted alkenyl", "optionally substituted alkynyl", "optionally substituted acyl", and "optionally substituted heterocycle" mean that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O), two hydrogen atoms are replaced. In this regard, substituents include, but are not limited to, oxo, halogen, heterocycle, -CN, $-OR^x$, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$, and $-SO_nNR^xR^y$, wherein n is 0, 1, or 2, R^x and R^y are the same or different and are independently hydrogen, alkyl, or heterocycle, and each of the alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, $-OR^x$, heterocycle, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$, and $-SO_nNR^xR^y$. The term "optionally substituted," when used before a list of substituents, means that each of the substituents in the list may be optionally substituted as described herein.

[0080] The term "halogen" includes fluoro, chloro, bromo, and iodo.

[0081] The term "fusogenic" refers to the ability of a lipid particle, such as a SNALP, to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0082] As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

[0083] As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

[0084] "Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0085] "Serum-stable" in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

[0086] "Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent such as an interfering RNA (*e.g.,* siRNA) within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by

first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0087] "Local delivery," as used herein, refers to delivery of an active agent such as an interfering RNA (*e.g.,* siRNA) directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site, other target site, or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0088] The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

III. Description of the Embodiments

[0089] The present invention provides therapeutic nucleic acids such as interfering RNA that target *APOC3* gene expression, lipid particles comprising one or more (*e.g.,* a cocktail) of the therapeutic nucleic acids, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.,* for the prevention or treatment of dyslipidemia and/or atherosclerosis).

[0090] In one aspect, the present invention provides interfering RNA molecules that target *APOC3* expression. Non-limiting examples of interfering RNA molecules include siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, miRNA, and mixtures thereof. In certain instances, the present invention provides compositions comprising a combination (*e.g.,* a cocktail, pool, or mixture) of siRNAs that target different regions of the *APOC3* gene and/or multiple genes (*e.g.,* a cocktail of siRNAs that silence *APOC3* and *APOB* expression). The interfering RNA (*e.g.,* siRNA) molecules of the present invention are capable of reducing *APOC3* mRNA *in vitro* (*e.g.,* in primary hepatocytes) or *in vivo* (*e.g.,* in liver tissue).

[0091] In particular embodiments, the present invention provides an siRNA that silences *APOC3* gene expression, wherein the siRNA comprises a sense strand and a complementary antisense strand, and wherein the siRNA comprises a double-stranded region of about 15 to about 60 nucleotides in length (*e.g.,* about 15-60, 15-30, 15-25, 19-30, or 19-25 nucleotides in length, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length).

[0092] In some embodiments, the antisense strand comprises one of the antisense strand sequences set forth in Tables 1-10. In related embodiments, the antisense strand comprises at least 15 contiguous nucleotides (*e.g.,* at least 15, 16, 17, 18, or 19 contiguous nucleotides) of

one of the antisense strand sequences set forth in Tables 1-10. In one particular embodiment, the antisense strand comprises nucleotides 1-19 of one of the antisense strand sequences set forth in Tables 1-10. In further embodiments, the sense strand comprises one of the sense strand sequences set forth in Tables 1-10. In related embodiments, the sense strand comprises at least 15 contiguous nucleotides (*e.g.,* at least 15, 16, 17, 18, or 19 contiguous nucleotides) of one of the sense strand sequences set forth in Tables 1-10. In one particular embodiment, the sense strand comprises nucleotides 1-19 of one of the sense strand sequences set forth in Tables 1-10. In other embodiments, the antisense strand specifically hybridizes to one of the target sequences set forth in Tables 1-10. In additional embodiments, the *APOC3* siRNA targets one of the target sequences set forth in Tables 7-10.

[0093] In certain embodiments, the *APOC3* siRNA of the invention may comprise at least one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides such as 2'OMe nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region of the siRNA. Preferably, uridine and/or guanosine nucleotides in the siRNA are modified with 2'OMe nucleotides. In certain instances, the siRNA contains 2'OMe nucleotides in both the sense and antisense strands and comprises at least one 2'OMe-uridine nucleotide and at least one 2'OMe-guanosine nucleotide in the double-stranded region. In some embodiments, the sense and/or antisense strand of the siRNA may further comprise modified (*e.g.,* 2'OMe-modified) adenosine and/or modified (*e.g.,* 2'OMe-modified) cytosine nucleotides, *e.g.,* in the double-stranded region of the siRNA.

[0094] In one embodiment, the antisense strand of the *APOC3* siRNA comprises one of the 2'OMe-modified sequences set forth in Table 1. The antisense strand sequence of *APOC3* siRNA "262" shown in Table 7 sets forth the unmodified version of the 2'OMe-modified sequences set forth in Table 1. Nucleotides 1-19 of the antisense strand sequence of the hAPOC3_260 siRNA shown in Table 10 also correspond to the unmodified version of the 2'OMe-modified sequences set forth in Table 1.

Table 1

5'-CUUAACGG**U**GC**U**CCAG**U**AG-3'	5'-C**U**UAACGGU**G**C**U**CCAG**U**AG-3'
5'-C**U**UAACGG**U**GC**U**CCAG**U**AG-3'	5'-C**U**UAAC**G**GU**G**C**U**CCAG**U**AG-3'
5'-CU**U**AACGG**U**GC**U**CCAG**U**AG-3'	5'-C**U**UAAC**G**GU**G**C**U**CCAG**U**A**G**-3'
5'-C**UU**AACGG**U**GC**U**CCAG**U**AG-3'	5'-CU**U**AACGG**U**GC**U**CCAG**U**A**G**-3'
5'-CUUAACGGU**G**CUCCA**G**UA**G**-3'	5'-CU**U**AAC**G**G**U**GC**U**CCAG**U**AG-3'
5'-CUUAAC**G**GUGCUCCA**G**UA**G**-3'	5'-CU**U**AACG**GU**GC**U**CCAG**U**A**G**-3'
5'-CUUAAC**G**GU**G**CUCCA**G**UA**G**-3'	5'-CU**U**AACG**GU**GC**U**CCAG**U**AG-3'

5′-CUUAACG**G**U**G**CUCCA**G**UA**G**-3′	5′-CU**U**AACGG**UG**C**U**CCAG**U**A**G**-3′
5′-CUUAAC**GG**U**G**CUCCA**G**UA**G**-3′	5′-CU**U**AACGG**UG**C**U**CCAG**U**AG-3′
5′-CUUAAC**G**G**U**GC**U**CCAG**U**AG-3′	5′-CU**U**AAC**G**G**U**GC**U**CCA**GU**A**G**-3′
5′-CUUAACGG**U**GC**U**CCAG**U**A**G**-3′	5′-CU**U**AAC**G**G**U**GC**U**CCA**GU**AG-3′
5′-CUUAAC**G**G**U**GC**U**CCAG**U**A**G**-3′	5′-CU**U**AACGG**U**GC**U**CCA**GU**AG-3′
5′-C**U**UAAC**G**G**U**GC**U**CCAG**U**A**G**-3′	5′-CU**U**AACG**GU**GC**U**CCAG**U**AG-3′
5′-CU**U**AAC**G**G**U**GC**U**CCAG**U**A**G**-3′	5′-CU**U**AACGG**UG**C**U**CCAG**U**AG-3′
5′-CUUAAC**G**G**U**GC**U**CCA**GU**AG-3′	5′-CUUAACG**GU**GC**U**CCAG**U**AG-3′
5′-CUUAAC**GG**U**G**C**U**CCAG**U**A**G**-3′	5′-CUUAACG**GU**GC**U**CCAG**U**A**G**-3′
5′-C**U**UAAC**G**GUGC**U**CCAG**U**AG-3′	5′-CUUAACGG**UG**C**U**CCAG**U**AG-3′
5′-C**UU**AAC**G**GUGC**U**CCAG**U**AG-3′	5′-CUUAACGG**UG**C**U**CCAG**U**A**G**-3′
5′-C**UU**AACG**G**UGC**U**CCAG**U**AG-3′	5′-CUUAACGG**U**GC**U**CCA**GU**A**G**-3′
5′-C**UU**AACG**G**UGC**U**CCAG**U**A**G**-3′	5′-CUUAAC**G**G**UG**C**U**CCAG**U**A**G**-3′
5′-C**U**UAAC**G**G**U**GC**U**CCA**GU**A**G**-3′	5′-CUUAAC**G**G**UG**C**U**CCA**GU**A**G**-3′
5′-CUUAACG**G**U**G**CUCCAG**U**AG-3′	5′-CUUAACG**G**U**G**C**U**CCAG**U**AG-3′
5′-CUUAACG**G**UGC**U**CCAG**U**A**G**-3′	5′-CUUAACG**G**U**G**C**U**CCA**GU**AG-3′
5′-CUUAACG**G**U**G**C**U**CCA**GU**A**G**-3′	5′-CU**U**AACG**G**U**G**C**U**CCAG**U**AG-3′
5′-CU**U**AACG**G**UGC**U**CCAG**U**A**G**-3′	5′-CU**U**AACG**G**U**G**C**U**CCAG**U**A**G**-3′
5′-CUUAAC**GG**UGC**U**CCAG**U**AG-3′	5′-CUUAAC**GG**U**G**C**U**CCAG**U**AG-3′
5′-CUUAACGG**U**G**C**U**C**CA**GU**AG-3′	5′-CU**U**AAC**GG**U**G**C**U**CCAG**U**AG-3′
5′-CUUAACG**G**U**G**C**U**CCAG**U**A**G**-3′	5′-CUUAAC**GG**UGC**U**CCAG**U**A**G**-3′

2'OMe nucleotides are indicated in bold and underlined.

[0095] In particular embodiments, the 2'OMe-modified sequence set forth in Table 1 corresponds to the antisense strand sequence present in the double-stranded region of the siRNA. In some embodiments, the 2'OMe-modified sequence set forth in Table 1 comprises a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other embodiments, the 2'OMe-modified sequence set forth in Table 1 further comprises at least one, two, three, four, five, six, or more 2'OMe-modified adenosine and/or modified 2'OMe-modified cytosine nucleotides. Each of the 2'OMe-modified antisense strand sequences set forth in Table 1 may comprise the complementary strand of any of the 2'OMe-modified sense strand sequences set forth in Table 2 or the unmodified *APOC3* siRNA "262" sense strand sequence shown in Table 7.

[0096] In another embodiment, the sense strand of the *APOC3* siRNA comprises one of the 2'OMe-modified sequences set forth in Table 2. The sense strand sequence of *APOC3* siRNA "262" shown in Table 7 sets forth the unmodified version of the 2'OMe-modified sequences set forth in Table 2. Nucleotides 1-19 of the sense strand sequence of the

hAPOC3_260 siRNA shown in Table 10 also correspond to the unmodified version of the 2'OMe-modified sequences set forth in Table 2.

Table 2

5'-C**U**AC**U**GGAGCACCG**U**UAAG-3'	5'-C**U**AC**U**G**G**A**G**CACCG**U**UAA**G**-3'
5'-C**U**AC**U**GGAGCACCGU**U**AAG-3'	5'-C**U**AC**U**G**G**AGCACCGU**U**AAG-3'
5'-C**U**AC**U**GGAGCACCG**UU**AAG-3'	5'-C**U**AC**U**GGA**G**CACCGU**U**AAG-3'
5'-CUAC**U**GGAGCACCG**U**UAAG-3'	5'-C**U**AC**U**GGAGCACCGU**U**AA**G**-3'
5'-CUAC**U**GGAGCACCGU**U**AAG-3'	5'-C**U**AC**U**G**G**A**G**CACCGU**U**AAG-3'
5'-CUACU**G**GA**G**CACC**G**UUAAG-3'	5'-C**U**AC**U**G**G**AGCACCGU**U**AA**G**-3'
5'-CUACU**G**GA**G**CACCGUUAA**G**-3'	5'-C**U**AC**U**GGA**G**CACCGU**U**AA**G**-3'
5'-CUACUG**G**A**G**CACC**G**UUAAG-3'	5'-C**U**AC**U**G**G**A**G**CACCGU**U**AA**G**-3'
5'-CUACUG**G**A**G**CACCGUUAA**G**-3'	5'-CUAC**U**G**G**A**G**CACCG**U**UAAG-3'
5'-CUACU**G**GA**G**CACC**G**UUAA**G**-3'	5'-CUAC**U**G**G**AGCACCG**U**UAA**G**-3'
5'-CUACUG**G**A**G**CACC**G**UUAA**G**-3'	5'-CUAC**U**GGA**G**CACCG**U**UAA**G**-3'
5'-CUACU**GG**A**G**CACC**G**UUAA**G**-3'	5'-CUAC**U**G**G**A**G**CACCG**U**UAA**G**-3'
5'-C**U**AC**U**G**G**AGCACCG**U**UAAG-3'	5'-CUAC**U**G**G**A**G**CACC**GU**UAA**G**-3'
5'-C**U**AC**U**GGA**G**CACCG**U**UAAG-3'	5'-CUAC**U**G**G**A**G**CACCG**UU**AA**G**-3'
5'-C**U**AC**U**GGAGCACCG**U**UAA**G**-3'	5'-CUAC**UG**GA**G**CACCG**U**UAA**G**-3'
5'-C**U**AC**U**G**G**A**G**CACCG**U**UAAG-3'	5'-CUAC**U**G**G**A**G**CACCGU**U**AAG-3'
5'-C**U**AC**U**G**G**AGCACCG**U**UAA**G**-3'	5'-CUAC**U**G**G**AGCACCGU**U**AA**G**-3'
5'-C**U**AC**U**GGA**G**CACCG**U**UAA**G**-3'	5'-CUAC**U**GGA**G**CACCGU**U**AA**G**-3'
5'-C**U**AC**U**GGA**G**CACCG**UU**AA**G**-3'	5'-CUAC**U**G**G**A**G**CACCGU**U**AA**G**-3'
5'-C**U**ACU**G**GA**G**CACCG**U**UAAG-3'	5'-C**U**ACU**G**GA**G**CACCG**U**UAA**G**-3'
5'-C**U**ACU**G**GA**G**CACCGU**U**AAG-3'	5'-C**U**ACU**G**GA**G**CACCGU**U**AA**G**-3'
5'-C**U**AC**U**G**G**AGCACC**G**U**U**AAG-3'	5'-C**U**AC**U**G**G**AGCACC**G**U**U**AA**G**-3'
5'-C**U**AC**U**GGA**G**CACC**G**U**U**AAG-3'	5'-C**U**AC**U**GGA**G**CACC**G**U**U**AA**G**-3'
5'-C**U**AC**U**GGAGCACC**G**U**U**AA**G**-3'	5'-C**U**AC**U**G**G**A**G**CACC**G**U**U**AA**G**-3'
5'-C**U**AC**U**G**G**A**G**CACC**G**U**U**AAG-3'	5'-CUAC**U**G**G**AGCACC**GU**UAA**G**-3'
5'-C**U**AC**U**G**G**A**G**CACC**GU**UAAG-3'	5'-CUAC**U**G**G**A**G**CACC**GU**UAAG-3'

2'OMe nucleotides are indicated in bold and underlined.

[0097] In particular embodiments, the 2'OMe-modified sequence set forth in Table 2 corresponds to the sense strand sequence present in the double-stranded region of the siRNA. In some embodiments, the 2'OMe-modified sequence set forth in Table 2 comprises a modified (*e.g.*, 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other embodiments, the 2'OMe-modified sequence set forth in Table 2 further comprises at least one, two, three, four, five, six, or more 2'OMe-modified adenosine and/or modified 2'OMe-

modified cytosine nucleotides. Each of the 2'OMe-modified sense strand sequences set forth in Table 2 may comprise the complementary strand of any of the 2'OMe-modified antisense strand sequences set forth in Table 1 or the unmodified *APOC3* siRNA "262" antisense strand sequence shown in Table 7.

[0098] In yet another embodiment, the antisense strand of the *APOC3* siRNA comprises one of the 2'OMe-modified sequences set forth in Table 3. The antisense strand sequence of *APOC3* siRNA "314" shown in Table 7 sets forth the unmodified version of the 2'OMe-modified sequences set forth in Table 3. Nucleotides 1-19 of the antisense strand sequence of the hAPOC3_312 siRNA shown in Table 10 also correspond to the unmodified version of the 2'OMe-modified sequences set forth in Table 3.

Table 3

5'-C**U**GAAG**U**UGG**U**CUGACCUC-3'	5'-C**U**GAAG**U**U**G**G**U**CU**G**ACCUC-3'
5'-C**U**GAAGU**U**GG**U**CUGACCUC-3'	5'-CU**G**AAG**U**UGG**U**C**U**GACC**U**C-3'
5'-C**U**GAAG**U**UGG**U**C**U**GACCUC-3'	5'-CUGAAG**U**U**G**G**U**C**U**GACC**U**C-3'
5'-C**U**GAAGU**U**GG**U**C**U**GACCUC-3'	5'-CU**G**AAG**U**U**G**G**U**C**U**GACC**U**C-3'
5'-C**U**GAAG**U**UGG**U**C**U**GACC**U**C-3'	5'-CUGAAG**U**UG**GU**C**U**GACC**U**C-3'
5'-C**U**GAAGU**U**GG**U**C**U**GACC**U**C-3'	5'-CUGAAG**U**UGG**U**C**UG**ACC**U**C-3'
5'-C**U**GAAG**UU**GG**U**C**U**GACC**U**C-3'	5'-CU**G**AAG**U**UG**GU**C**U**GACC**U**C-3'
5'-CUGAAG**U**UGG**U**C**U**GACC**U**C-3'	5'-CU**G**AAGU**U**GG**U**C**U**GACC**U**C-3'
5'-CUGAAGU**U**GG**U**C**U**GACC**U**C-3'	5'-CUGAAGU**UG**G**U**C**U**GACC**U**C-3'
5'-CUGAAG**UU**GG**U**C**U**GACC**U**C-3'	5'-CU**G**AAGU**UG**G**U**C**U**GACC**U**C-3'
5'-CU**G**AA**G**UU**G**GUCUGACCUC-3'	5'-CUGAAGU**U**G**GU**C**U**GACC**U**C-3'
5'-CU**G**AA**G**UUG**G**UCUGACCUC-3'	5'-CUGAAGU**U**GG**U**C**UG**ACC**U**C-3'
5'-CU**G**AA**G**UUG**G**UCU**G**ACCUC-3'	5'-CU**G**AAGU**U**G**GU**C**U**GACC**U**C-3'
5'-CU**G**AA**G**UUG**G**UCU**G**ACCUC-3'	5'-CU**G**AA**G**UUG**G**UC**U**GACC**U**C-3'
5'-CU**G**AA**G**UU**GG**UCU**G**ACCUC-3'	5'-CU**G**AA**G**UU**G**GU**C**UGACC**U**C-3'
5'-CUGAA**G**UUG**G**UCU**G**ACCUC-3'	5'-CU**G**AA**G**UU**G**G**U**C**U**GACC**U**C-3'
5'-CUGAA**G**UUG**G**UCU**G**ACCUC-3'	5'-CU**G**AAGUU**G**G**U**C**U**GACC**U**C-3'
5'-C**U**GAAG**U**U**G**G**U**CUGACCUC-3'	5'-CU**G**AA**G**UUGG**U**C**U**GACC**U**C-3'
5'-C**U**GAAG**U**UGG**U**CU**G**ACCUC-3'	5'-CUGAAGUUGG**U**C**U**GACC**U**C-3'
5'-CU**G**AAGU**U**G**G**U**C**U**G**ACC**U**C-3'	5'-CU**G**AA**G**UUG**G**UC**U**GACC**U**C-3'

2'OMe nucleotides are indicated in bold and underlined.

[0099] In particular embodiments, the 2'OMe-modified sequence set forth in Table 3 corresponds to the antisense strand sequence present in the double-stranded region of the siRNA. In some embodiments, the 2'OMe-modified sequence set forth in Table 3 comprises

a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other embodiments, the 2'OMe-modified sequence set forth in Table 3 further comprises at least one, two, three, four, five, six, or more 2'OMe-modified adenosine and/or modified 2'OMe-modified cytosine nucleotides. Each of the 2'OMe-modified antisense strand sequences set forth in Table 3 may comprise the complementary strand of any of the 2'OMe-modified sense strand sequences set forth in Table 4 or the unmodified *APOC3* siRNA "314" sense strand sequence shown in Table 7.

[0100] In still yet another embodiment, the sense strand of the *APOC3* siRNA comprises one of the 2'OMe-modified sequences set forth in Table 4. The sense strand sequence of *APOC3* siRNA "314" shown in Table 7 sets forth the unmodified version of the 2'OMe-modified sequences set forth in Table 4. Nucleotides 1-19 of the sense strand sequence of the hAPOC3_312 siRNA shown in Table 10 also correspond to the unmodified version of the 2'OMe-modified sequences set forth in Table 4.

Table 4

5′-GAGG**U**CAGACCAAC**UU**CAG-3′	5′-**G**A**G**G**U**CA**G**ACCAACU**U**CAG-3′
5′-**G**A**G**GUCA**G**ACCAACUUCAG-3′	5′-**G**A**G**G**U**CAGACCAACU**U**CA**G**-3′
5′-**G**AG**G**UCA**G**ACCAACUUCAG-3′	5′-**G**AG**GU**CAGACCAACU**U**CA**G**-3′
5′-**G**A**G**GUCAGACCAACUUCA**G**-3′	5′-**G**AG**GU**CA**G**ACCAACU**U**CA**G**-3′
5′-**G**AG**G**UCAGACCAACUUCA**G**-3′	5′-**G**A**G**G**U**CA**G**ACCAACU**U**CA**G**-3′
5′-**G**AGGUCA**G**ACCAACUUCA**G**-3′	5′-GAGG**U**CA**G**ACCAAC**U**UCAG-3′
5′-**G**A**GG**UCA**G**ACCAACUUCAG-3′	5′-GAGG**U**CA**G**ACCAAC**U**UCA**G**-3′
5′-**G**A**GG**UCAGACCAACUUCA**G**-3′	5′-GA**G**G**U**CAGACCAAC**U**UCAG-3′
5′-**G**A**GG**UCA**G**ACCAACUUCA**G**-3′	5′-GA**G**G**U**CA**G**ACCAAC**U**UCAG-3′
5′-**G**AGG**U**CA**G**ACCAAC**U**UCAG-3′	5′-GA**G**G**U**CAGACCAAC**U**UCA**G**-3′
5′-**G**AGG**U**CA**G**ACCAAC**U**UCA**G**-3′	5′-GAG**GU**CAGACCAAC**U**UCA**G**-3′
5′-**G**A**G**G**U**CAGACCAAC**U**UCAG-3′	5′-GAG**GU**CA**G**ACCAAC**U**UCA**G**-3′
5′-**G**AG**GU**CAGACCAAC**U**UCA**G**-3′	5′-GA**G**G**U**CAGACCAACU**U**CAG-3′
5′-**G**AG**GU**CA**G**ACCAAC**U**UCA**G**-3′	5′-GA**G**G**U**CA**G**ACCAACU**U**CAG-3′
5′-**G**A**G**G**U**CA**G**ACCAAC**U**UCA**G**-3′	5′-GA**G**G**U**CA**G**ACCAACU**U**CA**G**-3′
5′-**G**AGG**U**CA**G**ACCAACU**U**CAG-3′	5′-GAG**GU**CAGACCAAC**U**UCA**G**-3′
5′-**G**AGG**U**CA**G**ACCAACU**U**CA**G**-3′	5′-GAG**GU**CA**G**ACCAAC**U**UCA**G**-3′
5′-**G**A**G**G**U**CAGACCAACU**U**CAG-3′	5′-**G**A**G**G**U**CAGACCAAC**UU**CAG-3′
5′-GA**G**G**U**CAGACCAAC**UU**CA**G**-3′	5′-GA**G**G**U**CA**G**ACCAAC**UU**CAG-3′

2'OMe nucleotides are indicated in bold and underlined.

[0101] In particular embodiments, the 2'OMe-modified sequence set forth in Table 4 corresponds to the sense strand sequence present in the double-stranded region of the siRNA. In some embodiments, the 2'OMe-modified sequence set forth in Table 4 comprises a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other embodiments, the 2'OMe-modified sequence set forth in Table 4 further comprises at least one, two, three, four, five, six, or more 2'OMe-modified adenosine and/or modified 2'OMe-modified cytosine nucleotides. Each of the 2'OMe-modified sense strand sequences set forth in Table 4 may comprise the complementary strand of any of the 2'OMe-modified antisense strand sequences set forth in Table 3 or the unmodified *APOC3* siRNA "314" antisense strand sequence shown in Table 7.

[0102] In yet another embodiment, the antisense strand of the *APOC3* siRNA comprises one of the 2'OMe-modified sequences set forth in Table 5. The antisense strand sequence of *APOC3* siRNA "268" shown in Table 7 sets forth the unmodified version of the 2'OMe-modified sequences set forth in Table 5. Nucleotides 1-19 of the antisense strand sequence of the hAPOC3_266 siRNA shown in Table 10 also correspond to the unmodified version of the 2'OMe-modified sequences set forth in Table 5.

Table 5

5'-C**U**UGUCC**U**UAACGG**U**GCUC-3'	5'-CU**U**GUCC**U**UAAC**G**GU**G**C**U**C-3'
5'-C**U**UGUCC**U**UAACGGUGC**U**C-3'	5'-CU**U**GUCCU**U**AAC**G**GUGC**U**C-3'
5'-C**U**UGUCC**U**UAACGG**U**GC**U**C-3'	5'-CU**U**GUCC**U**UAAC**G**G**U**GC**U**C-3'
5'-CU**U**GUCC**U**UAACGG**U**GCUC-3'	5'-CU**U**GUCCU**U**AAC**G**G**U**GC**U**C-3'
5'-CU**U**GUCCU**U**AACGG**U**GCUC-3'	5'-CU**U**GUCCU**U**AAC**G**GU**G**C**U**C-3'
5'-CU**U**GUCC**U**UAACGGUGC**U**C-3'	5'-CU**U**GUCC**UU**AAC**G**G**U**GCUC-3'
5'-CU**U**GUCCU**U**AACGGUGC**U**C-3'	5'-CU**U**GUCC**UU**AACGGU**G**C**U**C-3'
5'-CU**U**GUCC**U**UAACGG**U**GC**U**C-3'	5'-CU**U**GUCC**UU**AAC**G**G**U**GC**U**C-3'
5'-CU**U**GUCCU**U**AACGG**U**GC**U**C-3'	5'-CUUGUCC**UU**AAC**G**G**U**GC**U**C-3'
5'-CU**U**GUCC**UU**AACGG**U**GCUC-3'	5'-C**UU**GUCC**UU**AAC**G**G**U**GC**U**C-3'
5'-CU**U**GUCC**UU**AACGGUGC**U**C-3'	5'-CU**U**GUCC**UU**AAC**G**GU**G**C**U**C-3'
5'-CU**U**GUCC**UU**AACGG**U**GC**U**C-3'	5'-CU**U**GUCC**UU**AACG**G**U**G**C**U**C-3'
5'-CUUGUCC**UU**AACGG**U**GC**U**C-3'	5'-CUU**G**UCC**UU**AAC**G**G**U**GC**U**C-3'
5'-C**UU**GUCC**UU**AACGG**U**GC**U**C-3'	5'-CUUG**U**CC**U**UAACGG**U**GC**U**C-3'
5'-CUU**G**UCCUUAAC**G**GU**G**CUC-3'	5'-CUUG**U**CC**U**UAAC**G**G**U**GC**U**C-3'
5'-CUU**G**UCCUUAACG**G**U**G**CUC-3'	5'-CUUG**U**CC**U**UAACG**GU**GC**U**C-3'
5'-CUUGUCCUUAAC**GG**U**G**CUC-3'	5'-CUUG**U**CC**U**UAACGG**UG**C**U**C-3'
5'-CUU**G**UCCUUAAC**GG**U**G**CUC-3'	5'-CUUG**U**CC**UU**AACGG**U**GC**U**C-3'
5'-CU**U**GUCC**U**UAAC**G**G**U**GCUC-3'	5'-CUUG**U**CC**UU**AAC**G**G**U**GC**U**C-3'

5′-CU**U**GUCCU**U**AAC**G**G**U**GCUC-3′	5′-CUUG**U**CC**UU**AAC**G**G**U**GCUC-3′
5′-CU**U**GUCC**U**UAAC**G**GUGC**U**C-3′	5′-CUUG**U**CC**UU**AAC**G**GU**G**C**U**C-3′
5′-CUU**G**UCC**U**UAACGG**U**GC**U**C-3′	5′-CUU**GU**CC**U**UAACGG**U**GC**U**C-3′
5′-CUU**G**UCC**U**UAAC**G**G**U**GC**U**C-3′	5′-CUU**GU**CCU**U**AACGG**U**GC**U**C-3′

2'OMe nucleotides are indicated in bold and underlined.

[0103] In particular embodiments, the 2'OMe-modified sequence set forth in Table 5 corresponds to the antisense strand sequence present in the double-stranded region of the siRNA. In some embodiments, the 2'OMe-modified sequence set forth in Table 5 comprises a modified (*e.g.*, 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other embodiments, the 2'OMe-modified sequence set forth in Table 5 further comprises at least one, two, three, four, five, six, or more 2'OMe-modified adenosine and/or modified 2'OMe-modified cytosine nucleotides. Each of the 2'OMe-modified antisense strand sequences set forth in Table 5 may comprise the complementary strand of any of the 2'OMe-modified sense strand sequences set forth in Table 6 or the unmodified *APOC3* siRNA "268" sense strand sequence shown in Table 7.

[0104] In still yet another embodiment, the sense strand of the *APOC3* siRNA comprises one of the 2'OMe-modified sequences set forth in Table 6. The sense strand sequence of *APOC3* siRNA "268" shown in Table 7 sets forth the unmodified version of the 2'OMe-modified sequences set forth in Table 6. Nucleotides 1-19 of the sense strand sequence of the hAPOC3_266 siRNA shown in Table 10 also correspond to the unmodified version of the 2'OMe-modified sequences set forth in Table 6.

