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(54) Title: LDLR VARIANTS AND THEIR USE IN COMPOSITIONS FOR REDUCING CHOLESTEROL LEVELS

(57) Abstract: A recombinant vector having an expression cassette comprising a modified human low density lipoprotein receptor (hLDLR) gene is provided, wherein said hLDLR gene encodes a modified hLDLR comprising (a) one or more of the following amino acid substitutions: L318H, N295D, H306D, V307D, N309A, D310N, L318H, and/or L318D; or (b) an amino acid substitution of any of (a) in combination with one or more of the following amino acid substitutions: K796, K809R and/or C818A. Also provided are pharmaceutical compositions containing this vector and uses therefor in lowering cholesterol and/or treating familial hypercholesterolemia.

LDLR VARIANTS AND THEIR USE IN COMPOSITIONS FOR REDUCING CHOLESTEROL LEVELS

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN ELECTRONIC FORM

[0001] Applicant hereby incorporates by reference the Sequence Listing material filed in electronic form herewith. This file is labeled "14-7025PCT_ST25.txt".

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This work was supported in part by a grant from the National Institutes of Health, Heart. Lung and Blood Institute, P01-HL059407-15. The US government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Familial Hypercholesterolemia (FH) is an autosomal co-dominant disorder characterized by absence of the receptor for low-density lipoproteins (LDLR); a single chain glycoprotein containing 839 amino acids in its mature form. Hussain MM, et al, Annu Rev Nutr. 1999;19:141-172. Patients with one abnormal allele, heterozygous FH (heFH) have moderate elevations in plasma LDL and suffer from premature coronary artery disease (CAD), whereas homozygous FH patients (hoFH) have high serum cholesterol (LDL-C > 24 mmol/L) that often results in the early onset of life-threatening cardio vascular disease (CVD). Marais AD, Clin Biochem Rev. 2004;25:49-68. Current treatment options to reduce excess serum cholesterol include LDL apheresis [McGowan MP. J Clin Lipidol. 2013;7:S21-26] and treatment with cholesterol lowering drugs. Hovingh GK, et al, Eur Heart J. 2013;34:962-971. Orthotopic liver transplantation can lead to long term correction [Raal FJ, 2012; 223:262-268], although, it is associated with substantial treatment related morbidity and mortality.

[0004] Liver-directed gene therapy using adeno-associated viral vectors (AAV) has been demonstrated in preclinical models to stably correct several metabolic disorders and is

currently being pursued in clinical trials for treatment of hemophilia A and B, ornithine transcarbamylase deficiency (OTC) and alpha1-antitrypsin (A1AT) deficiency. Wang L, et al, Mol Genet Metab. 2012;105:203-211; Brantly ML, et al, Proc Natl Acad Sci U S A. 2009;106:16363-16368; Nathwani AC, et al., N Engl J Med. 2011;365:2357-2365; Ward NJ, et al, Blood. 2011; 117:798-807]. Recently, the effectiveness of AAV mediated gene therapy in correcting serum cholesterol levels in humanized mouse models of FH has been demonstrated. Kassim SH, et al, PLoS One. 2010;5:e13424. In these mice, systemic administration of AAV8 expressing human LDLR (AAV8.hLDLR) led to a lowering of cholesterol to normal levels by day 7 which was sustained for over a year and led to regression of pre-existing atherosclerosis. However, AAV8.LDLR transduction was dose dependent and statistically significant correction was only achieved at a vector dose of $1.5x10^{11}$ GC/kg or above. For clinical gene therapy, minimizing the vector dose is critical for many reasons, including vector injection volume, toxicity, immune response and manufacturing and cost of goods constraints.

[0005] Hepatic LDLR expression is modulated by multiple pathways within the cell: LDLR transcription is regulated by the sterol response element binding proteins (SREBPs), and HMGcoA reductase inhibitors (statins) activate SREBPs by inhibiting cholesterol synthesis within hepatocytes [Blumenthal RS, Am Heart J. 2000;139:577-583].

[0006] A second pathway of LDLR regulation, involving pro-protein convertase subtilisin kexin 9 (PCSK9), was discovered based on human genetics gain-of-function mutations that caused high LDL-C levels [Abifadel M, et al., Nat Genet. 2003;34:154-156] and loss-of-function mutations that caused low LDL-C levels [Cohen J, et al., Nat Genet. 2005;37:161-1653]. The loss of PCSK9 function was associated with an 88% reduction in cardiovascular disease and has led to the development of a new class of cholesterol lowering drugs based on the inhibition of PCSK9 [Fitzgerald K, et al, Lancet. 2014;383:60-68; Giugliano RP, et al, Lancet. 2012; 380:2007-2017]. Patients with FH have significantly higher plasma levels of PCSK9 [Raal F, et al., J Am Heart Assoc. 2013;2:e000028].

[0007] A third pathway of LDLR regulation was discovered by Zelcher et al, [Zelcher N, et al., Science. 2009; 325:100-104] who demonstrated the degradation of LDLR by IDOL (inducible degrader of LDLR). An E3 ubiquitin ligase, IDOL was induced following activation of liver X receptors (LXRs) and subsequently interacted with the cytoplasmic tail

of LDLR in mediating receptor ubiquitination and degradation. Furthermore, screening of subjects with low LDL-C identified loss-of-function mutations in IDOL that prevented degradation of LDLR [Sorrentino V, et al., Eur Heart J. 2013;34:1292-1297].

[0008] Compositions useful for effectively lowering cholesterol in subjects, particularly those having familial hypercholesterolemia, are needed.

SUMMARY OF THE INVENTION

[0009] Novel engineered human low density lipoprotein receptor (hLDLR) variants are provided herein, which have increased efficacy as compared to prior art "wild-type" LDLR, due to PCSK9 and/or IDOL resistance. These engineered variants of hLDLR are suitably characterized by a reduced affinity for PCSK9 and/or IDOL, an increased systemic half-life, and are useful for lowering cholesterol as compared to the native hLDLR. These variants can be delivered to subjects in need thereof via a number of routes, and particularly by expression *in vivo* mediated by a recombinant vector such as a recombinant adeno-associated virus (rAAV) vector.

[00010] In some embodiments, a synthetic or recombinant vector comprising a modified hLDLr gene is provided. In some embodiments, the modified hLDLR gene encodes a modified hLDLR that reduces cholesterol following expression. In some embodiments, the modified hLDLR comprises one or more amino acid substitutions that interfere with the wild-type hLDLR IDOL pathway and/or one or more amino acid substitutions which are resistant to degradation of hLDLR by interfering with the PCSK9 pathway.

[00011] In certain embodiments, the synthetic or recombinant vector encodes a modified hLDLR that comprises an amino acid substitution at amino acid position N295, H306, V307, N309, D310, L318, L796, K809 and/or C818. These amino acid positions are based on the numbering of SEQ ID NO:1 (the LDLR without the signal peptide). In a specific embodiment, the one or more amino acid substitutions are N295D, H306D, V307D, N309A, D310N, L318H, and/or L318D, which are examples of amino acid substitutions that interfere with the wild-type hLDLR IDOL pathway. In another specific embodiment, the one or more amino acid substitutions are L769R, K809R and/or C818A, which are examples of amino acid substitutions which are resistant to degradation of hLDLR by interfering with the

PCSK9 pathway. In another specific embodiment, the recombinant vector encodes a modified hLDLR that comprises one or more of amino acid substitutions N295D, H306D, V307D, N309A, D310N, L318H, and/or L318D in combination with one or more of amino acid substitutions L7696R, K809R and/or C818A (numbering based on SEQ ID NO:1).

[00012] In some embodiments, the recombinant vectors provided herein have an expression cassette comprising the modified hLDLR. In some embodiments, the expression cassette comprises a promoter which specifically directs expression of the modified hLDLR in liver cells.

[00013] In some embodiments, the recombinant vector is a recombinant adeno-associated virus (rAAV) vector. In some embodiments, the rAAV has a capsid selected from AAV8, rh64R1, AAV9, or rh10. In a particular embodiment, an rAAV vector is provided that has an expression cassette comprising a modified hLDLR gene, wherein said hLDLR gene encodes a modified hLDLR comprising an L318D amino acid substitution. In a specific embodiment, the modified hLDLR further comprises a K809R and/or C818A amino acid substitution. In a specific embodiment, the rAAV vector comprises an expression cassette comprising a promoter which specifically directs expression of the modified hLDLr in liver cells.

[00014] In certain embodiments, the hLDLR gene encodes a modified hLDLr having three substitutions: L318D/ K809R/C818A (numbering based on SEQ ID NO: 1). Other combinations of substitutions may be selected.

[00015] In some embodiments, a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a recombinant vector as described herein is provided. Also provided are methods for reducing circulating cholesterol levels by administering to a subject in need thereof a recombinant vector described herein that has an expression cassette, wherein said expression cassette further comprises regulatory control sequences which direct expression of modified hLDLr in the subject.

[00016] In yet another embodiment, methods for increasing the circulating half-life of a hLDLR are provided, comprising modifying the hLDLR at one or more amino acid positions (position numbers based on SEQ ID NO: 1) selected from: N295, H306, V307, N309, D310, L318, L7696, K809 and/or C818. In a specific embodiment, the hLDLR is modified to comprise one or more amino acid substitutions selected from: N295D, H306D,

V307D, N309A, D310N, L318H, and/or L318D. In another specific embodiment, the hLDLR is further modified to comprise a K769R, K809R and/or C818A amino acid substitution.

[00017] The recombinant vectors described above can be used in a regimen for treating familial hypercholesterolemia.

[00018] Other aspects and advantages of the invention will be readily apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[00019] FIGS. 1A and 1B provide the results of *in vitro* evaluation of LDLR variants that escape hPCSK9 regulation. Plasmids expressing wild type hLDLR or one of the LDLR variants were co-transfected along with hPCSK9 into HEK293 cells. 24 hours (hr) after transfection, cells were pulsed with BODIPY™-LDL [Molecular Probes] for 2 hr and then evaluated by flow cytometry for fluorescent LDL positive cells. FIG. 1A is a bar chart showing the percentage of BODIPY™-LDL positive cells when co-transfected with hLDLR or hLDLR along with hPCSK9. The experiment was controlled by transfecting cells with an irrelevant plasmid (Mock). FIG. 1B is a bar chart showing the fold change in BODIPY-LDL positive cells in hLDLR plus hPCSK9 co-transfected cells relative to hLDLR only transfected cells. FIG. 1C is a bar chart showing in vivo results in a mouse model expressing mLDLR. The results indicate some level of interaction between hPCSK9 and mLDLR.

[00020] FIGS. 2A- 2C provide results from a study showing *in vivo* overexpression of hPCSK9 leads to an increase in serum cholesterol in animals dosed with wild type hLDLR. $LDLR^{-/-}$, APOBEC-$1^{-/-}$ double knock-out (DKO) mice (n=4/group) were administered intravenously with a dose of $5x10^{10}$ GC of AAV8.TBG.hLDLR or AAV8.TBG.hLDLR along with $5x10^{10}$ GC of AAV9.hPCSK9 vector. Serum from animals before and 30 days after vector administration was analyzed for total serum cholesterol and HDL cholesterol. Non-HDL cholesterol levels were determined by subtracting the HDL component from total cholesterol. FIG. 2A is a bar chart showing the percent change in day 30 non-HDL serum cholesterol relative to baseline levels in animals that received hLDLR with or without hPCSK9. FIG. 2B is a line graph showing the time course of hPCSK9 expression in serum from mice that received hLDLR or hLDLR plus hPCSK9. hPCSK9 expression was

evaluated using a sandwich ELISA. The reported reference average hPCSK9 levels in humans is also shown on the graph. FIG. 2C is an immunoblot of hLDLR expression in mice treated with hLDLR or hLDLR with hPCSK9. Total liver lysates from two representative animals per group were electrophoresed on a 4-12% gradient SDS gel and probed with a polyclonal anti-hLDLR goat polyclonal antibody. Mouse tubulin expression was used as a loading control. All values are expressed as mean ± SEM. ***$p < 0.001$.

[00021] FIGS. 3A and 3B illustrate that mice transduced with hLDLR-L318D are resistant to hPCSK9 mediated regulation. DKO mice (n=4/group) were co-transduced with $5x10^{10}$ GC of AAV8.hLDLR or hLDLR-L318D along with $5x10^{10}$ GC of AA9.hPCSK9. Serum from animals pre and 30 days post vector administration was evaluated for total cholesterol and HDL cholesterol. FIG. 3A is a bar chart showing the percent change in day 30 non-HDL serum cholesterol levels relative to pre-vector administration in DKO mice co-administered with hLDLR or hLDLR-L318D, along with hPCSK9. FIG. 3B show total liver lysates from two animals per group which were electrophoresed on a 4-12% SDS PAGE gel and probed for hLDLR expression. Mouse tubulin expression was used as a loading control. All values are expressed as mean ± SEM. ***$p < 0.001$. ns $p>0.05$.

