AAV Vectors Expressing LDLR Gain-of-Function Variants Demonstrate Increased Efficacy in Mouse Models of Familial Hypercholesterolemia

Suryanarayan Somanathan, Frank Jacobs, Qiang Wang, Alexandra L. Hanlon, James M. Wilson, Daniel J. Rader

**Rationale:** Familial hypercholesterolemia is a genetic disorder that arises because of loss-of-function mutations in the low-density lipoprotein receptor (LDLR) and homozygous familial hypercholesterolemia is a candidate for gene therapy using adeno-associated viral vectors. Proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of LDLR (IDOL) negatively regulate LDLR protein and could dampen adeno-associated viral vector encoded LDLR expression.

**Objective:** We sought to create vectors expressing gain-of-function human LDLR variants that are resistant to degradation by human PCSK9 (hPCSK9) and IDOL and thereby enhance hepatic LDLR protein abundance and plasma LDL cholesterol reduction.

**Methods and Results:** Amino acid substitutions were introduced into the coding sequence of human LDLR cDNA to reduce interaction with hPCSK9 and human IDOL. A panel of mutant human LDLRs was initially screened in vitro for escape from PCSK9. The variant human LDLR-L318D was further evaluated using a mouse model of homozygous familial hypercholesterolemia lacking endogenous LDLR and apolipoprotein B mRNA editing enzyme catalytic, APOBEC-1 (double knockout). Administration of wild-type human LDLR to double knockout mice, expressing hPCSK9, led to diminished LDLR activity. However, LDLR-L318D was resistant to hPCSK9-mediated degradation and effectively reduced cholesterol levels. Similarly, the LDLR-K809R/C818A construct avoided human IDOL regulation and achieved stable reductions in serum cholesterol. An adeno-associated viral vector serotype 8.LDLR-L318DK809RC818A vector that carried all 3 amino acid substitutions conferred partial resistance to both hPCSK9- and human IDOL-mediated degradation.

**Conclusions:** Amino acid substitutions in the human LDLR confer partial resistance to PCSK9 and IDOL regulatory pathways with improved reduction in cholesterol levels and improve on a potential gene therapeutic approach to treat homozygous familial hypercholesterolemia subjects. (Circ Res. 2014;115:591-599.)

**Key Words:** genetic therapy ■ hyperlipoproteinemia type II ■ low-density lipoprotein receptor, human ■ PCSK9 protein, human

Familial hypercholesterolemia (FH) is an autosomal codominant disorder characterized by the absence of the receptor for low-density lipoproteins (LDLR), a single chain glycoprotein containing 839 amino acids in its mature form. Patients with 1 abnormal allele (heterozygous FH) have moderate elevations in plasma LDL and have premature coronary artery disease, whereas patients with homozygous FH (hoFH) have high serum LDL-C concentrations that cause early onset of life-threatening cardiovascular disease. Current treatment options for hoFH include treatment with cholesterol-lowering drugs, LDL apheresis, and orthotopic liver transplantation, all of which have substantial limitations.

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Liver-directed gene therapy using adeno-associated viral vectors (AAV) has been demonstrated in preclinical models to correct several metabolic disorders stably and is currently being pursued in clinical trials for treatment of hemophilia A and B, ornithine transcarbamylase deficiency, and alpha-1-antitrypsin deficiency. Recently, we have demonstrated the
Nonstandard Abbreviations and Acronyms

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<th>Abbreviation</th>
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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
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<tr>
<td>DKO</td>
<td>double knockout</td>
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<td>FH</td>
<td>familial hypercholesterolemia</td>
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<td>hoFH</td>
<td>homozygous FH</td>
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<td>HDL</td>
<td>high-density lipoproteins</td>
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<td>hIDOL</td>
<td>human inducible degrader of LDLR</td>
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<td>hLDLR</td>
<td>human low-density lipoprotein receptor</td>
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<tr>
<td>hPCSK9</td>
<td>human proprotein convertase subtilisin/kexin type 9</td>
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<td>LDL-C</td>
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effectiveness of AAV-mediated gene therapy in correcting serum cholesterol levels in humanized mouse models of FH. 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 more than a year and led to the regression of preexisting atherosclerosis. However, AAV8.LDLR transduction was dose dependent, and statistically significant correction was only achieved at a vector dose of ≥1.5×10^{11} GC/kg. For clinical gene therapy, minimizing the vector dose is critical for many reasons, including vector toxicity, immune response, and manufacturing constraints.