Table 6

5′-GAGCACCG**UU**AAGGACAAG-3′	5′-**G**A**G**CACCGU**U**AA**G**GACAAG-3′
5′-**G**A**G**CACC**G**UUAA**G**GACAA**G**-3′	5′-**G**A**G**CACCGU**U**AAG**G**ACAAG-3′
5′-**G**A**G**CACC**G**UUAAG**G**ACAA**G**-3′	5′-GA**G**CACCGU**U**AA**G**GACAA**G**-3′
5′-GA**G**CACC**G**UUAA**G**GACAA**G**-3′	5′-GA**G**CACCGU**U**AAG**G**ACAA**G**-3′
5′-GA**G**CACC**G**UUAAG**G**ACAA**G**-3′	5′-**G**A**G**CACCGU**U**AA**G**GACAA**G**-3′
5′-GA**G**CACCG**U**UAAGGACAAG-3′	5′-**G**A**G**CACCGU**U**AAG**G**ACAA**G**-3′
5′-**G**A**G**CACC**G**UUAAG**G**ACAAG-3′	5′-GA**G**CACC**G**U**U**AAG**G**ACAAG-3′
5′-GA**G**CACC**G**UUAAG**G**ACAA**G**-3′	5′-GA**G**CACC**G**U**U**AAG**G**ACAA**G**-3′
5′-**G**A**G**CACC**G**UUAAG**G**ACAA**G**-3′	5′-**G**AGCACCG**U**UAA**G**GACAA**G**-3′
5′-**G**A**G**CACC**G**UUAAG**G**ACAA**G**-3′	5′-**G**AGCACCG**U**UAAG**G**ACAA**G**-3′
5′-**G**A**G**CACCG**U**UAAG**G**ACAA**G**-3′	5′-**G**AGCACCG**U**UAA**GG**ACAAG-3′
5′-**G**A**G**CACCG**U**UAAG**G**ACAA**G**-3′	5′-**G**AGCACCG**U**UAA**GG**ACAA**G**-3′

5′-**G**A**G**CACCG**U**UAA**GG**ACAA**G**-3′	5′-**G**AGCACC**GU**UAA**G**GACAA**G**-3′
5′-GA**G**CACC**GU**UAAG**G**ACAAG-3′	5′-**G**AGCACC**GU**UAAG**G**ACAA**G**-3′
5′-GA**G**CACC**GU**UAAG**G**ACAA**G**-3′	5′-GAGCACC**G**U**U**AA**G**GACAA**G**-3′
5′-**G**A**G**CACCG**UU**AA**G**GACAAG-3′	5′-GAGCACC**G**U**U**AAG**G**ACAA**G**-3′
5′-**G**A**G**CACCG**UU**AAG**G**ACAA**G**-3′	5′-GAGCACC**G**U**U**AA**GG**ACAAG-3′
5′-GA**G**CACCG**UU**AA**G**GACAA**G**-3′	5′-GAGCACC**G**U**U**AA**GG**ACAA**G**-3′
5′-GA**G**CACCG**UU**AAG**G**ACAA**G**-3′	5′-GAGCACCG**UU**AAG**G**ACAA**G**-3′
5′-**G**A**G**CACCG**UU**AA**GG**ACAA**G**-3′	5′-GAGCACCG**UU**AA**GG**ACAA**G**-3′
5′-**G**A**G**CACCG**UU**AAG**G**ACAA**G**-3′	5′-**G**A**G**CACC**GUU**AA**GG**ACAA**G**-3′
5′-**G**A**G**CACCG**UU**AA**GG**ACAA**G**-3′	5′-GA**G**CACCGU**U**AA**GG**ACAA**G**-3′

2'OMe nucleotides are indicated in bold and underlined.

[0105] In particular embodiments, the 2'OMe-modified sequence set forth in Table 6 corresponds to the sense strand sequence present in the double-stranded region of the siRNA. In some embodiments, the 2'OMe-modified sequence set forth in Table 6 comprises a modified (*e.g.,* 2'OMe) and/or unmodified 3' overhang of 1, 2, 3, or 4 nucleotides. In other embodiments, the 2'OMe-modified sequence set forth in Table 6 further comprises at least one, two, three, four, five, six, or more 2'OMe-modified adenosine and/or modified 2'OMe-modified cytosine nucleotides. Each of the 2'OMe-modified sense strand sequences set forth in Table 6 may comprise the complementary strand of any of the 2'OMe-modified antisense strand sequences set forth in Table 5 or the unmodified *APOC3* siRNA "268" antisense strand sequence shown in Table 7.

[0106] One of skill in the art will understand that the sequences set forth in Tables 1-6 can also be modified in accordance with the selective modification patterns described herein (*e.g.,* at alternative uridine and/or guanosine nucleotides, and optionally at adenosine and/or cytosine nucleotides, within the siRNA duplex), and screened for RNAi activity as well as immune stimulation, such that the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. Similarly, one of skill in the art will understand that the sequences set forth in Tables 7-10 can be modified in accordance with the selective modification patterns described herein (*e.g.,* at uridine and/or guanosine nucleotides, and optionally at adenosine and/or cytosine nucleotides, within the siRNA duplex), and screened for RNAi activity as well as immune stimulation, such that the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity.

[0107] In preferred embodiments, the *APOC3* siRNA of the present invention (*e.g.,* siRNA comprising nucleotides 1-19 of one of the sense and/or antisense strand sequences set forth in Tables 1-10) comprises a 3' overhang of 1, 2, 3, or 4 nucleotides in one or both strands of the siRNA. In certain instances, the siRNA may contain at least one blunt end. In particular embodiments, the 3' overhangs in one or both strands of the siRNA molecule may each independently comprise 1, 2, 3, or 4 modified and/or unmodified deoxythymidine ("t" or "dT") nucleotides, 1, 2, 3, or 4 modified (*e.g.,* 2'OMe) and/or unmodified uridine ("U") ribonucleotides, or 1, 2, 3, or 4 modified (*e.g.,* 2'OMe) and/or unmodified ribonucleotides or deoxyribonucleotides having complementarity to the target *APOC3* sequence (3' overhang in antisense strand) or the complementary strand thereof (3' overhang in sense strand).

[0108] In another embodiment, the present invention provides a composition comprising a cocktail (*e.g.,* at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more) of the unmodified and/or modified siRNA sequences set forth in Tables 1-10. In particular embodiments, the present invention provides a composition comprising one or more of the siRNA sequences set forth in Tables 1-10 in combination with one or more siRNAs that target one or more other genes (*e.g.,* additional genes associated with liver diseases or disorders such as dyslipidemia or atherosclerosis). In certain embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more (*e.g.,* all) of these siRNA sequences are chemically modified (*e.g.,* 2'OMe-modified) as described herein.

[0109] The present invention also provides a pharmaceutical composition comprising one or more (*e.g.,* a cocktail) of the siRNA molecules described herein and a pharmaceutically acceptable carrier.

[0110] In another aspect, the present invention provides a nucleic acid-lipid particle (*e.g.,* SNALP) that targets *APOC3* gene expression. The nucleic acid-lipid particles (*e.g.,* SNALP) typically comprise one or more (*e.g.,* a cocktail) of the siRNAs described herein, a cationic lipid, and a non-cationic lipid. In certain instances, the nucleic acid-lipid particles (*e.g.,* SNALP) further comprise a conjugated lipid that inhibits aggregation of particles. Preferably, the nucleic acid-lipid particles (*e.g.,* SNALP) comprise one or more (*e.g.,* a cocktail) of the siRNAs described herein, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In particular embodiments, the nucleic acid-lipid particles (*e.g.,* SNALP) of the invention comprise 1, 2, 3, 4, 5, 6, 7, 8, or more unmodified and/or modified siRNAs that silence 1, 2, 3, 4, 5, 6, 7, 8, or more different genes associated with liver diseases or disorders (*e.g., APOC3*, alone or in combination with other

genes expressed in the liver), a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles.

[0111] In some embodiments, the siRNA molecules of the invention are fully encapsulated in the nucleic acid-lipid particle (*e.g.,* SNALP). With respect to formulations comprising an siRNA cocktail, the different types of siRNA species present in the cocktail (*e.g.,* siRNA compounds with different sequences) may be co-encapsulated in the same particle, or each type of siRNA species present in the cocktail may be encapsulated in a separate particle. The siRNA cocktail may be formulated in the particles described herein using a mixture of two or more individual siRNAs (each having a unique sequence) at identical, similar, or different concentrations or molar ratios. In one embodiment, a cocktail of siRNAs (corresponding to a plurality of siRNAs with different sequences) is formulated using identical, similar, or different concentrations or molar ratios of each siRNA species, and the different types of siRNAs are co-encapsulated in the same particle. In another embodiment, each type of siRNA species present in the cocktail is encapsulated in different particles at identical, similar, or different siRNA concentrations or molar ratios, and the particles thus formed (each containing a different siRNA payload) are administered separately (*e.g.,* at different times in accordance with a therapeutic regimen), or are combined and administered together as a single unit dose (*e.g.,* with a pharmaceutically acceptable carrier). The particles described herein are serum-stable, are resistant to nuclease degradation, and are substantially non-toxic to mammals such as humans.

[0112] The cationic lipid in the nucleic acid-lipid particles of the present invention (*e.g.,* SNALP) may comprise, *e.g.,* one or more cationic lipids of Formula I-II or any other cationic lipid species. In one particular embodiment, the cationic lipid is selected from the group consisting of 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), salts thereof, and mixtures thereof.

[0113] The non-cationic lipid in the nucleic acid-lipid particles of the present invention (*e.g.,* SNALP) may comprise, *e.g.,* one or more anionic lipids and/or neutral lipids. In some embodiments, the non-cationic lipid comprises one of the following neutral lipid components: (1) a mixture of a phospholipid and cholesterol or a derivative thereof; (2) cholesterol or a derivative thereof; or (3) a phospholipid. In certain preferred embodiments, the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC),

or a mixture thereof. In a particularly preferred embodiment, the non-cationic lipid is a mixture of DPPC and cholesterol.

[0114] The lipid conjugate in the nucleic acid-lipid particles of the invention (*e.g.,* SNALP) inhibits aggregation of particles and may comprise, *e.g.,* one or more of the lipid conjugates described herein. In one particular embodiment, the lipid conjugate comprises a PEG-lipid conjugate. Examples of PEG-lipid conjugates include, but are not limited to, PEG-DAG conjugates, PEG-DAA conjugates, and mixtures thereof. In certain embodiments, the PEG-DAA conjugate in the lipid particle may comprise a PEG-didecyloxypropyl (C_{10}) conjugate, a PEG-dilauryloxypropyl (C_{12}) conjugate, a PEG-dimyristyloxypropyl (C_{14}) conjugate, a PEG-dipalmityloxypropyl (C_{16}) conjugate, a PEG-distearyloxypropyl (C_{18}) conjugate, or mixtures thereof.

[0115] In some embodiments, the present invention provides nucleic acid-lipid particles (*e.g.,* SNALP) comprising: (a) one or more (*e.g.,* a cocktail) siRNA molecules that target *APOC3* gene expression; (b) one or more cationic lipids or salts thereof comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0116] In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.,* a cocktail) siRNA molecules that target *APOC3* gene expression; (b) a cationic lipid or a salt thereof comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation. In one particular embodiment, the 1:57 formulation is a four-component system comprising about 1.4 mol % PEG-lipid conjugate (*e.g.,* PEG2000-C-DMA), about 57.1 mol % cationic lipid (*e.g.,* DLinDMA) or a salt thereof, about 7.1 mol % DPPC (or DSPC), and about 34.3 mol % cholesterol (or derivative thereof).

[0117] In another aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.,* a cocktail) siRNA molecules that target *APOC3* gene expression; (b) a cationic lipid or a salt thereof comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about

31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation. In one particular embodiment, the 1:62 formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 61.5 mol % cationic lipid (*e.g.*, DLinDMA) or a salt thereof, and about 36.9 mol % cholesterol (or derivative thereof).

[0118] Additional embodiments related to the 1:57 and 1:62 formulations are described in PCT Publication No. WO 09/127060 and U.S. Provisional Application No. 61/184,652, filed June 5, 2009.

[0119] In other embodiments, the present invention provides nucleic acid-lipid particles (*e.g.*, SNALP) comprising: (a) one or more (*e.g.*, a cocktail) siRNA molecules that target *APOC3* gene expression; (b) one or more cationic lipids or salts thereof comprising from about 2 mol % to about 50 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 5 mol % to about 90 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 20 mol % of the total lipid present in the particle.

[0120] In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules that target *APOC3* gene expression; (b) a cationic lipid or a salt thereof comprising from about 30 mol % to about 50 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 47 mol % to about 69 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 3 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "2:40" formulation. In one particular embodiment, the 2:40 formulation is a four-component system which comprises about 2 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 40 mol % cationic lipid (*e.g.*, DLinDMA) or a salt thereof, about 10 mol % DPPC (or DSPC), and about 48 mol % cholesterol (or derivative thereof).

[0121] The present invention also provides pharmaceutical compositions comprising a nucleic acid-lipid particle such as a SNALP and a pharmaceutically acceptable carrier.

[0122] The nucleic acid-lipid particles of the invention are useful for the therapeutic delivery of interfering RNA (*e.g.*, siRNA) molecules that silence the expression of one or

more genes associated with liver diseases or disorders (*e.g., APOC3*). In some embodiments, a cocktail of siRNAs that target one or more genes expressed in the liver is formulated into the same or different nucleic acid-lipid particles, and the particles are administered to a mammal (*e.g.,* a human) requiring such treatment. In certain instances, a therapeutically effective amount of the nucleic acid-lipid particles can be administered to the mammal, *e.g.,* for treating, preventing, reducing the risk of developing, or delaying the onset of a lipid disorder such as dyslipidemia (*e.g.,* elevated triglyceride and/or cholesterol levels) or atherosclerosis. In particular embodiments, administration of the nucleic acid-lipid particles of the invention does not alter (*e.g.,* reduce) hepatic triglyceride levels, *e.g.,* liver triglyceride levels are not significantly changed upon particle administration.

[0123] Non-limiting examples of lipid disorders suitable for prevention and/or treatment with the nucleic acid-lipid particles of the invention (*e.g.,* SNALP) include dyslipidemia (*e.g.,* hyperlipidemias such as elevated triglyceride levels (hypertriglyceridemia) and/or elevated cholesterol levels (hypercholesterolemia)), atherosclerosis, low HDL-cholesterol, high LDL-cholesterol, coronary heart disease, coronary artery disease, atherosclerotic cardiovascular disease (CVD), fatty liver disease (hepatic steatosis), abnormal lipid metabolism, abnormal cholesterol metabolism, pancreatitis (*e.g.,* acute pancreatitis associated with severe hypertriglyceridemia), diabetes (including Type 2 diabetes), obesity, cardiovascular disease, and other disorders relating to abnormal metabolism.

[0124] In some embodiments, the interfering RNA (*e.g.,* siRNA) molecules described herein are used in methods for silencing *APOC3* gene expression, *e.g.,* in a cell such as a liver cell. In particular, it is an object of the invention to provide methods for treating, preventing, reducing the risk of developing, or delaying the onset of a lipid disorder in a mammal by downregulating or silencing the transcription and/or translation of the *APOC3* gene. In certain embodiments, the present invention provides a method for introducing one or more interfering RNA (*e.g.,* siRNA) molecules described herein into a cell by contacting the cell with a nucleic acid-lipid particle described herein (*e.g.,* a SNALP formulation). In one particular embodiment, the cell is a liver cell such as, *e.g.,* a hepatocyte present in the liver tissue of a mammal (*e.g.,* a human). In another embodiment, the present invention provides a method for the *in vivo* delivery of one or more interfering RNA (*e.g.,* siRNA) molecules described herein to a liver cell (*e.g.,* hepatocyte) by administering to a mammal (*e.g.,* human) a nucleic acid-lipid particle described herein (*e.g.,* a SNALP formulation).

[0125] In some embodiments, the nucleic acid-lipid particles described herein (*e.g.,* SNALP) are administered by one of the following routes of administration: oral, intranasal,

intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. In particular embodiments, the nucleic acid-lipid particles are administered systemically, *e.g.,* via enteral or parenteral routes of administration.

[0126] In particular embodiments, the nucleic acid-lipid particles of the invention (*e.g.,* SNALP) can preferentially deliver a payload such as an interfering RNA (*e.g.,* siRNA) to the liver as compared to other tissues, *e.g.,* for the treatment of a liver disease or disorder such as dyslipidemia or atherosclerosis.

[0127] In certain aspects, the present invention provides methods for silencing *APOC3* gene expression in a mammal (*e.g.,* human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.,* a SNALP formulation) comprising one or more interfering RNAs (*e.g.,* siRNAs) described herein (*e.g.,* siRNAs targeting the *APOC3* gene). In some embodiments, administration of nucleic acid-lipid particles comprising one or more *APOC3*-targeting siRNAs reduces liver *APOC3* mRNA levels by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% (or any range therein) relative to liver *APOC3* mRNA levels detected in the absence of the siRNA (*e.g.,* buffer control or irrelevant non-*APOC3* targeting siRNA control). In other embodiments, administration of nucleic acid-lipid particles comprising one or more *APOC3*-targeting siRNAs reduces liver *APOC3* mRNA levels for at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 days or more (or any range therein) relative to a negative control such as, *e.g.,* a buffer control or an irrelevant non-*APOC3* targeting siRNA control. The *APOC3*-targeting siRNAs may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.,* 2'OMe-modified) form.

[0128] In certain other aspects, the present invention provides methods for treating, preventing, reducing the risk or likelihood of developing (*e.g.,* reducing the susceptibility to), delaying the onset of, and/or ameliorating one or more symptoms associated with a lipid disorder in a mammal (*e.g.,* human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.,* a SNALP formulation) comprising one or more interfering RNA molecules (*e.g.,* siRNAs) described herein (*e.g.,* one or more siRNAs targeting the *APOC3* gene). Non-limiting examples of lipid disorders are described above and include dyslipidemia and atherosclerosis. The *APOC3*-

targeting siRNAs may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.,* 2'OMe-modified) form.

[0129] In a related aspect, the present invention provides a method for treating and/or ameliorating one or more symptoms associated with atherosclerosis or a dyslipidemia such as hyperlipidemia (*e.g.,* elevated levels of triglycerides and/or cholesterol) in a mammal (*e.g.,* human) in need thereof (*e.g.,* a mammal with atheromatous plaques, elevated triglyceride levels, and/or elevated cholesterol levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.,* a SNALP formulation) comprising one or more interfering RNAs (*e.g.,* siRNAs) described herein (*e.g.,* siRNAs targeting the *APOC3* gene). In some embodiments, administration of nucleic acid-lipid particles comprising one or more *APOC3*-targeting siRNA molecules reduces the level of atherosclerosis (*e.g.,* decreases the size and/or number of atheromatous plaques or lesions) or blood (*e.g.,* serum and/or plasma) triglyceride and/or cholesterol levels by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to the level of atherosclerosis, blood triglyceride levels, or blood cholesterol levels detected in the absence of the siRNA (*e.g.,* buffer control or irrelevant non-*APOC3* targeting siRNA control). The *APOC3*-targeting siRNAs may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.,* 2'OMe-modified) form.

[0130] In another related aspect, the present invention provides a method for reducing the risk or likelihood of developing (*e.g.,* reducing the susceptibility to) atherosclerosis or a dyslipidemia such as hyperlipidemia (*e.g.,* elevated levels of triglycerides and/or cholesterol) in a mammal (*e.g.,* human) at risk of developing atherosclerosis or dyslipidemia, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.,* a SNALP formulation) comprising one or more interfering RNAs (*e.g.,* siRNAs) described herein (*e.g.,* siRNAs targeting the *APOC3* gene). In some embodiments, administration of nucleic acid-lipid particles comprising one or more *APOC3*-targeting siRNAs reduces the risk or likelihood of developing atherosclerosis or dyslipidemia by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to the risk or likelihood of developing atherosclerosis or dyslipidemia in the absence of the siRNA (*e.g.,* buffer control or irrelevant non-*APOC3* targeting siRNA control). The *APOC3*-targeting siRNAs may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.,* 2'OMe-modified) form.

[0131] In yet another related aspect, the present invention provides a method for preventing or delaying the onset of atherosclerosis or a dyslipidemia such as hyperlipidemia (*e.g.*, elevated levels of triglycerides and/or cholesterol) in a mammal (*e.g.*, human) at risk of developing atherosclerosis or dyslipidemia, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.*, a SNALP formulation) comprising one or more interfering RNAs (*e.g.*, siRNAs) described herein (*e.g.*, siRNAs targeting the *APOC3* gene). The *APOC3*-targeting siRNA molecules may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.*, 2'OMe-modified) form.

[0132] In a further related aspect, the present invention provides a method for lowering or reducing cholesterol levels in a mammal (*e.g.*, human) in need thereof (*e.g.*, a mammal with elevated blood cholesterol levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.*, a SNALP formulation) comprising one or more interfering RNAs (*e.g.*, siRNAs) described herein (*e.g.*, siRNAs targeting the *APOC3* gene). In particular embodiments, administration of nucleic acid-lipid particles (*e.g.*, SNALP) comprising one or more *APOC3*-targeting siRNA molecules lowers or reduces blood (*e.g.*, serum and/or plasma) cholesterol levels. In some embodiments, administration of nucleic acid-lipid particles (*e.g.*, SNALP) comprising one or more *APOC3*-targeting siRNA reduces blood cholesterol levels by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to blood cholesterol levels detected in the absence of the siRNA (*e.g.*, buffer control or irrelevant non-*APOC3* targeting siRNA control). In certain instances, administration of nucleic acid-lipid particles (*e.g.*, SNALP) comprising one or more *APOC3*-targeting siRNA molecules elevates HDL-cholesterol levels and/or reduces LDL-cholesterol levels. The *APOC3*-targeting siRNAs may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.*, 2'OMe-modified) form.

[0133] In another related aspect, the present invention provides a method for lowering or reducing triglyceride levels in a mammal (*e.g.*, human) in need thereof (*e.g.*, a mammal with elevated blood triglyceride levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.*, a SNALP formulation) comprising one or more interfering RNAs (*e.g.*, siRNAs) described herein (*e.g.*, siRNAs targeting the *APOC3* gene). In particular embodiments, administration of nucleic acid-lipid particles (*e.g.*, SNALP) comprising one or more *APOC3*-targeting siRNA molecules lowers or reduces blood (*e.g.*, serum and/or plasma) triglyceride levels. In certain embodiments,

administration of nucleic acid-lipid particles comprising one or more *APOC3*-targeting siRNA reduces blood triglyceride levels by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to blood triglyceride levels detected in the absence of the siRNA (*e.g.*, buffer control or irrelevant non-*APOC3* targeting siRNA control). In other embodiments, administration of nucleic acid-lipid particles of the invention lowers or reduces hepatic (*i.e.*, liver) triglyceride levels. The *APOC3*-targeting siRNAs may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.*, 2'OMe-modified) form.

[0134] In an additional related aspect, the present invention provides a method for lowering or reducing glucose levels in a mammal (*e.g.*, human) in need thereof (*e.g.*, a mammal with elevated blood glucose levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.*, a SNALP formulation) comprising one or more interfering RNAs (*e.g.*, siRNAs) described herein (*e.g.*, siRNAs targeting the *APOC3* gene). In particular embodiments, administration of nucleic acid-lipid particles (*e.g.*, SNALP) comprising one or more *APOC3*-targeting siRNA lowers or reduces blood (*e.g.*, serum and/or plasma) glucose levels. In some embodiments, administration of nucleic acid-lipid particles comprising one or more *APOC3*-targeting siRNA reduces blood glucose levels by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to blood glucose levels detected in the absence of the siRNA (*e.g.*, buffer control or irrelevant non-*APOC3* targeting siRNA control). The *APOC3*-targeting siRNAs may comprise at least one of the sequences set forth in Tables 1-10 in unmodified or modified (*e.g.*, 2'OMe-modified) form.

IV. Therapeutic Nucleic Acids

[0135] The term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60 nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucletoides of the invention are from about 15 to about 60 nucleotides in length. In some embodiments, nucleic acid is associated with a carrier system such as the lipid particles described herein. In certain embodiments, the nucleic acid is fully encapsulated in the lipid particle. Nucleic acid may be administered alone in the lipid particles of the present invention, or in combination (*e.g.*, co-administered) with lipid particles comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0136] In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

[0137] Oligonucleotides are generally classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5' and 3' carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

[0138] The nucleic acid can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. In preferred embodiments, the nucleic acid is double-stranded RNA. Examples of double-stranded RNA are described herein and include, *e.g.,* siRNA and other RNAi agents such as Dicer-substrate dsRNA, shRNA, aiRNA, and pre-miRNA. In other embodiments, the nucleic acid is single-stranded. Single-stranded nucleic acids include, *e.g.,* antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides.

[0139] Nucleic acids of the invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 60 nucleotides, from about 15 to about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0140] In particular embodiments, an oligonucleotide (or a strand thereof) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In

preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.,* under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

A. siRNA

[0141] The unmodified and modified siRNA molecules of the invention are capable of silencing *APOC3* gene expression, *e.g.,* to reduce plasma triglyceride levels and/or plasma cholesterol levels. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. The modified nucleotides can be present in one strand (*i.e.,* sense or antisense) or both strands of the siRNA. In some preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified (*e.g.,* 2'OMe-modified) in one strand (*i.e.,* sense or antisense) or both strands of the siRNA. In these embodiments, the modified siRNA can further comprise one or more modified (*e.g.,* 2'OMe-modified) adenosine and/or modified (*e.g.,* 2'OMe-modified) cytosine nucleotides. In other preferred embodiments, only uridine and/or guanosine nucleotides are modified (*e.g.,* 2'OMe-modified) in one strand (*i.e.,* sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell,* 107:309 (2001)), or may lack overhangs (*i.e.*, have blunt ends).

[0142] In particular embodiments, the selective incorporation of modified nucleotides such as 2'OMe uridine and/or guanosine nucleotides into the double-stranded region of either or both strands of the *APOC3* siRNA reduces or completely abrogates the immune response to that siRNA molecule. In certain instances, the immunostimulatory properties of *APOC3*

siRNA sequences and their ability to silence *APOC3* gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the double-stranded region of the siRNA duplex. This can be achieved at therapeutically viable siRNA doses without cytokine induction, toxicity, and off-target effects associated with the use of unmodified siRNA.

[0143] The modified siRNA generally comprises from about 1% to about 100% (*e.g.,* about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In certain other embodiments, some or all of the modified nucleotides in the double-stranded region of the siRNA are 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides apart from each other. In one preferred embodiment, none of the modified nucleotides in the double-stranded region of the siRNA are adjacent to each other (*e.g.,* there is a gap of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 unmodified nucleotides between each modified nucleotide).

[0144] In some embodiments, less than about 50% (*e.g.,* less than about 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, or 36%, preferably less than about 35%, 34%, 33%, 32%, 31%, or 30%) of the nucleotides in the double-stranded region of the siRNA comprise modified (*e.g.,* 2'OMe) nucleotides. In one aspect of these embodiments, less than about 50% of the uridine and/or guanosine nucleotides in the double-stranded region of one or both strands of the siRNA are selectively (*e.g.,* only) modified. In another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein 2'OMe-guanosine nucleotides and 2'OMe-uridine nucleotides are the only 2'OMe nucleotides present in the double-stranded region. In yet another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides

in the double-stranded region. In a further aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the 2'OMe nucleotides in the double-stranded region are not adjacent to each other.

[0145] In other embodiments, from about 1% to about 50% (*e.g.,* from about 5%-50%, 10%-50%, 15%-50%, 20%-50%, 25%-50%, 30%-50%, 35%-50%, 40%-50%, 45%-50%, 5%-45%, 10%-45%, 15%-45%, 20%-45%, 25%-45%, 30%-45%, 35%-45%, 40%-45%, 5%-40%, 10%-40%, 15%-40%, 20%-40%, 25%-40%, 25%-39%, 25%-38%, 25%-37%, 25%-36%, 26%-39%, 26%-38%, 26%-37%, 26%-36%, 27%-39%, 27%-38%, 27%-37%, 27%-36%, 28%-39%, 28%-38%, 28%-37%, 28%-36%, 29%-39%, 29%-38%, 29%-37%, 29%-36%, 30%-40%, 30%-39%, 30%-38%, 30%-37%, 30%-36%, 31%-39%, 31%-38%, 31%-37%, 31%-36%, 32%-39%, 32%-38%, 32%-37%, 32%-36%, 33%-39%, 33%-38%, 33%-37%, 33%-36%, 34%-39%, 34%-38%, 34%-37%, 34%-36%, 35%-40%, 5%-35%, 10%-35%, 15%-35%, 20%-35%, 21%-35%, 22%-35%, 23%-35%, 24%-35%, 25%-35%, 26%-35%, 27%-35%, 28%-35%, 29%-35%, 30%-35%, 31%-35%, 32%-35%, 33%-35%, 34%-35%, 30%-34%, 31%-34%, 32%-34%, 33%-34%, 30%-33%, 31%-33%, 32%-33%, 30%-32%, 31%-32%, 25%-34%, 25%-33%, 25%-32%, 25%-31%, 26%-34%, 26%-33%, 26%-32%, 26%-31%, 27%-34%, 27%-33%, 27%-32%, 27%-31%, 28%-34%, 28%-33%, 28%-32%, 28%-31%, 29%-34%, 29%-33%, 29%-32%, 29%-31%, 5%-30%, 10%-30%, 15%-30%, 20%-34%, 20%-33%, 20%-32%, 20%-31%, 20%-30%, 21%-30%, 22%-30%, 23%-30%, 24%-30%, 25%-30%, 25%-29%, 25%-28%, 25%-27%, 25%-26%, 26%-30%, 26%-29%, 26%-28%, 26%-27%, 27%-30%, 27%-29%, 27%-28%, 28%-30%, 28%-29%, 29%-30%, 5%-25%, 10%-25%, 15%-25%, 20%-29%, 20%-28%, 20%-27%, 20%-26%, 20%-25%, 5%-20%, 10%-20%, 15%-20%, 5%-15%, 10%-15%, or 5%-10%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In one aspect of these embodiments, from about 1% to about 50% of the uridine and/or guanosine nucleotides in the

double-stranded region of one or both strands of the siRNA are selectively (*e.g.*, only) modified. In another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein 2'OMe-guanosine nucleotides and 2'OMe-uridine nucleotides are the only 2'OMe nucleotides present in the double-stranded region. In yet another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In a further aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the 2'OMe nucleotides in the double-stranded region are not adjacent to each other.

[0146] Additional ranges, percentages, and patterns of modifications that may be introduced into siRNA are described in U.S. Patent Publication No. 20070135372.

1. Selection of siRNA Sequences

[0147] Suitable siRNA sequences can be identified using any means known in the art. Typically, the methods described in Elbashir *et al.*, *Nature,* 411:494-498 (2001) and Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds *et al.*, *Nature Biotech.,* 22(3):326-330 (2004).

[0148] As a non-limiting example, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest may be scanned for dinucleotide sequences (*e.g.*, AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see*, *e.g.*, Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.*, a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments, the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences, *e.g.,* in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

[0149] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can be found. One of skill in the art

will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0150] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.*, to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0151] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.,* Khvorova *et al., Cell*, 115:209-216 (2003); and Schwarz *et al.*, *Cell*, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.,* Luo *et al.*, *Biophys. Res. Commun.*, 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0152] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.,* using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA sequence such as GU-rich motifs (*e.g.,* 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory. Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naïve mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.*, a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable immune response may comprise production of a cytokine or growth factor such as, *e.g.*, TNF-

α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% (*e.g.,* less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0153] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.*, *J. Biol. Chem.,* 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines *et al.*, *J. Biol. Chem.,* 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks *et al.*, *Clin. Exp. Immunol.,* 39:477 (1980)); and neutralization of activity (Bowen-Pope *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

[0154] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.,* Judge *et al.*, *Mol. Ther.*, 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g.,* mouse and human IFN-α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF-α (eBioscience; San Diego, CA); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, CA)).

[0155] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in the art (*see*, *e.g.*, Kohler *et al.*, *Nature*, 256: 495-497 (1975) and Harlow and Lane,

ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring *et al.*, in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g.*, with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means) to facilitate detection.

1. Generating siRNA Molecules

[0156] siRNA can be provided in several forms including, *e.g.*, as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. In some embodiments, siRNA may be produced enzymatically or by partial/total organic synthesis, and modified ribonucleotides can be introduced by *in vitro* enzymatic or organic synthesis. In certain instances, each strand is prepared chemically. Methods of synthesizing RNA molecules are known in the art, *e.g.,* the chemical synthesis methods as described in Verma and Eckstein (1998, "Modified Oligionucleotides: Synthesis and Strategy for Users", Annual Review of Biochemistry 67:99-134) or as described herein.