[00022] FIGS. 4A - 4D illustrate AAV8.hLDLR-K809R\C818A escapes *in vivo* hIDOL mediated regulation. HEK293 cells were transiently transfected with plasmids expressing either hLDLR or hLDLR-K809R\C818A along with hIDOL. 24 hr later, cells were pulsed with BODIPY-LDL for 2 hr and then evaluated for fluorescent LDL uptake using a flow cytometer. FIG. 4A is a bar chart showing the percent BODIPY™-LDL positive cells transfected with hLDLR or hLDLR-K809R\C818A along with hIDOL. FIG. 4B is a line graph showing data from $LDLR^{+/-}$, $Apobec^{-/-}$, Tg-hApoB100 (LAHB) heterozygous FH (heFH) mice (n=4) which were systemically administered with $1x10^{11}$ GC of AAV9hPCSK9 vector. Time course of non-HDL cholesterol levels following vector administration. FIG. 4C is a bar chart showing homozygous FH (hoFH) DKO mice (n=4\group) systemically administered with $3x10^{9}$GC AAV8.hLDLR, or AAV8.hLDLR-K809R\C818A, along with AAV9.hIDOL $5X10^{10}$ GC. Serum from animals pre- and 30 days post vector administration was evaluated for total serum cholesterol. Percent change in serum non-HDL levels at 30 day relative to pre-administration baseline levels. All values are expressed as mean ± SEM. ***$p<0.0001$.*$p < 0.05$. ns $p>0.05$. FIG. 4D provides the total cell lysates of transfected cells

(FIG. 4A) electrophoresed on a 4-12% SDS gel and probed using anti-hLDLR antibody. The location of mature (M) and processed (P) forms of LDLR along with the tubulin loading control is shown.

[00023] FIGS. 5A - 5B illustrate the AAV8.hLDLR-L318D\K809R\C818A variant encoding three amino acid substitutions escapes both PCSK9 and IDOL mediated regulation. FIG. 5A illustrates the results in a study in which DKO mice (n=4) were intravenously administered with $3x10^9$ GC of hLDLR or hLDLR-L318D\K809R\C818A. Additional groups of mice also received a simultaneous administration of AAV9.hIDOL. Total serum cholesterol levels were evaluated before and 30 days after vector administration. Percent decrease in non-HDL cholesterol relative to baseline. Total liver lysates from 2 representative animals per group were electrophoresed on a SDS PAGE gel and probed using an anti-hLDLR antibody along with tubulin as a loading control. FIG. 5B illustrates the results following coadministration of AAV8.hLDLR ($5x10^{10}$ GC) or hLDLR-L318D\K809R\C818A along with AAV9.hPCSK9 ($5x10^{10}$ GC). Percent decrease in day 30 non-HDL cholesterol relative to baseline is shown along with an immunoblot of hLDLR expression in livers. n ***$p < 0.001$.

[00024] FIG. 6 is a bar chart illustrating the hLDLR activity of variants that escape PCSK9 regulation in DKO mice. DKO mice (n=4/group) were injected with $3x10^{10}$ GC of AAV8.TBG.hLDLR or AAV8 vectors expressing one of nine hPCSK9 escape variants. Serum from animals was analyzed before and 30 days after vector administration and percent reduction in non-HDL cholesterol at day 30 day compared to baseline is shown along with SD.

[00025] FIG. 7 is a bar chart illustrating that AAV.hLDLR overcome hIDOL mediated inhibition when administered at higher dose. DKO mice (n=4/group) were administered with of $5x10^{10}$ GC of AAV8.TBG.hLDLR or AAV8.TBG.hLDLR-K809R\C818A. Additional groups of mice received hLDLR along with of $5x10^{10}$ GC of AAV9.TBG.hIDOL. Percent non-HDL cholesterol levels on day 30 compared to baseline is shown along with SD.

[00026] FIG. 8 is a bar chart illustrating the hLDLR activity of variants that escape PCSK9 regulation in a LDLR -/-, ApoBec -/-, double-knock out a mouse model. DKO mice (n=5/group) were injected (tail vein) with $5x10^{10}$ GC of AAV8.TBG.hLDLR or AAV8 vectors expressing one of nine hPCSK9 escape variants along with 5×10^{10} GC of

AAV9.TBG.hPCSK9 vectors. Serum from animals was analyzed before and 30 days after vector administration. Percent reduction in non-HDL cholesterol at day 30 day compared to baseline is shown along with SD. Control mice received only the LDLR vector without co-administration of PCSK9 (bars to left in each pair).

DETAILED DESCRIPTION OF THE INVENTION

[00027] The novel engineered human low density lipoprotein receptor (hLDLR) variants described herein are characterized by increased half-life and increased efficacy in decreasing cholesterol levels as compared to the native hLDLR due at least in part to their ability to substantially avoid degradation by pro-protein convertase subtilisin kexin 9 (PCSK9) and/or substantially avoid degradation by the inducible degrader of LDLR (IDOL).

[00028] Delivery of these variants to subjects in need thereof via a number of routes, and particularly by expression *in vivo* mediated by a recombinant vector such as a rAAV vector, are described. Also provided are methods of using these variants in regimens for lowering cholesterol levels in subject in need thereof, treating familial hypercholesterolemia, treating atherosclerosis, decreasing the risk of premature coronary artery disease and/or decreasing early onset of cardio vascular disease. Advantageously, compositions provided herein are useful for simultaneously targeting multiple pathways in these treatments and regimens.

[00029] As used herein, the term familial hypercholesterolemia (FH) refers to a genetic disorder of lipid metabolism. Unless otherwise specified herein, both homozygous FH (hoFH) subjects and heterozygous FH (heFH) subjects are encompassed within the term FH.

[00030] As used herein, the term "lowering cholesterol levels" may encompass decreasing serum cholesterol levels and/or decreasing low-density lipoprotein levels (e.g., in plasma). Treating atherosclerosis may include decreasing number and/or volume of plaques and/or preventing further accumulation of atherosclerotic plaques.

[00031] The amino acid sequence of the mature "wild-type" hLDLR (isoform 1) is reproduced herein as SEQ ID NO: 1 for convenience and provides a reference for the numbering of the amino acid variants provided herein. While the sequence numbering provided herein refers to the mature hLDLR protein (a single chain glycoprotein of 839 amino acids), it will be understood that wild-type hLDLR leader sequence (amino acids 1-21

of SEQ ID NO:2) may be used or a heterologous leader sequence may be selected for use in the constructs described herein. Additionally, or optionally, one or more of the other hLDLR isoforms 2, 3, 4, 5 and 6, the sequences of which are available, *e.g.*, from http://www.uniprot.org/uniprot/P01130, and the amino acid substitutions described herein may be incorporated into these isoforms (*see also*, SEQ ID NO: 3 - 7 where the sequences of these isoforms are reproduced for convenience). In the following descriptions, substitutions may be written as (first amino acid identified by single letter code)- residue position # - (second amino acid identified by single letter code) whereby the first amino acid is the substituted amino acid and the second amino acid is the substituting amino acid at the specified position with reference to isoform 1; however, by conventional alignment steps, the corresponding amino acid residues identified herein with respect to the numbering of isoform 1 can be located in the other isoforms or hLDLR proteins identified herein.

[00032] The term "amino acid substitution" and its synonyms described above are intended to encompass modification of an amino acid sequence by replacement of an amino acid with another, substituting, amino acid. The substitution may be a conservative substitution. It may also be a non-conservative substitution. The term conservative, in referring to two amino acids, is intended to mean that the amino acids share a common property recognized by one of skill in the art. For example, amino acids having hydrophobic nonacidic side chains, amino acids having hydrophobic acidic side chains, amino acids having hydrophilic nonacidic side chains, amino acids having hydrophilic acidic side chains, and amino acids having hydrophilic basic side chains. Common properties may also be amino acids having hydrophobic side chains, amino acids having aliphatic hydrophobic side chains, amino acids having aromatic hydrophobic side chains, amino acids with polar neutral side chains, amino acids with electrically charged side chains, amino acids with electrically charged acidic side chains, and amino acids with electrically charged basic side chains. Both naturally occurring and non-naturally occurring amino acids are known in the art and may be used as substituting amino acids in embodiments. Methods for replacing an amino acid are well known to the skilled in the art and include, but are not limited to, mutations of the nucleotide sequence encoding the amino acid sequence. Reference to "one or more" herein is intended to encompass the individual embodiments of, for example, 1, 2, 3, 4, 5, 6, or more.

[00033] As described herein, the hLDLR variants provided herein are engineered to reduce the PCSK9 degradation characteristic of the wild-type LDLR. In one embodiment, the variant is a human LDLR having an amino acid substation at position 318, in which the native leucine (Leu) is modified. In one example, the L318 is modified to histidine (His, H). However, other substitutions (*e.g.,* an L318D) may be made at this position. Alternatively or additionally, other hLDLR variants resistant to PCSK9 degradation may be selected from among those identified herein. These may include, *e.g.,* substitutions of N295, H306, V307, N309, and/or D310 (position numbers based on SEQ ID NO:1). Methods of determining resistance to PCSK9 degradation and/or determining increased circulating half-life as compared to the wild-type hLDLR are known in the art, and at least one these assays is illustrated in the examples below.

[00034] Additionally, the PCSK9-resistant LDLR variants described herein may be further engineered to include resistance to degradation by IDOL. Suitable substitutions for conferring this characteristic include substitutions at position K796 (abbreviated K6 in sequence listing), K809 and C818. The substitutions illustrated herein are K809R and C818A. However, other IDOL-resistant substitutions may be selected. Methods of determining resistance to IDOL degradation and/or determining increased circulating half-life as compared to the wild-type hLDLR are known in the art, and at least one these assays is illustrated in the examples below.

[00035] Other modifications to the hLDLR isoform 1 amino acid sequence, which incorporate one or more of the above variants, are encompassed within the invention. For example, the corresponding modification to the amino acid sequence of any of isoforms 2 (SEQ ID NO:3), isoform 3 (SEQ ID NO: 4), isoform 4 (SEQ ID NO: 5), isoform 5 (SEQ ID NO: 6), and isoform 7 (SEQ ID NO: 7) may be utilized. These isoforms are reproduced in the Sequence Listing herein. In another example, the hLDLR variant described herein may be engineered to contain the hLDLR leader sequence. Alternatively, a heterologous leader sequence may be engineered to the N-terminus of the hLDLR variant. Alternatively, still other variations, which may include up to about 5% variation (about 95% identity to about 99.9 % identity to the variant sequence, or about 97% to about 98% identity) to the hLDLR variants provided herein (excluding the leader sequence) may be selected which retain one or

more of the therapeutic functions of the hLDLR variants described herein, and which are characterized by PCSK9-resitstance and/or IDOL-resistance.

[00036] In the examples section of this description, while a number of constructs did escape PCSK9 regulation in initial *in vitro* screening, the studies focused on the L318D amino acid substitution. Among the variants provided herein, the L318D modification has been demonstrated to confer protection from PCSK9 both *in vitro* and *in vivo*. In the examples provided herein, L318D (position number based on SEQ ID NO:1, illustrative construct with leader sequence in SEQ ID NO: 26) conferred protection following hepatic expression in mice overexpressing PCSK9 and led to a significant decrease in serum cholesterol; whereas, wild-type LDLR was less efficient and more readily degraded by PCSK9.

[00037] As illustrated in the examples below, the K809R/C818A hLDLR double mutant (position numbers based on SEQ ID NO:1, illustrative construct with leader sequence in SEQ ID NO: 36] conferred protection following hepatic expression in mice expressing hIDOL and led to a significant decrease in serum cholesterol; whereas, wild-type LDLR was less efficient and more readily degraded by IDOL. These data thus establish that the amino acid modifications in the LDLR can also overcome in vivo IDOL mediated suppression. Factors that lead to LDLR degradation are expected to be higher in subjects lacking endogenous receptor expression due to lack of a substrate to remove the inhibitors. The usefulness of LDLR variants in overcoming negative cellular regulatory pathways, known to exist in FH subjects, is demonstrated herein. The findings presented here demonstrate for the first time the successful use of an AAV encoded 'gain-of-function' transgene in reducing cholesterol in humanized mouse models expressing high levels of inhibitory factors which is useful in gene therapy products for FH.

[00038] In addition to the hLDLR protein variants provided herein, nucleic acid sequences encoding these hLDLR protein variants are provided. The coding sequences for these variants may be generating using site-directed mutagenesis of the wild-type nucleic acid sequence. Alternatively or additionally, web-based or commercially available computer programs, as well as service based companies may be used to back translate the amino acids sequences to nucleic acid coding sequences, including both RNA and/or cDNA. *See, e.g.,* backtranseq by EMBOSS, http://www.ebi.ac.uk/Tools/st/ ; Gene Infinity

(http://www.geneinfinity.org/sms-/sms_backtranslation.html); ExPasy (http://www.expasy.org/tools/). In one embodiment, the RNA and/or cDNA coding sequences are designed for optimal expression in human cells.