Hepatic LDLR expression is modulated by multiple pathways within the cell: LDLR transcription is regulated by the serum response element binding proteins, and HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase inhibitors (statins) activate serum response element binding proteins by inhibiting cholesterol synthesis within hepatocytes. A second pathway of LDLR regulation, involving proprotein convertase subtilisin/kexin type 9 (PCSK9), was discovered based on human genetic gain-of-function mutations that caused high LDL-C levels and loss-of-function mutations that caused low LDL-C levels. Importantly, the loss of PCSK9 function was associated with a 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.

A third pathway of LDLR regulation was discovered by Zelcer et al. who demonstrated the degradation of LDLR by inducible degrader of LDLR (IDOL). An E3 ubiquitin ligase, IDOL, was induced after the activation of liver X receptors 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.

It is likely that AAV8.LDLR expression is influenced by multiple regulatory pathways that control receptor expression. Importantly, patients with FH have significantly higher plasma levels of PCSK9. The negative effect of post-transcriptional receptor regulation may be more pronounced when transduction efficiencies are suboptimal or when administering vector at lower doses, which is the expected results in humans with current AAV technology. In the present study, we evaluated the hypothesis that engineered variants of human LDLR with reduced affinity for PCSK9 or IDOL are more effective in lowering cholesterol in hoFH mice stably expressing human PCSK9 or human IDOL.

Methods
Animal studies were performed in accordance with the University of Pennsylvania, Institutional Review Board. LDLR^{−/−}, APOBEC-1^{−/−} double knockout (DKO) and LDLR^{−/−}, APOBEC-1^{−/−}, human ApoB100 transgenic mice were injected via tail vein for vector administration and serum collected by retro-orbital bleeds. At the end of study, animals were euthanized and livers were harvested for analysis. Vectors were obtained from the Vector Core at the University of Pennsylvania and expressed cDNAs for hLDLR, hPCSK9, or hIDOL driven from a thymoxine-binding globulin promoter. Mutations were introduced into wild-type hLDLR using the Quick Change lightning kit (Agilent). Serum cholesterol levels were analyzed on an MIRA analyzer (Roche). Western blotting was done using precast mini gels (Invitrogen) and probed using a polyclonal hLDLR antibody. In vitro LDLR assay was performed by transiently transfecting human embryonic kidney (HEK293) cells and pulsing the cells the following day with BODIPY-labeled LDL (Invitrogen) and probing with a polyclonal hLDLR antibody. In vitro LDLR assay was performed by transiently transfecting human embryonic kidney (HEK293) cells and pulsing the cells the following day with BODIPY-labeled LDL (Invitrogen) and probing with a polyclonal hLDLR antibody. In vitro LDLR assay was performed by transiently transfecting human embryonic kidney (HEK293) cells and pulsing the cells the following day with BODIPY-labeled LDL (Invitrogen) and probing with a polyclonal hLDLR antibody.

Table 1. Vectors Used in the Study

<table>
<thead>
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<td>AAV8.hLDLR</td>
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<td>AAV8.hLDLR-N295D</td>
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<td>AAV8.hLDLR-D299N</td>
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<td>AAV8.hLDLR-H306G</td>
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<td>AAV8.hLDLR-V307D</td>
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<td>AAV8.hLDLR-N309A</td>
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<td>AAV8.hLDLR-D310N</td>
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<td>AAV8.hLDLR-L311T</td>
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<td>AAV8.hLDLR-L318D</td>
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<td>AAV8.hLDLR-L318H</td>
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<td>AAV8.hLDLR-K809R/C818A</td>
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<td>AAV8.hLDLR-L318D/K809R/C818A</td>
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<td>AAV9.hPCSK9</td>
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<td>AAV9.hIDOL</td>
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All vectors expressed the transgene from a thymoxine-binding globulin promoter and had a bovine growth hormone polyadenylation signal. hIDOL indicates human inducible degrader of LDLR; hLDLR, human low-density lipoprotein receptor; and hPCSK9, human proprotein convertase subtilisin/kexin type 9.