[0157] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.*), or can represent a single target sequence. RNA can be naturally occurring (*e.g.*, isolated from tissue or cell samples), synthesized *in vitro (e.g.,* using T7 or SP6 polymerase and PCR products or a cloned cDNA), or chemically synthesized.

[0158] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occuring RNA population is used, the RNA complements are also provided (*e.g.*, to form dsRNA for digestion by *E. coli* RNAse III or Dicer), *e.g.*, by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0159] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,* Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al.,*

supra), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994).

[0160] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques known in the art, such as those described in Usman *et al.*, *J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al.*, *Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al.*, *Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μmol scale protocol. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

[0161] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or strands that hybridize to form the siRNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary

sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

3. Modifying siRNA Sequences

[0162] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (*e.g.,* less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0163] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, *e.g.,* Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (*e.g.,* 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides), 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'Cl) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (*see, e.g.,* Lin *et al., J. Am. Chem. Soc.,* 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (*see, e.g.,* Loakes, *Nucl. Acids Res.,* 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0164] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, *threo*-pentofuranosyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (*see, e.g.,* U.S. Patent No. 5,998,203; Beaucage *et al.*, *Tetrahedron* 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e.,* resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (*see, e.g.,* Hunziker *et al.*, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417 (1995); Mesmaeker *et al.*, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA.

[0165] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.,* 1, 2, 3, or 4) 2'-deoxy ribonucleotides, modified (*e.g.,* 2'OMe) and/or unmodified uridine ribonucleotides, and/or any other combination of modified (*e.g.,* 2'OMe) and unmodified nucleotides.

[0166] Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g.,* in UK Patent No. GB

2,397,818 B and U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372.

[0167] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both strands of the siRNA. As used herein, the term "non-nucleotide" refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0168] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.,* a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.,* through a carbamate group or other linking group (*see, e.g.,* U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.,* folic acid, folate analogs and derivatives thereof), sugars (*e.g.,* galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc.*), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.,* U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described

in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models.

2. Target Genes

[0169] The siRNA molecules of the invention can be used to downregulate or silence the translation (*i.e.*, expression) of the *APOC3* gene, alone or in combination with one or more additional genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders). In certain embodiments, the invention provides a cocktail of siRNA molecules that silences the expression of the *APOC3* gene, wherein each siRNA present in the cocktail is complementary to a different part of the *APOC3* mRNA sequence. Each *APOC3* siRNA present in the cocktail may target a distinct region of the *APOC3* mRNA sequence, or there may be some degree of overlap between two or more *APOC3* siRNAs present in the cocktail. In certain other embodiments, the present invention provides a cocktail of siRNA molecules that silences the expression of the *APOC3* gene and one or more additional genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders). In some instances, the cocktail of siRNA molecules is fully encapsulated in a lipid particle such as a nucleic acid-lipid particle (*e.g.*, SNALP). The siRNA molecules present in the cocktail may be co-encapsulated in the same lipid particle, or each siRNA species present in the cocktail may be formulated in separate particles.

[0170] Examples of additional genes associated with metabolic diseases and disorders (*e.g.*, disorders in which the liver is the target and liver diseases and disorders) include, but are not limited to, genes expressed in dyslipidemia, such as, *e.g.*, apolipoprotein B (ApoB) (Genbank Accession No. NM_000384), apolipoprotein E (ApoE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612), proprotein convertase subtilisin/kexin type 9 (PCSK9) (Genbank Accession No. NM_174936), diacylglycerol O-acyltransferase type 1 (DGAT1) (Genbank Accession No. NM_012079), diacylglyerol O-acyltransferase type 2 (DGAT2) (Genbank Accession No. NM_032564), liver X receptors such as LXRα (Genbank Accession Nos. NM_001130101, NM_001130102, and NM_005693) and LXRβ

(Genback Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase); and genes expressed in diabetes, such as, *e.g.*, glucose 6-phosphatase (*see*, *e.g.*, Forman *et al., Cell,* 81:687 (1995); Seol *et al.*, *Mol. Endocrinol.,* 9:72 (1995), Zavacki *et al., Proc. Natl. Acad. Sci. USA*, 94:7909 (1997); Sakai *et al., Cell,* 85:1037-1046 (1996); Duncan *et al., J. Biol. Chem.*, 272:12778-12785 (1997); Willy *et al., Genes Dev.,* 9:1033-1045 (1995); Lehmann *et al., J. Biol. Chem.*, 272:3137-3140 (1997); Janowski *et al., Nature,* 383:728-731 (1996); and Peet *et al., Cell,* 93:693-704 (1998)).

[0171] One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the *APOB* gene include those described in U.S. Patent Publication Nos. 20060134189 and 20060105976, and PCT Publication No. WO 04/091515. Non-limiting examples of siRNA molecules targeting the *PCSK9* gene include those described in U.S. Patent Publication Nos. 20070173473, 20080113930, and 20080306015. Exemplary siRNA molecules targeting the *DGAT1* gene may be designed using the antisense compounds described in U.S. Patent Publication No. 20040185559. Exemplary siRNA molecules targeting the *DGAT2* gene may be designed using the antisense compounds described in U.S. Patent Publication No. 20050043524.

[0172] In addition to its utility in silencing *APOC3* gene expression for therapeutic purposes, the siRNAs described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications.

5. Exemplary siRNA Embodiments

[0173] In some embodiments, each strand of the siRNA molecule comprises from about 15 to about 60 nucleotides in length (*e.g.,* about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). In one particular embodiment, the siRNA is chemically synthesized. The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

[0174] In other embodiments, the siRNA comprises at least one modified nucleotide. In certain embodiments, the siRNA comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In particular embodiments, less than about 50% (*e.g.,* less than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In preferred embodiments, from about 1% to about 50% (*e.g.,* from about 5%-50%, 10%-50%, 15%-50%, 20%-50%, 25%-50%, 30%-50%, 35%-50%, 40%-50%, 45%-50%, 5%-45%, 10%-45%, 15%-45%, 20%-45%, 25%-45%, 30%-45%, 35%-45%, 40%-45%, 5%-40%, 10%-40%, 15%-40%, 20%-40%, 25%-40%, 30%-40%, 35%-40%, 5%-35%, 10%-35%, 15%-35%, 20%-35%, 25%-35%, 30%-35%, 5%-30%, 10%-30%, 15%-30%, 20%-30%, 25%-30%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, 5%-20%, 10%-20%, 15%-20%, 5%-15%, 10%-15%, or 5%-10%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0175] In further embodiments, the siRNA comprises modified nucleotides including, but not limited to, 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.,* 2'OMe purine and/or pyrimidine nucleotides) such as, *e.g.,* 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, or mixtures thereof. In one particular embodiment, the siRNA comprises at least one 2'OMe-guanosine nucleotide, 2'OMe-uridine nucleotide, or mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0176] In certain embodiments, the siRNA comprises modified nucleotides in one strand (*i.e.,* sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the

double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0177] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.,* by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0178] In some embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.,* within the double-stranded region of the siRNA duplex. In certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF-α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

[0179] In other embodiments, a modified siRNA molecule has an IC_{50} (*i.e.,* half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.*, the modified siRNA has an IC_{50} that is less than or equal to ten-times the IC_{50} of

the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC_{50} less than or equal to three-fold that of the corresponding unmodified siRNA sequence. In yet other embodiments, the modified siRNA has an IC_{50} less than or equal to two-fold that of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC_{50} values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

[0180] In another embodiment, an unmodified or modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the expression of the target sequence (*e.g., APOC3*) relative to a negative control (*e.g.,* buffer only, an siRNA sequence that targets a different gene, a scrambled siRNA sequence, *etc.*).

[0181] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the expression of the target sequence (*e.g., APOC3*) relative to the corresponding unmodified siRNA sequence.

[0182] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0183] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0184] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.,* within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0185] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack

overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. In certain embodiments, the 3' overhang on the sense and/or antisense strand independently comprises one, two, three, four, or more modified nucleotides such as 2'OMe nucleotides and/or any other modified nucleotide described herein or known in the art.

[0186] In particular embodiments, siRNAs targeting *APOC3* mRNA are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more siRNA molecules targeting the *APOC3* gene; (b) a cationic lipid (*e.g.,* DLinDMA, DLenDMA, and/or DLin-K-C2-DMA); and (c) a non-cationic lipid (*e.g.,* DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.,* PEG-DAA).

B. Dicer-Substrate dsRNA

[0187] As used herein, the term "Dicer-substrate dsRNA" or "precursor RNAi molecule" is intended to include any precursor molecule that is processed *in vivo* by Dicer to produce an active siRNA which is incorporated into the RISC complex for RNA interference of a target gene.

[0188] In one embodiment, the Dicer-substrate dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA. According to this embodiment, the Dicer-substrate dsRNA comprises (i) a first oligonucleotide sequence (also termed the sense strand) that is between about 25 and about 60 nucleotides in length (*e.g.,* about 25-60, 25-55, 25-50, 25-45, 25-40, 25-35, or 25-30 nucleotides in length), preferably between about 25 and about 30 nucleotides in length (*e.g.,* 25, 26, 27, 28, 29, or 30 nucleotides in length), and (ii) a second oligonucleotide sequence (also termed the antisense strand) that anneals to the first sequence under biological conditions, such as the conditions found in the cytoplasm of a cell. The second oligonucleotide sequence may be between about 25 and about 60 nucleotides in length (*e.g.,* about 25-60, 25-55, 25-50, 25-45, 25-40, 25-35, or 25-30 nucleotides in length), and is preferably between about 25 and about 30 nucleotides in length (*e.g.,* 25, 26, 27, 28, 29, or 30 nucleotides in length). In addition, a region of one of the sequences, particularly of the antisense strand, of the Dicer-substrate dsRNA has a sequence length of at least about 19 nucleotides, for example, from about 19 to about 60 nucleotides (*e.g.,* about 19-60, 19-55, 19-50, 19-45, 19-40, 19-35, 19-30, or 19-25 nucleotides), preferably from about 19 to about 23 nucleotides (*e.g.,* 19, 20, 21, 22, or 23 nucleotides) that are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene to trigger an RNAi response.

[0189] In a second embodiment, the Dicer-substrate dsRNA has several properties which enhance its processing by Dicer. According to this embodiment, the dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA and has at least one of the following properties: (i) the dsRNA is asymmetric, *e.g.,* has a 3'-overhang on the antisense strand; and/or (ii) the dsRNA has a modified 3'-end on the sense strand to direct orientation of Dicer binding and processing of the dsRNA to an active siRNA. According to this latter embodiment, the sense strand comprises from about 22 to about 28 nucleotides and the antisense strand comprises from about 24 to about 30 nucleotides.

[0190] In one embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the antisense strand. In another embodiment, the sense strand is modified for Dicer binding and processing by suitable modifiers located at the 3'-end of the sense strand. Suitable modifiers include nucleotides such as deoxyribonucleotides, acyclonucleotides, and the like, and sterically hindered molecules such as fluorescent molecules and the like. When nucleotide modifiers are used, they replace ribonucleotides in the dsRNA such that the length of the dsRNA does not change. In another embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the antisense strand and the sense strand is modified for Dicer processing. In another embodiment, the 5'-end of the sense strand has a phosphate. In another embodiment, the 5'-end of the antisense strand has a phosphate. In another embodiment, the antisense strand or the sense strand or both strands have one or more 2'-O-methyl (2'OMe) modified nucleotides. In another embodiment, the antisense strand contains 2'OMe modified nucleotides. In another embodiment, the antisense stand contains a 3'-overhang that is comprised of 2'OMe modified nucleotides. The antisense strand could also include additional 2'OMe modified nucleotides. The sense and antisense strands anneal under biological conditions, such as the conditions found in the cytoplasm of a cell. In addition, a region of one of the sequences, particularly of the antisense strand, of the Dicer-substrate dsRNA has a sequence length of at least about 19 nucleotides, wherein these nucleotides are in the 21-nucleotide region adjacent to the 3'-end of the antisense strand and are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene. Further, in accordance with this embodiment, the Dicer-substrate dsRNA may also have one or more of the following additional properties: (a) the antisense strand has a right shift from the typical 21-mer (*i.e.,* the antisense strand includes nucleotides on the right side of the molecule when compared to the typical 21-mer); (b) the strands may not be completely complementary, *i.e.,* the strands may contain simple mismatch pairings; and (c)

base modifications such as locked nucleic acid(s) may be included in the 5'-end of the sense strand.

[0191] In a third embodiment, the sense strand comprises from about 25 to about 28 nucleotides (*e.g.,* 25, 26, 27, or 28 nucleotides), wherein the 2 nucleotides on the 3'-end of the sense strand are deoxyribonucleotides. The sense strand contains a phosphate at the 5'-end. The antisense strand comprises from about 26 to about 30 nucleotides (*e.g.,* 26, 27, 28, 29, or 30 nucleotides) and contains a 3'-overhang of 1-4 nucleotides. The nucleotides comprising the 3'-overhang are modified with 2'OMe modified ribonucleotides. The antisense strand contains alternating 2'OMe modified nucleotides beginning at the first monomer of the antisense strand adjacent to the 3'-overhang, and extending 15-19 nucleotides from the first monomer adjacent to the 3'-overhang. For example, for a 27-nucleotide antisense strand and counting the first base at the 5'-end of the antisense strand as position number 1, 2'OMe modifications would be placed at bases 9, 11, 13, 15, 17, 19, 21, 23, 25, 26, and 27. In one embodiment, the Dicer-substrate dsRNA has the following structure:

5′-pXXXXXXXXXXXXXXXXXXXXXXXXXDD-3′
3′-YXXXXXXXXXXXXXXXXXXXXXXXXXp-5′

wherein "X" = RNA, "p" = a phosphate group, "X" = 2'OMe RNA, "Y" is an overhang domain comprised of 1, 2, 3, or 4 RNA monomers that are optionally 2'OMe RNA monomers, and "D" = DNA. The top strand is the sense strand, and the bottom strand is the antisense strand.

[0192] In a fourth embodiment, the Dicer-substrate dsRNA has several properties which enhance its processing by Dicer. According to this embodiment, the dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA and at least one of the following properties: (i) the dsRNA is asymmetric, *e.g.,* has a 3'-overhang on the sense strand; and (ii) the dsRNA has a modified 3'-end on the antisense strand to direct orientation of Dicer binding and processing of the dsRNA to an active siRNA. According to this embodiment, the sense strand comprises from about 24 to about 30 nucleotides (*e.g.,* 24, 25, 26, 27, 28, 29, or 30 nucleotides) and the antisense strand comprises from about 22 to about 28 nucleotides (*e.g.,* 22, 23, 24, 25, 26, 27, or 28 nucleotides). In one embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the sense strand. In another embodiment, the antisense strand is modified for Dicer binding and processing by suitable modifiers located at the 3'-end of the antisense strand. Suitable modifiers include nucleotides such as

deoxyribonucleotides, acyclonucleotides, and the like, and sterically hindered molecules such as fluorescent molecules and the like. When nucleotide modifiers are used, they replace ribonucleotides in the dsRNA such that the length of the dsRNA does not change. In another embodiment, the dsRNA has an overhang on the 3'-end of the sense strand and the antisense strand is modified for Dicer processing. In one embodiment, the antisense strand has a 5'-phosphate. The sense and antisense strands anneal under biological conditions, such as the conditions found in the cytoplasm of a cell. In addition, a region of one of the sequences, particularly of the antisense strand, of the dsRNA has a sequence length of at least 19 nucleotides, wherein these nucleotides are adjacent to the 3'-end of antisense strand and are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene. Further, in accordance with this embodiment, the Dicer-substrate dsRNA may also have one or more of the following additional properties: (a) the antisense strand has a left shift from the typical 21-mer (*i.e.,* the antisense strand includes nucleotides on the left side of the molecule when compared to the typical 21-mer); and (b) the strands may not be completely complementary, *i.e.,* the strands may contain simple mismatch pairings.

[0193] In a preferred embodiment, the Dicer-substrate dsRNA has an asymmetric structure, with the sense strand having a 25-base pair length, and the antisense strand having a 27-base pair length with a 2 base 3'-overhang. In certain instances, this dsRNA having an asymmetric structure further contains 2 deoxynucleotides at the 3'-end of the sense strand in place of two of the ribonucleotides. In certain other instances, this dsRNA having an asymmetric structure further contains 2'OMe modifications at positions 9, 11, 13, 15, 17, 19, 21, 23, and 25 of the antisense strand (wherein the first base at the 5'-end of the antisense strand is position 1). In certain additional instances, this dsRNA having an asymmetric structure further contains a 3'-overhang on the antisense strand comprising 1, 2, 3, or 4 2'OMe nucleotides (*e.g.,* a 3'-overhang of 2'OMe nucleotides at positions 26 and 27 on the antisense strand).

[0194] In another embodiment, Dicer-substrate dsRNAs may be designed by first selecting an antisense strand siRNA sequence having a length of at least 19 nucleotides. In some instances, the antisense siRNA is modified to include about 5 to about 11 ribonucleotides on the 5'-end to provide a length of about 24 to about 30 nucleotides. When the antisense strand has a length of 21 nucleotides, 3-9, preferably 4-7, or more preferably 6 nucleotides may be added on the 5'-end. Although the added ribonucleotides may be complementary to the target gene sequence, full complementarity between the target sequence and the antisense siRNA is not required. That is, the resultant antisense siRNA is sufficiently complementary

with the target sequence. A sense strand is then produced that has about 22 to about 28 nucleotides. The sense strand is substantially complementary with the antisense strand to anneal to the antisense strand under biological conditions. In one embodiment, the sense strand is synthesized to contain a modified 3'-end to direct Dicer processing of the antisense strand. In another embodiment, the antisense strand of the dsRNA has a 3'-overhang. In a further embodiment, the sense strand is synthesized to contain a modified 3'-end for Dicer binding and processing and the antisense strand of the dsRNA has a 3'-overhang.

[0195] In a related embodiment, the antisense siRNA may be modified to include about 1 to about 9 ribonucleotides on the 5'-end to provide a length of about 22 to about 28 nucleotides. When the antisense strand has a length of 21 nucleotides, 1-7, preferably 2-5, or more preferably 4 ribonucleotides may be added on the 3'-end. The added ribonucleotides may have any sequence. Although the added ribonucleotides may be complementary to the target gene sequence, full complementarity between the target sequence and the antisense siRNA is not required. That is, the resultant antisense siRNA is sufficiently complementary with the target sequence. A sense strand is then produced that has about 24 to about 30 nucleotides. The sense strand is substantially complementary with the antisense strand to anneal to the antisense strand under biological conditions. In one embodiment, the antisense strand is synthesized to contain a modified 3'-end to direct Dicer processing. In another embodiment, the sense strand of the dsRNA has a 3'-overhang. In a further embodiment, the antisense strand is synthesized to contain a modified 3'-end for Dicer binding and processing and the sense strand of the dsRNA has a 3'-overhang.

[0196] Suitable Dicer-substrate dsRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. In particular embodiments, Dicer-substrate dsRNAs targeting *APOC3* mRNA are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more Dicer-substrate dsRNA molecules targeting the *APOC3* gene; (b) a cationic lipid (*e.g.,* DLinDMA, DLenDMA, and/or DLin-K-C2-DMA); and (c) a non-cationic lipid (*e.g.,* DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.,* PEG-DAA).

[0197] Additional embodiments related to the Dicer-substrate dsRNAs of the invention, as well as methods of designing and synthesizing such dsRNAs, are described in U.S. Patent Publication Nos. 20050244858, 20050277610, and 20070265220, and U.S. Provisional

Application No. 61/184,652, filed June 5, 2009.

C. shRNA

[0198] A "small hairpin RNA" or "short hairpin RNA" or "shRNA" includes a short RNA sequence that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNAs of the invention may be chemically synthesized or transcribed from a transcriptional cassette in a DNA plasmid. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC).

[0199] The shRNAs of the invention are typically about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.,* each complementary sequence of the double-stranded shRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded shRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably about 18-22, 19-20, or 19-21 base pairs in length). shRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides on the antisense strand and/or 5'-phosphate termini on the sense strand. In some embodiments, the shRNA comprises a sense strand and/or antisense strand sequence of from about 15 to about 60 nucleotides in length (*e.g.,* about 15-60, 15-55, 15-50, 15-45, 15-40, 15-35, 15-30, or 15-25 nucleotides in length), preferably from about 19 to about 40 nucleotides in length (*e.g.,* about 19-40, 19-35, 19-30, or 19-25 nucleotides in length), more preferably from about 19 to about 23 nucleotides in length (*e.g.,* 19, 20, 21, 22, or 23 nucleotides in length).

[0200] Non-limiting examples of shRNA include a double-stranded polynucleotide molecule assembled from a single-stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; and a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions. In preferred embodiments, the sense and antisense strands of the shRNA are linked by a loop structure comprising from about 1 to about 25 nucleotides, from about 2 to about 20 nucleotides, from about 4 to about 15 nucleotides, from about 5 to about 12 nucleotides, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more nucleotides.

[0201] Suitable shRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. In particular embodiments, shRNAs targeting *APOC3* mRNA are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more shRNA molecules targeting the *APOC3* gene; (b) a cationic lipid (*e.g.,* DLinDMA, DLenDMA, and/or DLin-K-C2-DMA); and (c) a non-cationic lipid (*e.g.,* DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.,* PEG-DAA).

[0202] Additional embodiments related to the shRNAs of the invention, as well as methods of designing and synthesizing such shRNAs, are described in U.S. Provisional Application No. 61/184,652, filed June 5, 2009.

D aiRNA

[0203] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun *et al.*, *Nat. Biotech.*, 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0204] In another embodiment, aiRNA duplexes of various lengths (*e.g.,* about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more

typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0205] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.,* "AA", "UU", "dTdT", *etc.*). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.,* "AA", "UU", "dTdT", *etc.*). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.,* in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0206]In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.,* one of the siRNA molecules described herein.

[0207] In particular embodiments, aiRNAs targeting *APOC3* mRNA are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more aiRNA molecules targeting the *APOC3* gene; (b) a cationic lipid (*e.g.,* DLinDMA, DLenDMA, and/or DLin-K-C2-DMA); and (c) a non-cationic lipid (*e.g.,* DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.,* PEG-DAA).

[0208] Suitable aiRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. Additional embodiments related to the aiRNA molecules of the invention are described in U.S. Patent Publication No. 20090291131 and PCT Publication No. WO 09/127060.

E. miRNA

[0209] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more

messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.,* in Lagos-Quintana *et al.*, *Science*, 294:853-858 (2001); Lau *et al.*, *Science*, 294:858-862 (2001); and Lee *et al.*, *Science*, 294:862-864 (2001).

[0210] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli *et al.*, *Nature*, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein *et al.*, *Nature*, 409:363-366 (2001). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

[0211] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall *et al.*, *Curr. Biol.*, 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory *et al.*, *Cell*, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

[0212] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi). miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0213] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more

modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0214] In particular embodiments, miRNAs targeting *APOC3* mRNA are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more miRNA molecules targeting the *APOC3* gene; (b) a cationic lipid (*e.g.,* DLinDMA, DLenDMA, and/or DLin-K-C2-DMA); and (c) a non-cationic lipid (*e.g.,* DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.,* PEG-DAA).

[0215] In other embodiments, one or more agents that block the activity of an miRNA targeting *APOC3* mRNA are administered using a lipid particle of the invention (*e.g.,* a nucleic acid-lipid particle). Examples of blocking agents include, but are not limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

[0216] Additional embodiments related to the miRNA molecules of the invention are described in U.S. Patent Publication No. 20090291131 and PCT Publication No. WO 09/127060.

V. Carrier Systems Containing Therapeutic Nucleic Acids

[0217] In one aspect, the present invention provides carrier systems containing one or more therapeutic nucleic acids (*e.g.,* interfering RNA such as siRNA). In some embodiments, the carrier system is a lipid-based carrier system such as a lipid particle (*e.g.,* SNALP), a cationic lipid or liposome nucleic acid complex (*i.e.,* lipoplex), a liposome, a micelle, a virosome, or a mixture thereof. In other embodiments, the carrier system is a polymer-based carrier system such as a cationic polymer-nucleic acid complex (*i.e.,* polyplex). In additional embodiments, the carrier system is a cyclodextrin-based carrier system such as a cyclodextrin polymer-nucleic acid complex. In further embodiments, the carrier system is a protein-based carrier system such as a cationic peptide-nucleic acid complex. Preferably, the carrier system is a lipid particle such as a SNALP. One skilled in the art will appreciate that the therapeutic nucleic acids of the present invention can also be delivered as a naked molecule.

A. Lipid Particles

[0218] In certain aspects, the present invention provides lipid particles comprising one or more therapeutic nucleic acids (*e.g.,* interfering RNA such as siRNA) and one or more of cationic (amino) lipids or salts thereof. In some embodiments, the lipid particles of the invention further comprise one or more non-cationic lipids. In other embodiments, the lipid particles further comprise one or more conjugated lipids capable of reducing or inhibiting particle aggregation.

[0219] The lipid particles of the invention preferably comprise a therapeutic nucleic acid such as an interfering RNA (*e.g.,* siRNA), a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the therapeutic nucleic acid is fully encapsulated within the lipid portion of the lipid particle such that the therapeutic nucleic acid in the lipid particle is resistant in aqueous solution to nuclease degradation. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the invention typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm. The lipid particles of the invention also typically have a lipid:therapeutic agent (*e.g.,* lipid:nucleic acid) ratio (mass/mass ratio) of from about 1:1 to about 100:1, from about 1:1 to about 50:1, from about 2:1 to about 25:1, from about 3:1 to about 20:1, from about 5:1 to about 15:1, or from about 5:1 to about 10:1.

[0220] In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.,* siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, and/or miRNA), a cationic lipid (*e.g.*, one or more cationic lipids of Formula I-II or salts thereof as set forth herein), a non-cationic lipid (*e.g.,* mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.,* one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering RNA (*e.g.,* siRNA) molecules that target the *APOC3* gene. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.,* U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964.

[0221] In the nucleic acid-lipid particles of the invention, the nucleic acid may be fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the nucleic acid is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the nucleic acid-lipid particle compositions are substantially non-toxic to mammals such as humans.

[0222] The term "fully encapsulated" indicates that the nucleic acid in the nucleic acid-lipid particle is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free DNA or RNA. In a fully encapsulated system, preferably less than about 25% of the nucleic acid in the particle is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than about 10%, and most preferably less than about 5% of the nucleic acid in the particle is degraded. "Fully encapsulated" also indicates that the nucleic acid-lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0223] In the context of nucleic acids, full encapsulation may be determined by performing a membrane-impermeable fluorescent dye exclusion assay, which uses a dye that has enhanced fluorescence when associated with nucleic acid. Specific dyes such as OliGreen® and RiboGreen® (Invitrogen Corp.; Carlsbad, CA) are available for the quantitative determination of plasmid DNA, single-stranded deoxyribonucleotides, and/or single- or double-stranded ribonucleotides. Encapsulation is determined by adding the dye to a liposomal formulation, measuring the resulting fluorescence, and comparing it to the fluorescence observed upon addition of a small amount of nonionic detergent. Detergent-mediated disruption of the liposomal bilayer releases the encapsulated nucleic acid, allowing it to interact with the membrane-impermeable dye. Nucleic acid encapsulation may be calculated as $E = (I_o - I)/I_o$, where I and I_o refer to the fluorescence intensities before and after the addition of detergent (*see,* Wheeler *et al.*, *Gene Ther.*, 6:271-281 (1999)).

[0224] In other embodiments, the present invention provides a nucleic acid-lipid particle (*e.g.*, SNALP) composition comprising a plurality of nucleic acid-lipid particles.

[0225] In some instances, the SNALP composition comprises nucleic acid that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the particles have the nucleic acid encapsulated therein.

[0226] In other instances, the SNALP composition comprises nucleic acid that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the input nucleic acid is encapsulated in the particles.

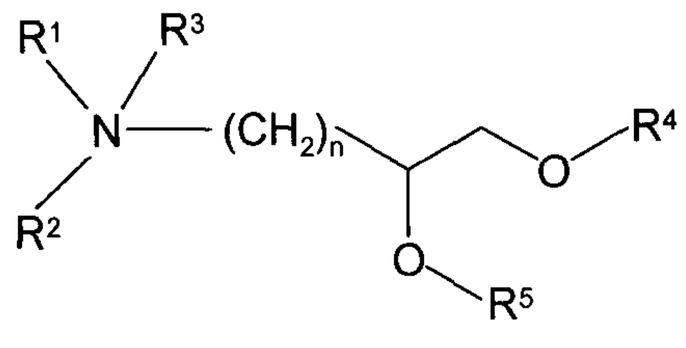
[0227] Depending on the intended use of the lipid particles of the invention, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.*, an endosomal release parameter (ERP) assay.

1. Cationic Lipids

[0228] Any of a variety of cationic lipids or salts thereof may be used in the lipid particles of the present invention (*e.g.*, SNALP), either alone or in combination with one or more other

cationic lipid species or non-cationic lipid species. The cationic lipids include the (R) and/or (S) enantiomers thereof.

[0229] In one aspect, cationic lipids of Formula I having the following structure are useful in the present invention:



(I),

or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently hydrogen (H) or an optionally substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, or C_2-C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1-C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{10}-C_{24} alkyl, C_{10}-C_{24} alkenyl, C_{10}-C_{24} alkynyl, or C_{10}-C_{24} acyl, wherein at least one of R^4 and R^5 comprises at least two sites of unsaturation; and

n is 0, 1, 2, 3, or 4.

[0230] In some embodiments, R^1 and R^2 are independently an optionally substituted C_1-C_4 alkyl, C_2-C_4 alkenyl, or C_2-C_4 alkynyl. In one preferred embodiment, R^1 and R^2 are both methyl groups. In other preferred embodiments, n is 1 or 2. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1-C_4 alkyl to provide a quaternary amine. In further embodiments, R^4 and R^5 are independently an optionally substituted C_{12}-C_{20} or C_{14}-C_{22} alkyl, C_{12}-C_{20} or C_{14}-C_{22} alkenyl, C_{12}-C_{20} or C_{14}-C_{22} alkynyl, or C_{12}-C_{20} or C_{14}-C_{22} acyl, wherein at least one of R^4 and R^5 comprises at least two or at least three sites of unsaturation.

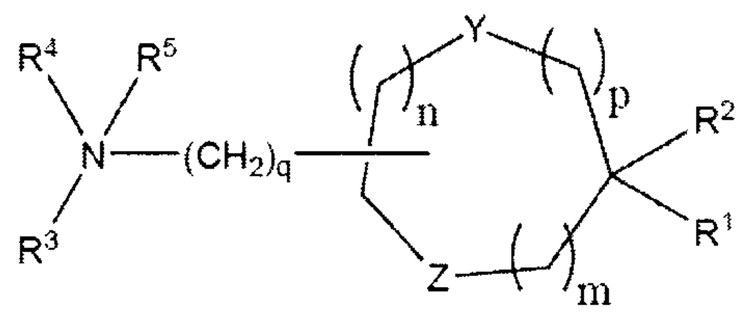
[0231] In certain embodiments, R^4 and R^5 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl

moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, an arachidonyl moiety, and a docosahexaenoyl moiety, as well as acyl derivatives thereof. In certain instances, the octadecadienyl moiety is a linoleyl moiety. In certain other instances, the octadecatrienyl moiety is a linolenyl moiety. In certain embodiments, R^4 and R^5 are both linoleyl moieties or linolenyl moieties. In particular embodiments, the cationic lipid of Formula I is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or mixtures thereof.