[00039] Codon-optimized coding regions can be designed by various different methods. This optimization may be performed using methods which are available on-line, published methods , or a company which provides codon optimizing services. One codon optimizing method is described, *e.g.,* in US Patent Application No. 61/817,110, which is incorporated by reference herein. Briefly, the nucleic acid sequence encoding the product is modified with synonymous codon sequences. Suitably, the entire length of the open reading frame (ORF) for the product is modified. However, in some embodiments, only a fragment of the ORF may be altered. By using one of these methods, one can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide.

[00040] The terms "percent (%) identity", "sequence identity", "percent sequence identity", or "percent identical" in the context of nucleic acid sequences refers to the bases in the two sequences which are the same when aligned for correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, or as desired. However, identity among smaller fragments, *e.g.* of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired. Multiple sequence alignment programs are also available for nucleic acid sequences. Examples of such programs include, "Clustal W", "CAP Sequence Assembly", "BLAST", "MAP", and "MEME", which are accessible through Web Servers on the internet. Other sources for such programs are known to those of skill in the art. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a program in GCG Version 6.1. Fasta™ provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM

factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference.

[00041] The terms "percent (%) identity", "sequence identity", "percent sequence identity", or "percent identical" in the context of amino acid sequences refers to the residues in the two sequences which are the same when aligned for correspondence. Percent identity may be readily determined for amino acid sequences over the full-length of a protein, polypeptide, about 32 amino acids, about 330 amino acids, or a peptide fragment thereof or the corresponding nucleic acid sequence coding sequencers. A suitable amino acid fragment may be at least about 8 amino acids in length, and may be up to about 700 amino acids. Generally, when referring to "identity", "homology", or "similarity" between two different sequences, "identity", "homology" or "similarity" is determined in reference to "aligned" sequences. "Aligned" sequences or "alignments" refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, *e.g.,* the "Clustal X", "MAP", "PIMA", "MSA", "BLOCKMAKER", "MEME", and "Match-Box" programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, *e.g.,* J. D. Thomson et al, Nucl. Acids. Res., "A comprehensive comparison of multiple sequence alignments", 27(13):2682-2690 (1999).

[00042] In one embodiment, the nucleic acid sequences encoding the hLDLR variants (*e.g.,* LDLR variant gene) described herein are engineered into any suitable genetic element, *e.g.,* naked DNA, phage, transposon, cosmid, RNA molecule (*e.g.,* mRNA), episome, *etc.,* which transfers the hLDLR sequences carried thereon to a host cell, *e.g.,* for generating nanoparticles carrying DNA or RNA, viral vectors in a packaging host cell and/or for delivery to a host cells in subject. In one embodiment, the genetic element is a plasmid. The selected genetic element may be delivered by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated

pellets, viral infection and protoplast fusion. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. *See, e.g.,* Green and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012).

[00043] As used herein, an "expression cassette" refers to a nucleic acid molecule which comprises the hLDLR variant coding sequences, promoter, and may include other regulatory sequences therefor, which cassette may be engineered into a genetic element and/or packaged into the capsid of a viral vector (*e.g.,* a viral particle). Typically, such an expression cassette for generating a viral vector contains the hLDLR sequences described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described herein.

[00044] The expression cassette typically contains a promoter sequence as part of the expression control sequences. The illustrative plasmid and vector described herein uses the liver-specific promoter thyroxin binding globulin (TBG). Alternatively, other liver-specific promoters may be used [*see, e.g.,* The Liver Specific Gene Promoter Database, Cold Spring Harbor, http://rulai.schl.edu/LSPD, alpha 1 anti-trypsin (A1AT); human albumin Miyatake et al., J. Virol., 71:5124 32 (1997), humAlb; and hepatitis B virus core promoter, Sandig *et al.,* Gene Ther., 3:1002 9 (1996)]. TTR minimal enhancer/promoter, alpha-antitrypsin promoter, LSP (845 nt)25(requires intron-less scAAV). Although less desired, other promoters, such as viral promoters, constitutive promoters, regulatable promoters [*see, e.g.,* WO 2011/126808 and WO 2013/04943], or a promoter responsive to physiologic cues may be used may be utilized in the vectors described herein.

[00045] In addition to a promoter, an expression cassette and/or a vector may contain other appropriate transcription initiation, termination, enhancer sequences, efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.,* Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. Examples of suitable polyA sequences include, *e.g.,* SV40, bovine growth hormone (bGH), and TK polyA. Examples of suitable enhancers include, *e.g.,* the alpha fetoprotein enhancer, the TTR minimal promoter/enhancer,

LSP (TH-binding globulin promoter/alpha1-microglobulin/bikunin enhancer), amongst others.

[00046] These control sequences are "operably linked" to the hLDLR gene sequences. As used herein, the term "operably linked" refers to both expression control sequences that are contiguous with the gene of interest and expression control sequences that act *in trans* or at a distance to control the gene of interest.

[00047] The expression cassette may be engineered onto a plasmid which is used for production of a viral vector. The minimal sequences required to package the expression cassette into an AAV viral particle are the AAV 5' and 3' ITRs, which may be of the same AAV origin as the capsid, or which of a different AAV origin (to produce an AAV pseudotype). In one embodiment, the ITR sequences from AAV2, or the deleted version thereof (ΔITR), are used for convenience and to accelerate regulatory approval. However, ITRs from other AAV sources may be selected. Where the source of the ITRs is from AAV2 and the AAV capsid is from another AAV source, the resulting vector may be termed pseudotyped. Typically, an expression cassette for an AAV vector comprises an AAV 5' ITR, the hLDLR coding sequences and any regulatory sequences, and an AAV 3' ITR. However, other configurations of these elements may be suitable. A shortened version of the 5' ITR, termed ΔITR, has been described in which the D-sequence and terminal resolution site (trs) are deleted. In other embodiments, the full-length AAV 5' and 3' ITRs are used.

[00048] The abbreviation "sc" refers to self-complementary. "Self-complementary AAV" refers a plasmid or vector having an expression cassette in which a coding region carried by a recombinant AAV nucleic acid sequence has been designed to form an intra-molecular double-stranded DNA template. Upon infection, rather than waiting for cell mediated synthesis of the second strand, the two complementary halves of scAAV will associate to form one double stranded DNA (dsDNA) unit that is ready for immediate replication and transcription. *See, e.g.,* D M McCarty *et al*, "Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis", Gene Therapy, (August 2001), Vol 8, Number 16, Pages 1248-1254. Self-complementary AAVs are described in, *e.g.,* U.S. Patent Nos. 6,596,535; 7,125,717; and 7,456,683, each of which is incorporated herein by reference in its entirety.

[00049] An adeno-associated virus (AAV) viral vector is an AAV DNase-resistant particle having an AAV protein capsid into which is packaged nucleic acid sequences for delivery to target cells. An AAV capsid is composed of 60 capsid protein subunits, VP1, VP2, and VP3, that are arranged in an icosahedral symmetry in a ratio of approximately 1:1:10 to 1:1:20, depending upon the selected AAV. AAV serotypes may be selected as sources for capsids of AAV viral vectors (DNase resistant viral particles) including, e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAV8, AAV9, rh10, AAVrh64R1, AAVrh64R2, rh8 [*See, e.g.,* US Published Patent Application No. 2007-0036760-A1; US Published Patent Application No. 2009-0197338-A1; EP 1310571]. *See also*, WO 2003/042397 (AAV7 and other simian AAV), US Patent 7790449 and US Patent 7282199 (AAV8), WO 2005/033321 and US 7,906,111 (AAV9), and WO 2006/110689], and rh10 [WO 2003/042397] or yet to be discovered, or a recombinant AAV based thereon, may be used as a source for the AAV capsid. These documents also describe other AAV which may be selected for generating AAV and are incorporated by reference. In some embodiments, an AAV cap for use in the viral vector can be generated by mutagenesis (i.e., by insertions, deletions, or substitutions) of one of the aforementioned AAV Caps or its encoding nucleic acid. In some embodiments, the AAV capsid is chimeric, comprising domains from two or three or four or more of the aforementioned AAV capsid proteins. In some embodiments, the AAV capsid is a mosaic of Vpl, Vp2, and Vp3 monomers from two or three different AAVs or recombinant AAVs. In some embodiments, an rAAV composition comprises more than one of the aforementioned Caps.

[00050] For packaging an expression cassette into virions, the ITRs are the only AAV components required in cis in the same construct as the gene. In one embodiment, the coding sequences for the replication (rep) and/or capsid (cap) are removed from the AAV genome and supplied *in trans* or by a packaging cell line in order to generate the AAV vector. For example, as described above, a pseudotyped AAV may contain ITRs from a source which differs from the source of the AAV capsid. Additionally or alternatively, a chimeric AAV capsid may be utilized. Still other AAV components may be selected. Sources of such AAV sequences are described herein and may also be isolated or obtained from academic, commercial, or public sources (e.g., the American Type Culture Collection, Manassas, VA). Alternatively, the AAV sequences may be obtained through synthetic or other suitable means

by reference to published sequences such as are available in the literature or in databases such as, e.g., GenBank®, PubMed®, or the like.

[00051] Methods for generating and isolating AAV viral vectors suitable for delivery to a subject are known in the art. *See, e.g.,* US Patent 7790449; US Patent 7282199; WO 2003/042397; WO 2005/033321, WO 2006/110689; and US 7588772 B2]. In a one system, a producer cell line is transiently transfected with a construct that encodes the transgene flanked by ITRs and a construct(s) that encodes rep and cap. In a second system, a packaging cell line that stably supplies rep and cap is transiently transfected with a construct encoding the transgene flanked by ITRs. In each of these systems, AAV virions are produced in response to infection with helper adenovirus or herpesvirus, requiring the separation of the rAAVs from contaminating virus. More recently, systems have been developed that do not require infection with helper virus to recover the AAV - the required helper functions (*i.e.,* adenovirus E1, E2a, VA, and E4 or herpesvirus UL5, UL8, UL52, and UL29, and herpesvirus polymerase) are also supplied, *in trans*, by the system. In these newer systems, the helper functions can be supplied by transient transfection of the cells with constructs that encode the required helper functions, or the cells can be engineered to stably contain genes encoding the helper functions, the expression of which can be controlled at the transcriptional or posttranscriptional level. In yet another system, the transgene flanked by ITRs and rep/cap genes are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, *e.g.*, Zhang et al., 2009, "Adenovirus-adeno-associated virus hybrid for large-scale recombinant adeno-associated virus production," Human Gene Therapy 20:922-929, the contents of each of which is incorporated herein by reference in its entirety. Methods of making and using these and other AAV production systems are also described in the following U.S. patents, the contents of each of which is incorporated herein by reference in its entirety: 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514; 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065. *See generally, e.g.*, Grieger & Samulski, 2005, "Adeno-associated virus as a gene therapy vector: Vector development, production and clinical applications," Adv. Biochem. Engin/Biotechnol. 99: 119-145; Buning et al., 2008, "Recent developments in adeno-associated virus vector technology," J. Gene Med. 10:717-733; and the references cited below, each of which is incorporated herein by reference in its entirety. The methods used to

construct any embodiment of this invention are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. *See, e.g.,* Green and Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (2012). Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present invention. See, e.g., K. Fisher et al, (1993) J. Virol., 70:520-532 and US Patent No. 5,478,745.

[00052] Optionally, the hLDLR genes described herein may be delivered via viral vectors other than rAAV. Such other viral vectors may include any virus suitable for gene therapy may be used, including but not limited to adenovirus; herpes virus; lentivirus; retrovir*us; etc.* Suitably, where one of these other vectors is generated, it is produced as a replication-defective viral vector.

[00053] A “replication-defective virus" or "viral vector" refers to a synthetic or artificial viral particle in which an expression cassette containing a gene of interest is packaged in a viral capsid or envelope, where any viral genomic sequences also packaged within the viral capsid or envelope are replication-deficient; *i.e.,* they cannot generate progeny virions but retain the ability to infect target cells. In one embodiment, the genome of the viral vector does not include genes encoding the enzymes required to replicate (the genome can be engineered to be "gutless" - containing only the transgene of interest flanked by the signals required for amplification and packaging of the artificial genome), but these genes may be supplied during production. Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication.

[00054] The pharmaceutical compositions described herein are designed for delivery to subjects in need thereof by any suitable route or a combination of different routes. Direct delivery to the liver (optionally via intravenous, via the hepatic artery, or by transplant), oral, inhalation, intranasal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, and other parental routes of administration. The viral vectors described herein may be delivered in a single composition or multiple compositions. Optionally, two or more different AAV may be delivered, or multiple viruses [*see, e.g.*, WO

2011/126808 and WO 2013/049493]. In another embodiment, multiple viruses may contain different replication-defective viruses (*e.g.,* AAV and adenovirus).