Results
Amino Acid Substitutions in hLDLR Confer PCSK9 Resistance
Nine LDLR variants with potentially decreased binding to PCSK9 (N295D, D299N, H306G, V307D, N309A, D310N, L311T, L318D, and L318H; Table 1) were initially screened in HEK293 cells using an in vitro assay for uptake of fluorescent boron-dipyrrromethene labeled-LDL (BODIPY-LDL) in the presence or in the absence of hPCSK9. A list of the amino acid substitutions and their role in reducing LDLR–PCSK9 interaction is presented in Table 2. We performed the studies in HEK293 cells that have low levels of endogenous expression of hLDLR and hPCSK9. As a source of exogenous hPCSK9, cells were cotransfected 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.
from PCSK9 although some constructs were less efficient in BODIPY-LDL uptake in the absence of PCSK9 when compared with 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 (Figure 1B). In contrast, the L318H substitution led to reduced receptor activity and BODIPY-LDL uptake was lower when compared with wild-type hLDLR in the absence of hPCSK9 (30% versus 6%; hLDLR versus hLDLR-L318H). Next, we evaluated all constructs in vivo using a humanized mouse model of hoFH phenotype (lacking LDLR and APOBEC-1 by virtue of germ-line interruption). Systemic administration of 3×10^{10} GC of AAV8 expressing wild-type human LDLR led to a decline in serum non–high-density lipoprotein (HDL) cholesterol by day 30 to 16% of baseline levels (Figure 1C). Mice administered with the novel AAV8.hLDLR variants also demonstrated reductions in non-HDL levels that varied from 10% to 20% of baseline. Interestingly, variants that did not perform well in the in vitro assay were still efficient when administered in vivo (eg, D295D). However, on the basis of in vitro and in vivo studies, we decided to investigate only the L318D variant further.

**Overexpression of hPCSK9 in Mice Downregulates AAV-Expressed hLDLR**

Evaluating the activity of wild-type 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. We used our partially humanized mouse model of hoFH and overexpressed hPCSK9 [after intravenous injection of an AAV9 vector expressing hPCSK9 via the liver-specific promoter thyroxine binding globulin [AAV9.hPCSK9]]. Expression of AAV9.hPCSK9 vector was first evaluated in C57BL/6 mice, which received increasing doses of hPCSK9 (Online Figure I). At high-dose

<table>
<thead>
<tr>
<th>Amino Acid Substitution</th>
<th>LDLR–PCSK9 Interaction</th>
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<tr>
<td>N295D</td>
<td>Prevent hydrogen bonding with PCSK9 Asp-238</td>
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<tr>
<td>D299N</td>
<td>Affects salt bridge with PCSK9 Ser-153</td>
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<tr>
<td>H306G</td>
<td>Affects salt bridge with PCSK9 Asp-374</td>
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<td>V307D</td>
<td>Prevents hydrophobic interaction with PCSK9 Val-380</td>
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<tr>
<td>N309A</td>
<td>Prevent hydrogen bonding with PCSK9 Thr-377</td>
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<tr>
<td>D310N</td>
<td>Affects salt bridge with PCSK9 Arg-194</td>
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<td>L311T</td>
<td>Prevent hydrogen bonding with PCSK9 Thr-377</td>
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<td>L318D</td>
<td>Hydrophobic interaction with PCSK9 Cys-378</td>
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<td>L318H</td>
<td>Hydrophobic interaction with PCSK9 Cys-378</td>
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In silico analysis was used to determine human LDLR amino acid substitutions likely to interfere with PCSK9 binding. The amino acid substitutions and the LDLR–PCSK9 interaction that is expected to be affected are presented. LDLR indicates low-density lipoprotein receptor; and PCSK9, proprotein convertase subtilisin/kexin type 9.
vector (ie, 5×10¹⁰ GC) serum non-HDL cholesterol increased =2.5-fold (P<0.0015), indicating some level of interaction between hPCSK9 and mLDLR.