[0232] In some embodiments, the cationic lipid of Formula I forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula I is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

[0233] The synthesis of cationic lipids such as DLinDMA and DLenDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060083780.

[0234] In another aspect, cationic lipids of Formula II having the following structure (or salts thereof) are useful in the present invention:



(II),

wherein R^1 and R^2 are either the same or different and are independently an optionally substituted C_{12}-C_{24} alkyl, C_{12}-C_{24} alkenyl, C_{12}-C_{24} alkynyl, or C_{12}-C_{24} acyl; R^3 and R^4 are either the same or different and are independently an optionally substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, or C_2-C_6 alkynyl, or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; R^5 is either absent or is hydrogen (H) or a C_1-C_6 alkyl to provide a quaternary amine; m, n, and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and are independently O, S, or NH. In a preferred embodiment, q is 2.

[0235] In some embodiments, the cationic lipid of Formula II is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2" or "C2K"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA; "C3K"), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA; "C4K"), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-

[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-dioleoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DO-K-DMA), 2,2-distearoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DS-K-DMA), 2,2-dilinoleyl-4-N-morpholino-[1,3]-dioxolane (DLin-K-MA), 2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane chloride (DLin-K-TMA.Cl), 2,2-dilinoleyl-4,5-bis(dimethylaminomethyl)-[1,3]-dioxolane (DLin-K^2-DMA), 2,2-dilinoleyl-4-methylpiperzine-[1,3]-dioxolane (D-Lin-K-N-methylpiperzine), or mixtures thereof. In preferred embodiments, the cationic lipid of Formula II is DLin-K-C2-DMA.

[0236] In some embodiments, the cationic lipid of Formula II forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula II is the oxalate (*e.g.,* hemioxalate) salt thereof, which is preferably a crystalline salt.

[0237] The synthesis of cationic lipids such as DLin-K-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086558. The synthesis of cationic lipids such as DLin-K-C2-DMA, DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, DLin-K-MPZ, DO-K-DMA, DS-K-DMA, DLin-K-MA, DLin-K-TMA.Cl, DLin-K^2-DMA, and D-Lin-K-N-methylpiperzine, as well as additional cationic lipids, is described in PCT Application No. PCT/US2009/060251, entitled "Improved Amino Lipids and Methods for the Delivery of Nucleic Acids," filed October 9, 2009.

[0238] Examples of other cationic lipids or salts thereof which may be included in the lipid particles of the present invention include, but are not limited to, cationic lipids such as those described in U.S. Provisional Application No. 61/222,462, entitled "Improved Cationic Lipids and Methods for the Delivery of Nucleic Acids," filed July 1, 2009, as well as cationic lipids such as N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-

oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-dioeylcarbamoyloxy-3-dimethylaminopropane (DO-C-DAP), 1,2-dimyristoleoyl-3-dimethylaminopropane (DMDAP), 1,2-dioleoyl-3-trimethylaminopropane chloride (DOTAP.Cl), dilinoleylmethyl-3-dimethylaminopropionate (DLin-M-K-DMA; also known as DLin-M-DMA), and mixtures thereof. Additional cationic lipids or salts thereof which may be included in the lipid particles of the present invention are described in U.S. Patent Publication No. 20090023673.

[0239] The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554. The synthesis of cationic lipids such as DLin-C-DAP, DLinDAC, DLinMA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLinTMA.Cl, DLinTAP.Cl, DLinMPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086558. The synthesis of cationic lipids such as DO-C-DAP, DMDAP, DOTAP.Cl, DLin-M-K-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US2009/060251, entitled "Improved Amino Lipids and Methods for the Delivery of Nucleic Acids," filed October 9, 2009. The synthesis of a number of other cationic lipids and related analogs has been described in U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390

Additionally, a number of commercial preparations of cationic lipids can be used, such as, *e.g.*, LIPOFECTIN® (including DOTMA and DOPE, available from Invitrogen); LIPOFECTAMINE® (including DOSPA and DOPE, available from Invitrogen); and TRANSFECTAM® (including DOGS, available from Promega Corp.).

[0240] In some embodiments, the cationic lipid comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, from about 50 mol % to about 60 mol %, from about 55 mol % to about 65 mol %, or from about 55 mol % to about 70 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In particular embodiments, the cationic lipid comprises about 50 mol %, 51 mol %, 52 mol %, 53 mol %, 54 mol %, 55 mol %, 56 mol %, 57 mol %, 58 mol %, 59 mol %, 60 mol %, 61 mol %, 62 mol %, 63 mol %, 64 mol %, or 65 mol % (or any fraction thereof) of the total lipid present in the particle.

[0241] In other embodiments, the cationic lipid comprises from about 2 mol % to about 60 mol %, from about 5 mol % to about 50 mol %, from about 10 mol % to about 50 mol %, from about 20 mol % to about 50 mol %, from about 20 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, or about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0242] Additional percentages and ranges of cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Provisional Application No. 61/184,652, filed June 5, 2009, U.S. Provisional Application No. 61/222,462, filed July 1, 2009, and U.S. Provisional Application No. 61/222,469, filed July 1, 2009.

[0243] It should be understood that the percentage of cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of cationic lipid present in the formulation may vary, for example, by ± 5 mol %. For example, in the 1:57 lipid particle (*e.g.*, SNALP) formulation, the target amount of cationic lipid is 57.1 mol %, but the actual amount of cationic lipid may be ± 5 mol %, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle).

2. Non-cationic Lipids

[0244] The non-cationic lipids used in the lipid particles of the invention (*e.g.,* SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0245] Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g.,* lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0246] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof. Non-limiting examples of cholesterol derivatives include polar analogues such as 5α-cholestanol, 5β-coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as 5α-cholestane, cholestenone, 5α-cholestanone, 5β-cholestanone, and cholesteryl decanoate; and mixtures thereof. In preferred embodiments, the cholesterol derivative is a polar analogue such as cholesteryl-(4'-hydroxy)-butyl ether. The synthesis of cholesteryl-(2'-hydroxy)-ethyl ether is described in PCT Publication No. WO 09/127060.

[0247] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.,* SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof. In other embodiments, the non-cationic lipid present in the lipid particles

(*e.g.*, SNALP) comprises or consists of one or more phospholipids, *e.g.*, a cholesterol-free lipid particle formulation. In yet other embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.*, a phospholipid-free lipid particle formulation.

[0248] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0249] In some embodiments, the non-cationic lipid comprises from about 10 mol % to about 60 mol %, from about 20 mol % to about 55 mol %, from about 20 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 50 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 50 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, from about 37 mol % to about 42 mol %, or about 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0250] In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

[0251] In some embodiments, the phospholipid component in the mixture may comprise from about 2 mol % to about 20 mol %, from about 2 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 4 mol % to about 15 mol %, or from about 4 mol % to about 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the phospholipid component in the mixture comprises from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a 1:57 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (or any

fraction thereof), *e.g.,* in a mixture with cholesterol or a cholesterol derivative at about 34 mol % (or any fraction thereof) of the total lipid present in the particle.

[0252] In other embodiments, the cholesterol component in the mixture may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 27 mol % to about 37 mol %, from about 25 mol % to about 30 mol %, or from about 35 mol % to about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the cholesterol component in the mixture comprises from about 25 mol % to about 35 mol %, from about 27 mol % to about 35 mol %, from about 29 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 30 mol % to about 34 mol %, from about 31 mol % to about 33 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, or 35 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. Typically, a 1:57 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise cholesterol or a cholesterol derivative at about 34 mol % (or any fraction thereof), *e.g.,* in a mixture with a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof) of the total lipid present in the particle.

[0253] In embodiments where the lipid particles are phospholipid-free, the cholesterol or derivative thereof may comprise up to about 25 mol %, 30 mol %, 35 mol %, 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

[0254] In some embodiments, the cholesterol or derivative thereof in the phospholipid-free lipid particle formulation may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 31 mol % to about 39 mol %, from about 32 mol % to about 38 mol %, from about 33 mol % to about 37 mol %, from about 35 mol % to about 45 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 40 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, or 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a 1:62 lipid particle formulation may comprise cholesterol at about 37 mol % (or any fraction thereof) of the total lipid present in the particle.

[0255] In other embodiments, the non-cationic lipid comprises from about 5 mol % to about 90 mol %, from about 10 mol % to about 85 mol %, from about 20 mol % to about 80 mol %, about 10 mol % (*e.g.,* phospholipid only), or about 60 mol % (*e.g.,* phospholipid and

cholesterol or derivative thereof) (or any fraction thereof or range therein) of the total lipid present in the particle.

[0256] Additional percentages and ranges of non-cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Provisional Application No. 61/184,652, filed June 5, 2009, U.S. Provisional Application No. 61/222,462, filed July 1, 2009, and U.S. Provisional Application No. 61/222,469, filed July 1, 2009.

[0257] It should be understood that the percentage of non-cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of non-cationic lipid present in the formulation may vary, for example, by ± 5 mol %. For example, in the 1:57 lipid particle (*e.g.,* SNALP) formulation, the target amount of phospholipid is 7.1 mol % and the target amount of cholesterol is 34.3 mol %, but the actual amount of phospholipid may be ± 2 mol %, ± 1.5 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, and the actual amount of cholesterol may be ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle).

3. Lipid Conjugates

[0258] In addition to cationic and non-cationic lipids, the lipid particles of the invention (*e.g.,* SNALP) may further comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0259] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.,* PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.,* U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.,* U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof.

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[0260] Additional PEG-lipids suitable for use in the invention include, without limitation, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Publication No. WO 09/086558. Yet additional suitable PEG-lipid conjugates include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969.

[0261] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, but are not limited to, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM), as well as such compounds containing a terminal hydroxyl group instead of a terminal methoxy group (*e.g.,* HO-PEG-S, HO-PEG-S-NHS, HO-PEG-NH_2, *etc.*). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.,* mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0262] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.,* from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons.

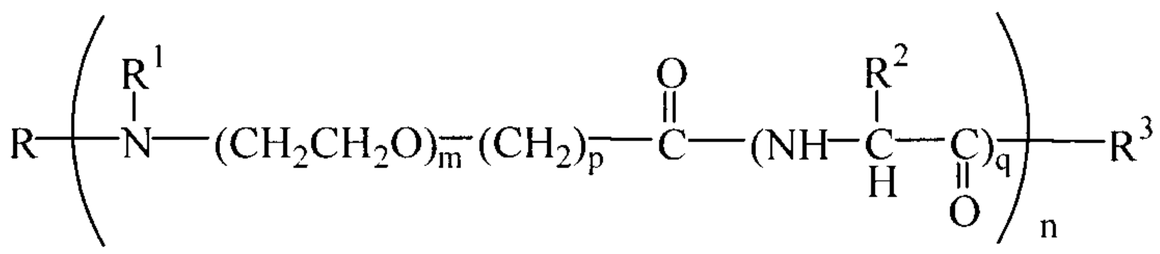
[0263] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used

including, *e.g.,* non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-(O)CCH_2CH_2C(O)-), succinamidyl (-NHC(O)CH_2CH_2C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

[0264] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.,* carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

[0265] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

[0266] The term "ATTA" or "polyamide" includes, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559. These compounds include a compound having the formula:

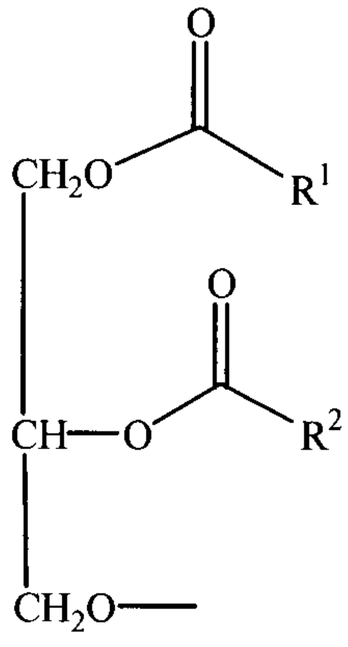


(III),

wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1

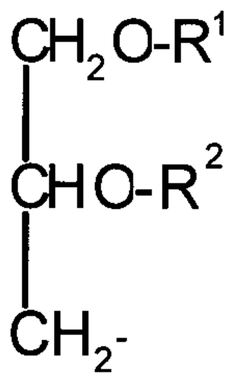
and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

[0267] The term "diacylglycerol" or "DAG" includes a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauroyl (C_{12}), myristoyl (C_{14}), palmitoyl (C_{16}), stearoyl (C_{18}), and icosoyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristoyl (*i.e.*, dimyristoyl), R^1 and R^2 are both stearoyl (*i.e.*, distearoyl), *etc.* Diacylglycerols have the following general formula:



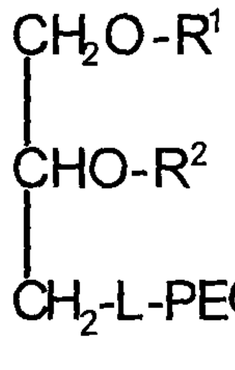
(IV).

[0268] The term "dialkyloxypropyl" or "DAA" includes a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(V).

[0269] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



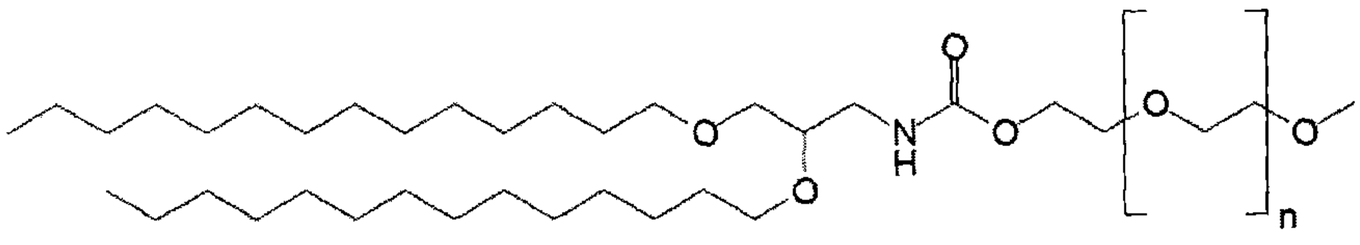
(VI),

wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, decyl (C_{10}), lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.*

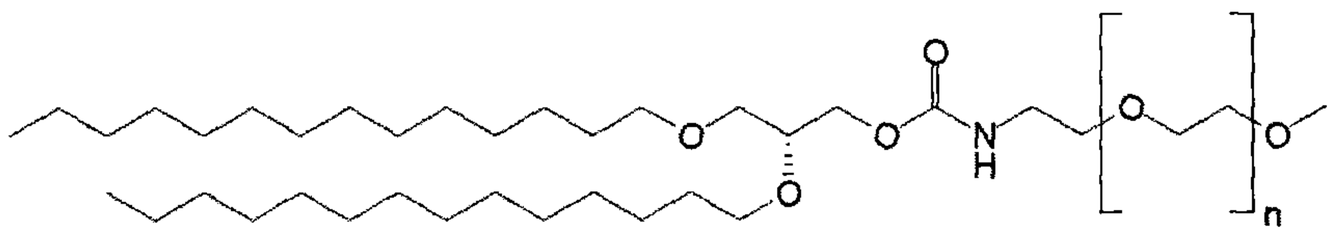
[0270] In Formula VI above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl groups. In certain instances, the terminal hydroxyl group is substituted with a methoxy or methyl group.

[0271] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-*C*-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-*A*-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-*S*-DAA conjugate).

[0272] In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and



(PEG-C-DOMG).

[0273] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.,* March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.,* Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0274] Preferably, the PEG-DAA conjugate is a PEG-didecyloxypropyl (C_{10}) conjugate, a PEG-dilauryloxypropyl (C_{12}) conjugate, a PEG-dimyristyloxypropyl (C_{14}) conjugate, a PEG-dipalmityloxypropyl (C_{16}) conjugate, or a PEG-distearyloxypropyl (C_{18}) conjugate. In these embodiments, the PEG preferably has an average molecular weight of about 2,000 daltons. In one particularly preferred embodiment, the PEG-lipid conjugate comprises PEG2000-C-DMA, wherein the "2000" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In particular embodiments, the terminal hydroxyl group of the PEG is substituted with a methyl group. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

[0275] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0276] In addition to the foregoing components, the lipid particles (*e.g.,* SNALP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.,* Chen *et al., Bioconj. Chem.,* 11:433-437 (2000); U.S. Patent No. 6,852,334; PCT Publication No. WO 00/62813).

[0277] Suitable CPLs include compounds of Formula VII:

A-W-Y (VII),

wherein A, W, and Y are as described below.

[0278] With reference to Formula VII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0279] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0280] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0281] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.,* a charge spike. If the charge density is distributed

on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

[0282] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.,* U.S. Patent Nos. 6,320,017 and 6,586,559), an amide bond will form between the two groups.

[0283] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0284] In some embodiments, the lipid conjugate (*e.g.,* PEG-lipid) comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to about 1.5 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0285] In other embodiments, the lipid conjugate (*e.g.,* PEG-lipid) comprises from about 0 mol % to about 20 mol %, from about 0.5 mol % to about 20 mol %, from about 2 mol % to about 20 mol %, from about 1 mol % to about 15 mol %, from about 1.5 mol % to about 18 mol %, from about 2 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 5 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, or about 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0286] Additional percentages and ranges of lipid conjugates suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Provisional Application No. 61/184,652, filed June 5, 2009, U.S. Provisional Application No. 61/222,462, filed July 1, 2009, and U.S. Provisional Application No. 61/222,469, filed July 1, 2009.

[0287] It should be understood that the percentage of lipid conjugate (*e.g.,* PEG-lipid) present in the lipid particles of the invention is a target amount, and that the actual amount of lipid conjugate present in the formulation may vary, for example, by ± 2 mol %. For example, in the 1:57 lipid particle (*e.g.,* SNALP) formulation, the target amount of lipid conjugate is 1.4 mol %, but the actual amount of lipid conjugate may be ± 0.5 mol %, ± 0.4 mol %, ± 0.3 mol %, ± 0.2 mol %, ± 0.1 mol %, or ± 0.05 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle).

[0288] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the lipid particle is to become fusogenic.

[0289] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the lipid particle and, in turn, the rate at which the lipid particle becomes fusogenic. For instance, when a PEG-DAA conjugate is used as the lipid conjugate, the rate at which the lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the alkyl groups on the PEG-DAA conjugate. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the lipid particle becomes fusogenic will become apparent to those of skill in

the art upon reading this disclosure. Also, by controlling the composition and concentration of the lipid conjugate, one can control the lipid particle (*e.g.,* SNALP) size.

B. Additional Carrier Systems

[0290] Non-limiting examples of additional lipid-based carrier systems suitable for use in the present invention include lipoplexes (*see, e.g.,* U.S. Patent Publication No. 20030203865; and Zhang *et al.*, *J. Control Release*, 100:165-180 (2004)), pH-sensitive lipoplexes (*see, e.g.,* U.S. Patent Publication No. 20020192275), reversibly masked lipoplexes (*see, e.g.,* U.S. Patent Publication Nos. 20030180950), cationic lipid-based compositions (*see, e.g.,* U.S. Patent No. 6,756,054; and U.S. Patent Publication No. 20050234232), cationic liposomes (*see, e.g.,* U.S. Patent Publication Nos. 20030229040, 20020160038, and 20020012998; U.S. Patent No. 5,908,635; and PCT Publication No. WO 01/72283), anionic liposomes (*see, e.g.,* U.S. Patent Publication No. 20030026831), pH-sensitive liposomes (*see, e.g.,* U.S. Patent Publication No. 20020192274; and AU 2003210303), antibody-coated liposomes (*see, e.g.,* U.S. Patent Publication No. 20030108597; and PCT Publication No. WO 00/50008), cell-type specific liposomes (*see, e.g.,* U.S. Patent Publication No. 20030198664), liposomes containing nucleic acid and peptides (*see, e.g.,* U.S. Patent No. 6,207,456), liposomes containing lipids derivatized with releasable hydrophilic polymers (*see, e.g.,* U.S. Patent Publication No. 20030031704), lipid-entrapped nucleic acid (*see, e.g.,* PCT Publication Nos. WO 03/057190 and WO 03/059322), lipid-encapsulated nucleic acid (*see, e.g.,* U.S. Patent Publication No. 20030129221; and U.S. Patent No. 5,756,122), other liposomal compositions (*see, e.g.,* U.S. Patent Publication Nos. 20030035829 and 20030072794; and U.S. Patent No. 6,200,599), stabilized mixtures of liposomes and emulsions (*see, e.g.,* EP1304160), emulsion compositions (*see, e.g.,* U.S. Patent No. 6,747,014), and nucleic acid micro-emulsions (*see, e.g.,* U.S. Patent Publication No. 20050037086).

[0291] Examples of polymer-based carrier systems suitable for use in the present invention include, but are not limited to, cationic polymer-nucleic acid complexes (*i.e.,* polyplexes). To form a polyplex, a nucleic acid (*e.g.,* interfering RNA) is typically complexed with a cationic polymer having a linear, branched, star, or dendritic polymeric structure that condenses the nucleic acid into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. In some embodiments, the polyplex comprises nucleic acid (*e.g.,* interfering RNA) complexed with a cationic polymer such as polyethylenimine (PEI) (*see, e.g.,* U.S. Patent No. 6,013,240; commercially available from Qbiogene, Inc. (Carlsbad, CA) as *In vivo* jetPEI™, a linear form of PEI),

polypropylenimine (PPI), polyvinylpyrrolidone (PVP), poly-L-lysine (PLL), diethylaminoethyl (DEAE)-dextran, poly(β-amino ester) (PAE) polymers (*see, e.g.,* Lynn *et al.*, *J. Am. Chem. Soc.*, 123:8155-8156 (2001)), chitosan, polyamidoamine (PAMAM) dendrimers (*see, e.g.,* Kukowska-Latallo *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:4897-4902 (1996)), porphyrin (*see, e.g.,* U.S. Patent No. 6,620,805), polyvinylether (*see, e.g.,* U.S. Patent Publication No. 20040156909), polycyclic amidinium (*see, e.g.,* U.S. Patent Publication No. 20030220289), other polymers comprising primary amine, imine, guanidine, and/or imidazole groups (*see, e.g.,* U.S. Patent No. 6,013,240; PCT Publication No. WO/9602655; PCT Publication No. WO95/21931; Zhang *et al.*, *J. Control Release*, 100:165-180 (2004); and Tiera *et al.*, *Curr. Gene Ther.*, 6:59-71 (2006)), and a mixture thereof. In other embodiments, the polyplex comprises cationic polymer-nucleic acid complexes as described in U.S. Patent Publication Nos. 20060211643, 20050222064, 20030125281, and 20030185890, and PCT Publication No. WO 03/066069; biodegradable poly(β-amino ester) polymer-nucleic acid complexes as described in U.S. Patent Publication No. 20040071654; microparticles containing polymeric matrices as described in U.S. Patent Publication No. 20040142475; other microparticle compositions as described in U.S. Patent Publication No. 20030157030; condensed nucleic acid complexes as described in U.S. Patent Publication No. 20050123600; and nanocapsule and microcapsule compositions as described in AU 2002358514 and PCT Publication No. WO 02/096551.

[0292] In certain instances, the interfering RNA may be complexed with cyclodextrin or a polymer thereof. Non-limiting examples of cyclodextrin-based carrier systems include the cyclodextrin-modified polymer-nucleic acid complexes described in U.S. Patent Publication No. 20040087024; the linear cyclodextrin copolymer-nucleic acid complexes described in U.S. Patent Nos. 6,509,323, 6,884,789, and 7,091,192; and the cyclodextrin polymer-complexing agent-nucleic acid complexes described in U.S. Patent No. 7,018,609. In certain other instances, the interfering RNA may be complexed with a peptide or polypeptide. An example of a protein-based carrier system includes, but is not limited to, the cationic oligopeptide-nucleic acid complex described in PCT Publication No. WO95/21931.

VI. Preparation of Lipid Particles

[0293] The lipid particles of the present invention, *e.g.,* SNALP, in which a nucleic acid such as an interfering RNA (*e.g.,* siRNA) is entrapped within the lipid portion of the particle and is protected from degradation, can be formed by any method known in the art including,

but not limited to, a continuous mixing method, a direct dilution process, and an in-line dilution process.

[0294] In particular embodiments, the cationic lipids may comprise lipids of Formula I and II or salts thereof, alone or in combination with other cationic lipids. In other embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.,* PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, derivatives thereof, or combinations thereof.

[0295] In certain embodiments, the present invention provides nucleic acid-lipid particles (*e.g.,* SNALP) produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid (*e.g.*, interfering RNA) in a first reservoir, providing an organic lipid solution in a second reservoir (wherein the lipids present in the organic lipid solution are solubilized in an organic solvent, *e.g.*, a lower alkanol such as ethanol), and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a lipid vesicle (*e.g.,* liposome) encapsulating the nucleic acid within the lipid vesicle. This process and the apparatus for carrying out this process are described in detail in U.S. Patent Publication No. 20040142025.

[0296] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a lipid vesicle substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid

solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0297] The nucleic acid-lipid particles formed using the continuous mixing method typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0298] In another embodiment, the present invention provides nucleic acid-lipid particles (*e.g.*, SNALP) produced via a direct dilution process that includes forming a lipid vesicle (*e.g.*, liposome) solution and immediately and directly introducing the lipid vesicle solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of lipid vesicle solution introduced thereto. As a non-limiting example, a lipid vesicle solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0299] In yet another embodiment, the present invention provides nucleic acid-lipid particles (*e.g.*, SNALP) produced via an in-line dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the lipid vesicle (*e.g.*, liposome) solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the lipid vesicle solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180° (*e.g.*, about 90°). A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of lipid vesicle solution introduced thereto from the first

mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the lipid vesicle solution in the second mixing region, and therefore also the concentration of lipid vesicle solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0300] These processes and the apparatuses for carrying out these direct dilution and in-line dilution processes are described in detail in U.S. Patent Publication No. 20070042031.

[0301] The nucleic acid-lipid particles formed using the direct dilution and in-line dilution processes typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0302] If needed, the lipid particles of the invention (*e.g.*, SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0303] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0304] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a

relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0305] In some embodiments, the nucleic acids present in the particles are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103.

[0306] In other embodiments, the methods may further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brand name POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0307] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed nucleic acid-lipid particle (*e.g.*, SNALP) will range from about 0.01 to about 0.2, from about 0.05 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials (input) also falls within this range. In other embodiments, the particle preparation uses about 400 µg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 µg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0308] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed nucleic acid-lipid particle (*e.g.*, SNALP) will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), or about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), 15 (15:1), 16 (16:1), 17 (17:1), 18 (18:1), 19 (19:1), 20 (20:1), 21 (21:1), 22 (22:1), 23 (23:1), 24 (24:1), or 25 (25:1), or any fraction thereof or range therein. The ratio of the starting materials (input) also falls within this range.

[0309] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include the "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making SNALP-CPLs are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813.

VII. Kits

[0310] The present invention also provides lipid particles (*e.g.,* SNALP) in kit form. In some embodiments, the kit comprises a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). Preferably, the kit comprises a container (*e.g.,* a vial or ampoule) which holds the lipid particles of the invention (*e.g.,* SNALP), wherein the particles are produced by one of the processes set forth herein. In certain embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the particle compositions of the invention, either as a suspension in a pharmaceutically acceptable carrier or in dehydrated form, with instructions for their rehydration (if lyophilized) and administration.

[0311] The SNALP formulations of the present invention can be tailored to preferentially target particular tissues or organs of interest. Preferential targeting of SNALP may be carried out by controlling the composition of the SNALP itself. For instance, it has been found that the 1:57 SNALP formulation can be used to preferentially target the liver. In particular embodiments, the kits of the invention comprise these lipid particles, wherein the particles are present in a container as a suspension or in dehydrated form. Such kits are particularly advantageous for use in providing effective treatment of a lipid disorder such as dyslipidemia or atherosclerosis.

[0312] In certain instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

VIII. Administration of Lipid Particles

[0313] Once formed, the lipid particles of the invention (*e.g.*, SNALP) are particularly useful for the introduction of nucleic acids (*e.g.*, interfering RNA such as siRNA) into cells. Accordingly, the present invention also provides methods for introducing a nucleic acid (*e.g.*, interfering RNA) into a cell. In particular embodiments, the nucleic acid (*e.g.*, interfering RNA) is introduced into an *APOC3*-expressing cell such as a hepatocyte or other liver cell. The methods described herein may be carried out *in vitro* or *in vivo* by first forming the lipid particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the nucleic acid to the cells to occur.

[0314] The lipid particles of the invention (*e.g.*, SNALP) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid (*e.g.*, interfering RNA) portion of the particle can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

[0315] The lipid particles of the invention (*e.g.*, SNALP) can be administered either alone or in a mixture with a pharmaceutically acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc*. Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically acceptable" refers to molecular entities and

compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0316] The pharmaceutically acceptable carrier is generally added following lipid particle formation. Thus, after the lipid particle (*e.g.,* SNALP) is formed, the particle can be diluted into pharmaceutically acceptable carriers such as normal buffered saline.

[0317] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0318] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol, and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

[0319] In some embodiments, the lipid particles of the invention (*e.g.,* SNALP) are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.,* siRNA). In particular, it is an object of this invention to provide *in vitro* and *in vivo* methods for the treatment of *APOC3*-mediated diseases and disorders in a mammal (*e.g.,* a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of *APOC3*, alone or in combination with one or more additional target nucleic acid sequences or genes of interest. As a non-limiting example, the methods of the present invention are useful for the *in vivo* delivery of interfering RNA (*e.g.,* siRNA) to the liver cells (*e.g.,* hepatocytes) of a mammal such as a human for the treatment of a lipid disorder such as

dyslipidemia or atherosclerosis. In certain embodiments, the *APOC3*-mediated disease or disorder is associated with expression and/or overexpression of *APOC3* and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.,* siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle may be administered to the mammal. In some instances, one, two, three, or more interfering RNA molecules (*e.g.,* siRNA molecules targeting different regions of the *APOC3* gene) are formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA is delivered *in vitro* (*e.g.,* using a SNALP described herein), and the cells are reinjected into the patient.

A. *In vivo* Administration

[0320] Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453. The present invention also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are non-immunogenic, are small in size, and are suitable for repeat dosing.

[0321] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intransal or intratracheal), transdermal application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see*, *e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques,* 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see*, *e.g.*, US Patent Publication No. 20050118253).

[0322] In embodiments where the lipid particles of the present invention (*e.g.,* SNALP) are administered intravenously, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the particles is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles is detectable at least about 1 hour after administration of the particle. In some embodiments, the presence of a therapeutic nucleic acid such as an interfering RNA molecule (*e.g.,* siRNA) is detectable in cells (*e.g.,* liver cells) at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence, such as an *APOC3* sequence, by an interfering RNA (*e.g.,* siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence, such as an *APOC3* sequence, by an interfering RNA (*e.g.,* siRNA) occurs preferentially in liver cells. In further embodiments, the presence or effect of an interfering RNA (*e.g.,* siRNA) in cells at a site proximal or distal to the site of administration is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.,* SNALP) of the invention are administered parenterally or intraperitoneally.

[0323] The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am. J. Sci.*, 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0324] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.,* in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent

No. 5,780,045.

[0325] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0326] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0327] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos.