[00055] The replication-defective viruses can be formulated with a physiologically acceptable carrier for use in gene transfer and gene therapy applications. In the case of AAV viral vectors, quantification of the genome copies ("GC") may be used as the measure of the dose contained in the formulation. Any method known in the art can be used to determine the genome copy (GC) number of the replication-defective virus compositions of the invention. One method for performing AAV GC number titration is as follows: Purified AAV vector samples are first treated with DNase to eliminate un-encapsidated AAV genome DNA or contaminating plasmid DNA from the production process. The DNase resistant particles are then subjected to heat treatment to release the genome from the capsid. The released genomes are then quantitated by real-time PCR using primer/probe sets targeting specific region of the viral genome (usually poly A signal).

[00056] Also, the replication-defective virus compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0×10^9 GC to about 1.0×10^{15} GC (to treat an average subject of 70 kg in body weight), and preferably 1.0×10^{12} GC to 1.0×10^{14} GC for a human patient. In another embodiment, the dose is less than about 1.5×10^{11} GC/kg. For example, the dose of AAV virus may be about 1×10^9 GC, about 5×10^9 GC, about 1×10^{10} GC, about 5×10^{10} GC, or about 1×10^{11} GC. In another example, the variants may be delivered in an amount of about 0.001 mg to about 10 mg/kg.

[00057] The above-described recombinant vectors may be delivered to host cells according to published methods. The rAAV, preferably suspended in a physiologically compatible carrier, may be administered to a human or non-human mammalian subject. Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the transfer virus is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present invention.

[00058] Optionally, the compositions of the invention may contain, in addition to the rAAV and/or variants and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

[00059] The viral vectors and other constructs described herein may be used in preparing a medicament for delivering a LDLR variant to a subject in need thereof, supplying LDLR variant having an increased half-life to a subject, and/or for treating elevated cholesterol levels, elevated high density lipoprotein (HDL), elevated triglycerides, familial hypercholesterolemia, atherosclerosis, coronary artery disease, cardiovascular disease, and/or another lipoprotein metabolic disorder.

[00060] A course of treatment may optionally involve repeat administration of the same viral vector (*e.g.,* an AAV8 vector) or a different viral vector (*e.g.,* an AAV8 and an AAVrh10). Still other combinations may be selected using the viral vectors described herein. Optionally, the composition described herein may be combined in a regimen involving other anti-lipid drugs (*e.g.,* statins, monoclonal antibodies, etc), or protein-based therapies (including, *e.g.,* delivery of a composition containing one or more LDLR variants as described herein).

[00061] It is to be noted that the term "a" or "an" refers to one or more. As such, the terms "a" (or "an"), "one or more," and "at least one" are used interchangeably herein.

[00062] The words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively. The words "consist", "consisting", and its variants, are to be interpreted exclusively, rather than inclusively. While various embodiments in the specification are presented using “comprising” language, under other circumstances, a related embodiment is also intended to be interpreted and described using “consisting of” or “consisting essentially of” language.

[00063] As used herein, the term "about" means a variability of 10% from the reference given, unless otherwise specified.

[00064] The term "regulation" or variations thereof as used herein refers to the ability of a composition to inhibit one or more components of a biological pathway.

[00065] A "subject" is a mammal, *e.g.*, a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, or non-human primate, such as a monkey, chimpanzee, baboon or gorilla.

[00066] As used herein, "disease", "disorder" and "condition" are used interchangeably, to indicate an abnormal state in a subject.

[00067] Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

[00068] The following examples are illustrative only and are not intended to limit the present invention.

EXAMPLES -

Example 1 - AAV Vectors Expressing LDLR gain-of-function variants demonstrate increased efficacy in mouse models of familial hypercholesterolemia

A. Experimental animals

[00069] All animal studies were approved by the institutional review board (IRB) at the University of Pennsylvania. $LDLR^{-/-}$, $APOBEC\text{-}1^{-/-}$ double knockout mice (DKO) and $LDLR^{-/-}$, $APOBEC\text{-}1^{-/-}$, human ApoB100 transgenic (LAHB) were maintained at the University of Pennsylvania. These mice overexpress hPCSK9. The absence of endogenous mouse LDLR expression in this animal model permits evaluation of hLDLR transgene expression without interference from mouse LDLR. Overexpression of hPCSK9 is achieved by coadministering an AAV vector expressing hPCSK9 (AAV9.TBG.hPCSK9), the preparation of which is described in Part C of this Example.

[00070] 6-8 week old male mice were injected intravenously (tail vein) with vector diluted in phosphate buffered saline (PBS) in a total volume of 100 µL. Serum was collected pre and post vector administration by retro orbital bleeds. At the end of the study all animals were sacrificed and the livers harvested for analysis of vector genomes and transgene expression. Serum samples from animals were analyzed for total cholesterol (Tc), LDL, HDL

and total triglycerides (Tg) using a MIRA analyzer (Roche). Non-HDL cholesterol was derived by subtracting the Tg from Tc. Livers from animals were harvested and homogenized using RIPA buffer. 25 µg of total liver lysate was electrophoresed on a 4-12% PAGE gel and probes with a polyclonal anti-hLDLR antibody.

B. LDLR variants

[00071] Amino acid residues (position numbers based on SEQ ID NO:1) targeted for mutagenesis were as follows:

Table 1. Amino acid substitutions and affected LDLR-PCSK9 interaction

Amino acid substitution	*Predicted LDLR-PCSK9 interaction*
N295D	Prevent hydrogen bonding with PCSK9 Asp-238
D299N	Affects salt bridge with PCSK9 Ser-153
H306G	Affects salt bridge with PCSK9 Asp-374
V307D	Prevents hydrophobic interaction with PCSK9 Val-380
N309A	Prevent hydrogen bonding with PCSK9 Thr-377
D310N	Affects salt bridge with PCSK9 Arg-194
L311T	Prevent hydrogen bonding with PCSK9 Thr-377
L318D	Hydrophobic interaction with PCSK9 Cys-378
L318H	Hydrophobic interaction with PCSK9 Cys-378

[00072] In addition, amino acid substitutions, K809R and C818A, in the C-terminal cytoplasmic domain of LDLR that prevent IDOL mediated degradation were selected.

C. Vector

[00073] The AAV8 vector expressing wild type hLDLR cDNA from a liver-specific thyroxine binding globulin (TBG) promoter has been previously described and was obtained from the Vector Core at the University of Pennsylvania. Briefly, HEK293 cells were triple transfected using AAV cis- and trans-plasmid along with the Ad helper plasmids. AAV particles were purified from the culture supernatant and quantified using primers to the bGH polyadenylation sequence. Vector preparations were analyzed for DNA structure by

restriction digests and endotoxin contamination (<20 EU/mL) before injection into animals. The wild type hLDLR cDNA was used as a template for site directed mutagenesis to introduce amino acid substitutions using the Quickchange XL kit (Stratagene) as per the manufacturers' recommendations.

[00074] The sequences of plasmids used for production of AAV vectors as described herein are provided in the appended sequence listing. The plasmid constructs having the TBG promoter were used in the animal (mice) studies; those with the CB promoter were used for in vitro screening.

[00075] The cDNA sequences encoding hPCSK9 and hIDOL were purchased (Origene, MD), cloned, and vectored to express from an AAV9 vector behind a TBG promoter and a bovine growth hormone (bGH) polyadenylation signal. An AAV9 vector expressing human alpha1-antitrypsin (A1AT) also expressed from a TBG promoter was used as a control in studies that required an irrelevant transgene.

[00076] Table 2. *Vectors*

AAV8.hLDLR
AAV8.hLDLR-N295D
AAV8.hLDLR-D299N
AAV8.hLDLR-H306G
AAV8.hLDLR-V307D
AAV8.hLDLR-N309A
AAV8.hLDLR-D310N
AAV8.hLDLR-L311T
AAV8.hLDLR-L318D
AAV8.hLDLR-L318H
AAV8.hLDLR-K809R\C818A
AAV8.hLDLR-L318D\K809R\C818A
AAV9.hPCSK9
AAV9.hIDOL

D. *In vitro* LDLR assay

[00077] HEK 293 cells growing in 6 well plates were transfected overnight with plasmids expressing hLDLR along with hPCSK9 or hIDOL. All cDNAs were cloned behind

a cytomegalovirus promoter (CMV) to obtain expression in HEK293 cells. Control cells were transfected with hLDLR plus a plasmid expressing an irrelevant transgene (A1AT). In studies where the dose of one vector was titrated lower an irrelevant plasmid was added to ensure that the total amount of plasmid did not vary from one experimental well to another. The following day cells were pulsed with BODIPY-LDL (Invitrogen) at a concentration of 4 μg/mL. Cells were removed after 2 hr and evaluated for fluorescent LDL uptake using a flow cytometer (FC500, Beckman Coulter).

E. Immunoblotting and Enzyme linked Immune assays

[00078] 50 μg of total cell lysates prepared from cells or mouse livers expressing human LDLR were electrophoresed on a 4-12% gradient precast mini gel (Invitrogen) before transferring to PVDF membrane (Invitrogen). An anti-hLDLR goat polyclonal antibody (Invitrogen) was used to probe the membrane (1/1000 dilution) followed by a secondary anti-goat antibody conjugated to alkaline phosphatase (Invitrogen). Human PCSK9 expression levels in mouse serum were analyzed using an ELISA kit (R&D) as per the manufacturers' instructions.

F. Statistical analysis

[00079] All experiments were analyzed using one-way Analysis of Variance models with pair-wise group differences in mean cholesterol level assessed using Tukey's post-hoc tests. However, for experiments evaluating the effect of PCSK9 in C57BL/6 mice, a linear mixed effects model was used to assess group differences in cholesterol level while taking into account correlation between repeated measurements on the same mouse. Similarly, analysis of PCSK9 on AAV transduced hLDLR relied on Analysis of Covariance modeling, with post-cholesterol level regressed on pre-cholesterol level and group. Statistical significance was taken at the 0.05 level for all experiments.

Results

G. Amino acid substitutions in hLDLR confer PCSK9 resistance

[00080] Nine LDLR variants with potentially decreased binding to PCSK9 (N295D, D299N, H306G, V307D, N309A, D310N, L311T, L318D and L318H *see* Table 1, position numbers based on numbering of SEQ ID NO:1) were initially screened in HEK293 cells using an *in vitro* assay for uptake of fluorescently labeled-LDL (BODPIY-LDL™), in the presence or absence of hPCSK9.

[00081] Studies in HEK293 cells that have low levels of endogenous expression of hLDLR and hPCSK9 were performed. As a source of exogenous hPCSK9, cells were co-transfected with a plasmid expressing hPCSK9 along with the hLDLR constructs. Mock transfected cells expressed low levels of LDLR based on immunoblotting which failed to detect LDLR protein (data not shown); moreover, mock transfected cells failed to demonstrate uptake of BODIPY™-LDL (FIG 1A). In contrast, transient transfection of wild type hLDLR into HEK293 cells led to internalization of BODIPY™-LDL in 30% of cells which was reduced to 18% when co-transfected with hPCSK9 (FIG 1A). Among the mutant constructs co-expressed with hPCSK9, only the D299N and L311T amino acid substitutions failed to afford any protection to PCSK9 mediated degradation in that BODIPY™-LDL uptake was reduced to a similar extent as wild type LDLR. All other amino acid substitutions afforded varying degrees of protection from PCSK9, although some constructs were less efficient in BODIPY™-LDL uptake in the absence of PCSK9 when compared to wild-type hLDLR. As an example, although the L318D and L318H substitutions were both resistant to hPCSK9 degradation, only L318D showed normal BODIPY™-LDL uptake in the absence of PCSK9 (FIG 1B). In contrast, the L318H substitution led to reduced receptor activity and BODIPY™-LDL uptake was lower when compared to wild type hLDLR in the absence of hPCSK9 (30% vs 6%; hLDLR vs hLDLR-L318H). For this reason the hLDLR-L318D vector was selected for further *in vivo* evaluation in mice.

H. Overexpression of hPCSK9 in mice downregulates AAV expressed hLDLR

[00082] Evaluating the activity of wildtype and L318D forms of hLDLR in mice was complicated because of potential diminished interactions between the exogenous hLDLR protein and the endogenous mouse PCSK9 protein. A fully humanized mouse model with the hoFH phenotype (lacking LDLR and APOBEC-1 by virtue of germ line interruption) and overexpressing hPCSK9 [following i.v. injection of an AAV9 vector expressing hPCSK9 via the liver specific promoter TBG (AAV9.hPCSK9)] was created (the LDLR-/-, ApoBec -/- double knock-out (DKO) mice described in Part A of this Example. Expression of AAV9.hPCSK9 vector was first evaluated in C57BL/6 mice who received increasing doses of hPCSK9. At high dose vector (*i.e.*, $5x10^{10}$ GC) serum non-HDL cholesterol increased

approximately 2.5-fold (p=0.0015), indicating some level of interaction between hPCSK9 and mLDLR. See, FIG. 1C.