Before 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 after the administration of 5×10¹⁰ GC of AAV8, hLDLR. Non-HDL levels were stabilized and were only 37±7 mg/dL by day 30, which was 9% of baseline levels (P=0.037; Figure 2A). Next, we evaluated the performance of this vector in DKO mice expressing hPCSK9 by coadministering (intravenous) an equal dose (5×10¹⁰ GC) of AAV9, hPCSK9 along with AAV8.hLDLR. After the vector administration, serum levels of hPCSK9 rose steadily and reached peak levels (7500±1496 ng/mL) by day 30. Concomitantly, non-HDL levels in mice cotransduced with hPCSK9 were significantly higher (P=0.0008) when compared with animals that only received hLDLR (Figure 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 cotransduction with PCSK9 resulted in reduced hLDLR protein in the liver (Figure 2B and 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.19 No reduction in hLDLR expression was observed in animals cotransduced with an AAV9 vector expressing an irrelevant transgene (Online Figure II).


We used a similar strategy 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) whereas cotransduction with hPCSK9 resulted in reduced hLDLR activity with non-HDL cholesterol levels only 23% of baseline (P<0.0001; Figure 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% versus 14%; P=0.1337). Immunoblotting of livers collected at the end of the study (day 30) revealed that hLDLR protein levels were significantly decreased in animals that received wild-type hLDLR along with hPCSK9 when compared with those that received hLDLR alone (Figure 3B). However, liver levels of hLDLR-L318D were unaffected by coexpression with hPCSK9 and were comparable with those observed with wild-type hLDLR in the absence of hPCSK9 (Figure 3B). To confirm that the observed differences did not arise from changes in mRNA expression, we analyzed hLDLR transcripts in livers using a quantitative polymerase chain reaction assay. These studies indicated only a modest decrease in wild-type hLDLR-treated mice that were substantially less than the decrease in hLDLR protein (Figure 3B; Online Figure III).

hLDLR-K809R\C818A Escapes hIDOL Regulation

LDLR expression is also subject to regulation by IDOL, an E3 ubiquitin ligase transcriptionally upregulated by liver X
receptors after an increase in intracellular concentrations of oxysterols. Activated IDOL interacts with the cytoplasmic tail region of LDLR leading to receptor degradation. Amino acid residues, lysine (K809) and cysteine (C818), in the cytoplasmic tail region of hLDLR, have previously been identified constructs to hIDOL (Figure 4C).

Next, we evaluated the activity of the hLDLR-K809R\C818A construct in DKO mice overexpressing human IDOL. Similar to our strategy with the PCSK9 studies, we created a phenotype of mice overexpressing human IDOL in liver by administering an AAV vector expressing human IDOL under control of a liver-specific promoter. In pilot studies, we assessed the efficacy of human IDOL in regulating endogenous LDLR expression in mice by administering (intravenous) $5 \times 10^{10}$ GC of AAV9.hIDOL to FH mice heterozygous for LDLR expression (heterozygous FH). This strain of mice (human ApoB100 transgenic mice) is deficient in APOBEC-1, heterozygous for mouse LDLR and transgenic for human ApoB100, which leads to higher serum cholesterol. After the administration of AAV9.hIDOL, non-HDL levels increased by day 7 and reached stable levels by day 30 ($P < 0.0001$; Figure 4B). These results confirmed that AAV expressed hIDOL was active in mouse livers and can cause the loss of endogenous mLDLR. Next, we investigated the effect of hIDOL overexpression on vector encoded hLDLR in DKO mice. In pilot studies, we noted that only low-dose hLDLR vector administrations were significantly affected by human IDOL. At higher doses, vector expressed LDLR was able to overcome IDOL inhibition (Online Figure V). Hence, we coadministered mice with $3 \times 10^{10}$ GC of AAV8.hLDLR and $5 \times 10^{10}$ GC of AAV9.hIDOL. At this low dose, hLDLR and hLDLR-K809R\C818A vectors were functionally similar ($P = 0.9$) and induced a modest reduction (20% of baseline) in serum cholesterol in the absence of hIDOL (Figure 4C). However, coadministration of human IDOL ablated wild-type hLDLR activity, and no correction was seen in non-HDL cholesterol levels, which remained at pretreatment baseline levels ($P = 0.0248$; Figure 4C). In contrast, non-HDL cholesterol levels in mice that received hLDLR-K809R\C818A in the presence or in the absence of hIDOL were similar ($P > 0.05$), demonstrating the in vivo resistance of the modified constructs to hIDOL (Figure 4C).