5,641,515, 5,580,579, and 5,792,451). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0328] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0329] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic nucleic acid (*e.g.,* interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic nucleic acid (*e.g.,* interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a therapeutic nucleic acid (*e.g.,* interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic nucleic acid in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic nucleic acid, carriers known in the art.

[0330] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid

particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0331] When preparing pharmaceutical preparations of the lipid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0332] The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0333] The amount of particles administered will depend upon the ratio of therapeutic nucleic acid (*e.g.,* interfering RNA) to lipid, the particular therapeutic nucleic acid used, the disease or disorder being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about 10^8-10^{10} particles per administration (*e.g.,* injection).

B. *In vitro* Administration

[0334] For *in vitro* applications, the delivery of therapeutic nucleic acids (*e.g.,* interfering RNA) can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

[0335] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37ºC) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0336] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2 x 10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

[0337] To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed.,

Wiley-Liss, New York (1994), Kuchler *et al.*, Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

[0338] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP or other lipid particle based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.,* siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.*, the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0339] The compositions and methods of the present invention are particularly well suited for treating any of a variety of *APOC3*-mediated diseases and disorders by targeting *APOC3* gene expression *in vivo*. The present invention can be practiced on a wide variety of cell types from any vertebrate species, including mammals, such as, *e.g,* canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, mice, rats, and guinea pigs), lagomorphs, swine, and primates (*e.g.* monkeys, chimpanzees, and humans). Suitable cells include, but are not limited to, liver cells such as hepatocytes, hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells (*e.g.,* intestinal epithelial cells), bone cells,

and the like. In preferred embodiments, an interfering RNA (*e.g.,* siRNA) is delivered to hepatocytes.

D. Detection of Lipid Particles

[0340] In some embodiments, the lipid particles of the present invention (*e.g.,* SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention (*e.g.,* SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g.*, by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (*e.g.,* siRNA) sequence, detection of the target sequence of interest (*i.e.*, by detecting expression or reduced expression of the sequence of interest), detection of a compound modulated by apoC-III (*e.g.*, serum triglycerides or cholesterol), or a combination thereof.

1. Detection of Particles

[0341] Lipid particles of the invention such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc.*, digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*; enzymes such as horseradish peroxidase, alkaline phosphatase, *etc.*; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0342] Nucleic acids (*e.g.*, interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography.

Additional analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0343] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g.*, "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

[0344] The sensitivity of the hybridization assays may be enhanced through the use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Qβ-replicase amplification, and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; The *Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation.

[0345] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of polynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic polynucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

[0346] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

IX. Combination Therapy

[0347] In some embodiments, the present invention provides methods for treating a lipid disorder associated with elevated triglycerides, cholesterol, and/or glucose by administering a therapeutic nucleic acid that targets the *APOC3* gene (*e.g., APOC3* interfering RNA such as *APOC3* siRNA) in combination with one or more therapeutic nucleic acids that target other genes (*e.g., APOB* siRNA). In one particular embodiment, the present invention provides methods for preventing and/or ameliorating hepatic steatosis (*e.g.*, fatty liver or triglyceride accumulation) induced by silencing *APOB* gene expression by co-administering an *APOC3* siRNA together with an *APOB* siRNA. In a preferred embodiment, the combination of therapeutic nucleic acids is delivered to a liver cell in a mammal such as a human.

[0348] In other embodiments, the present invention provides methods for treating a lipid disorder associated with elevated triglycerides, cholesterol, and/or glucose by administering a therapeutic nucleic acid that targets the *APOC3* gene (*e.g., APOC3* interfering RNA such as *APOC3* siRNA) in combination with a lipid-lowering agent. Non-limiting examples of lipid-

lowering agents include, but are not limited to, statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, and fish oil. The methods can be carried out *in vivo* by administering the therapeutic nucleic acid and lipid-lowering agent as described herein or using any means known in the art. In one preferred embodiment, the combination of therapeutic agents is delivered to a liver cell in a mammal such as a human.

[0349] In certain aspects, a patient about to begin therapy with either a lipid-lowering agent or a therapeutic nucleic acid that targets another gene (*e.g., APOB* siRNA) is first pretreated with a suitable dose of one or more lipid particles (*e.g.,* SNALP) containing a therapeutic nucleic acid that targets the *APOC3* gene (*e.g., APOC3* siRNA). The patient can be pretreated with a suitable dose of lipid particles targeting the *APOC3* gene at any reasonable time prior to administration of the lipid-lowering agent or other therapeutic nucleic acid. As non-limiting examples, the dose of one or more lipid particles targeting *APOC3* expression can be administered about 96, 84, 72, 60, 48, 36, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 hours, or any interval thereof, before administration of the lipid-lowering agent or other therapeutic nucleic acid.

[0350] Additionally, a patient about to begin therapy with either a lipid-lowering agent or a therapeutic nucleic acid that targets another gene (*e.g., APOB* siRNA) can be pretreated with more than one dose of lipid particles (*e.g.,* SNALP) containing a therapeutic nucleic acid that targets the *APOC3* gene (*e.g., APOC3* siRNA) at different times before administration of the lipid-lowering agent or other therapeutic nucleic acid. As such, the methods of the present invention can further comprise administering a second dose of lipid particles targeting the *APOC3* gene prior to administration of the lipid-lowering agent or other therapeutic nucleic acid. In certain instances, the lipid particles of the first dose are the same as the lipid particles of the second dose. In certain other instances, the lipid particles of the first dose are different from the lipid particles of the second dose. Preferably, the two pretreatment doses use the same lipid particles, *e.g.,* SNALP containing the same therapeutic nucleic acid that targets the *APOC3* gene (*e.g., APOC3* siRNA). One skilled in the art will appreciate that the second dose of lipid particles can occur at any reasonable time following the first dose. As a non-limiting example, if the first dose was administered about 12 hours before administration of the lipid-lowering agent or other therapeutic nucleic acid, the second dose can be administered about 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 hours, or any interval thereof, before administration of the lipid-lowering agent or other therapeutic nucleic acid. One skilled in the art will also appreciate that the second dose of

lipid particles can be the same or a different dose. In additional embodiments of the present invention, the patient can be pretreated with a third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, or more dose of the same or different lipid particles targeting the *APOC3* gene prior to administration of the lipid-lowering agent or other therapeutic nucleic acid.

[0351] A patient can also be treated with a suitable dose of one or more lipid particles (*e.g.*, SNALP) containing a therapeutic nucleic acid that targets the *APOC3* gene (*e.g., APOC3* siRNA) at any reasonable time during administration of either a lipid-lowering agent or a therapeutic nucleic acid that targets another gene (*e.g., APOB* siRNA). As such, the methods of the present invention can further comprise administering a dose of lipid particles targeting the *APOC3* gene during administration of the lipid-lowering agent or other therapeutic nucleic acid. One skilled in the art will appreciate that more than one dose of such lipid particles can be administered at different times during administration of the lipid-lowering agent or other therapeutic nucleic acid. As a non-limiting example, lipid particles (*e.g.*, SNALP) containing one or more unmodified and/or modified *APOC3* siRNA sequences can be administered at the beginning of administration of the lipid-lowering agent or other therapeutic nucleic acid, while administration of the lipid-lowering agent or other therapeutic nucleic acid is in progress, and/or at the end of administration of the lipid-lowering agent or other therapeutic nucleic acid. One skilled in the art will also appreciate that the pretreatment and intra-treatment (*i.e.*, during administration of the lipid-lowering agent or other therapeutic nucleic acid) doses of lipid particles targeting *APOC3* gene expression can be the same or a different dose.

[0352] In addition, a patient can be treated with a suitable dose of one or more nucleic acid-lipid particles (*e.g.*, SNALP) containing a therapeutic nucleic acid that targets the *APOC3* gene (*e.g., APOC3* siRNA) at any reasonable time following administration of either a lipid-lowering agent or a therapeutic nucleic acid that targets another gene (*e.g., APOB* siRNA). As such, the methods of the present invention can further comprise administering a dose of lipid particles targeting the *APOC3* gene after administration of the lipid-lowering agent or other therapeutic nucleic acid. As non-limiting examples, the dose of one or more such lipid particles can be administered about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 36, 48, 60, 72, 84, 96, 108, or more hours, or any interval thereof, after administration of the lipid-lowering agent or other therapeutic nucleic acid. In certain instances, the same lipid particle targeting the *APOC3* gene is used before and after administration of the lipid-lowering agent or other therapeutic nucleic acid. In certain other instances, a different lipid particle targeting the *APOC3* gene is

used following administration of the lipid-lowering agent or other therapeutic nucleic acid. One skilled in the art will appreciate that more than one dose of the lipid particles targeting *APOC3* gene expression can be administered at different times following administration of the lipid-lowering agent or other therapeutic nucleic acid. One skilled in the art will also appreciate that the pretreatment and posttreatment (*i.e.*, following administration of the lipid-lowering agent or other therapeutic nucleic acid) doses of lipid particles targeting the *APOC3* gene can be the same or a different dose.

[0353] Lipid-lowering agents or therapeutic nucleic acid (*e.g.,* interfering RNA) molecules that target other genes can be administered with a suitable pharmaceutical excipient as necessary and can be carried out via any of the accepted modes of administration. Thus, administration can be, for example, oral, buccal, sublingual, gingival, palatal, intravenous, topical, subcutaneous, transcutaneous, transdermal, intramuscular, intra-joint, parenteral, intra-arteriole, intradermal, intraventricular, intracranial, intraperitoneal, intravesical, intrathecal, intralesional, intranasal, rectal, vaginal, or by inhalation. By "co-administer" it is meant that the therapeutic nucleic acid targeting *APOC3* expression is administered at the same time, just prior to, or just after the administration of the lipid-lowering agent or therapeutic nucleic acid that targets another gene.

[0354] A therapeutically effective amount of a lipid-lowering agent may be administered repeatedly, *e.g.,* at least 2, 3, 4, 5, 6, 7, 8, or more times, or the dose may be administered by continuous infusion. The dose may take the form of solid, semi-solid, lyophilized powder, or liquid dosage forms, such as, for example, tablets, pills, pellets, capsules, powders, solutions, suspensions, emulsions, suppositories, retention enemas, creams, ointments, lotions, gels, aerosols, foams, or the like, preferably in unit dosage forms suitable for simple administration of precise dosages. One skilled in the art will appreciate that administered dosages of lipid-lowering agents will vary depending on a number of factors, including, but not limited to, the particular lipid-lowering agent or set of lipid-lowering agents to be administered, the mode of administration, the type of application, the age of the patient, and the physical condition of the patient. Preferably, the smallest dose and concentration required to produce the desired result should be used. Dosage should be appropriately adjusted for children, the elderly, debilitated patients, and patients with cardiac and/or liver disease. Further guidance can be obtained from studies known in the art using experimental animal models for evaluating dosage.

[0355] As used herein, the term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a

predetermined quantity of a lipid-lowering agent calculated to produce the desired onset, tolerability, and/or therapeutic effects, in association with a suitable pharmaceutical excipient (*e.g.,* an ampoule). In addition, more concentrated dosage forms may be prepared, from which the more dilute unit dosage forms may then be produced. The more concentrated dosage forms thus will contain substantially more than, *e.g.,* at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times the amount of the lipid-lowering agent.

[0356] Methods for preparing such dosage forms are known to those skilled in the art (*see, e.g., REMINGTON'S PHARMACEUTICAL SCIENCES*, 18TH ED., Mack Publishing Co., Easton, PA (1990)). The dosage forms typically include a conventional pharmaceutical carrier or excipient and may additionally include other medicinal agents, carriers, adjuvants, diluents, tissue permeation enhancers, solubilizers, and the like. Appropriate excipients can be tailored to the particular dosage form and route of administration by methods well known in the art (*see, e.g., REMINGTON'S PHARMACEUTICAL SCIENCES, supra*).

[0357] Examples of suitable excipients include, but are not limited to, lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, saline, syrup, methylcellulose, ethylcellulose, hydroxypropylmethylcellulose, and polyacrylic acids such as Carbopols, *e.g.,* Carbopol 941, Carbopol 980, Carbopol 981, *etc.* The dosage forms can additionally include lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying agents; suspending agents; preserving agents such as methyl-, ethyl-, and propyl-hydroxy-benzoates (*i.e.,* the parabens); pH adjusting agents such as inorganic and organic acids and bases; sweetening agents; and flavoring agents. The dosage forms may also comprise biodegradable polymer beads, dextran, and cyclodextrin inclusion complexes.

[0358] For oral administration, the therapeutically effective dose can be in the form of tablets, capsules, emulsions, suspensions, solutions, syrups, sprays, lozenges, powders, and sustained-release formulations. Suitable excipients for oral administration include pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, gelatin, sucrose, magnesium carbonate, and the like.

[0359] In some embodiments, the therapeutically effective dose takes the form of a pill, tablet, or capsule, and thus, the dosage form can contain, along with a lipid-lowering agent, any of the following: a diluent such as lactose, sucrose, dicalcium phosphate, and the like; a disintegrant such as starch or derivatives thereof; a lubricant such as magnesium stearate and the like; and a binder such a starch, gum acacia, polyvinylpyrrolidone, gelatin, cellulose and

derivatives thereof. A lipid-lowering agent can also be formulated into a suppository disposed, for example, in a polyethylene glycol (PEG) carrier.

[0360] Liquid dosage forms can be prepared by dissolving or dispersing a lipid-lowering agent and optionally one or more pharmaceutically acceptable adjuvants in a carrier such as, for example, aqueous saline (*e.g.,* 0.9% w/v sodium chloride), aqueous dextrose, glycerol, ethanol, and the like, to form a solution or suspension, *e.g.,* for oral, topical, or intravenous administration. A lipid-lowering agent can also be formulated into a retention enema.

[0361] For topical administration, the therapeutically effective dose can be in the form of emulsions, lotions, gels, foams, creams, jellies, solutions, suspensions, ointments, and transdermal patches. For administration by inhalation, a lipid-lowering agent can be delivered as a dry powder or in liquid form via a nebulizer. For parenteral administration, the therapeutically effective dose can be in the form of sterile injectable solutions and sterile packaged powders. Preferably, injectable solutions are formulated at a pH of from about 4.5 to about 7.5.

[0362] The therapeutically effective dose can also be provided in a lyophilized form. Such dosage forms may include a buffer, *e.g.,* bicarbonate, for reconstitution prior to administration, or the buffer may be included in the lyophilized dosage form for reconstitution with, *e.g.,* water. The lyophilized dosage form may further comprise a suitable vasoconstrictor, *e.g.,* epinephrine. The lyophilized dosage form can be provided in a syringe, optionally packaged in combination with the buffer for reconstitution, such that the reconstituted dosage form can be immediately administered to a subject.

X. Examples

[0363] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Exemplary siRNA molecules targeting *APOC3*.

[0364] Table 7 provides non-limiting examples of siRNA molecules that are suitable for modulating (*e.g.,* silencing) *APOC3* gene expression. In some embodiments, the sense strand comprises or consists of one of the target *APOC3* sequences set forth in Table 7. In related embodiments, the sense strand comprises at least 15 contiguous nucleotides (*e.g.,* at least 15,

16, 17, 18, or 19 contiguous nucleotides) of one of the target *APOC3* sequences set forth in Table 7. In other embodiments, the antisense strand comprises or consists of one of the antisense strand sequences set forth in Table 7. In related embodiments, the antisense strand comprises at least 15 contiguous nucleotides (*e.g.,* at least 15, 16, 17, 18, or 19 contiguous nucleotides) of one of the antisense strand sequences set forth in Table 7. In further embodiments, the antisense strand specifically hybridizes to one of the target *APOC3* sequences set forth in Table 7.

Table 7. siRNA sequences that target human *APOC3* expression.

siRNA	Target or Sense Strand Sequence (5' → 3')	Antisense Strand Sequence (5' → 3')
1	UGCUCAGUUCAUCCCUAGA	UCUAGGGAUGAACUGAGCA
2	GCUCAGUUCAUCCCUAGAG	CUCUAGGGAUGAACUGAGC
3	CUCAGUUCAUCCCUAGAGG	CCUCUAGGGAUGAACUGAG
4	UCAGUUCAUCCCUAGAGGC	GCCUCUAGGGAUGAACUGA
5	CAGUUCAUCCCUAGAGGCA	UGCCUCUAGGGAUGAACUG
6	AGUUCAUCCCUAGAGGCAG	CUGCCUCUAGGGAUGAACU
7	GUUCAUCCCUAGAGGCAGC	GCUGCCUCUAGGGAUGAAC
8	UUCAUCCCUAGAGGCAGCU	AGCUGCCUCUAGGGAUGAA
9	UCAUCCCUAGAGGCAGCUG	CAGCUGCCUCUAGGGAUGA
10	CAUCCCUAGAGGCAGCUGC	GCAGCUGCCUCUAGGGAUG
11	AUCCCUAGAGGCAGCUGCU	AGCAGCUGCCUCUAGGGAU
12	UCCCUAGAGGCAGCUGCUC	GAGCAGCUGCCUCUAGGGA
13	CCCUAGAGGCAGCUGCUCC	GGAGCAGCUGCCUCUAGGG
14	CCUAGAGGCAGCUGCUCCA	UGGAGCAGCUGCCUCUAGG
15	CUAGAGGCAGCUGCUCCAG	CUGGAGCAGCUGCCUCUAG
16	UAGAGGCAGCUGCUCCAGG	CCUGGAGCAGCUGCCUCUA
17	AGAGGCAGCUGCUCCAGGA	UCCUGGAGCAGCUGCCUCU
18	GAGGCAGCUGCUCCAGGAA	UUCCUGGAGCAGCUGCCUC
19	AGGCAGCUGCUCCAGGAAC	GUUCCUGGAGCAGCUGCCU
20	GGCAGCUGCUCCAGGAACA	UGUUCCUGGAGCAGCUGCC
21	GCAGCUGCUCCAGGAACAG	CUGUUCCUGGAGCAGCUGC
22	CAGCUGCUCCAGGAACAGA	UCUGUUCCUGGAGCAGCUG
23	AGCUGCUCCAGGAACAGAG	CUCUGUUCCUGGAGCAGCU
24	GCUGCUCCAGGAACAGAGG	CCUCUGUUCCUGGAGCAGC
25	CUGCUCCAGGAACAGAGGU	ACCUCUGUUCCUGGAGCAG
26	UGCUCCAGGAACAGAGGUG	CACCUCUGUUCCUGGAGCA
27	GCUCCAGGAACAGAGGUGC	GCACCUCUGUUCCUGGAGC
28	CUCCAGGAACAGAGGUGCC	GGCACCUCUGUUCCUGGAG
29	UCCAGGAACAGAGGUGCCA	UGGCACCUCUGUUCCUGGA
30	CCAGGAACAGAGGUGCCAU	AUGGCACCUCUGUUCCUGG
31	CAGGAACAGAGGUGCCAUG	CAUGGCACCUCUGUUCCUG
32	AGGAACAGAGGUGCCAUGC	GCAUGGCACCUCUGUUCCU
33	GGAACAGAGGUGCCAUGCA	UGCAUGGCACCUCUGUUCC
34	GAACAGAGGUGCCAUGCAG	CUGCAUGGCACCUCUGUUC
35	AACAGAGGUGCCAUGCAGC	GCUGCAUGGCACCUCUGUU
36	ACAGAGGUGCCAUGCAGCC	GGCUGCAUGGCACCUCUGU
37	CAGAGGUGCCAUGCAGCCC	GGGCUGCAUGGCACCUCUG
38	AGAGGUGCCAUGCAGCCCC	GGGGCUGCAUGGCACCUCU
39	GAGGUGCCAUGCAGCCCCG	CGGGGCUGCAUGGCACCUC
40	AGGUGCCAUGCAGCCCCGG	CCGGGGCUGCAUGGCACCU
41	GGUGCCAUGCAGCCCCGGG	CCCGGGGCUGCAUGGCACC
42	GUGCCAUGCAGCCCCGGGU	ACCCGGGGCUGCAUGGCAC
43	UGCCAUGCAGCCCCGGGUA	UACCCGGGGCUGCAUGGCA
44	GCCAUGCAGCCCCGGGUAC	GUACCCGGGGCUGCAUGGC
45	CCAUGCAGCCCCGGGUACU	AGUACCCGGGGCUGCAUGG

46	CAUGCAGCCCCGGGUACUC	GAGUACCCGGGGCUGCAUG
47	AUGCAGCCCCGGGUACUCC	GGAGUACCCGGGGCUGCAU
48	UGCAGCCCCGGGUACUCCU	AGGAGUACCCGGGGCUGCA
49	GCAGCCCCGGGUACUCCUU	AAGGAGUACCCGGGGCUGC
50	CAGCCCCGGGUACUCCUUG	CAAGGAGUACCCGGGGCUG
51	AGCCCCGGGUACUCCUUGU	ACAAGGAGUACCCGGGGCU
52	GCCCCGGGUACUCCUUGUU	AACAAGGAGUACCCGGGGC
53	CCCCGGGUACUCCUUGUUG	CAACAAGGAGUACCCGGGG
54	CCCGGGUACUCCUUGUUGU	ACAACAAGGAGUACCCGGG
55	CCGGGUACUCCUUGUUGUU	AACAACAAGGAGUACCCGG
56	CGGGUACUCCUUGUUGUUG	CAACAACAAGGAGUACCCG
57	GGGUACUCCUUGUUGUUGC	GCAACAACAAGGAGUACCC
58	GGUACUCCUUGUUGUUGCC	GGCAACAACAAGGAGUACC
59	GUACUCCUUGUUGUUGCCC	GGGCAACAACAAGGAGUAC
60	UACUCCUUGUUGUUGCCCU	AGGGCAACAACAAGGAGUA
61	ACUCCUUGUUGUUGCCCUC	GAGGGCAACAACAAGGAGU
62	CUCCUUGUUGUUGCCCUCC	GGAGGGCAACAACAAGGAG
63	UCCUUGUUGUUGCCCUCCU	AGGAGGGCAACAACAAGGA
64	CCUUGUUGUUGCCCUCCUG	CAGGAGGGCAACAACAAGG
65	CUUGUUGUUGCCCUCCUGG	CCAGGAGGGCAACAACAAG
66	UUGUUGUUGCCCUCCUGGC	GCCAGGAGGGCAACAACAA
67	UGUUGUUGCCCUCCUGGCG	CGCCAGGAGGGCAACAACA
68	GUUGUUGCCCUCCUGGCGC	GCGCCAGGAGGGCAACAAC
69	UUGUUGCCCUCCUGGCGCU	AGCGCCAGGAGGGCAACAA
70	UGUUGCCCUCCUGGCGCUC	GAGCGCCAGGAGGGCAACA
71	GUUGCCCUCCUGGCGCUCC	GGAGCGCCAGGAGGGCAAC
72	UUGCCCUCCUGGCGCUCCU	AGGAGCGCCAGGAGGGCAA
73	UGCCCUCCUGGCGCUCCUG	CAGGAGCGCCAGGAGGGCA
74	GCCCUCCUGGCGCUCCUGG	CCAGGAGCGCCAGGAGGGC
75	CCCUCCUGGCGCUCCUGGC	GCCAGGAGCGCCAGGAGGG
76	CCUCCUGGCGCUCCUGGCC	GGCCAGGAGCGCCAGGAGG
77	CUCCUGGCGCUCCUGGCCU	AGGCCAGGAGCGCCAGGAG
78	UCCUGGCGCUCCUGGCCUC	GAGGCCAGGAGCGCCAGGA
79	CCUGGCGCUCCUGGCCUCU	AGAGGCCAGGAGCGCCAGG
80	CUGGCGCUCCUGGCCUCUG	CAGAGGCCAGGAGCGCCAG
81	UGGCGCUCCUGGCCUCUGC	GCAGAGGCCAGGAGCGCCA
82	GGCGCUCCUGGCCUCUGCC	GGCAGAGGCCAGGAGCGCC
83	GCGCUCCUGGCCUCUGCCC	GGGCAGAGGCCAGGAGCGC
84	CGCUCCUGGCCUCUGCCCG	CGGGCAGAGGCCAGGAGCG
85	GCUCCUGGCCUCUGCCCGA	UCGGGCAGAGGCCAGGAGC
86	CUCCUGGCCUCUGCCCGAG	CUCGGGCAGAGGCCAGGAG
87	UCCUGGCCUCUGCCCGAGC	GCUCGGGCAGAGGCCAGGA
88	CCUGGCCUCUGCCCGAGCU	AGCUCGGGCAGAGGCCAGG
89	CUGGCCUCUGCCCGAGCUU	AAGCUCGGGCAGAGGCCAG
90	UGGCCUCUGCCCGAGCUUC	GAAGCUCGGGCAGAGGCCA
91	GGCCUCUGCCCGAGCUUCA	UGAAGCUCGGGCAGAGGCC
92	GCCUCUGCCCGAGCUUCAG	CUGAAGCUCGGGCAGAGGC
93	CCUCUGCCCGAGCUUCAGA	UCUGAAGCUCGGGCAGAGG
94	CUCUGCCCGAGCUUCAGAG	CUCUGAAGCUCGGGCAGAG
95	UCUGCCCGAGCUUCAGAGG	CCUCUGAAGCUCGGGCAGA
96	CUGCCCGAGCUUCAGAGGC	GCCUCUGAAGCUCGGGCAG
97	UGCCCGAGCUUCAGAGGCC	GGCCUCUGAAGCUCGGGCA
98	GCCCGAGCUUCAGAGGCCG	CGGCCUCUGAAGCUCGGGC
99	CCCGAGCUUCAGAGGCCGA	UCGGCCUCUGAAGCUCGGG
100	CCGAGCUUCAGAGGCCGAG	CUCGGCCUCUGAAGCUCGG
101	CGAGCUUCAGAGGCCGAGG	CCUCGGCCUCUGAAGCUCG
102	GAGCUUCAGAGGCCGAGGA	UCCUCGGCCUCUGAAGCUC
103	AGCUUCAGAGGCCGAGGAU	AUCCUCGGCCUCUGAAGCU
104	GCUUCAGAGGCCGAGGAUG	CAUCCUCGGCCUCUGAAGC
105	CUUCAGAGGCCGAGGAUGC	GCAUCCUCGGCCUCUGAAG
106	UUCAGAGGCCGAGGAUGCC	GGCAUCCUCGGCCUCUGAA
107	UCAGAGGCCGAGGAUGCCU	AGGCAUCCUCGGCCUCUGA
108	CAGAGGCCGAGGAUGCCUC	GAGGCAUCCUCGGCCUCUG
109	AGAGGCCGAGGAUGCCUCC	GGAGGCAUCCUCGGCCUCU

110	GAGGCCGAGGAUGCCUCCC	GGGAGGCAUCCUCGGCCUC
111	AGGCCGAGGAUGCCUCCCU	AGGGAGGCAUCCUCGGCCU
112	GGCCGAGGAUGCCUCCCUU	AAGGGAGGCAUCCUCGGCC
113	GCCGAGGAUGCCUCCCUUC	GAAGGGAGGCAUCCUCGGC
114	CCGAGGAUGCCUCCCUUCU	AGAAGGGAGGCAUCCUCGG
115	CGAGGAUGCCUCCCUUCUC	GAGAAGGGAGGCAUCCUCG
116	GAGGAUGCCUCCCUUCUCA	UGAGAAGGGAGGCAUCCUC
117	AGGAUGCCUCCCUUCUCAG	CUGAGAAGGGAGGCAUCCU
118	GGAUGCCUCCCUUCUCAGC	GCUGAGAAGGGAGGCAUCC
119	GAUGCCUCCCUUCUCAGCU	AGCUGAGAAGGGAGGCAUC
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121	UGCCUCCCUUCUCAGCUUC	GAAGCUGAGAAGGGAGGCA
122	GCCUCCCUUCUCAGCUUCA	UGAAGCUGAGAAGGGAGGC
123	CCUCCCUUCUCAGCUUCAU	AUGAAGCUGAGAAGGGAGG
124	CUCCCUUCUCAGCUUCAUG	CAUGAAGCUGAGAAGGGAG
125	UCCCUUCUCAGCUUCAUGC	GCAUGAAGCUGAGAAGGGA
126	CCCUUCUCAGCUUCAUGCA	UGCAUGAAGCUGAGAAGGG
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133	CAGCUUCAUGCAGGGUUAC	GUAACCCUGCAUGAAGCUG
134	AGCUUCAUGCAGGGUUACA	UGUAACCCUGCAUGAAGCU
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146	GGUUACAUGAAGCACGCCA	UGGCGUGCUUCAUGUAACC
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159	ACGCCACCAAGACCGCCAA	UUGGCGGUCUUGGUGGCGU
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179	GAUGCACUGAGCAGCGUGC	GCACGCUGCUCAGUGCAUC
180	AUGCACUGAGCAGCGUGCA	UGCACGCUGCUCAGUGCAU
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187	GAGCAGCGUGCAGGAGUCC	GGACUCCUGCACGCUGCUC
188	AGCAGCGUGCAGGAGUCCC	GGGACUCCUGCACGCUGCU
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190	CAGCGUGCAGGAGUCCCAG	CUGGGACUCCUGCACGCUG
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194	GUGCAGGAGUCCCAGGUGG	CCACCUGGGACUCCUGCAC
195	UGCAGGAGUCCCAGGUGGC	GCCACCUGGGACUCCUGCA
196	GCAGGAGUCCCAGGUGGCC	GGCCACCUGGGACUCCUGC
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235	GACCGAUGGCUUCAGUUCC	GGAACUGAAGCCAUCGGUC
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387	AGCUCCUUGGGUCCUGCAA	UUGCAGGACCCAAGGAGCU
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404	AAUCUCCAGGGCUGCCCCU	AGGGGCAGCCCUGGAGAUU
405	AUCUCCAGGGCUGCCCCUG	CAGGGGCAGCCCUGGAGAU
406	UCUCCAGGGCUGCCCCUGU	ACAGGGGCAGCCCUGGAGA
407	CUCCAGGGCUGCCCCUGUA	UACAGGGGCAGCCCUGGAG
408	UCCAGGGCUGCCCCUGUAG	CUACAGGGGCAGCCCUGGA
409	CCAGGGCUGCCCCUGUAGG	CCUACAGGGGCAGCCCUGG
410	CAGGGCUGCCCCUGUAGGU	ACCUACAGGGGCAGCCCUG
411	AGGGCUGCCCCUGUAGGUU	AACCUACAGGGGCAGCCCU
412	GGGCUGCCCCUGUAGGUUG	CAACCUACAGGGGCAGCCC
413	GGCUGCCCCUGUAGGUUGC	GCAACCUACAGGGGCAGCC
414	GCUGCCCCUGUAGGUUGCU	AGCAACCUACAGGGGCAGC
415	CUGCCCCUGUAGGUUGCUU	AAGCAACCUACAGGGGCAG
416	UGCCCCUGUAGGUUGCUUA	UAAGCAACCUACAGGGGCA
417	GCCCCUGUAGGUUGCUUAA	UUAAGCAACCUACAGGGGC
418	CCCCUGUAGGUUGCUUAAA	UUUAAGCAACCUACAGGGG
419	CCCUGUAGGUUGCUUAAAA	UUUUAAGCAACCUACAGGG
420	CCUGUAGGUUGCUUAAAAG	CUUUUAAGCAACCUACAGG
421	CUGUAGGUUGCUUAAAAGG	CCUUUUAAGCAACCUACAG
422	UGUAGGUUGCUUAAAAGGG	CCCUUUUAAGCAACCUACA
423	GUAGGUUGCUUAAAAGGGA	UCCCUUUUAAGCAACCUAC
424	UAGGUUGCUUAAAAGGGAC	GUCCCUUUUAAGCAACCUA
425	AGGUUGCUUAAAAGGGACA	UGUCCCUUUUAAGCAACCU
426	GGUUGCUUAAAAGGGACAG	CUGUCCCUUUUAAGCAACC
427	GUUGCUUAAAAGGGACAGU	ACUGUCCCUUUUAAGCAAC
428	UUGCUUAAAAGGGACAGUA	UACUGUCCCUUUUAAGCAA
429	UGCUUAAAAGGGACAGUAU	AUACUGUCCCUUUUAAGCA