[00083] Prior to evaluating the effects of hPCSK9 on transgene derived hLDLR, the hoFH DKO mice were injected with AAV8.hLDLR alone. In these animals, baseline non-HDL levels on a chow diet were 417±23 mg/dl; which decreased by day 7 following administration of $5x10^{10}$ GC of AAV8.hLDLR. Non-HDL levels stabilized and were only 37±7 mg/dl by day 30 which was 9% of baseline levels (p=0.037, FIG 2A). Next, the performance of this vector in DKO mice expressing hPCSK9 were evaluated by co-administering (i.v.) an equal dose ($5x10^{10}$ GC) of AAV9.hPCSK9 along with AAV8.hLDLR. Following vector administration, serum levels of hPCSK9 rose steadily and reached peak levels (7500 ± 3000 ng/mL) by day 30 (FIG 2B). Concomitantly, non-HDL levels in mice co-transduced with hPCSK9 were significantly higher (p=0.0008) when compared to animals that only received hLDLR (FIG 2A). AAV8.hLDLR reduced non-HDL 10-fold in the absence of hPCSK9; however, this reduction was only 2.5-fold in the presence of hPCSK9. Immunoblotting of total liver lysates confirmed that co-transduction with PCSK9 resulted in reduced hLDLR protein in the liver (FIG 2C); whereas, levels of hLDLR messenger RNA remained unchanged between the experimental groups (data not shown). These findings are consistent with the reported mode of action of PCSK9 to bind and sequester LDLR in an intracellular compartment that increases receptor degradation [Wang, et al, J Lipid Res, 2012; 53: 1932-1943]. No reduction in hLDLR expression was observed in animals co-transduced with an AAV9 vector expressing an irrelevant transgene.

I. THE LDLR-L318D amino acid substitution confers resistance to human PCSK9 mediated degradation

[00084] A similar strategy was used to evaluate the activity of hLDLR-L318D in DKO mice overexpressing hPCSK9 and compared the results to mice transduced with wild type hLDLR. As expected, transduction with hLDLR resulted in a dramatic lowering of serum cholesterol by day 30 (10% of baseline); while, co-transduction with hPCSK9 resulted in reduced hLDLR activity with non-HDL cholesterol levels only 23% of baseline (p<0.0001, FIG 3A). In contrast, the L318D substitution apparently prevented receptor degradation in that differences in non-HDL levels between animals that received hLDLR-L318D or hLDLR-

L318D along with hPCSK9 was not statistically significant (10% vs 14%; p=0.1337). Moreover, immunoblotting of livers collected at the end of the study (Day 30) revealed that hLDLR protein levels were significantly decreased only in animals that received wild-type hLDLR along with hPCSK9 but not in those that received hLDLR alone (FIG 3B). However, liver levels of hLDLR-L318D were unaffected by co-expression with hPCSK9 and the same as observed with wild type hLDLR in the absence of hPCSK9 (FIG 3B). To confirm that the observed differences did not arise from changes in mRNA expression, hLDLR transcripts were analyzed in livers using a quantitative PCR assay. These studies indicated only a modest decrease in wild type hLDLR treated mice that was substantially less than the decrease in hLDLR protein (FIG 3B).

J. hLDLR-K809R\C818A escapes hIDOL regulation

[00085] LDLR expression is also subject to regulation by IDOL; an E3 ubiquitin ligase transcriptionally upregulated by liver X receptors (LXRs) following an increase in intracellular concentrations of oxysterols. Activated IDOL interacts with the cytoplasmic tail region of LDLR leading to receptor degradation [Zhang L, et al, Arterioscler Thromb Vasc Biol. 2012;32:2541-2546]. An AAV8 vector expressing hLDLR containing the K809R and C818A amino acid substitutions (AAV8.hLDLR-K808R\C818A) was constructed. This construct was first evaluated in HEK293 cells in the presence or absence of hIDOL; as a source of human IDOL, plasmids expressing hIDOL were co-expressed with hLDLR. As expected, transfection of wild type hLDLR resulted in LDL uptake in 28% of cells; however, co-transfection of hIDOL along with hLDLR dramatically reduced LDL positive cells to only 2% (FIG. 4A). The K808R\C818A amino acid substitutions did not impact receptor activity and the LDLR-K809R\C818A construct was as efficient as wild type hLDLR in internalizing LDL, in the absence of IDOL (LDLR vs LDLR-K809R\C818A, 28% vs 22%). However, differences between the two constructs did appear when co-transfected with hIDOL. The hLDLR-K809R\C818A construct was more resistant to the effects of hIDOL resulting in roughly 14% of cells taking up fluorescent LDL as opposed to 2% with wild type LDLR. Immunoblotting of whole cell lysates further confirmed that the observed differences in LDL uptake correlated with reduced levels of hLDLR protein, and not hLDLR-K809R\C818A, in the presence of hIDOL (FIG 4A).

[00086] Next the activity of the hLDLR-K809R\C818A construct in DKO mice overexpressing human IDOL was evaluated. A phenotype of mice overexpressing human IDOL in liver was created by administering an AAV9 vector expressing human IDOL under control of a liver specific promoter. In pilot studies the efficacy of human IDOL in regulating endogenous LDLR expression was evaluated in mice by administering (i.v.) $5x10^{10}$ GC of AAV9.hIDOL to FH mice heterozygous for LDLR expression (heFH). This strain of mice (LAHB mice) is deficient in *APOBEC-1*, heterozygous for mouse $LDLR^{+/-}$ and transgenic for human *ApoB100* which leads to higher serum cholesterol. Following administration of AAV9.hIDOL, non-HDL levels increased by day 7 and reached stable levels by day 30 ($p<0.0001$, FIG 4B). These results confirmed that AAV expressed hIDOL was active in mouse livers and can cause the loss of endogenous mLDLR. Next, the effect of hIDOL overexpression on vector encoded hLDLR was expressed in DKO mice. In pilot studies only low dose hLDLR vector administrations were significantly impacted by human IDOL; hence, mice were co-administered $3x10^{9}$ GC of AAV8.hLDLR and $5x10^{10}$ GC of AAV9.hIDOL. At this low dose, hLDLR and hLDLR-K809\C818A vectors were functionally similar ($p=0.9$) and induced a modest reduction (20% of baseline) in serum cholesterol in the absence of hIDOL (FIG 4C). However, co-administration of hIDOL ablated wild type hLDLR activity and no correction was seen in non-HDL cholesterol levels which remained at pre-treatment baseline levels ($p=0.0248$, FIG 4C). In contrast, non-HDL cholesterol levels in mice that received hLDLR-K809R\C818A in the presence or absence of hIDOL were similar ($p>0.05$) demonstrating the *in vivo* resistance of the modified constructs to hIDOL (FIG 4C).

Example 2 - hLDLR-L318D\K809R\C818A avoids regulation by both PCSK9 and IDOL

[00087] The L318D, K809R and C818A amino acid substitutions were cloned into a single vector to create a construct that would be resistant to regulation by both pathways. The vector was administered to DKO mice at a low dose ($3x10^{9}$ GC), when evaluating the IDOL escape mutations; or at a higher dose ($5x10^{10}$ GC), when evaluating the PCSK9 escape mutation. When administered at a low dose, hLDLR-L318D\K809R\C818A was comparable to wild type hLDLR ($p>0.05$) in that only a modest decrease in serum cholesterol was realized following either vector administration (FIG 5A). However when administered in the presence of hIDOL, only the mutant vector showed any resistance to hIDOL in that serum

cholesterol levels remained significantly lower than that seen in wild type hLDLR plus hIDOL (p=0.0002). Immunoblotting of liver samples confirmed that the mutant vector was more resistant to hIDOL mediated degradation (FIG 5A). In the parallel study where vectors were administered at a higher dose along with hPCSK9, the variant protein performed significantly better in reducing serum cholesterol than the control wild type LDLR in mice overexpressing hPCSK9 (p=0.0007, FIG 5B). Immunoblot analysis of livers demonstrated a nearly complete absence of wild type hLDLR in the presence of hPCSK9; in contrast, the mutant vector was protected and less degraded by hPCSK9.

Example 3 - Comparison of hLDLR variants in a mouse model of familial hypercholesterolemia.

[00088] The panel of hLDLR carrying single amino acid substitutions that were expected to avoid PCSK9 regulation were screened by administering to LDLR-/-, APOBEC-/- double knockout mice (DKOs). Animals were injected intravenously (i.v. tail vein) with $3x10^{10}$ GC of AAV8.TBG.hLDLR or one of the hLDLR variants that was expected to avoid hPCSK9 regulation. Reduction in serum levels of non-HDL cholesterol was used as a surrogate for comparing receptor activity from the different constructs. Serum was collected from animals by retro-orbital bleeds before and 30 days after vector administration and cholesterol levels analyzed using a MIRA analyzer (Roche). Non-HDL cholesterol levels were determined by subtracting the HDL component from total cholesterol. FIG 6 shows percent decline in non-HDL levels over baseline in animals following vector administration.

[00089] This study was repeated under the same conditions, with the exception that vector administered at a higher dose, *i.e.,* 5 x 10^{10} GC of AAV8.TBGF.hLDLR for each of variants (L318D, N295D, H306G, V307D, N309A, D310N, L311T, L318H) . Administration of 5 x 10^{10} GC of wild type hLDLR by itself led to a 90% decrease in baseline non-HDL cholesterol levels (Figure 8). With the exception of D299N, all other hLDLR variants also achieved similar reduction in non-HDL cholesterol. As expected, coadministration of hPCSK9 significantly reduced the efficacy of hLDLR vector. hPCSK9 overexpression had only a minimal effect on variants, L318D, N295D, H306G, V307D and N309A. Furthermore, immunoblotting of day 30 livers confirmed that with the exception of the H306G, these variants were significantly protected from degradation (not shown).

Example 4 - High dose AAV.hLDLR administration circumvents *in vivo* IDOL inhibition.

[00090] LDLR-/-, APOBEC-/- double knockout mice (DKOs) were injected with AAV8.TBG.hLDLR or AAV8.TBG.K809R\C818A at a dose of $5x10^{10}$ GC. In addition, some groups of mice were co-administered with an equal dose of an AAV9 vector expressing human IDOL (AAV9.TBG.hIDOL) to evaluate hLDLR activity in the presence of hIDOL. Non-HDL cholesterol levels were analyzed before and 30 days after vector administration. The percent non-HDL cholesterol at day 30 compared to baseline following vector administration is shown in FIG. 7.

[00091] All publications cited in this specification are incorporated herein by reference, as are US Provisional Patent Application Nos. 62/022,627, filed July 9, 2014 and 61/984,620, filed April 25, 2014. Similarly, the SEQ ID NOs which are referenced herein and which appear in the appended Sequence Listing are incorporated by reference. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

(Sequence Listing Free Text)

[00092] The following information is provided for sequences containing free text under numeric identifier <223>.

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13	<223> Synthetic Construct

SEQ ID NO: (containing free text)	Free text under <223>
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SEQ ID NO: (containing free text)	Free text under <223>
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SEQ ID NO: (containing free text)	Free text under <223>
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13	<223> Synthetic Construct
14	Synthetic plasmid with hLDLR (K25K, C29A).PGT(P2701)Q <220> <221> repeat_region <222> (1)..(130) <223> 5' ITR <220> <221> enhancer <222> (221)..(320) <223> alpha mic/bik <220> <221> enhancer <222> (327)..(426) <223> alpha mic/bik <220> <221> promoter <222> (442)..(901) <223> TBG promoter <220> <221> TATA_signal <222> (885)..(888) <220> <221> Intron <222> (1027)..(1159) <223> Intron 1 <220> <221> CDS <222> (1245)..(3827) <223> hLDLR with K25R-C29A variants

SEQ ID NO: (containing free text)	Free text under <223>
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SEQ ID NO: (containing free text)	Free text under <223>
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34	<223> Synthetic Construct
35	<223> synthetic hLDLR.K809R-818A with leader
36	<223> Synthetic Construct
37	<223> synthetic hLDLR (K25R, C29A, L318D) with leader
38	<223> Synthetic Construct
39	<223> synthetic K809R, C818A, L318D with leader
40	<223> Synthetic Construct

CLAIMS:

1. A recombinant vector having an expression cassette comprising a modified human low density lipoprotein receptor (hLDLR) gene, wherein said hLDLR gene encodes a modified hLDLR that reduces cholesterol following expression, and wherein said modified hLDLR comprises:

(a) one or more amino acid substitutions that interfere with the wild-type hLDLR IDOL pathway; and/or

(b) one or more amino acid substitutions which are resistant to degradation of hLDLR by interfering with the PCSK9 pathway.

2. The recombinant vector according to claim 1, wherein the modified hLDLR comprises (a) an amino acid substitution at one or more of N295, H306, V307, N309, D310, and/or L318, based on the numbering of SEQ ID NO:1.

3. The recombinant vector according to claim 2, wherein the hLDLR comprises one or more amino acid substitutions selected from: N295D, H306D, V307D, N309A, D310N, L318H, and/or L318D, based on the numbering of SEQ ID NO:1.