**hLDLR-L318D\K809R\C818A Avoids Regulation by Both PCSK9 and IDOL**

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 (Online Figure VI). We then administered the vector to DKO mice at a low dose ($3 \times 10^{10}$ GC), when evaluating the IDOL escape mutations, or at a high dose ($5 \times 10^{10}$ GC), when evaluating the PCSK9 escape mutation. When administered at a low dose, hLDLR-L318D\K809R\C818A was comparable with wild-type hLDLR ($P = 0.05$) in that only a modest decrease in serum cholesterol was realized after either vector administration (Figure 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 (Figure 5A). In the parallel study, where we administered vectors at a higher dose along with hPCSK9, the variant protein performed significantly better in reducing serum cholesterol than the

**Figure 4.** AAV8.hLDLR-K809R\(\text{C818A}\) escapes in vivo human inducible degrader of LDLR (hIDOL)-mediated regulation. HEK293 (human embryonic kidney) cells were transiently transfected with plasmids expressing either human low-density lipoprotein receptor (hLDLR) or hLDLR-K809R\(\text{C818A}\) along with hIDOL. Twenty-four hours later, cells were pulsed with boron-dipyrromethene-LDL (BODIPY-LDL) for 2 hours and then evaluated for fluorescent LDL uptake using a flow cytometer. A, Percentage BODIPY-LDL–positive cells transfected with hLDLR or hLDLR-K809R\(\text{C818A}\) along with hIDOL. Data represent averages from 3 studies. Total cell lysates of transfected cells from 1 representative study were electrophoresed on a 4% to 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. B, LDLR\(\text{−}\)/Apoec\(\text{−}\)/Tg-ApoB100 heterozygous FH (heFH) mice \(n=4\) per group) were systemically administered with \(3 \times 10^{10}\) GC of AAV8.hIDOL vector. Serum from animals before and 30 days after vector administration was evaluated for total serum cholesterol. Percentage change in serum non-HDL levels at 30 days relative to preadministration baseline levels. All values are expressed as mean±SEM. ***$P<0.0001$, \(^*P<0.05\), and ns $P > 0.05$.

**Figure 5.** The AAV8.hLDLR-L318D\.K809R\(\text{C818A}\) variant encoding 3 amino acid substitutions escapes both proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of LDLR (IDOL)–mediated regulation. A, Double knockout mice \(n=4\) per group) were intravenously administered with \(3 \times 10^{10}\) GC of human low-density lipoprotein receptor (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. Percentage decrease in non–high-density lipoprotein (HDL) cholesterol relative to baseline. Total liver lysates from 2 representative animals per group was electrophoresed on a SDS-PAGE gel and probed using an anti-hLDLR antibody along with tubulin as a loading control. B, Coadministration of AAV8.hLDLR \(5 \times 10^{10}\) GC or hLDLR-L318D\.K809R\.C818A along with AAV9.hPCSK9 \(5 \times 10^{10}\) GC. Percentage decrease in day 30 non–HDL cholesterol relative to baseline is shown along with an immunoblot of hLDLR expression in livers. ***$P<0.001$, \(^*P<0.05\), and ns $P > 0.05$. 
control wild-type LDLR in mice overexpressing hPCSK9 (P=0.0007; Figure 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.

Discussion
The attractiveness of AAV gene therapy is based on the premise of life-long correction of a metabolic disorder after the single administration of a vector encoding the correct protein. Although the new AAV vectors are much more efficient than the original AAV serotype 2–based vectors, the efficiency of transduction remains an important issue. This has led to a strategy to express proteins with increasing activity over the normal wild-type protein. For instance, the lipoprotein lipase gain-of-function naturally occurring mutation (S447X) has been demonstrated to prevent degradation by post-translational mechanisms; a finding that has resulted in the development of the first licensed AAV gene therapy product carrying this gain-of-function mutation for lipoprotein lipase deficiency. A similar approach has been pursued in gene therapy of hemophilia B using gene encoding hyperactive factor IX variant, which is in phase I studies.