430	GCUUAAAAGGGACAGUAUU	AAUACUGUCCCUUUUAAGC
431	CUUAAAAGGGACAGUAUUC	GAAUACUGUCCCUUUUAAG
432	UUAAAAGGGACAGUAUUCU	AGAAUACUGUCCCUUUUAA
433	UAAAAGGGACAGUAUUCUC	GAGAAUACUGUCCCUUUUA
434	AAAAGGGACAGUAUUCUCA	UGAGAAUACUGUCCCUUUU
435	AAAGGGACAGUAUUCUCAG	CUGAGAAUACUGUCCCUUU
436	AAGGGACAGUAUUCUCAGU	ACUGAGAAUACUGUCCCUU
437	AGGGACAGUAUUCUCAGUG	CACUGAGAAUACUGUCCCU
438	GGGACAGUAUUCUCAGUGC	GCACUGAGAAUACUGUCCC
439	GGACAGUAUUCUCAGUGCU	AGCACUGAGAAUACUGUCC
440	GACAGUAUUCUCAGUGCUC	GAGCACUGAGAAUACUGUC
441	ACAGUAUUCUCAGUGCUCU	AGAGCACUGAGAAUACUGU
442	CAGUAUUCUCAGUGCUCUC	GAGAGCACUGAGAAUACUG
443	AGUAUUCUCAGUGCUCUCC	GGAGAGCACUGAGAAUACU
444	GUAUUCUCAGUGCUCUCCU	AGGAGAGCACUGAGAAUAC
445	UAUUCUCAGUGCUCUCCUA	UAGGAGAGCACUGAGAAUA
446	AUUCUCAGUGCUCUCCUAC	GUAGGAGAGCACUGAGAAU
447	UUCUCAGUGCUCUCCUACC	GGUAGGAGAGCACUGAGAA
448	UCUCAGUGCUCUCCUACCC	GGGUAGGAGAGCACUGAGA
449	CUCAGUGCUCUCCUACCCC	GGGGUAGGAGAGCACUGAG
450	UCAGUGCUCUCCUACCCCA	UGGGGUAGGAGAGCACUGA
451	CAGUGCUCUCCUACCCCAC	GUGGGGUAGGAGAGCACUG
452	AGUGCUCUCCUACCCCACC	GGUGGGGUAGGAGAGCACU
453	GUGCUCUCCUACCCCACCU	AGGUGGGGUAGGAGAGCAC
454	UGCUCUCCUACCCCACCUC	GAGGUGGGGUAGGAGAGCA
455	GCUCUCCUACCCCACCUCA	UGAGGUGGGGUAGGAGAGC
456	CUCUCCUACCCCACCUCAU	AUGAGGUGGGGUAGGAGAG
457	UCUCCUACCCCACCUCAUG	CAUGAGGUGGGGUAGGAGA
458	CUCCUACCCCACCUCAUGC	GCAUGAGGUGGGGUAGGAG
459	UCCUACCCCACCUCAUGCC	GGCAUGAGGUGGGGUAGGA
460	CCUACCCCACCUCAUGCCU	AGGCAUGAGGUGGGGUAGG
461	CUACCCCACCUCAUGCCUG	CAGGCAUGAGGUGGGGUAG
462	UACCCCACCUCAUGCCUGG	CCAGGCAUGAGGUGGGGUA
463	ACCCCACCUCAUGCCUGGC	GCCAGGCAUGAGGUGGGGU
464	CCCCACCUCAUGCCUGGCC	GGCCAGGCAUGAGGUGGGG
465	CCCACCUCAUGCCUGGCCC	GGGCCAGGCAUGAGGUGGG
466	CCACCUCAUGCCUGGCCCC	GGGGCCAGGCAUGAGGUGG
467	CACCUCAUGCCUGGCCCCC	GGGGGCCAGGCAUGAGGUG
468	ACCUCAUGCCUGGCCCCCC	GGGGGGCCAGGCAUGAGGU
469	CCUCAUGCCUGGCCCCCCU	AGGGGGGCCAGGCAUGAGG
470	CUCAUGCCUGGCCCCCCUC	GAGGGGGGCCAGGCAUGAG
471	UCAUGCCUGGCCCCCCUCC	GGAGGGGGGCCAGGCAUGA
472	CAUGCCUGGCCCCCCUCCA	UGGAGGGGGGCCAGGCAUG
473	AUGCCUGGCCCCCCUCCAG	CUGGAGGGGGGCCAGGCAU
474	UGCCUGGCCCCCCUCCAGG	CCUGGAGGGGGGCCAGGCA
475	GCCUGGCCCCCCUCCAGGC	GCCUGGAGGGGGGCCAGGC
476	CCUGGCCCCCCUCCAGGCA	UGCCUGGAGGGGGGCCAGG
477	CUGGCCCCCCUCCAGGCAU	AUGCCUGGAGGGGGGCCAG
478	UGGCCCCCCUCCAGGCAUG	CAUGCCUGGAGGGGGGCCA
479	GGCCCCCCUCCAGGCAUGC	GCAUGCCUGGAGGGGGGCC
480	GCCCCCCUCCAGGCAUGCU	AGCAUGCCUGGAGGGGGGC
481	CCCCCCUCCAGGCAUGCUG	CAGCAUGCCUGGAGGGGGG
482	CCCCCUCCAGGCAUGCUGG	CCAGCAUGCCUGGAGGGGG
483	CCCCUCCAGGCAUGCUGGC	GCCAGCAUGCCUGGAGGGG
484	CCCUCCAGGCAUGCUGGCC	GGCCAGCAUGCCUGGAGGG
485	CCUCCAGGCAUGCUGGCCU	AGGCCAGCAUGCCUGGAGG
486	CUCCAGGCAUGCUGGCCUC	GAGGCCAGCAUGCCUGGAG
487	UCCAGGCAUGCUGGCCUCC	GGAGGCCAGCAUGCCUGGA
488	CCAGGCAUGCUGGCCUCCC	GGGAGGCCAGCAUGCCUGG
489	CAGGCAUGCUGGCCUCCCA	UGGGAGGCCAGCAUGCCUG
490	AGGCAUGCUGGCCUCCCAA	UUGGGAGGCCAGCAUGCCU
491	GGCAUGCUGGCCUCCCAAU	AUUGGGAGGCCAGCAUGCC
492	GCAUGCUGGCCUCCCAAUA	UAUUGGGAGGCCAGCAUGC
493	CAUGCUGGCCUCCCAAUAA	UUAUUGGGAGGCCAGCAUG

494	AUGCUGGCCUCCCAAUAAA	UUUAUUGGGAGGCCAGCAU
495	UGCUGGCCUCCCAAUAAAG	CUUUAUUGGGAGGCCAGCA
496	GCUGGCCUCCCAAUAAAGC	GCUUUAUUGGGAGGCCAGC
497	CUGGCCUCCCAAUAAAGCU	AGCUUUAUUGGGAGGCCAG
498	UGGCCUCCCAAUAAAGCUG	CAGCUUUAUUGGGAGGCCA
499	GGCCUCCCAAUAAAGCUGG	CCAGCUUUAUUGGGAGGCC
500	GCCUCCCAAUAAAGCUGGA	UCCAGCUUUAUUGGGAGGC
501	CCUCCCAAUAAAGCUGGAC	GUCCAGCUUUAUUGGGAGG
502	CUCCCAAUAAAGCUGGACA	UGUCCAGCUUUAUUGGGAG
503	UCCCAAUAAAGCUGGACAA	UUGUCCAGCUUUAUUGGGA
504	CCCAAUAAAGCUGGACAAG	CUUGUCCAGCUUUAUUGGG
505	CCAAUAAAGCUGGACAAGA	UCUUGUCCAGCUUUAUUGG
506	CAAUAAAGCUGGACAAGAA	UUCUUGUCCAGCUUUAUUG
507	AAUAAAGCUGGACAAGAAG	CUUCUUGUCCAGCUUUAUU
508	AUAAAGCUGGACAAGAAGC	GCUUCUUGUCCAGCUUUAU
509	UAAAGCUGGACAAGAAGCU	AGCUUCUUGUCCAGCUUUA
510	AAAGCUGGACAAGAAGCUG	CAGCUUCUUGUCCAGCUUU
511	AAGCUGGACAAGAAGCUGC	GCAGCUUCUUGUCCAGCUU
512	AGCUGGACAAGAAGCUGCU	AGCAGCUUCUUGUCCAGCU
513	GCUGGACAAGAAGCUGCUA	UAGCAGCUUCUUGUCCAGC
514	CUGGACAAGAAGCUGCUAU	AUAGCAGCUUCUUGUCCAG
515	UGGACAAGAAGCUGCUAUG	CAUAGCAGCUUCUUGUCCA

[0365] The number under "siRNA" in Table 7 refers to the nucleotide position of the 5' base of the target or sense strand sequence relative to the first nucleotide of the human *APOC3* mRNA sequence (Genbank Accession No. NM_000040.1). In certain embodiments, the sense and/or antisense strand comprises modified nucleotides such as 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. In particular embodiments, the sense and/or antisense strand comprises 2'OMe nucleotides in accordance with one or more of the selective modification patterns described herein. In some instances, the sense and/or antisense strand contains "dTdT" or "UU" 3' overhangs. In other instances, the sense and/or antisense strand contains 3' overhangs that have complementarity to the target sequence (3' overhang in the antisense strand) or the complementary strand thereof (3' overhang in the sense strand). In further embodiments, the 3' overhang on the sense strand, antisense strand, or both strands may comprise one, two, three, four, or more modified nucleotides such as those described herein (*e.g.*, 2'OMe nucleotides).

Example 2. Stable Nucleic Acid-Lipid Particle-Mediated Silencing of Apolipoprotein CIII Reduces Plasma Triglycerides in Mice.

[0366] This example illustrates that administration of stable nucleic acid-lipid particles (SNALP) containing fully encapsulated siRNA targeting the *Apoc3* gene to mice resulted in reductions in hepatic *Apoc3* mRNA levels, plasma triglycerides, and plasma cholesterol levels, without an increase in hepatic triglycerides. No measurable immune response was

induced with these formulations, minimizing the potential for nonspecific effects in models of chronic inflammatory disease, such as atherosclerosis.

Introduction

[0367] Apolipoprotein CIII (apoCIII) is implicated in atherogenesis through its association with hypertriglyceridemia and induction of endothelial dysfunction. This example shows that nucleic acid-lipid particles (*e.g.*, SNALP) facilitate RNAi-mediated silencing of apoCIII and other targets thought to be "non-druggable" with conventional medicines. Studies of siRNA-based silencing of *Apoc3* in mice supports further preclinical studies of apoCIII-targeting SNALP in mouse models of atherosclerosis.

Materials and Methods

[0368] **siRNA design.** siRNA sequences targeting mouse *Apoc3* (GenBank Accession No. NM_023114.3) were selected using an algorithm implemented by the Whitehead Institute for Biomedical Research that incorporates standard siRNA design guidelines (1-3). For 17 of the siRNA sequences, the following criteria were selected: (1) NNN21 target sequences; (2) thermodynamically unstable 5' antisense end ($\Delta G > -8.3$ kcal/mol); and (3) thermodynamically less stable 5' antisense end ($\Delta G_{sense} - \Delta G_{anti\text{-}sense} < -2.1$).

[0369] All selected sequences were assessed for potential sequence-specific targeting activity against other mouse genes using the BLASTN algorithm against the mouse mRNA Reference Sequence database at the National Center for Biotechnology Information. siRNAs were eliminated if they contained sequence complementary to a transcript other than *Apoc3* at positions 4 to 18 of the antisense strand.

[0370] Five single nucleotide polymorphisms (SNPs), rs32674708, rs32674710, rs32674712, rs8254931and rs29889677, located in the coding or UTR sequences of the mouse *Apoc3* gene, were identified in the NCBI SNP database and used to evaluate the panel of siRNAs. Several siRNAs were identified that contained a nucleotide complementary to one of the SNPs, including mApoc3_146 (rs8254931), mApoc3_232 and mApoc3_245 (rs32674712), mApoc3_344 (rs32674710), mApoc3_465, mApoc3_466, mApoc3_467, and mApoc3_484 (rs32674708); however, these siRNAs were kept in the panel because they were designed based on genomic sequence from the C57Bl/6 mouse strain, the same strain used for primary hepatocytes and *in vivo* studies.

[0371] In order to evaluate expected cross-reactivity of siRNAs, sequences from mouse *Apoc3* mRNA and human (GenBank Accession No. NM_000040.1) and cynomolgus monkey

(*Macaca fascicularis*; GenBank Accession No. X68359.1) *APOC3* mRNA were aligned using ClustalX (4), with manual editing when necessary. This sequence alignment was also used to identify 3 siRNAs, mApoc3_92, mApoc3_258, and mApoc3_501, that did not meet the original siRNA criteria, but instead were chosen based on an antisense (AS) sequence that contains only one mismatch to the *APOC3* transcript (*i.e.,* 95% complementary) in humans and cynomolgus monkeys. Selected sequences were verified and the positions within the mouse *Apoc3* target sequence were identified.

[0372] siRNA synthesis. All siRNA molecules used in this study were chemically synthesized by Integrated DNA Technologies (Coralville, IA). The siRNAs were desalted and annealed using standard procedures. Sequences of unmodified mouse *Apoc3* siRNAs are listed in Table 8. Sequences of modified mouse *Apoc3* siRNAs are listed in Table 9. Sequence numbers represent the nucleotide position of mouse *Apoc3* mRNA (Genbank Accession No. NM_023114.3) that is complementary to the 3' end of the antisense strand of the siRNA.

Table 8. Unmodified siRNA sequences that target mouse *Apoc3* expression.

siRNA	Target Sequence (5' → 3')	Sense Strand (5' → 3')	Antisense Strand (5' → 3')
mApoc3_92	CCUGGCAUCUGCCCGAGCU	CCUGGCAUCUGCCCGAGCUGA	AGCUCGGGCAGAUGCCAGGAG
mApoc3_146	ACAGGGCUACAUGGAACAA	ACAGGGCUACAUGGAACAAGC	UUGUUCCAUGUAGCCCUGUAC
mApoc3_232	GCUGGAUGGACAAUCACUU	GCUGGAUGGACAAUCACUUCA	AAGUGAUUGUCCAUCCAGCCC
mApoc3_245	UCACUUCAGAUCCCUGAAA	UCACUUCAGAUCCCUGAAAGG	UUUCAGGGAUCUGAAGUGAUU
mApoc3_258	CUGAAAGGCUACUGGAGCA	CUGAAAGGCUACUGGAGCAAG	UGCUCCAGUAGCCUUUCAGGG
mApoc3_262	AAGGCUACUGGAGCAAGUU	AAGGCUACUGGAGCAAGUUUA	AACUUGCUCCAGUAGCCUUUC
mApoc3_263	AGGCUACUGGAGCAAGUUU	AGGCUACUGGAGCAAGUUUAC	AAACUUGCUCCAGUAGCCUUU
mApoc3_264	GGCUACUGGAGCAAGUUUA	GGCUACUGGAGCAAGUUUACU	UAAACUUGCUCCAGUAGCCUU
mApoc3_265	GCUACUGGAGCAAGUUUAC	GCUACUGGAGCAAGUUUACUG	GUAAACUUGCUCCAGUAGCCU
mApoc3_274	GCAAGUUUACUGACAAGUU	GCAAGUUUACUGACAAGUUCA	AACUUGUCAGUAAACUUGCUC
mApoc3_323	CCAACCAACUCCAGCUAUU	CCAACCAACUCCAGCUAUUGA	AAUAGCUGGAGUUGGUUGGUC
mApoc3_324	CAACCAACUCCAGCUAUUG	CAACCAACUCCAGCUAUUGAG	CAAUAGCUGGAGUUGGUUGGU
mApoc3_344	GUCGUGAGACUUCUGUGUU	GUCGUGAGACUUCUGUGUUGC	AACACAGAAGUCUCACGACUC
mApoc3_465	UCCCUAGAUCUCACCUAAA	UCCCUAGAUCUCACCUAAACA	UUUAGGUGAGAUCUAGGGAGG
mApoc3_466	CCCUAGAUCUCACCUAAAC	CCCUAGAUCUCACCUAAACAU	GUUUAGGUGAGAUCUAGGGAG
mApoc3_467	CCUAGAUCUCACCUAAACA	CCUAGAUCUCACCUAAACAUG	UGUUUAGGUGAGAUCUAGGGA
mApoc3_484	CAUGCUGUCCCUAAUAAAG	CAUGCUGUCCCUAAUAAAGCU	CUUUAUUAGGGACAGCAUGUU
mApoc3_492	CCCUAAUAAAGCUGGAUAA	CCCUAAUAAAGCUGGAUAAGA	UUAUCCAGCUUUAUUAGGGAC
mApoc3_493	CCUAAUAAAGCUGGAUAAG	CCUAAUAAAGCUGGAUAAGAA	CUUAUCCAGCUUUAUUAGGGA
mApoc3_501	AGCUGGAUAAGAAGCUGCU	AGCUGGAUAAGAAGCUGCUGU	AGCAGCUUCUUAUCCAGCUUU

[0373] In Table 8 above, the last 2 nucleotides at the 3' ends of the sense and antisense strands correspond to the 3' overhang sequence. In other words, nucleotides 1-19 of each sense and antisense strand sequence depicted in Table 8 correspond to that portion of the

sense or antisense strand that is present in the double-stranded region of the siRNA duplex. In alternative embodiments, the 3' overhang on one or both strands of the siRNA molecule may comprise 1-4 (*e.g.,* 1, 2, 3, or 4) modified and/or unmodified deoxythymidine (t or dT) nucleotides, 1-4 (*e.g.,* 1, 2, 3, or 4) modified (*e.g.,* 2'OMe) and/or unmodified uridine (U) ribonucleotides, and/or 1-4 (*e.g.,* 1, 2, 3, or 4) modified (*e.g.,* 2'OMe) and/or unmodified ribonucleotides or deoxyribonucleotides having complementarity to the target sequence (3' overhang in the antisense strand) or the complementary strand thereof (3' overhang in the sense strand). In certain instances, the sense and/or antisense strand of the siRNA molecule lacks 3' overhangs (*i.e.,* does not contain the last 2 nucleotides at the 3' ends of the sense and/or antisense strand). In some embodiments, the sense and/or antisense strand comprises modified nucleotides such as 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. In particular embodiments, the sense and/or antisense strand comprises 2'OMe nucleotides in accordance with one or more of the selective modification patterns described herein.

Table 9. Mouse *Apoc3* siRNA sequences with 2'OMe modification patterns.

siRNA	Abbreviated name of siRNA	Sense Strand (5' → 3')	Antisense Strand (5' → 3')
mApoc3_465U2.1G1.1	465.1	**U**CCCUA**G**AUCUCACC**U**AAACA	UUUAGG**U**GAGAUCUA**G**GGAGG
mApoc3_465U2.2G1.1C1	465.2	**UC**CCUA**G**AUCUCACC**U**AAACA	U**U**UAGG**U**GAGAUCUA**G**GGAGG
mApoc3_467U3.1G0.1	467.1	CCUAGA**U**CUCACC**U**AAACA**U**G	UGUUUAG**G**UGAGAUC**U**AGGGA
mApoc3_467U3.1G0.2C1	467.2	C**C**UAGA**U**CUCACC**U**AAACA**U**G	U**G**UUUAG**G**UGAGAUC**U**AGGGA
mApoc3_492U3.1G0.1	492.1	CCCUAA**U**AAAGC**U**GGA**U**AAGA	UUAUCCAGCUU**U**AUUAGG**G**AC
mApoc3_492U3.2G0.1C1	492.2	C**C**CUAA**U**AAAGC**U**GGA**U**AAGA	U**U**AUCCAGCUU**U**AUUAGG**G**AC

2'OMe nucleotides are indicated in bold and underlined.

[0374] In Table 9 above, the last 2 nucleotides at the 3' ends of the sense and antisense strands correspond to the 3' overhang sequence. In other words, nucleotides 1-19 of each sense and antisense strand sequence depicted in Table 9 correspond to that portion of the sense or antisense strand that is present in the double-stranded region of the siRNA duplex. In alternative embodiments, the 3' overhang on one or both strands of the siRNA molecule may comprise 1-4 (*e.g.,* 1, 2, 3, or 4) modified and/or unmodified deoxythymidine (t or dT) nucleotides, 1-4 (*e.g.,* 1, 2, 3, or 4) modified (*e.g.,* 2'OMe) and/or unmodified uridine (U) ribonucleotides, and/or 1-4 (*e.g.,* 1, 2, 3, or 4) modified (*e.g.,* 2'OMe) and/or unmodified ribonucleotides or deoxyribonucleotides having complementarity to the target sequence (3' overhang in the antisense strand) or the complementary strand thereof (3' overhang in the

sense strand). In certain instances, the sense and/or antisense strand of the siRNA molecule lacks 3' overhangs (*i.e.,* does not contain the last 2 nucleotides at the 3' ends of the sense and/or antisense strand). In alternative embodiments, the 465.1, 467.1, or 492.1 sense strand sequence may be paired with the 465.2, 467.2, or 492.2 antisense strand sequence, respectively. In other alternative embodiments, the 465.2, 467.2, or 492.2 sense strand sequence may be paired with the 465.1, 467.1, or 492.1 antisense strand sequence, respectively.

[0375] Lipid Encapsulation of siRNA. siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: a lipid conjugate such as PEG-C-DMA (3-N-[(-Methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxy-propylamine); a cationic lipid such as DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); a phospholipid such as DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into stable nucleic acid-lipid particles ("SNALP") of the following "1:57" formulation: 1.4 mol % lipid conjugate (*e.g.,* PEG-C-DMA); 57.1 mol % cationic lipid (*e.g.,* DLinDMA); 7.1 mol % phospholipid (*e.g.,* DPPC); and 34.3 mol % cholesterol. For vehicle controls, empty particles with identical lipid composition are formed in the absence of siRNA. It should be understood that the 1:57 formulation is a target formulation, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two).

[0376] Hepatocyte isolation and culture. Primary hepatocytes were isolated from C57Bl/6J mice by standard procedures. Briefly, mice were anesthetized by intraperitoneal injection of Ketamine-Xylazine and the livers were perfused with Hanks' Buffered Salt Solution (Invitrogen) solution containing 0.5 M EDTA and 1 mg/ml insulin followed by Hanks' collagenase solution (100 U/ml). The hepatocytes were dispersed in Williams' Media E (Invitrogen) and washed two times in Hepatocyte Wash Medium (Invitrogen), then suspended in Williams' Media E containing 10% fetal bovine serum and plated on 96-well plates (2.5×10^4 cells/well). For the *in vitro* mouse siRNA silencing activity assay, hepatocytes were transfected with 2 nM or 20 nM of SNALP-formulated *Apoc3* siRNAs in

96-well plates. *Apoc3* mRNA levels were evaluated 24 h after transfection by bDNA assay (Panomics).

[0377] Animals and diet. Six- to seven-week-old C57Bl/6J wild-type mice and homozygous B6.129S7-*Ldlr*$^{tm1Her/J}$ mice were obtained from the Jackson Laboratory and subjected to at least a 1-week acclimation period prior to use. Mice received a standard laboratory rodent chow diet or Western diet (TD.88137; Harlan Teklad; Madison, WI). Mice were administered SNALP-formulated siRNAs in PBS via standard i.v. injection under normal pressure and low volume (0.01 mL/g) in the lateral tail vein for all experiments. For fenofibrate treatment, animals received fenofibrate (100 mg/kg body weight) daily by oral gavage for 2 days. All animal studies were performed at Tekmira Pharmaceuticals in accordance with Canadian Council on Animal Care guidelines and following protocols approval by the Institutional Animal Care and Use Committee of Tekmira Pharmaceuticals.

[0378] *In vivo* immune stimulation assays. SNALP-formulated siRNA were administered at 5 mg/kg to female C57Bl/6J mice at 8 weeks of age. Liver was collected into RNAlater (Sigma-Aldrich) for *Ifit1* mRNA analysis.

[0379] Lipid analysis. Mice were fasted for 4-6 hours prior to terminal anaesthesia, exsanguination, and collection of liver tissue. For hepatic triglyceride analysis, liver tissue was homogenized in PBS and total lipids extracted using Foldch solution (chloroform/methanol 2:1), dried under N_2, and resuspended in 2% Triton X-100. Plasma and liver lipid extracts were assayed for cholesterol and triglyceride concentrations by enzymatic assays with the use of commercially available reagents.

[0380] Mouse target mRNA quantitation. The QuantiGene® Reagent System (Panomics, Inc.; Fremont, CA) bDNA assay was used to quantify the reduction of mouse *Apoc3* mRNA levels relative to the mRNA levels of the housekeeping gene *Gapdh*. Primary hepatocytes were lysed 24 hours post SNALP treatment by adding 100 µL of 1x Lysis Mixture (Panomics) and 50 µg/mL proteinase K into each well followed by 30 minute incubation at 50°C. Murine liver was processed to quantitate *Apoc3* mRNA 48 hours after administration of SNALP. The QuantiGene® assay was performed according to the manufacturer's instructions. Relative *Apoc3* mRNA levels are expressed relative to cells treated with a Luciferase control siRNA or to animals that received a saline control injection.

[0381] Measurement of *Ifit1* mRNA in mouse tissues. Murine liver was processed for bDNA assay to quantitate *Ifit1* mRNA. The *Ifit1* probe set was specific to mouse *Ifit1* mRNA (positions 4-499 of NM_008331) and the *Gapdh* probe set was specific to mouse *Gapdh*

mRNA (positions 9-319 of NM_008084). Data is shown as the ratio of *Ifit1* relative light units (RLU) to *Gapdh* RLU.

[0382] Statistics. Data are presented as means plus or minus standard deviation. Analyses were performed using the unpaired two-tailed Student's *t*-test. Differences were deemed significant at $P < 0.05$.

Results

[0383] *Apoc3* siRNAs display dose-dependent activity *in vitro*. A panel of 20 siRNAs targeting mouse *Apoc3* was designed and screened for silencing activity in mouse primary hepatocytes. Treatment of hepatocytes with many of these siRNAs caused a dose-dependent reduction in levels of mouse *Apoc3* mRNA (Figure 1). This screen identified mApoc3_465, mApoc3_467, and mApoc3_492 as the most potent mouse siRNAs. Additional potent siRNAs include mApoc3_258, mApoc3_264, mApoc3_274, mApoc3_323, mApoc3_324, mApoc3_344, mApoc3_466, and mApoc3_493. Of these more potent siRNAs, mApoc3_258 is the most likely to be cross-reactive in primates based on an antisense (AS) sequence that contains only one mismatch to the *APOC3* transcript (*i.e.,* 95% complementary) in humans and cynomolgus monkeys.

[0384] 2'OMe-modified *Apoc3* siRNAs display only modest differences in activity compared with unmodified siRNA. Prior to the assessment of synthetic siRNA in animal models, it is important to consider the potential effects of immune stimulation and take steps to reduce this risk (Judge *et al.*, *Hum. Gene Ther.*, 19:111-24 (2008)). It has been shown that the selective incorporation of 2'-*O*-methyl (2'OMe) nucleotides into the constituent RNA oligonucleotides eliminates the capacity of the siRNA to activate a measurable immune response (Judge *et al.*, *Mol. Ther.*, 13:494-505 (2006); Robbins *et al.*, *Hum. Gene Ther.*, 19:991-9 (2008)). Therefore, 2'OMe-modified nucleotides were substituted into the native sense and AS oligonucleotides to form a panel of modified mApoc3_465, mApoc3_467, and mApoc3_492 duplexes. Figure 2 shows that 2'OMe-modified *Apoc3* siRNAs display only modest differences in silencing activity compared with the corresponding unmodified siRNA sequence.

[0385] *In vivo* gene silencing efficacy. Figure 3 shows that SNALP-mediated apoCIII silencing is potent and long-lasting. In particular, liver *Apoc3* mRNA levels were reduced by more than about 90% at doses of 0.5 and 5 mg/kg, and a reduction in liver *Apoc3* mRNA levels was observed for more than 21 days after a single 0.5 mg/kg treatment.

[0386] Immune response and hepatic TG *in vivo*. Figure 4 shows that 2'OMe-modified *Apoc3* siRNAs induce no measurable interferon response in mice. Figure 5 shows that SNALP-mediated apoCIII silencing does not increase liver triglyceride (TG) levels.

[0387] Plasma lipids in a dyslipidemic model. The LDLR-deficient hyperlipidemic mouse mimics human familial hypercholesterolemia and has been used in numerous studies as a model for the disrupted lipoprotein regulation and metabolic function that leads to diabetes and atherosclerosis (Getz *et al.*, *Arterioscler. Thromb. Vasc. Biol.*, 26:242-9 (2006)). LDLR-deficient mice develop features of the metabolic syndrome and atherosclerosis when fed a Western diet. Figure 6 shows that siRNA-based silencing of apoCIII improves plasma lipids in LDLR-deficient mice fed a Western diet. In particular, plasma triglyceride (TG) levels were reduced by about 35-60% for 2-14 days and plasma total cholesterol (TC) levels were reduced by about 20-25% for 7-14 days following SNALP administration. As such, this study demonstrates the therapeutic reduction of hyperlipidemia by systemic administration of a SNALP formulation containing fully encapsulated siRNA targeting the *Apoc3* gene.

Summary

[0388] This example demonstrates that SNALP-mediated silencing of apoCIII is potent and long-lasting. In particular, liver *Apoc3* mRNA levels were reduced by more than about 90% at doses of 0.5 and 5 mg/kg. In fact, a reduction in liver *Apoc3* mRNA levels was observed for more than 21 days after a single 0.5 mg/kg treatment. RACE PCR analysis also showed that *Apoc3*-targeting SNALP acted via a confirmed RNAi mechanism. Furthermore, this example illustrates that dyslipidemia in LDLR-deficient mice was ameliorated by siRNA-based silencing of apoCIII. In particular, plasma triglyceride (TG) levels were reduced by about 35-60% for 2-14 days and plasma total cholesterol (TC) levels were reduced by about 20-25% for 7-14 days. As such, amelioration of dyslipidemia associated with SNALP-mediated silencing of apoCIII advantageously reduces susceptibility to atherosclerosis in LDLR-deficient mice (*see,* Figure 7).

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Example 3. Silencing of human *APOC3* expression using RNA interference.