4. The recombinant vector according to claim 1, wherein the modified hLDLR comprises (b) an amino acid substitution of at least one of L769R, K809R and/or C818A, based on the numbering of SEQ ID NO:1, optionally in combination with an amino acid substitution of (a).

5. The recombinant vector according to any one of claims 1-4, wherein the vector is a recombinant adeno-associated virus (rAAV) vector.

6. The recombinant vector according to any one of claims 1-5, wherein the vector is a rAAV comprising a capsid selected from AAV8, rh64R1, AAV9, or rh10.

7. The recombinant vector according to any one of claims 1-6, wherein the expression cassette comprises a promoter which specifically directs expression of the modified hLDLr in liver cells.

8. A rAAV vector having an expression cassette comprising a modified hLDLR gene, wherein said hLDLR gene encodes a modified hLDLR comprising an L318D amino acid substitution, based on the numbering of SEQ ID NO:1.

9. The rAAV vector according to claim 8, wherein the modified hLDLR further comprises a K809R and/or C818A amino acid substitution, based on the numbering of SEQ ID NO:1.

10. The rAAV vector of claim 8 or 9, wherein the vector comprises a capsid selected from AAV8, rh64R1, AAV9, or rh10.

11. The rAAV vector according to any one of claims 8-10, wherein the expression cassette comprises a promoter which specifically directs expression of the modified hLDLr in liver cells.

12. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a recombinant vector according to any one of claims 1-11.

13. A method for reducing circulating cholesterol levels by administering to a subject in need thereof a recombinant vector according to any one of claims 1-6, wherein said expression cassette further comprises regulatory control sequences which direct expression of modified hLDLR in the subject.

14. A method for reducing circulating cholesterol levels by administering to a subject in need thereof a rAAV vector according to any one of claims 8-10, wherein said expression cassette further comprises regulatory control sequences which direct expression of modified hLDLR in the subject.

15. A method for increasing the circulating half-life of a hLDLR comprising modifying the hLDLR at one or more amino acid positions selected from: N295, H306, V307, N309, D310, and/or L318, based on the numbering of SEQ ID NO:1.

16. The method of claim 15, wherein the hLDLR is modified to comprise one or more amino acid substitutions selected from: N295D, H306D, V307D, N309A, D310N, L318H, and/or L318D. , based on the numbering of SEQ ID NO:1

17. The method according to claim 15 or 16, wherein the hLDLR is further modified to comprise a K769R, K809R and/or C818A amino acid substitution, based on the numbering of SEQ ID NO:1.

18. A recombinant vector according to any one of claims 1-7 for use in a regimen for treating familial hypercholesterolemia.

19. A rAAV vector according to any one of claims 8-11 for use in a regimen for treating familial hypercholesterolemia.

20. A synthetic or recombinant hLDLR comprising:

(a) an amino acid substitution at one or more of N295, H306, V307, N309, D310, L318, and/or L318, based on the numbering of SEQ ID NO:1.; or

(b) an amino acid substitution of any of (a) in combination with an amino acid substitution of at least one of K769R, K809R and/or C818A, based on the numbering of SEQ ID NO:1.

21. The synthetic or recombinant hLDLR of claim 20, wherein the one or more amino acid substitutions of (a) are selected from: N295D, H306D, V307D, N309A, D310N, L318H, and/or L318D, based on the numbering of SEQ ID NO:1.

22. A pharmaceutical composition comprising a synthetic or recombinant hLDLR protein according to claim 20 or 21 and a pharmaceutically acceptable carrier.

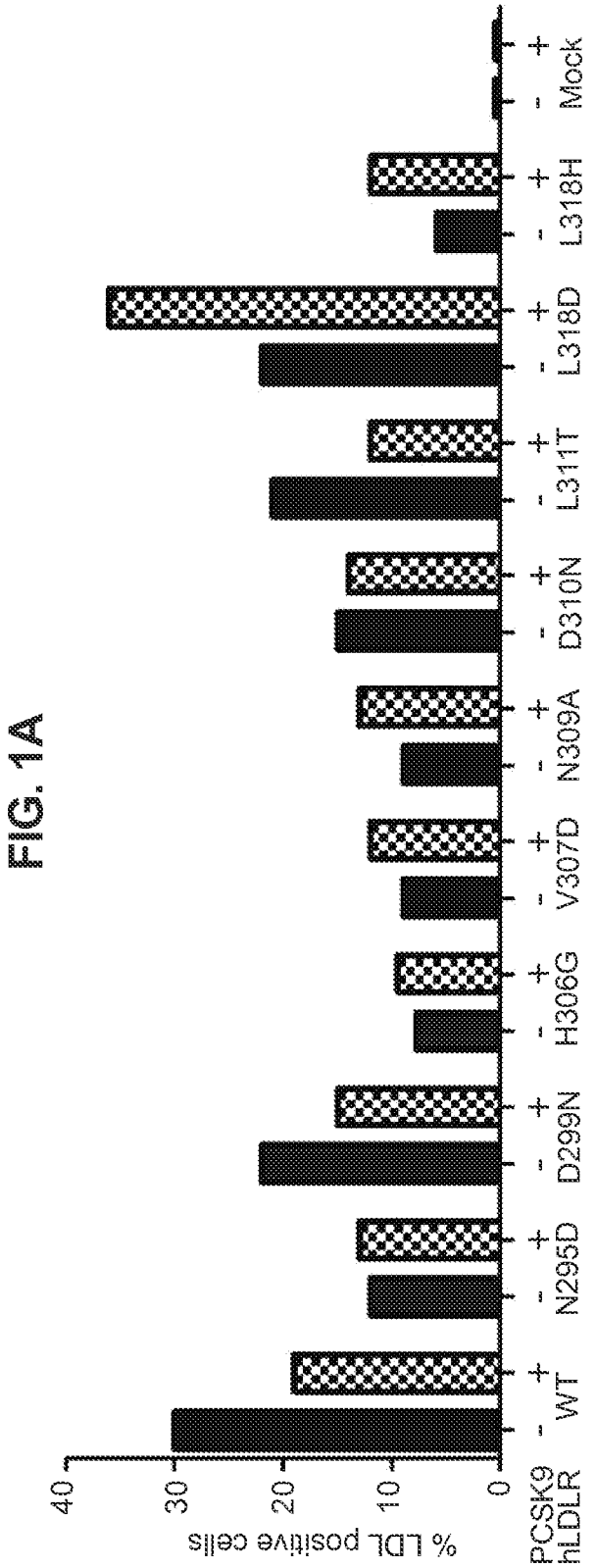


FIG. 1A

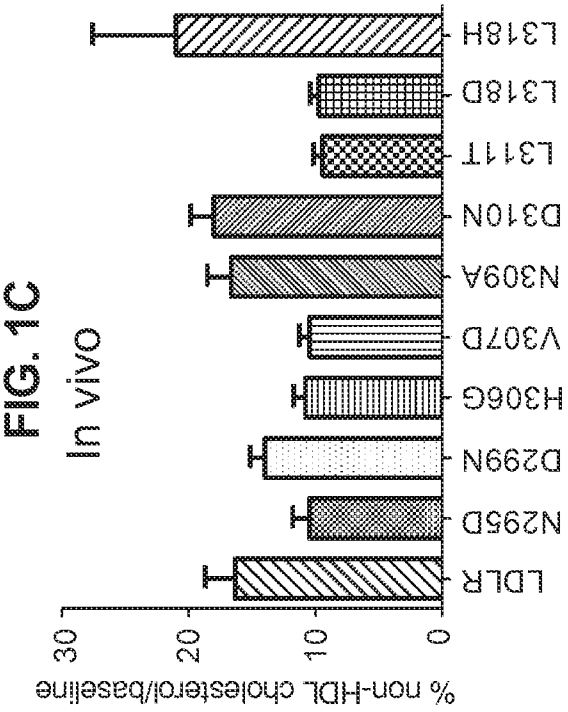


FIG. 1C
In vivo

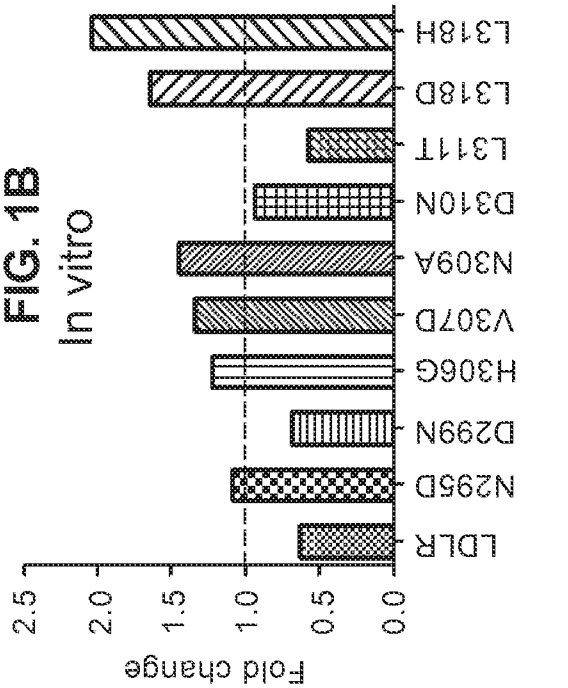


FIG. 1B
In vitro

FIG. 2A

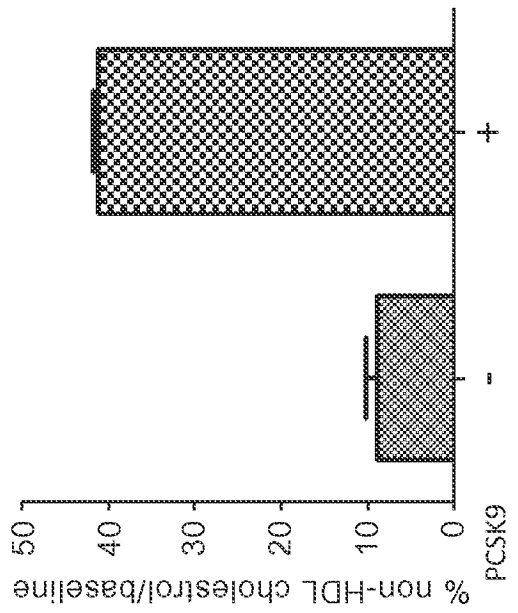


FIG. 2B

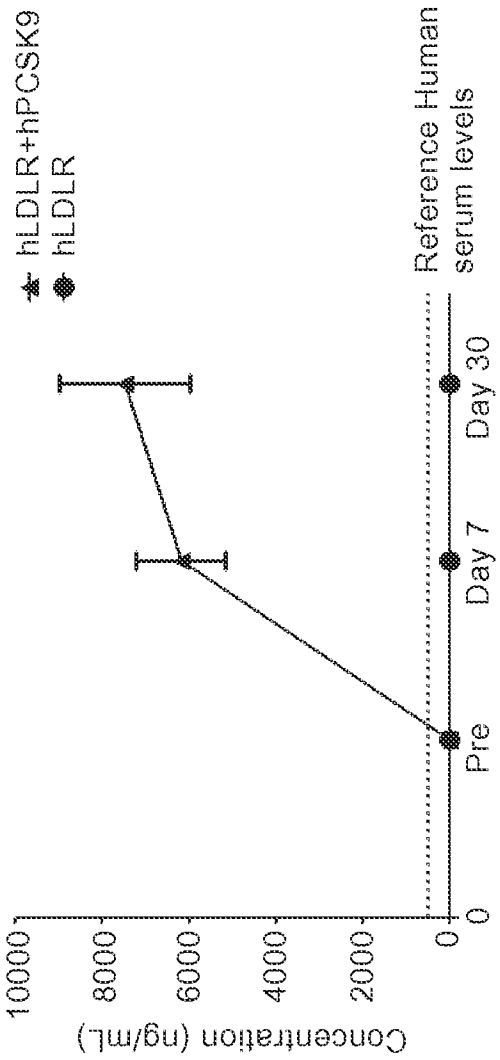
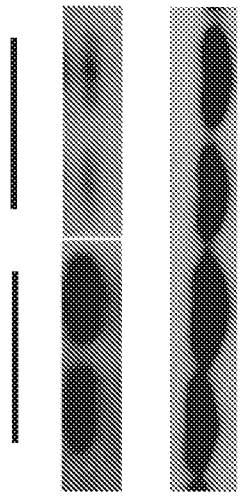