With these cases as precedent, we undertook studies to develop variants of hLDLR that are resistant to endogenous degradation pathways as a way to improve vector potency. AAV hepatic LDLR expression is the subject to multiple pathways of regulation; of these, regulation by PCSK9 has emerged as the leading candidate for the development of second-generation lipid-lowering drugs, and several clinical studies are underway to augment LDLR expression by interfering with PCSK9-mediated degradation of the receptor. More recently, a second form of post-transcriptional regulation of LDLR expression, based on the degradation of the receptor by the ubiquitin-proteasome pathway, has emerged as an additional target to increase receptor expression. Such strategies to increase LDLR expression were also bolstered by reports that subjects with naturally occurring loss-of-function mutations in PCSK9 and IDOL have lower LDL-C levels but have no disease associated with these mutations; therefore, avoiding LDLR degradation by these regulatory pathways should be considered to be safe and potentially helpful in improving gene therapy for hoFH.

In our previous studies, we noted that systemic administration of 5×10^{12} GC/kg of AAV8.LDLR led to a dose-dependent stable correction of serum cholesterol levels in humanized FH mice. In the present study, we investigated the potential of novel hLDLR variants to avoid degradation by PCSK9 and IDOL as a means to increase vector efficacy. Moreover, it is possible that pathways responsible for downregulating LDLRs may be more active in FH subjects. For instance, patients with homozygous FH and heterozygous FH have higher levels of PCSK9 when compared with normal subjects. It is unclear why FH subjects have increased expression of PCSK9. Although statins are known to increase PCSK9 expression, FH subjects have increased levels of PCSK9 even in the absence of statin therapy. It is possible that the increase could result from an inability to remove PCSK9 from circulation via binding to the missing LDLR.

The available crystal structure of LDLR-EGF-A domain with hPCSK9 allowed us to identify amino acid substitutions that abrogated the interaction. Among these, the L318D modification conferred protection from PCSK9 both in vitro and in vivo. Although several constructs did escape PCSK9 regulation in our in vitro studies and were functional in our in vivo screening, we focused only on the L318D amino acid substitution because it had also been previously described by Zhang et al. to reduce PCSK9 binding to LDLR. However, it is possible that other constructs may be more effective in avoiding degradation and could be considered in second-generation products. In our study, L318D conferred protection after 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. This establishes proof-of-concept that a PCSK9-resistant LDLR cDNA may be a more effective transgene for gene therapy for homozygous FH.

The K809R and C818A amino acid substitutions in hLDLR have been previously reported to prevent in vitro IDOL-mediated degradation of hLDLR. An interesting finding was the increase in serum cholesterol after the overexpression of human IDOL in LDLR“+” mice and suggests that hIDOL has some activity against mouse LDLR in terms of the endogenous ubiquitin-proteasome pathway. In our study, the K809R/C818A hLDLR double mutant conferred protection after 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. Our studies thus establish that amino acid modifications in LDLR can also overcome in vivo IDOL-mediated suppression. Escape from IDOL-mediated degradation was not complete and may indicate the involvement of other LDLR residues with IDOL. For instance, amino acids 816, 817, and 821 to 823 have all been reported to be involved in the interaction with IDOL, and substitution of these residues leads to decreased LDLR degradation by IDOL. Incorporating these additional amino acid substitutions may lower IDOL degradation of LDLR; however, multiple amino acid substitutions can efface transgene expression if the modified transgenes are sufficiently different to trigger an adaptive immune response. Moreover, we noted that overexpression of LDLR can overcome some of the negative regulatory effects of IDOL. However, AAV8.LDLR expression is expected to vary significantly in subjects and the incorporation of IDOL escape mutations may be more relevant in those subjects who receive lower doses of vector or have lower levels of LDLR expression.

Factors that lead to LDLR degradation are expected to be higher in subjects lacking endogenous receptor expression because of lack of a substrate to remove the inhibitors. This could argue for the consideration of these gain-of-function transgene variants to reduce clearance by these pathways. A potential caveat with this approach is the development of an adaptive immune response to the transgenes encompassing the mutated amino acids. However, systemically
administered AAV vectors have demonstrated to be safe and rarely induce an adaptive immune response to the transgenes; moreover, even when present, activated T cells are known to ignore AAV8 transduced hepatocytes. In support of these findings, administration of the hyperactive factor IX variant FIX-R338L to hemophilia B dogs did not elicit a T-cell response to the transgene. However, adaptive immunity accompanied by strong inflammatory signals is known to eliminate AAV transduced cells, suggesting that CTL responses to the new variants in the presence of inflammation need to be investigated in detail. In conclusion, we demonstrate here the usefulness of LDLR variants in overcoming negative cellular regulatory pathways, known to exist in FH subjects. The findings presented here demonstrate for the first time the successful use of an AAV encoded gain-of-function transgene in reducing cholesterol in partially humanized mouse models and could be considered in the development of more effective gene therapy products for hoFH.