[0389] This example provides an *in vitro* characterization of *APOC3* siRNA activity in human cells. ApoCIII is an important regulator of lipoprotein metabolism that has been implicated in the progression of atherosclerosis (1) through its association with hypertriglyceridemia (2-5) and its direct induction of endothelial dysfunction (6-7). A panel of 20 *APOC3* siRNAs were designed and screened for silencing activity in the human HepG2 hepatocellular carcinoma cell line. Treatment of HepG2 cells with many of these siRNAs caused a dose-dependent reduction in the levels of human *APOC3* mRNA (Figure 8). In particular, hAPOC3_260 was identified as the most potent human *APOC3* siRNA. Additional potent *APOC3* siRNAs include hAPOC3_312, hAPOC3_54, hAPOC3_266, hAPOC3_268, hAPOC3_287, and hAPOC3_427. Of these siRNAs, hAPOC3_260, hAPOC3_266, hAPOC3_268, and hAPOC3_427 are most likely to be cross-reactive in other primates based on an antisense sequence that is 100% complementary to the *APOC3* transcript in cynomolgus monkeys.

Materials and Methods

[0390] **siRNA design.** siRNA sequences targeting human *APOC3* (Genbank Accession No. NM_000040.1) were selected using an algorithm implemented by the Whitehead Institute for Biomedical Research that incorporates standard siRNA design guidelines (8-10). siRNA fulfilling the following criteria were selected: (1) NNN21 target sequences; (2) thermodynamically unstable 5' antisense end ($\Delta G > -8.2$ kcal/mol); (3) thermodynamically less stable 5' antisense end ($\Delta G_{sense} - \Delta G_{antisense} < -1.6$); (4) G/C content between 30-70%; (5) no stretches of four guanines in a row; and (6) no stretches of nine uracils or adenines in a row. Selected sequences were verified and the positions within the human *APOC3* target sequence were identified.

[0391] All selected sequences were assessed for potential sequence-specific targeting activity against other human genes using the BLASTN algorithm against the human mRNA Reference Sequence database at the National Center for Biotechnology Information. Transcripts other than *APOC3* that contain a sequence that is 100% complementary to positions 2 to 15 of the antisense strand of an siRNA were evaluated for gene expression in liver and other human tissues. Gene expression analysis was

performed using human gene expression data from the Genomics Institute of the Novartis Research Foundation (GNF), obtained from the human U133A+GNF1H microarray dataset and processed using the GC content adjusted robust multi-array algorithm (11). EST counts from different tissue source libraries were also extracted from the NCBI UniGene database. siRNAs were eliminated if they contained sequence complementary to a transcript that is expressed ubiquitously or at moderate to high levels in liver (*i.e.*, greater than two-fold higher than the global median over all tissues tested).

[0032] Four single nucleotide polymorphisms (SNPs), rs4225, rs4520, rs5128, and rs11540884, located in the coding or UTR sequences of the human *APOC3* gene, were identified in the NCBI SNP database and used to filter the panel of siRNAs. siRNAs were eliminated if their antisense strand contained a nucleotide complementary to one of these SNPs.

[0033] In order to evaluate expected cross-reactivity of siRNAs, *APOC3* sequences from human and cynomolgus monkey (*Macaca fascicularis*; Genbank Accession No. X68359.1) were aligned using ClustalX (12), with manual editing when necessary.

[0034] **siRNA synthesis.** All siRNA molecules used in this study were chemically synthesized by Integrated DNA Technologies (Coralville, IA). The siRNAs were desalted and annealed using standard procedures. Sequences of human *APOC3* siRNAs are listed in Table 10. Sequence numbers represent the nucleotide position of human *APOC3* mRNA (Genbank Accession No. NM_000040.1) that is complementary to the 3' end of the antisense strand of the siRNA.

Table 10. siRNA sequences that target human *APOC3* expression.

siRNA	Target Sequence (5' → 3')	Sense Strand (5' → 3')	Antisense Strand (5' → 3')
hAPOC3_54	CGGGUACUCCUUGUUGUUG	CGGGUACUCCUUGUUGUUGCC	CAACAACAAGGAGUACCCGGG
hAPOC3_120	GCCUCCCUUCUCAGCUUCA	GCCUCCCUUCUCAGCUUCAUG	UGAAGCUGAGAAGGGAGGCAU
hAPOC3_241	GCUUCAGUUCCCUGAAAGA	GCUUCAGUUCCCUGAAAGACU	UCUUUCAGGGAACUGAAGCCA
hAPOC3_259	ACUACUGGAGCACCGUUAA	ACUACUGGAGCACCGUUAAGG	UUAACGGUGCUCCAGUAGUCU
hAPOC3_260	CUACUGGAGCACCGUUAAG	CUACUGGAGCACCGUUAAGGA	CUUAACGGUGCUCCAGUAGUC
hAPOC3_266	GAGCACCGUUAAGGACAAG	GAGCACCGUUAAGGACAAGUU	CUUGUCCUUAACGGUGCUCCA
hAPOC3_267	AGCACCGUUAAGGACAAGU	AGCACCGUUAAGGACAAGUUC	ACUUGUCCUUAACGGUGCUCC
hAPOC3_268	GCACCGUUAAGGACAAGUU	GCACCGUUAAGGACAAGUUCU	AACUUGUCCUUAACGGUGCUC
hAPOC3_270	ACCGUUAAGGACAAGUUCU	ACCGUUAAGGACAAGUUCUCU	AGAACUUGUCCUUAACGGUGC
hAPOC3_277	AGGACAAGUUCUCUGAGUU	AGGACAAGUUCUCUGAGUUCU	AACUCAGAGAACUUGUCCUUA
hAPOC3_286	UCUCUGAGUUCUGGGAUUU	UCUCUGAGUUCUGGGAUUUGG	AAAUCCCAGAACUCAGAGAAC
hAPOC3_287	CUCUGAGUUCUGGGAUUUG	CUCUGAGUUCUGGGAUUUGGA	CAAAUCCCAGAACUCAGAGAA
hAPOC3_308	CCCUGAGGUCAGACCAACU	CCCUGAGGUCAGACCAACUUC	AGUUGGUCUGACCUCAGGGUC
hAPOC3_309	CCUGAGGUCAGACCAACUU	CCUGAGGUCAGACCAACUUCA	AAGUUGGUCUGACCUCAGGGU

hAPOC3_312	GAGGUCAGACCAACUUCAG	GAGGUCAGACCAACUUCAGCC	CUGAAGUUGGUCUGACCUCAG
hAPOC3_334	UGGCUGCCUGAGACCUCAA	UGGCUGCCUGAGACCUCAAUA	UUGAGGUCUCAGGCAGCCACG
hAPOC3_335	GGCUGCCUGAGACCUCAAU	GGCUGCCUGAGACCUCAAUAC	AUUGAGGUCUCAGGCAGCCAC
hAPOC3_337	CUGCCUGAGACCUCAAUAC	CUGCCUGAGACCUCAAUACCC	GUAUUGAGGUCUCAGGCAGCC
hAPOC3_388	UCCUUGGGUCCUGCAAUCU	UCCUUGGGUCCUGCAAUCUCC	AGAUUGCAGGACCCAAGGAGC
hAPOC3_427	UGCUUAAAAGGGACAGUAU	UGCUUAAAAGGGACAGUAUUC	AUACUGUCCCUUUUAAGCAAC

[0395] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_54 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "56" shown in Table 7.

[0396] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_120 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "122" shown in Table 7.

[0397] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_241 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "243" shown in Table 7.

[0398] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_259 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "261" shown in Table 7.

[0399] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_260 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "262" shown in Table 7.

[0400] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_266 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "268" shown in Table 7.

[0401] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_267 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "269" shown in Table 7.

[0402] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_268 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "270" shown in Table 7.

[0403] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_270 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "272" shown in Table 7.

[0404] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_277 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "279" shown in Table 7.

[0405] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_286 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "288" shown in Table 7.

[0406] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_287 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "289" shown in Table 7.

[0407] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_308 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "310" shown in Table 7.

[0408] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_309 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "311" shown in Table 7.

[0409] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_312 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "314" shown in Table 7.

[0410] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_334 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "336" shown in Table 7.

[0411] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_335 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "337" shown in Table 7.

[0412] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_337 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "339" shown in Table 7.

[0413] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_388 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "390" shown in Table 7.

[0414] Nucleotides 1-19 of the sense and antisense strand sequences of the hAPOC3_427 siRNA shown in Table 10 correspond to the sense and antisense strand sequences of *APOC3* siRNA "429" shown in Table 7.

[0415] In Table 10 above, the last 2 nucleotides at the 3' ends of the sense and antisense strands correspond to the 3' overhang sequence. In other words, nucleotides 1-19 of each sense and antisense strand sequence depicted in Table 10 correspond to that portion of the sense or antisense strand that is present in the double-stranded region of the siRNA duplex. In alternative embodiments, the 3' overhang on one or both strands of the siRNA comprises 1-4 (*e.g.*, 1, 2, 3, or 4) modified and/or unmodified deoxythymidine (t or dT) nucleotides, 1-4 (*e.g.*, 1, 2, 3, or 4) modified (*e.g.*, 2'OMe) and/or unmodified uridine (U) ribonucleotides, and/or 1-4 (*e.g.*, 1, 2, 3, or 4) modified (*e.g.*, 2'OMe) and/or unmodified ribonucleotides or deoxyribonucleotides having complementarity to the target sequence (3' overhang in the antisense strand) or the complementary strand thereof (3' overhang in the sense strand). In certain instances, the sense and/or antisense strand of the siRNA molecule lacks 3' overhangs (*i.e.*, does not contain the last 2 nucleotides at the 3' ends of the sense and/or antisense strand). In some embodiments, the sense and/or antisense strand sequence shown in Table 10 comprises modified nucleotides such as 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. In particular embodiments, the sense and/or antisense strand sequence shown in Table 10 comprises 2'OMe nucleotides in accordance with one or more of the selective modification patterns described herein.

[0416] Lipid Encapsulation of siRNA. siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: a lipid conjugate such as PEG-C-DMA (3-N-[(-Methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxy-propylamine); a cationic lipid such as DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); a phospholipid such as DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into stable nucleic acid-lipid particles ("SNALP") of the following "1:57" formulation: 1.4 mol % lipid conjugate (*e.g.*, PEG-C-DMA); 57.1 mol % cationic lipid (*e.g.*, DLinDMA); 7.1 mol % phospholipid (*e.g.*, DPPC); and 34.3 mol % cholesterol. For vehicle controls, empty particles with identical lipid composition are formed in the absence of siRNA. It should be understood that the 1:57 formulation is a target formulation, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with

the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two).

[0417] Cell culture. The HepG2 cell line was obtained from ATCC and cultured in complete media (Invitrogen GibcoBRL Minimal Essential Medium, 10% heat-inactivated FBS, 200 mM L-glutamine, 10mM MEM non-essential amino acids, 100 mM sodium pyruvate, 7.5% w/v sodium bicarbonate and 1% penicillin-streptomycin) in T175 flasks. For *in vitro* siRNA silencing activity assay, HepG2 cells from passage #28 were reverse transfected with 2.5 nM, 10 nM, and 40 nM of SNALP-formulated *APOC3* siRNAs in 96-well plates at an initial cell confluency of 50%. After 24 hours of treatment, media was removed and fresh complete media was added.

[0418] Target mRNA Quantitation. The QuantiGene® 2.0 Reagent System (Panomics, Inc., Fremont, CA) was used to quantify the reduction of human *APOC3* mRNA levels relative to the mRNA levels of the housekeeping gene *GAPDH* in lysates prepared from HepG2 cell cultures treated with SNALP. HepG2 Cells were lysed 48 hours post SNALP treatment by adding 100 µL of 1x Lysis Mixture (Panomics) into each well followed by 30 minute incubation at 37°C. The assay was performed according to the manufacturer's instructions. Relative *APOC3* mRNA levels are expressed relative to PBS-treated control cells.

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[0419] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled..

INFORMAL SEQUENCE LISTING

SEQ ID NO:1
Homo sapiens apolipoprotein C-III (APOC3) on chromosome 11, DNA.
NG_008949 REGION: 5001..8164

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   1 tgctcagttc atccctagag gcagctgctc caggtaatgc cctctgggga ggggaaagag
  61 gaggggagga ggatgaagag gggcaagagg agctccctgc ccagcccagc cagcaagcct
 121 ggagaagcac ttgctagagc taaggaagcc tcggagctgg acgggtgccc cccacccctc
 181 atcataacct gaagaacatg gaggcccggg aggggtgtca cttgcccaaa gctacacagg
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1441 cttcctttct ctctttcttt cttcttcttt ttttttaat ggagtctccc tctgtcacct
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1561 cattctcctg cctcagcctc ccaagtagct gggattacag gcacgcgcca ccacacccag
1621 ctaatttttg tatttttagc agagatgggg tttcaccatg ttggccaggt tggtcttgaa
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1861 tggttttggg atgaagctgt tccacctcag atcatcaggc attagattct cataaggagc
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2101 tgggggttgg ggacccctgg gctaggggtt tgccttggga ggccccacct gacccaattc
2161 aagcccgtga gtgcttctgc tttgttctaa gacctggggc cagtgtgagc agaagtgtgt
2221 ccttcctctc ccatcctgcc cctgcccatc agtactctcc tctcccctac tcccttctcc
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2521 ggcggtgctg tttgcacaga aaacaatgac agccttgacc tttcacatct ccccaccctg
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3001 tgcctatcca tcctgcgagc tccttgggtc ctgcaatctc cagggctgcc cctgtaggtt
3061 gcttaaaagg gacagtattc tcagtgctct cctaccccac ctcatgcctg gccccctcc
3121 aggcatgctg gcctcccaat aaagctggac aagaagctgc tatg
```

SEQ ID NO:2
Homo sapiens apolipoprotein C-III (APOC3), mRNA.
NM_000040.1