FIG. 2C



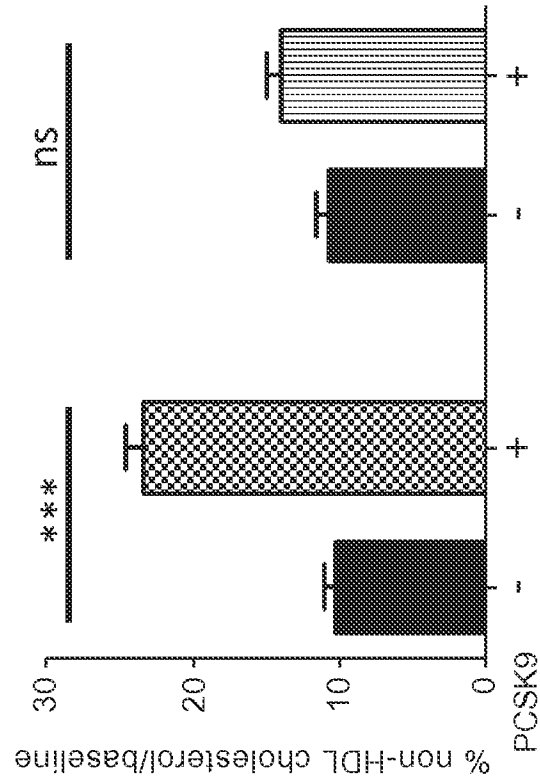

% non-HDL cholesterol/baseline
30
20
10
0
***
ns
PCSK9
-
+
-
+
LDLR
LDLR-L318D

FIG. 3A

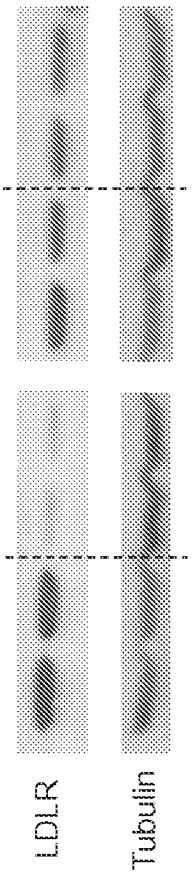

LDLR
Tubulin

FIG. 3B

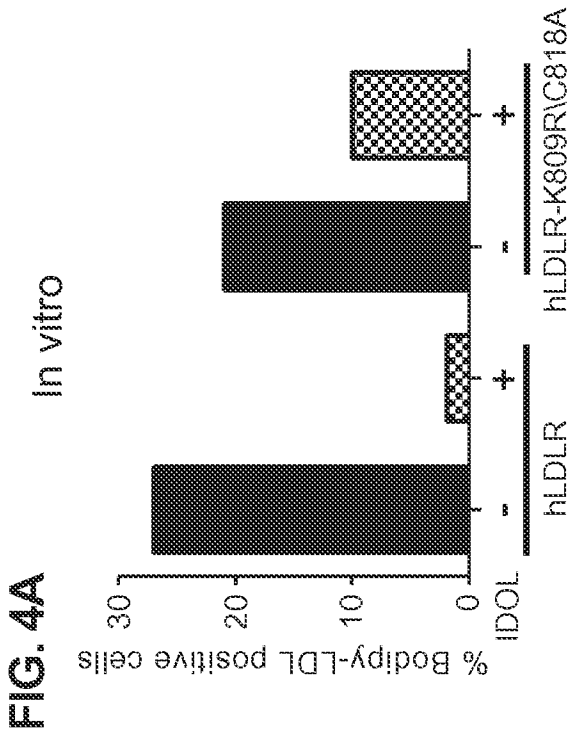


FIG. 4A
In vitro
% Bodipy-LDL positive cells
30
20
10
0
IDOL - + - +
hLDLR - - + +
hLDLR-K809R\C818A

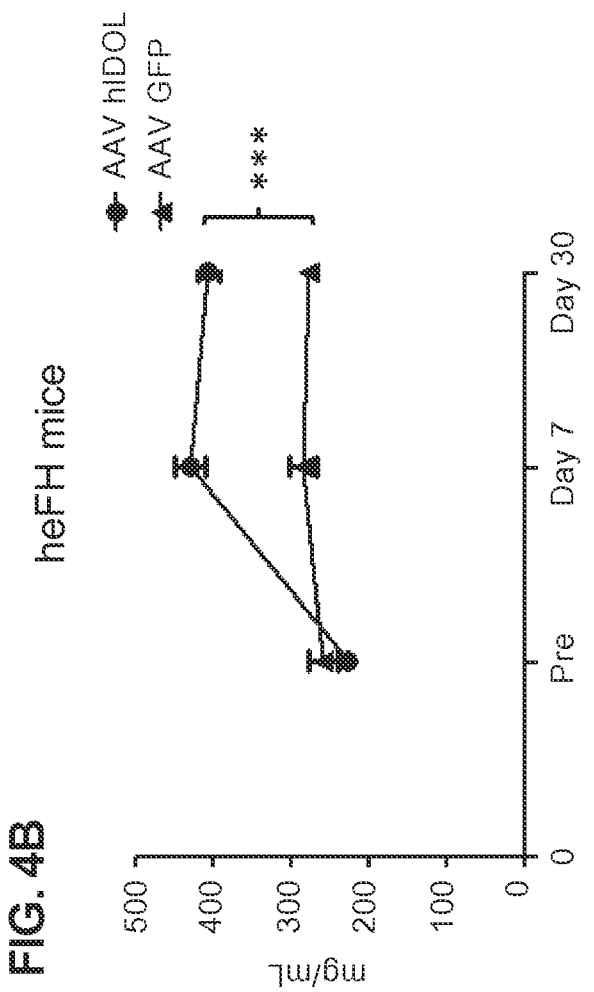


FIG. 4B
heFH mice
AAV hIDOL
AAV GFP
***
mg/mL
500
400
300
200
100
0
Pre
Day 7
Day 30

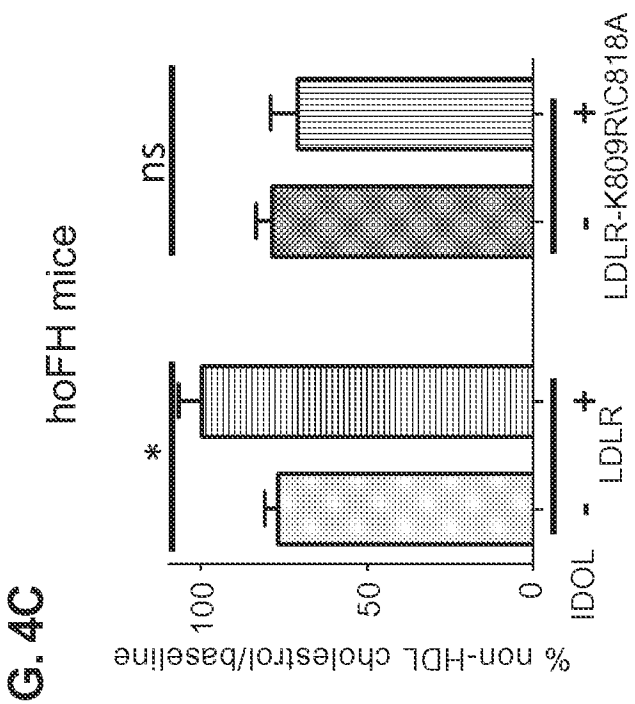


FIG. 4C
hoFH mice
ns
*
% non-HDL cholesterol/baseline
100
50
0
IDOL - + - +
LDLR - - + +
LDLR-K809R\C818A

FIG. 4D
M
P
Tubulin

FIG. 5A

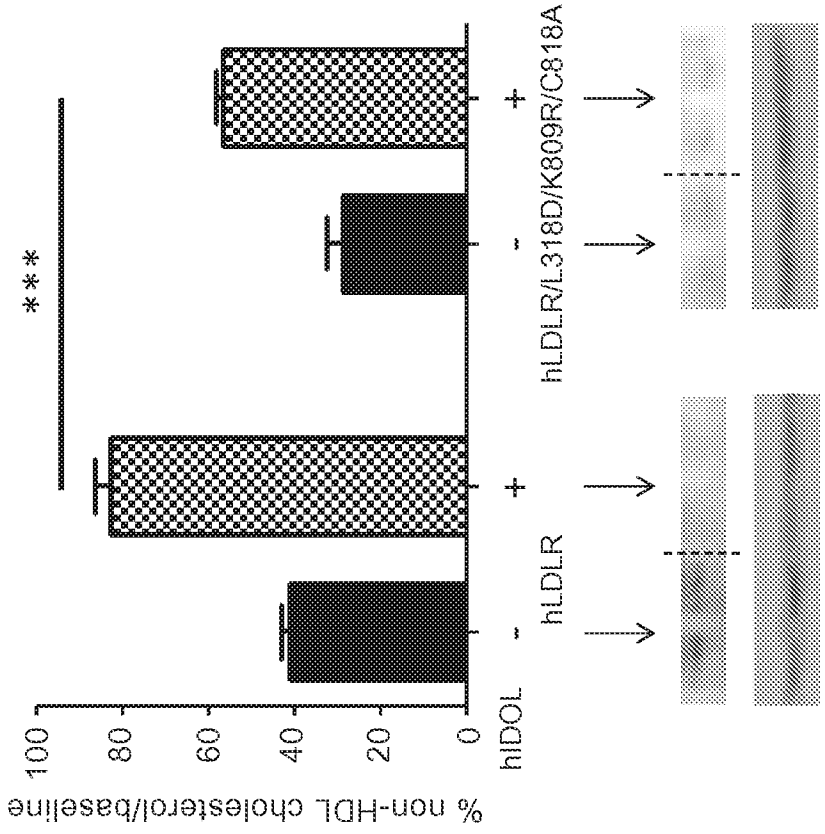

% non-HDL cholesterol/baseline
100
80
60
40
20
0
***
hIDOL - + - +
hLDLR
hLDLR/L318D/K809R/C818A

FIG. 5B

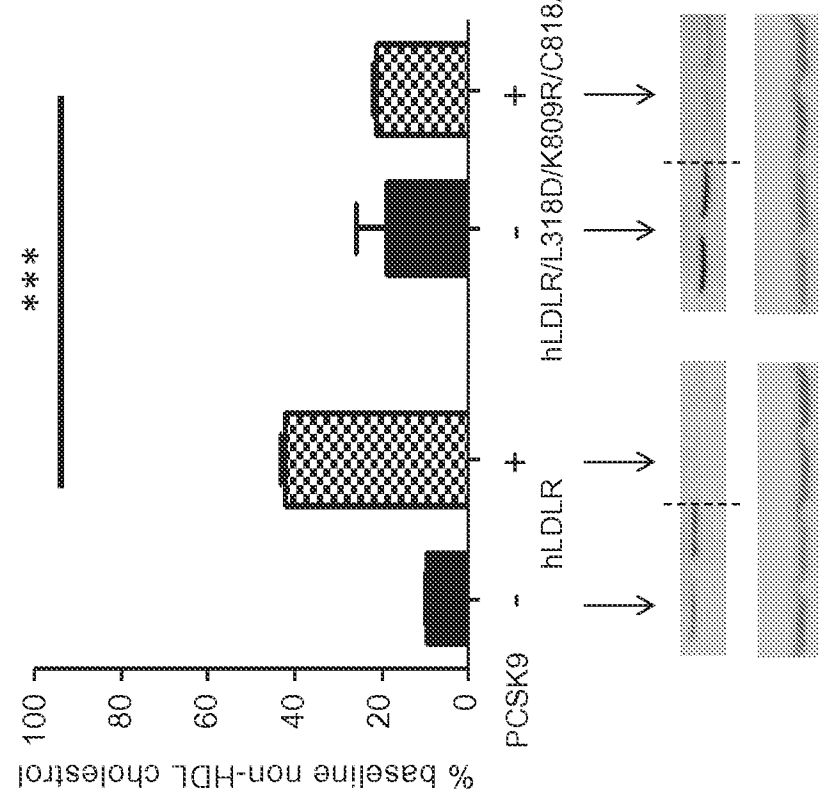

% baseline non-HDL cholesterol
100
80
60
40
20
0
***
PCSK9 - + - +
hLDLR
hLDLR/L318D/K809R/C818A