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Disclosures

J.M. Wilson is an advisor to ReGenX Biosciences and Dimension Therapeutics, and is a founder of, holds equity in, and receives grants from ReGenX Biosciences and Dimension Therapeutics; in addition, he is a consultant to several biopharmaceutical companies and is an inventor on patents licensed to various biopharmaceutical companies.

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**What Is Known?**
- Homozygous familial hypercholesterolemia (hoFH) is an autosomal dominant disorder characterized by high serum levels of LDL-cholesterol and markedly premature atherosclerotic cardiovascular disease.
- In mouse models of FH, gene transfer of the low-density lipoprotein receptor (LDLR) to liver with adeno-associated viral vector serotype 8 (AAV8.LDLR) leads to stable reduction in LDL-cholesterol levels, suggesting that this approach may be viable for treatment of hoFH in humans.
- Liver LDLR protein levels are the subject to negative regulation by proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of LDLR (IDOL), which could potentially limit the efficacy of liver-directed gene therapy for hoFH.

**What New Information Does This Article Contribute?**
- A liver-tropic AAV vector was used to create hypercholesterolemic mouse models overexpressing human PCSK9 or IDOL.
- Novel variants of the LDLR were engineered that avoid negative regulation by PCSK9 and/or IDOL.

**Novelty and Significance**
- Using AAV vectors, these novel variants were coexpressed in FH mice along with human PCSK9 and IDOL and were shown to be more effective in lowering LDL-cholesterol levels than wild-type LDLR.

Liver gene therapy with AAV8.LDLR has been shown to reduce the high LDL-cholesterol levels stably in mouse models of FH. However, multiple regulatory pathways control post-transcriptional expression of LDLR in the liver. In particular, downregulation by PCSK9 and IDOL is important in the control of liver LDLR expression and is likely to reduce the efficacy of gene therapy for hoFH with AAV8.LDLR in humans. We demonstrate that novel engineered LDLR transgenes were less degraded by PCSK9 and IDOL. Moreover, the novel constructs lowered cholesterol in mice with increased levels of PCSK9/IDOL more efficiently than wild-type LDLR. Our results demonstrate for the first time that a gene therapy product can be engineered to increase expression/activity by avoiding intracellular degradation and contrasts with current approaches to increase transgene activity by identifying hyperactive mutants. FH subjects have increased levels of PCSK9 and the novel variants described in our studies should be considered in the development of gene therapy for treating hoFH.
AAV Vectors Expressing LDLR Gain-of-Function Variants Demonstrate Increased Efficacy in Mouse Models of Familial Hypercholesterolemia
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Supplemental Methods

Animal studies

All animal studies were approved by the institutional review board (IRB) at the University of Pennsylvania. LDLR\textsuperscript{-/-}, APOBEC-1\textsuperscript{-/-} double knockout mice (DKO) and LDLR\textsuperscript{-/-}, APOBEC-1\textsuperscript{-/-}, human ApoB100 transgenic (LAHB) were maintained at the University of Pennsylvania. All animals were fed a chow diet throughout the study. 6-8 week old male mice were injected intravenously (tail vein) with vector diluted in PBS in a total volume of 100 uL. 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 was analyzed for LDL, HDL and triglycerides using a MIRA analyzer (Roche). Non-HDL cholesterol was derived by subtracting serum HDL from total cholesterol.