```
  1 tgctcagttc atccctagag gcagctgctc caggaacaga ggtgccatgc agccccgggt
 61 actccttgtt gttgccctcc tggcgctcct ggcctctgcc cgagcttcag aggccgagga
121 tgcctccctt ctcagcttca tgcagggtta catgaagcac gccaccaaga ccgccaagga
181 tgcactgagc agcgtgcagg agtcccaggt ggcccagcag gccaggggct gggtgaccga
241 tggcttcagt tccctgaaag actactggag caccgttaag gacaagttct ctgagttctg
301 ggatttggac cctgaggtca gaccaacttc agccgtggct gcctgagacc tcaatacccc
361 aagtccacct gcctatccat cctgcgagct ccttgggtcc tgcaatctcc agggctgccc
421 ctgtaggttg cttaaaaggg acagtattct cagtgctctc ctaccccacc tcatgcctgg
481 ccccccctcca ggcatgctgg cctcccaata aagctggaca agaagctgct atg
```

This description contains a sequence listing in electronic form in ASCII text format. A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

WHAT IS CLAIMED IS:

1. A small-interfering RNA (siRNA) that silences apolipoprotein C-III (APOC3) gene expression, wherein the siRNA comprises a sense strand and a complementary antisense strand, and wherein the siRNA comprises a double stranded region of about 19 to about 25 nucleotides in length, and wherein the antisense strand comprises a sequence set forth in SEQ ID NO:770 and/or wherein the sense strand comprises a sequence set forth in SEQ ID NO:769.

2. The siRNA of claim 1, wherein the antisense strand and/or sense strand comprises at least one modified nucleotide in the double-stranded region.

3. The siRNA of claim 2, wherein less than about 30% of the nucleotides in the double-stranded region comprise modified nucleotides.

4. The siRNA of claim 2, wherein the modified nucleotide is a 2'-O-methyl (2'OMe) nucleotide.

5. A nucleic acid-lipid particle comprising:

(a) an siRNA as defined in any one of claims 1 to 4;

(b) a cationic lipid; and

(c) a non-cationic lipid.

6. The nucleic acid-lipid particle of claim 5, wherein the non cationic lipid is a mixture of a phospholipid and cholesterol or a derivative thereof.

7. The nucleic acid-lipid particle of claim 5 or 6, further comprising a conjugated lipid that inhibits aggregation of particles.

8. Use of a nucleic acid-lipid particle as defined in claim 5, 6, or 7 for introducing an siRNA that silences APOC3 gene expression into a cell.

9. Use of a nucleic acid-lipid particle as defined in claim 5, 6, or 7 for silencing APOC3 gene expression in a cell.

10. The use of claim 8 or 9, wherein the cell is a mammalian cell.

11. Use of nucleic acid-lipid particle as defined in claim 5, 6, or 7 for treating and/or ameliorating one or more symptoms associated with atherosclerosis or dyslipidemia in a mammal.

12. Use of nucleic acid-lipid particle as defined in claim 5, 6, or 7 in the preparation of a medicament for treating and/or ameliorating one or more symptoms associated with atherosclerosis or dyslipidemia in a mammal.

13. Use of nucleic acid-lipid particle as defined in claim 5, 6, or 7 for reducing susceptibility to atherosclerosis or dyslipidemia in a mammal.

14. Use of nucleic acid-lipid particle as defined in claim 5, 6, or 7 in the preparation of a medicament for reducing susceptibility to atherosclerosis or dyslipidemia in a mammal.

15. Use of a nucleic acid-lipid particle as defined in claim 5, 6, or 7 for preventing or delaying the onset of atherosclerosis or dyslipidemia in a mammal.

16. Use of a nucleic acid-lipid particle as defined in claim 5, 6, or 7 in the preparation of a medicament for preventing or delaying the onset of atherosclerosis or dyslipidemia in a mammal.

17. Use of nucleic acid-lipid particle as defined in claim 5, 6, or 7 for lowering triglyceride levels in a mammal.

18. Use of nucleic acid-lipid particle as defined in claim 5, 6, or 7 in the preparation of a medicament for lowering triglyceride levels in a mammal.

19. Use of a nucleic acid-lipid particle as defined in claim 5, 6, or 7 for lowering cholesterol levels in a mammal.

20. Use of a nucleic acid-lipid particle as defined in claim 5, 6, or 7 in the preparation of a medicament for lowering cholesterol levels in a mammal.

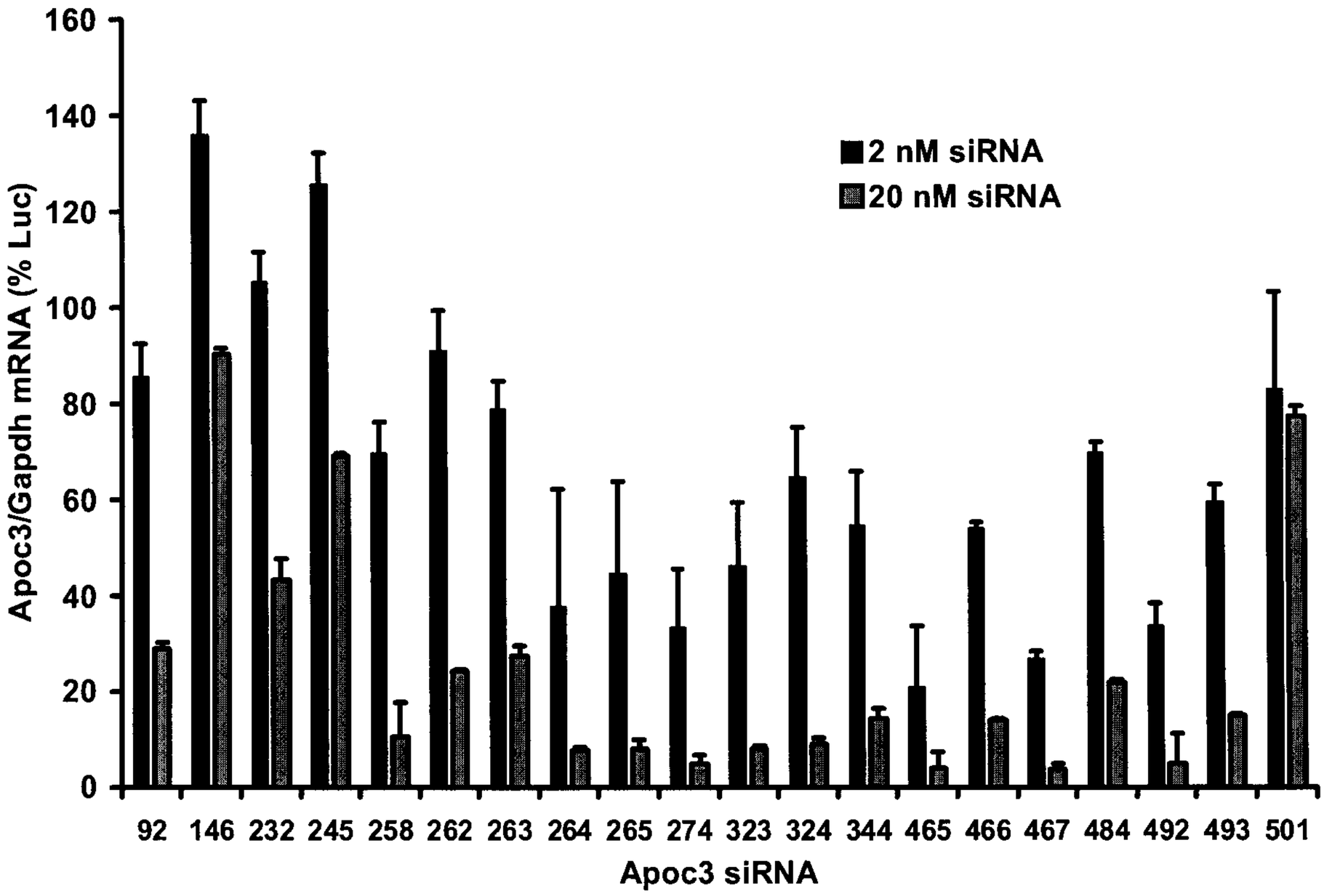

160
140
120
100
80
60
40
20
0
Apoc3/Gapdh mRNA (% Luc)
2 nM siRNA
20 nM siRNA
92
146
232
245
258
262
263
264
265
274
323
324
344
465
466
467
484
492
493
501
Apoc3 siRNA

FIG. 1

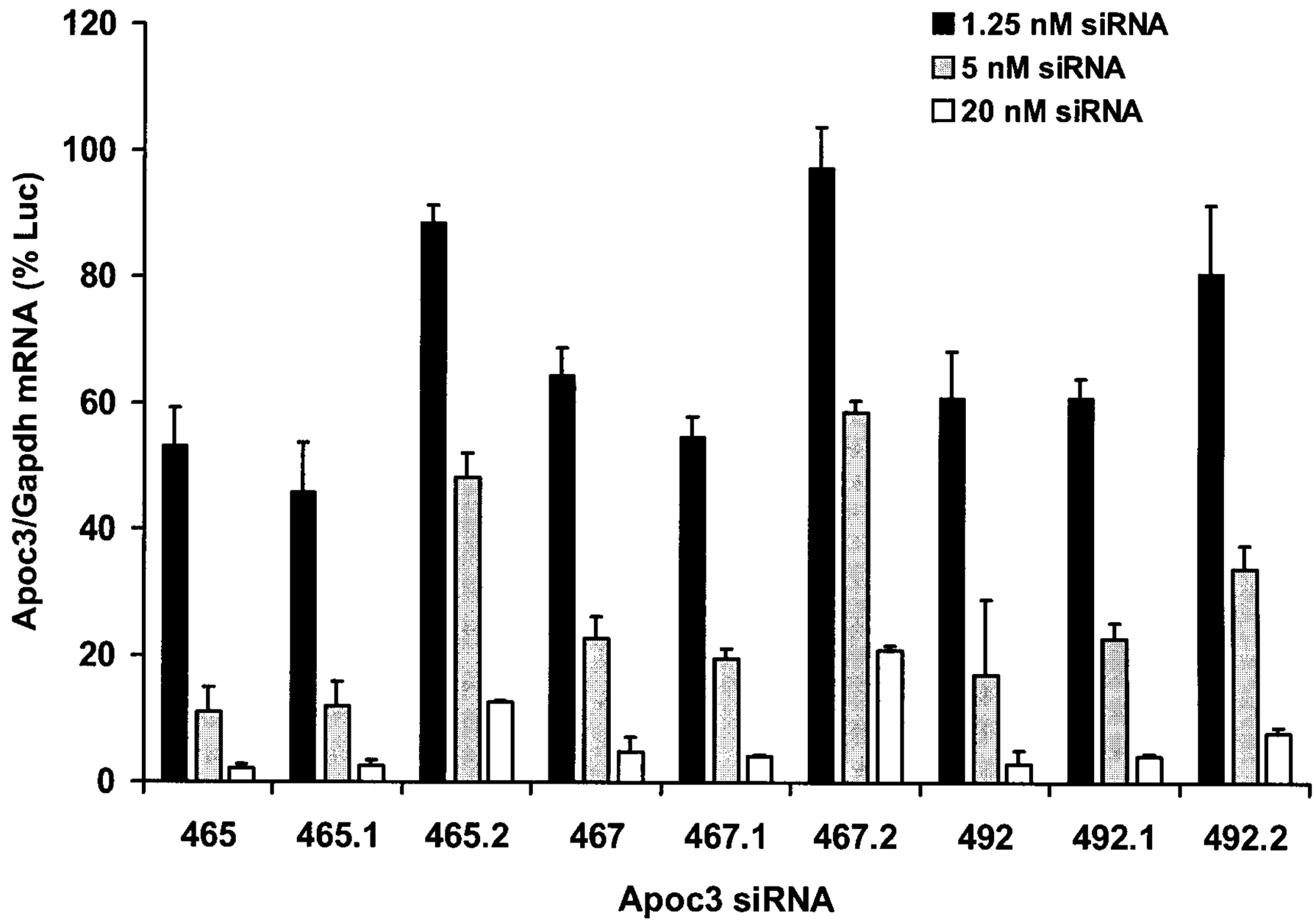

120
100
80
60
40
20
0
Apoc3/Gapdh mRNA (% Luc)
1.25 nM siRNA
5 nM siRNA
20 nM siRNA
465
465.1
465.2
467
467.1
467.2
492
492.1
492.2
Apoc3 siRNA

FIG. 2

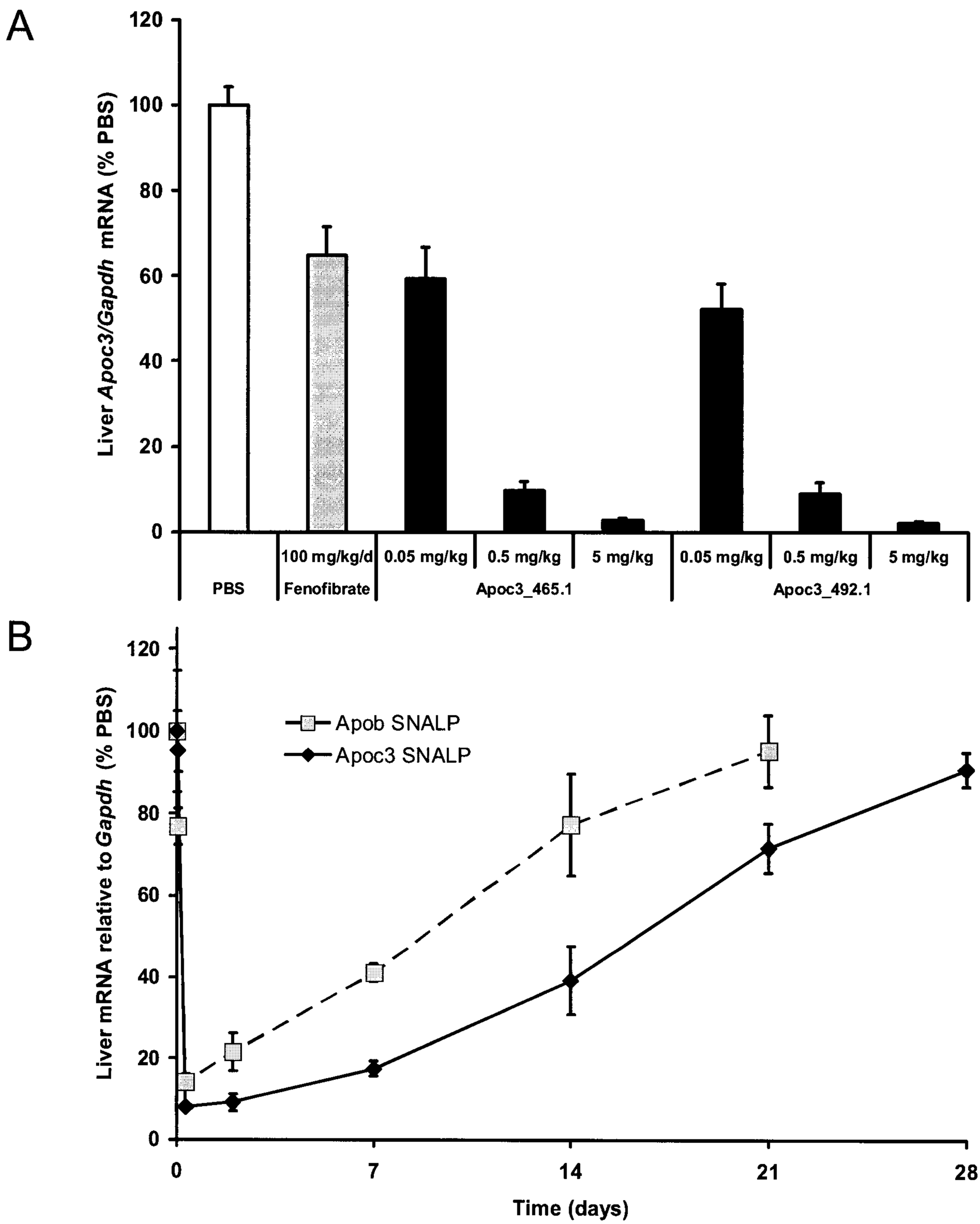

A
Liver Apoc3/Gapdh mRNA (% PBS)
120
100
80
60
40
20
0
PBS
100 mg/kg/d Fenofibrate
0.05 mg/kg
0.5 mg/kg
5 mg/kg
Apoc3_465.1
0.05 mg/kg
0.5 mg/kg
5 mg/kg
Apoc3_492.1
B
Liver mRNA relative to Gapdh (% PBS)
Apob SNALP
Apoc3 SNALP
0
7
14
21
28
Time (days)

FIG. 3

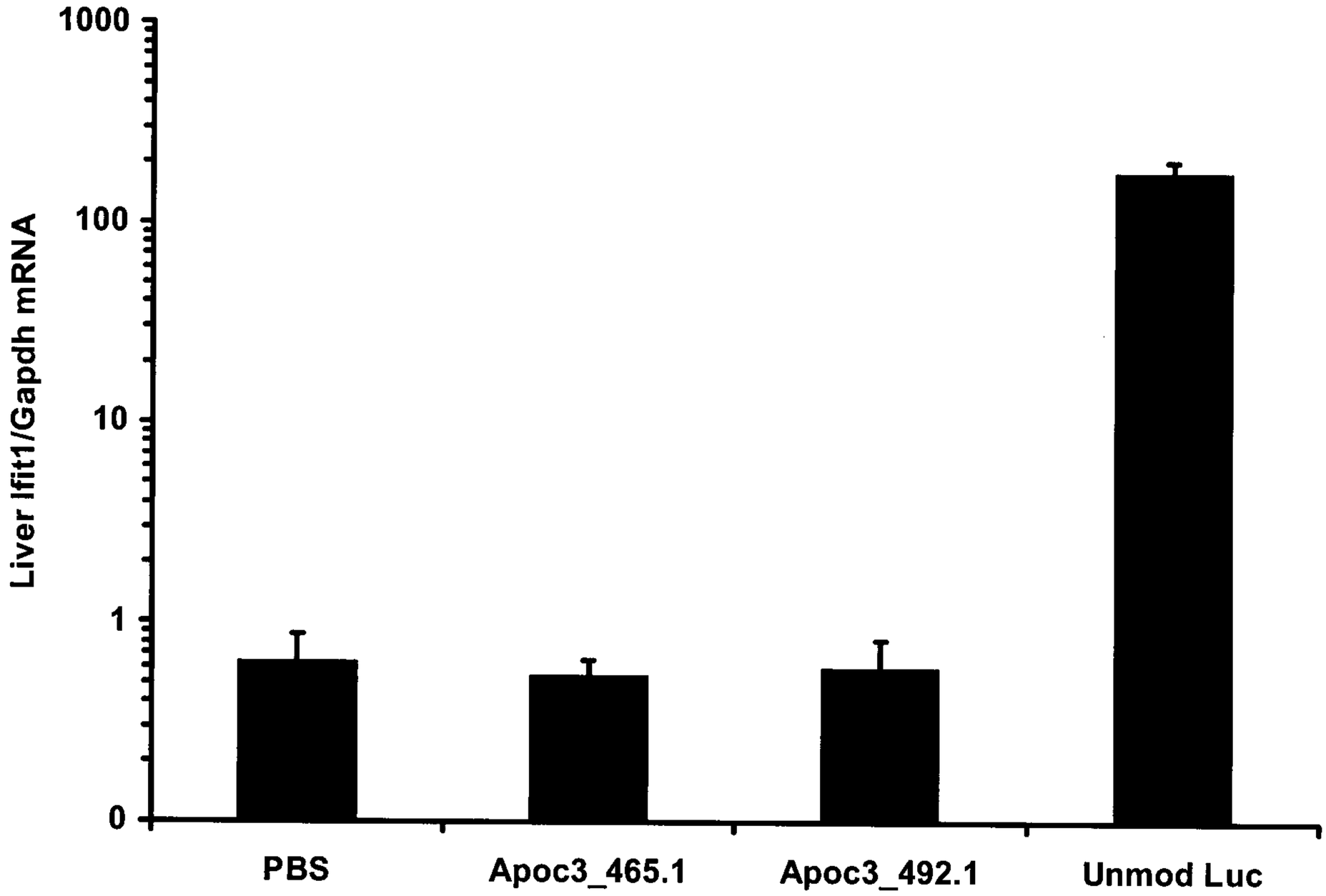

Liver Ifit1/Gapdh mRNA
1000
100
10
1
0
PBS
Apoc3_465.1
Apoc3_492.1
Unmod Luc

FIG. 4

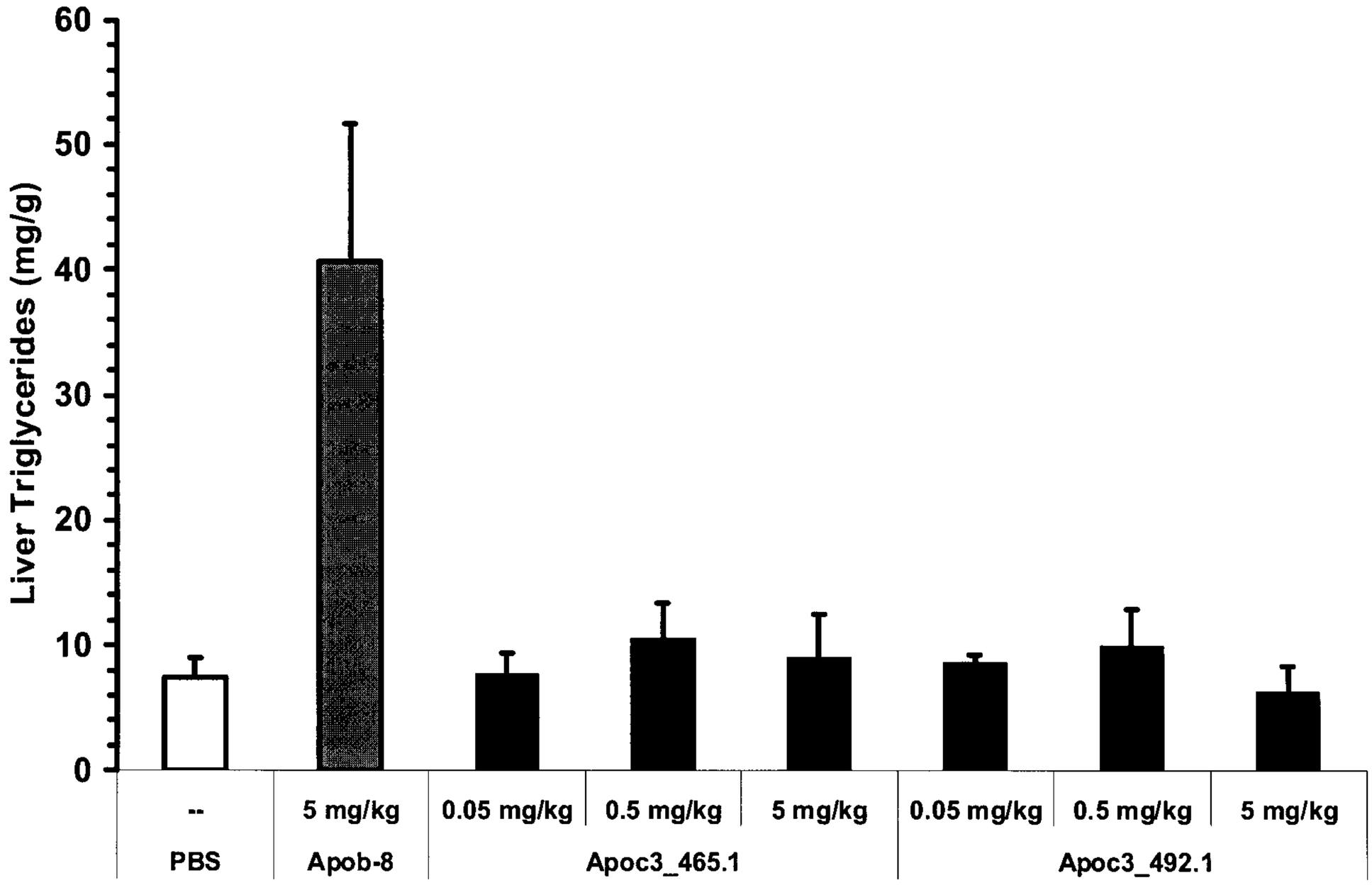
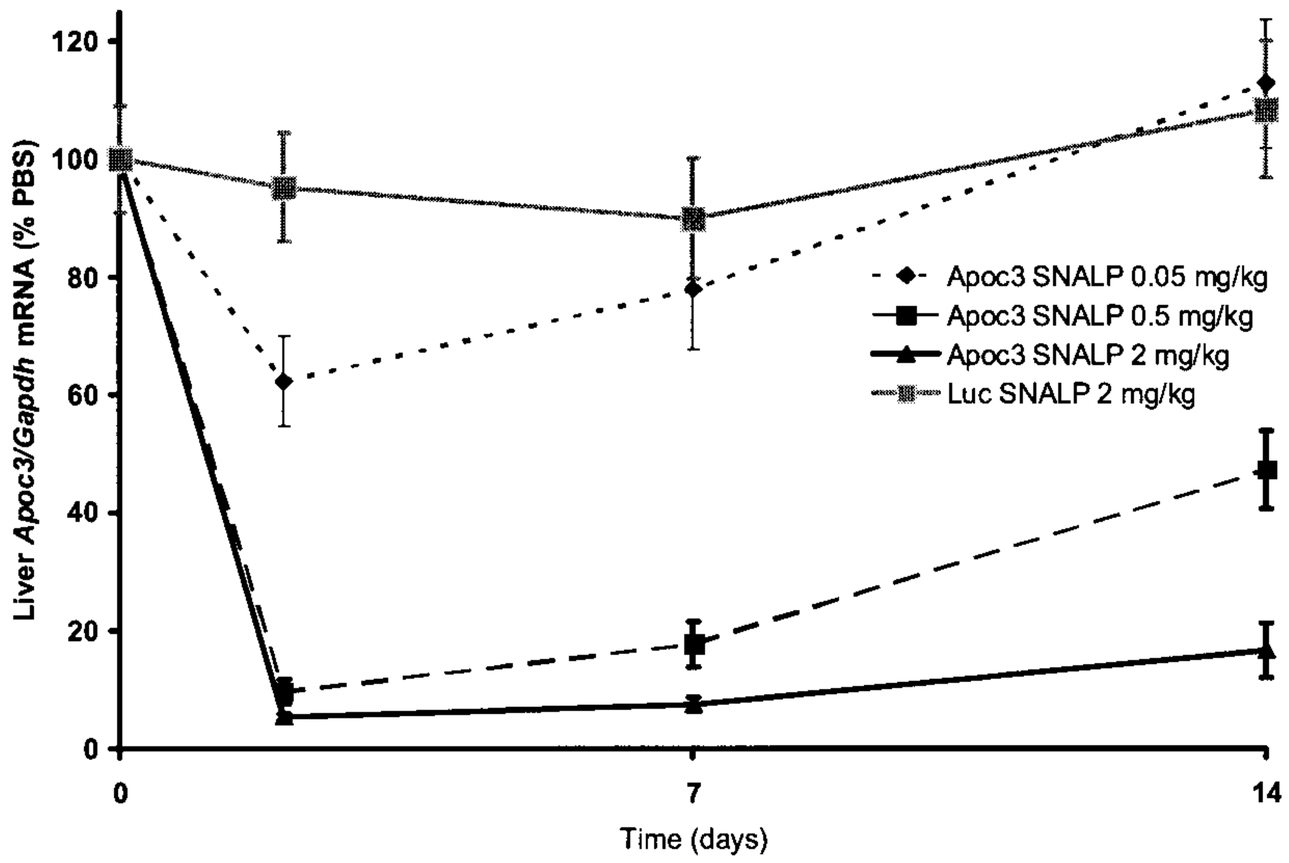
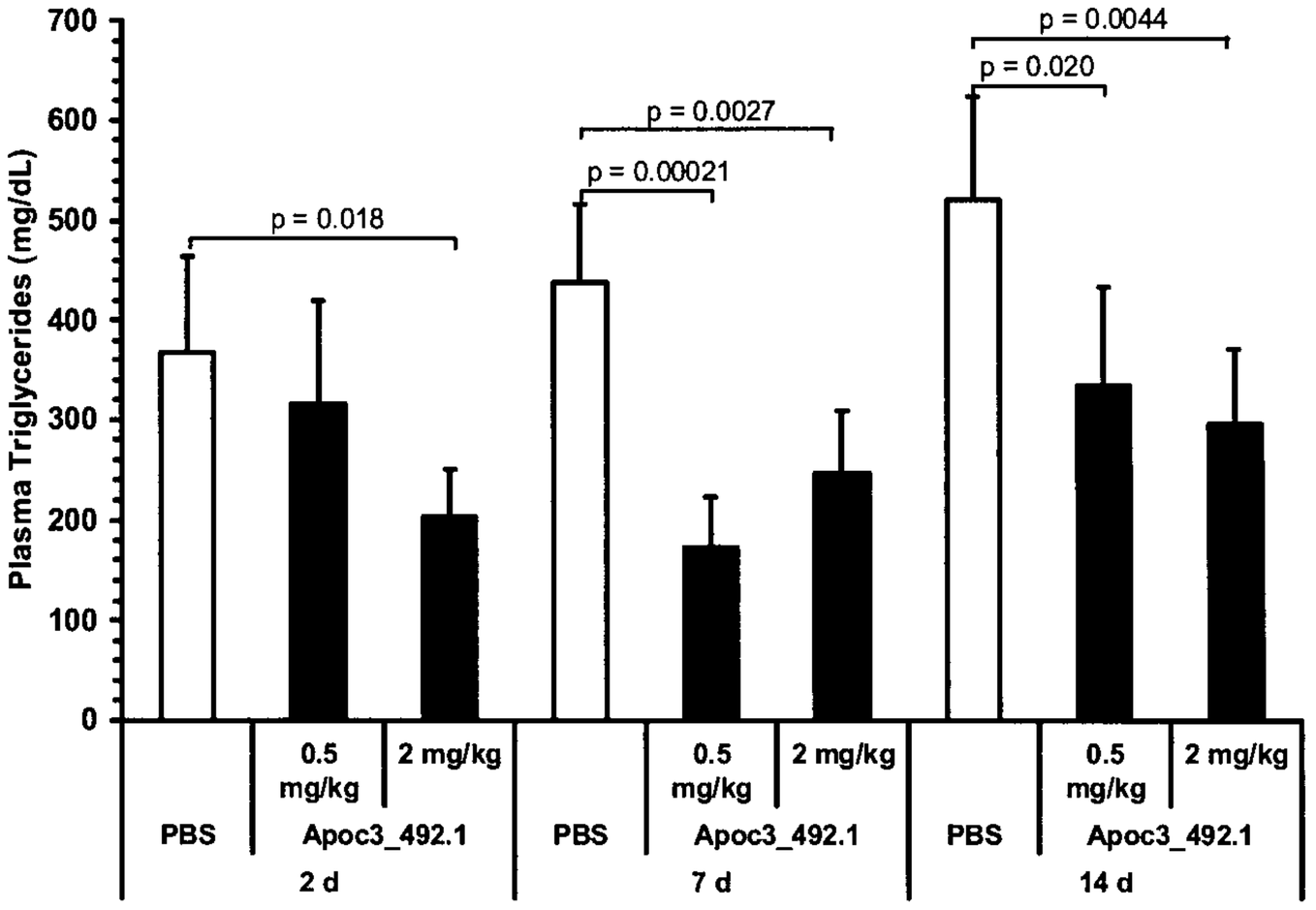

Liver Triglycerides (mg/g)
60
50
40
30
20
10
0
--
5 mg/kg
0.05 mg/kg
0.5 mg/kg
5 mg/kg
0.05 mg/kg
0.5 mg/kg
5 mg/kg
PBS
Apob-8
Apoc3_465.1
Apoc3_492.1

FIG. 5

A


Liver Apoc3/Gapdh mRNA (% PBS)
120
100
80
60
40
20
0
0
7
14
Time (days)
Apoc3 SNALP 0.05 mg/kg
Apoc3 SNALP 0.5 mg/kg
Apoc3 SNALP 2 mg/kg
Luc SNALP 2 mg/kg

B


Plasma Triglycerides (mg/dL)
700
600
500
400
300
200
100
0
p = 0.018
p = 0.00021
p = 0.0027
p = 0.020
p = 0.0044
0.5 mg/kg
2 mg/kg
PBS
Apoc3_492.1
2 d
7 d
14 d

C

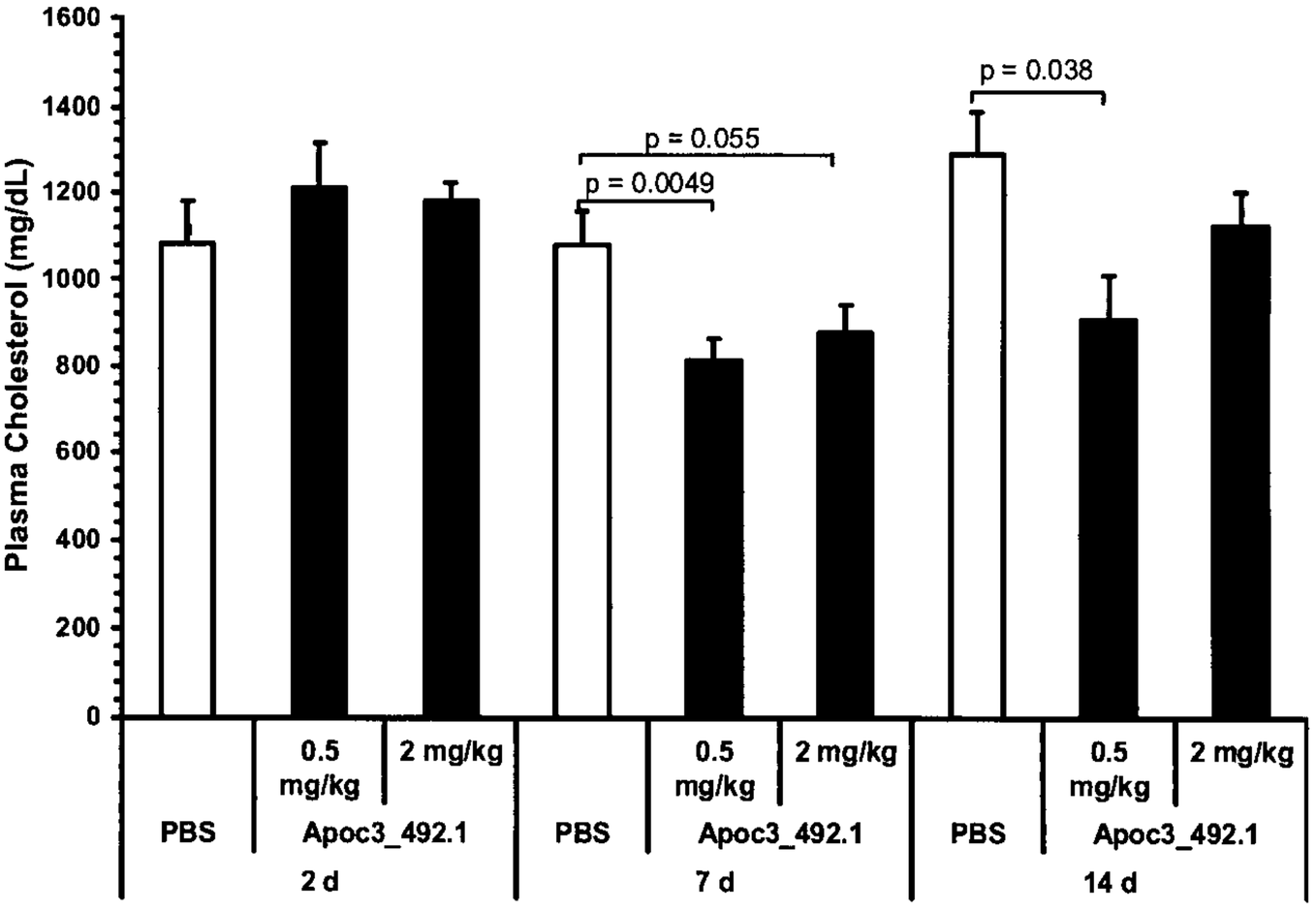

Plasma Cholesterol (mg/dL)
1600
1400
1200
1000
800
600
400
200
0
p = 0.055
p = 0.0049
p = 0.038
0.5 mg/kg
2 mg/kg
PBS
Apoc3_492.1
2 d
7 d
14 d

FIG. 6

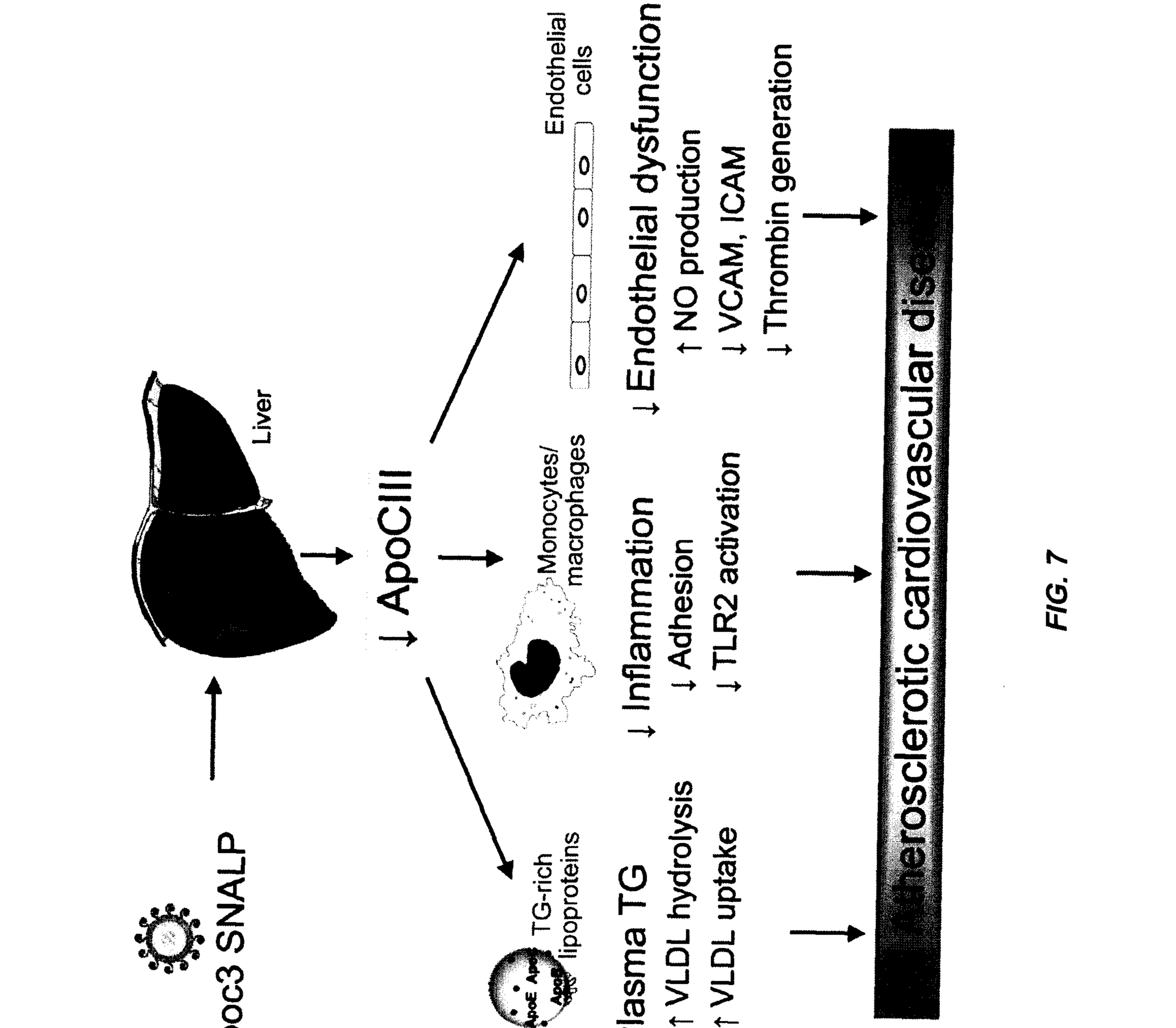

Apoc3 SNALP
Liver
↓ ApoCIII
TG-rich lipoproteins
Monocytes/ macrophages
Endothelial cells
↓ Plasma TG
↑ VLDL hydrolysis
↑ VLDL uptake
↓ Endothelial dysfunction
↑ NO production
↓ VCAM, ICAM
↓ Inflammation
↓ Adhesion
↓ TLR2 activation
↓ Thrombin generation
Atherosclerotic cardiovascular disease

FIG. 7

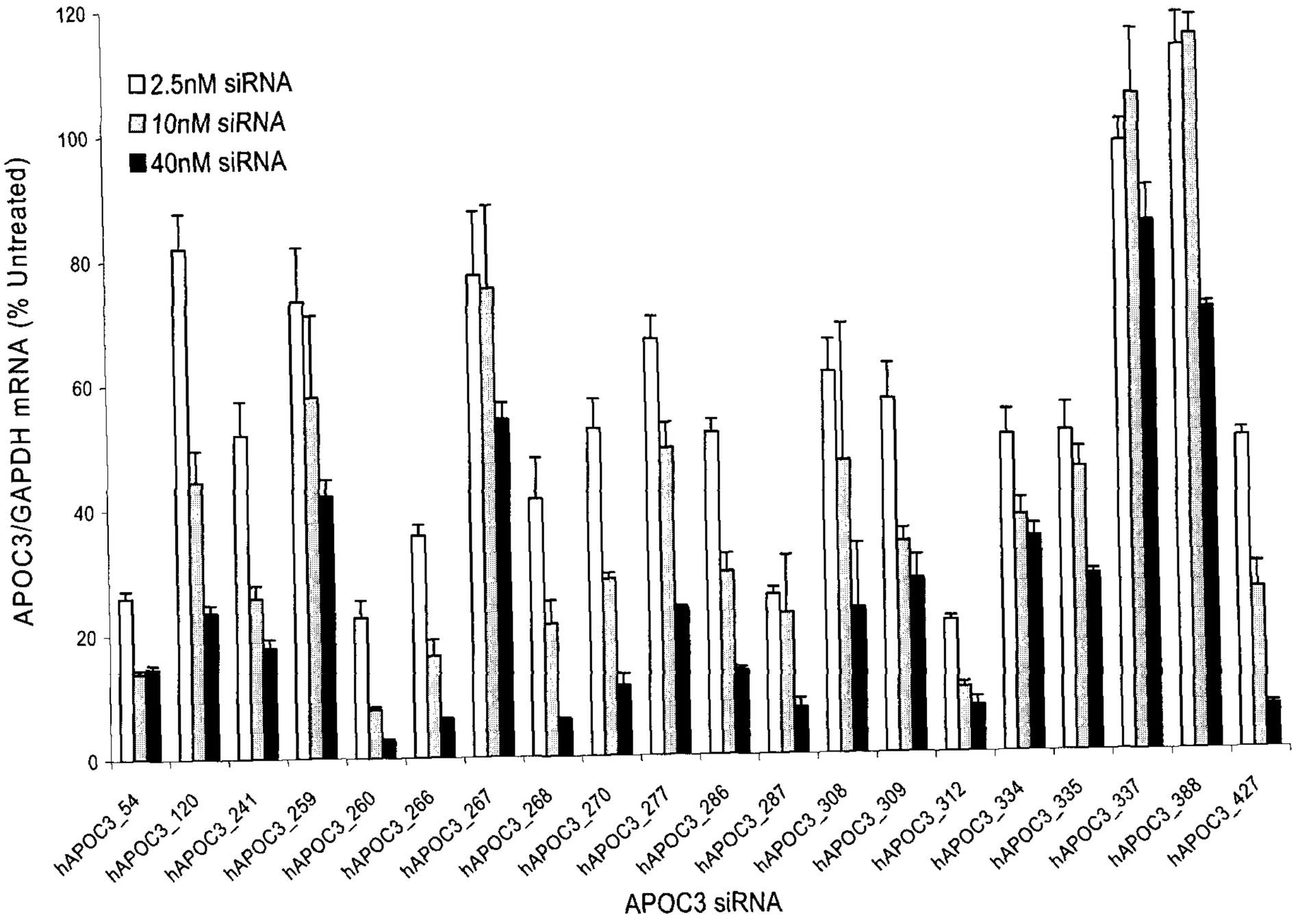
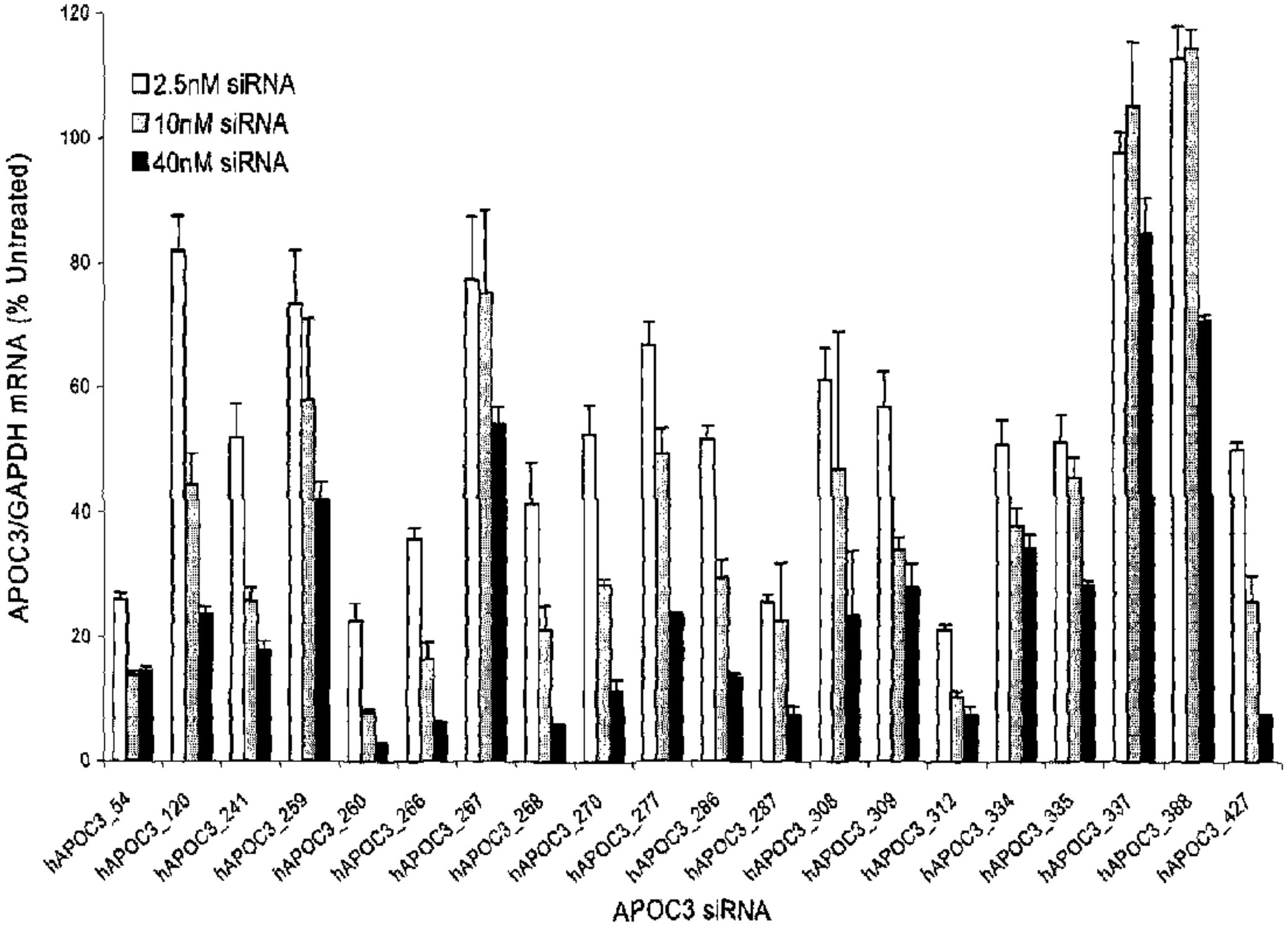

120
100
80
60
40
20
0
APOC3/GAPDH mRNA (% Untreated)
2.5nM siRNA
10nM siRNA
40nM siRNA
hAPOC3_54
hAPOC3_120
hAPOC3_241
hAPOC3_259
hAPOC3_260
hAPOC3_266
hAPOC3_267
hAPOC3_268
hAPOC3_270
hAPOC3_277
hAPOC3_286
hAPOC3_287
hAPOC3_308
hAPOC3_309
hAPOC3_312
hAPOC3_334
hAPOC3_335
hAPOC3_337
hAPOC3_388
hAPOC3_427
APOC3 siRNA

FIG. 8


120
100
80
60
40
20
0
APOC3/GAPDH mRNA (% Untreated)
2.5nM siRNA
10nM siRNA
40nM siRNA
hAPOC3_54
hAPOC3_120
hAPOC3_241
hAPOC3_259
hAPOC3_260
hAPOC3_266
hAPOC3_267
hAPOC3_268
hAPOC3_270
hAPOC3_277
hAPOC3_286
hAPOC3_287
hAPOC3_308
hAPOC3_309
hAPOC3_312
hAPOC3_334
hAPOC3_335
hAPOC3_337
hAPOC3_388
hAPOC3_427
APOC3 siRNA