FIG. 6

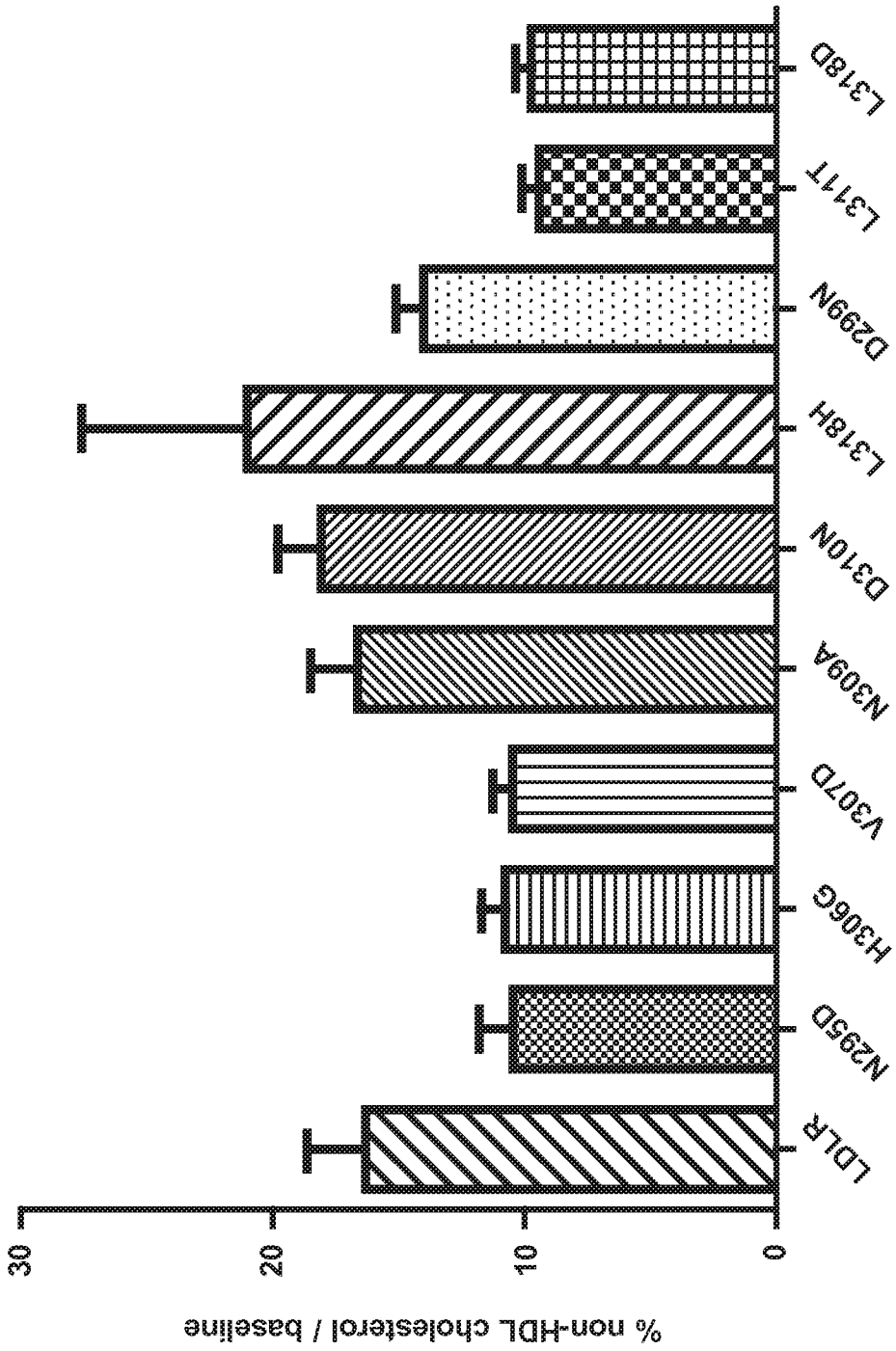

% non-HDL cholesterol / baseline
0
10
20
30
LDLR
N295D
H306G
V307D
N309A
D310N
L318H
D299N
L311T
L318D

FIG. 7

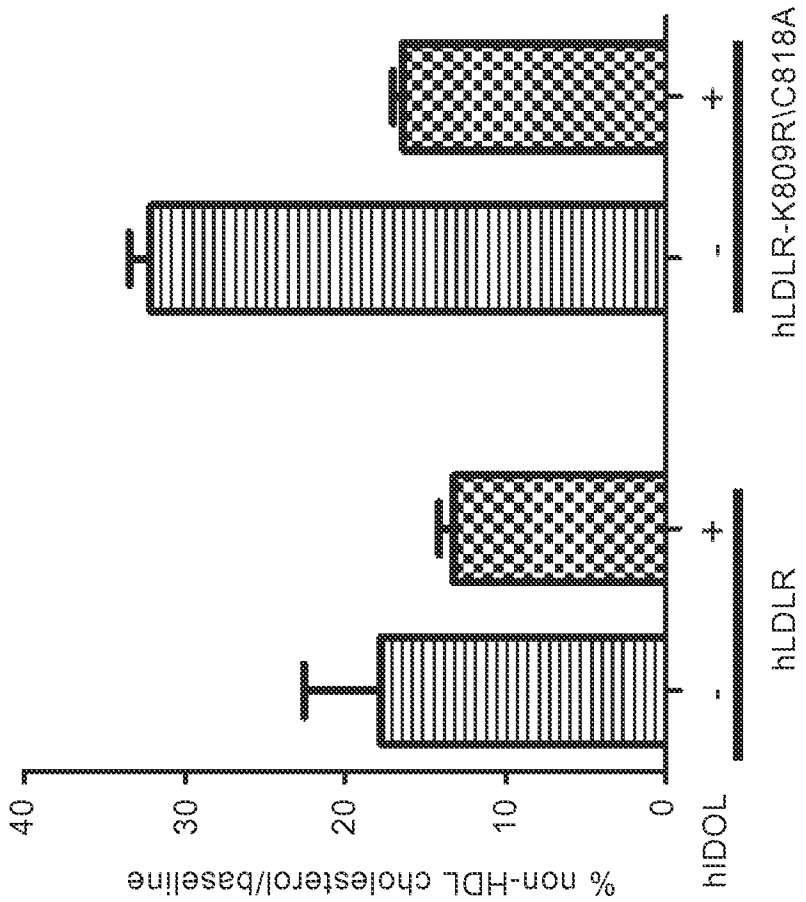
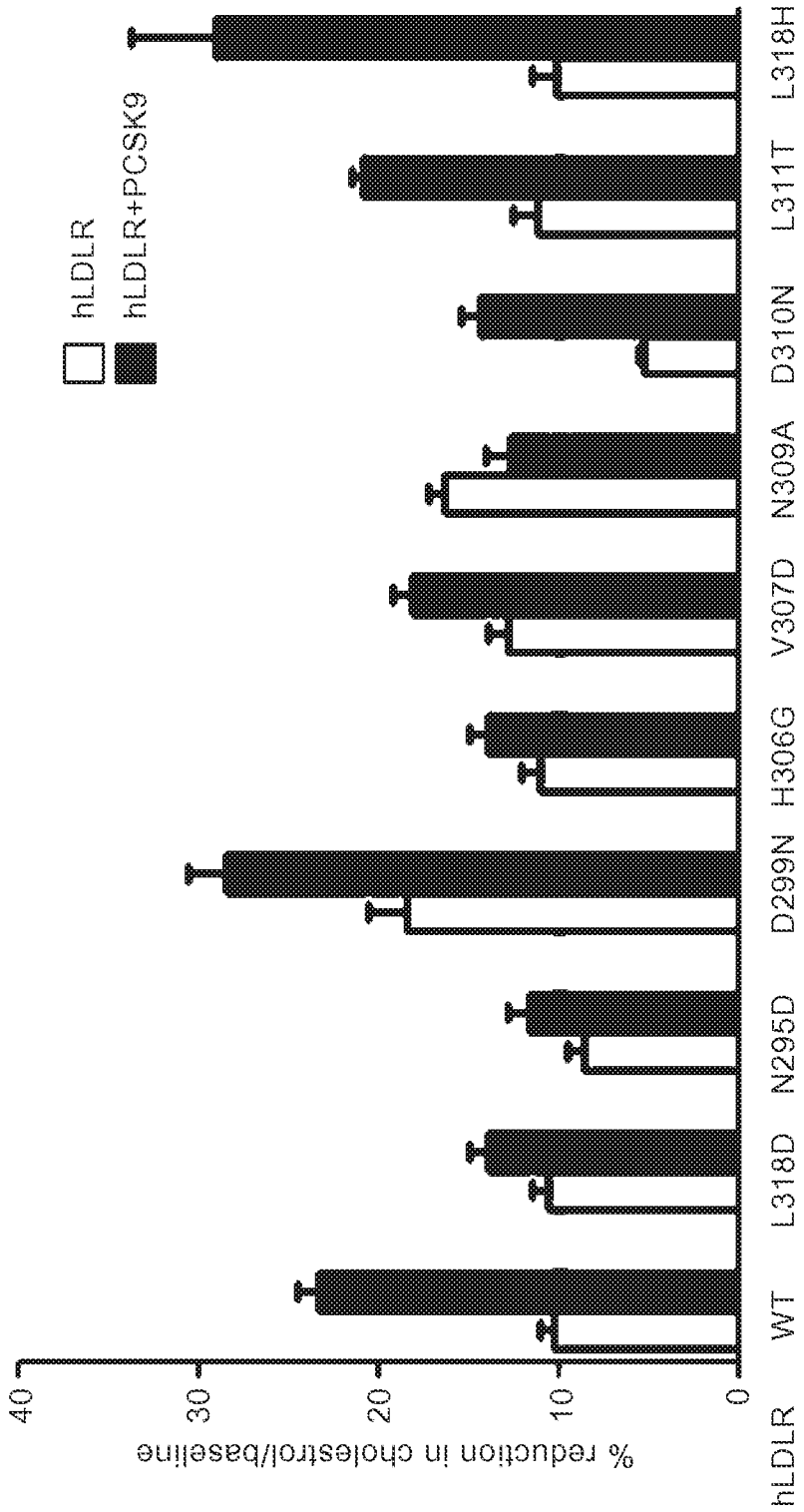

% non-HDL cholesterol/baseline
0
10
20
30
40
hIDOL
-
+
hLDLR
-
+
hLDLR-K809R\C818A

FIG. 8


hLDLR
hLDLR+PCSK9
% reduction in cholesterol/baseline
0
10
20
30
40
hLDLR
WT
L318D
N295D
D299N
H306G
V307D
N309A
D310N
L311T
L318H

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/027572

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705 A61K48/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NOAM ZELCER ET AL: "LXR Regulates Cholesterol Uptake Through Idol-Dependent Ubiquitination of the LDL Receptor", SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 325, 3 July 2009 (2009-07-03), pages 100-104, XP008150976, ISSN: 0036-8075 cited in the application	1,4,12, 20
Y	figure 4	5-7,13, 18,19
A		9,10,17
	----- -/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 July 2015	16/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Deleu, Laurent

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/027572

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	H.-M. GU ET AL: "Characterization of the role of EGF-A of low density lipoprotein receptor in PCSK9 binding", THE JOURNAL OF LIPID RESEARCH, vol. 54, no. 12, December 2013 (2013-12), pages 3345-3357, XP055199978, ISSN: 0022-2275, DOI: 10.1194/jlr.M041129	1-3,12, 20,21
A	abstract page 3346, left-hand column page 3354; figure 3	8-10, 14-17

X	WO 2012/177741 A1 (GENENTECH INC [US]; HOFFMANN LA ROCHE [CH]; KIRCHHOFER DANIEL [US]; LI) 27 December 2012 (2012-12-27)	20,22
Y	claims 1-9, 18-25	1,2,5-7, 13,18,19

Y	AL-ALLAF FAISAL A ET AL: "LDLR-Gene therapy for familial hypercholesterolaemia: problems, progress, and perspectives", INTERNATIONAL ARCHIVES OF MEDICINE, BIOMED CENTRAL LTD, LONDON UK, vol. 3, no. 1, 13 December 2010 (2010-12-13), page 36, XP021090935, ISSN: 1755-7682, DOI: 10.1186/1755-7682-3-36 page 12 - page 16	1,2,5-7, 13,18,19

A	JOHN MILLAR ET AL: "Overview of the LDL receptor: relevance to cholesterol metabolism and future approaches for the treatment of coronary heart disease", JOURNAL OF RECEPTOR, LIGAND AND CHANNEL RESEARCH, December 2009 (2009-12), pages 1-1, XP055200129, DOI: 10.2147/JRLCR.S6033 page 4 - page 6	1-22

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DA-WEI ZHANG ET AL: "Binding of Proprotein Convertase Subtilisin/Kexin Type 9 to Epidermal Growth Factor-like Repeat A of Low Density Lipoprotein Receptor Decreases Receptor Recycling and Increases Degradation*", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol. 282, no. 25, 22 June 2007 (2007-06-22), pages 18602-18612, XP007912352, ISSN: 0021-9258, DOI: 10.1074/JBC.M702027200 [retrieved on 2007-04-23] page 18607 - page 18611	8-10, 14-17
X,P	WO 2015/051214 A1 (MODERNA THERAPEUTICS INC [US]) 9 April 2015 (2015-04-09) claim 9	1-3,12, 13,15, 18-22
X,P	SOMANATHAN SURYANARAYAN ET AL: "AAV vectors expressing LDLR gain-of-function variants demonstrate increased efficacy in mouse models of familial hypercholesterolemia.", CIRCULATION RESEARCH, vol. 115, no. 6, 29 August 2014 (2014-08-29), pages 591-599, XP009185161, ISSN: 1524-4571 page 597 - page 598; table 1	1-22

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/027572

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012177741 A1	27-12-2012	CA 2837658 A1	27-12-2012
		CN 103717615 A	09-04-2014
		EP 2721063 A1	23-04-2014
		JP 2014519848 A	21-08-2014
		KR 20140041747 A	04-04-2014
		US 2014212431 A1	31-07-2014
		WO 2012177741 A1	27-12-2012
WO 2015051214 A1	09-04-2015	NONE	