LDLR variants

Amino acid residues targeted for mutagenesis were selected based on published reports of LDLR interaction with PCSK9 and IDOL as follows: A) The available crystal structure of PCSK9 bound to the Epidermal Growth Factor A domain (EGF-A) of LDLR was used to select amino acids that were involved in the formation of the complex via salt bridges and hydrogen bonds.\textsuperscript{1} \textit{In silico} modeling (CHIMERA software, UCSF) was then used to select single amino acid substitutions that were predicted to reduce PCSK9 interactions. B) Amino acid substitutions, K809R and C818A, in the C-terminal cytoplasmic domain of LDLR that prevent IDOL mediated degradation were selected based on published reports that have demonstrated these substitutions to prevent IDOL interaction.\textsuperscript{2,3}

Vector

The AAV8 vector expressing wild type hLDLR cDNA from a liver-specific thyroxine binding globulin (TBG) promoter (TBG) has been previously described\textsuperscript{21} 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. The cDNAs sequences encoding hPCSK9 and hIDOL were purchased (Origene, MD), cloned, and vectored to express from an AAV9 vector behind a TBG promoter. 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.
**In vitro LDLR assay**

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 mg/mL. Cells were removed after 2 hr and evaluated for fluorescent LDL uptake using a flow cytometer (FC500, Beckman Coulter).

**Immunoblotting and Enzyme linked Immune assays**

50 mg 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.

**Statistical analysis**

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.

**References**

Supplemental Figure Legends and Figures

Supplemental Figure I. Cholesterol levels in C57B/6 mice administered with increasing doses of AAV9hPCSK9. C57B/6 mice (n=4 per group) were administered (i.v.) with AAV9hPCSK9 at various doses (5x10⁹, 1.5x10¹⁰ and 5x10¹⁰ GC). Non-HDL serum cholesterol was analyzed on pre, day 7 and day 30 post vector administration along with levels in untreated mice and control mice treated with a vector expressing an irrelevant transgene.

Supplemental Figure II: Non-HDL cholesterol levels before (Pre) and 7 and 30 days post vector administration in DKO mice treated with hLDLR plus hPCSK9. DKO mice (n=4 per group) received AAV8hLDLR (blue), AAV8hLDLR and AAV9hPCSK9 (red) or AAV8hLDLR and AAV9hA1AT (black). Serum from animals at the various times as indicated in the figure were analyzed for non-HDL cholesterol. Levels in control (uninjected) animals is shown in green.

Supplemental Figure III: Fold change in hLDLR transcripts in mice co-administered with wildtype hLDLR or hLDLR-L318D, along with PCSK9. hLDLR mRNA transcript levels were evaluated using a Quantitative RT-PCR assay against the transgene from day 30 livers in animals that received vector. Data shows fold expression differences in hLDLR mRNA transcripts from individual animals normalized to levels in one mice that was administered only with hLDLR.

Supplemental Figure IV: Loss of hLDLR expression in HEK293 cells transiently transfected hLDLR or hLDLR-L809R, along with hIDOL. HEK293 cells were transfected overnight with vectors expressing hLDLR or hLDLR-L809R\C818A along with hIDOL at various levels as indicated by the symbol. 24 hr following transfection cells were removed and immunoblotted for hLDLR expression along with tubulin loading control. (mature,M; precursor,P).

Supplemental Figure V: High dose AAV8hLDLR administration overcomes IDOL regulation. Homozygous FH (hoFH) DKO mice (n=4 per group) were systemically administered with 5x10¹⁰ GC AAV8.hLDLR, or AAV8.hLDLR-K809R\C818A, along with AAV9.hIDOL 5x10¹⁰ 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. ns p>0.05.

Supplemental Figure VI: AAV8hLDLR construct indicating the location of various amino acid substitutions. TBG-thyroxine binding globulin promoter. LBD – ligand binding domain. A, B and C correspond to the three EGF homology domains.
Supplemental Figure II

The graph shows the change in non-HDL cholesterol levels (mg/dl) over time (Pre, Day 7, Day 30) for different treatment groups:
- **Control** (green line)
- **5E10 hPCSK9** (red line)
- **hLDLR + A1AT** (black line)
- **hLDLR** (blue line)

The y-axis represents non-HDL cholesterol levels, ranging from 0 to 500 mg/dl, and the x-axis represents time points (Pre, Day 7, Day 30).
Supplemental Figure V

% non-HDL cholesterol / baseline

IDOL

- LDLR

+ LDLR

- LDLR-K809R\C818A

+ LDLR-K809R\C818A

ns

ns