Increased Secretion of Lipoproteins in Transgenic Mice Expressing Human D374Y PCSK9 Under Physiological Genetic Control

Bronwen Herbert, Dilipkumar Patel, Simon N. Waddington, Emily R. Eden, Alexandra McAleenan, Xi-Ming Sun, Anne K. Soutar

Objective—To produce transgenic mice expressing the D374Y variant of the human proprotein convertase subtilisin/kexin type 9 (PCSK9) gene at physiological levels to investigate the mechanisms causing hypercholesterolemia and accelerated atherosclerosis.

Methods and Results—A bacterial artificial chromosome containing PCSK9 and its flanking regions was modified to introduce the D374Y mutation and a C-terminal myc tag. Transgenic mice that expressed 1 copy of the mutant or wild-type (WT) PCSK9 bacterial artificial chromosome were produced. Human PCSK9 mRNA was expressed at levels comparable to endogenous pcsk9 and with the same tissue specificity. The expression of D374Y or WT human PCSK9 increased the serum cholesterol level and reduced hepatic low-density lipoprotein receptor protein levels in the transgenic mice compared with bacterial artificial chromosome–negative controls; however, the effects were more marked in D374Y mice. The effect of a high-cholesterol diet on increasing serum cholesterol level was greater in D374Y mice, and atherosclerotic plaques after 15 weeks were more extensive in mice expressing D374Y than in WT PCSK9. D374Y mice secreted more triglyceride-rich lipoproteins into the circulation than WT mice.

Conclusion—The expression of human D374Y PCSK9 at physiological levels produced a phenotype that closely matched that found in heterozygous D374Y patients and suggested that reduced low-density lipoprotein receptor activity is not the sole cause of their hypercholesterolemia. (Arterioscler Thromb Vasc Biol. 2010;30:1333-1339.)

Key Words: atherosclerosis ■ genetically altered mice ■ hyperlipoproteinemia ■ lipoproteins ■ receptors

In 2003, linkage analysis in 2 families with autosomal dominant familial hypercholesterolemia (FH) of unknown etiology showed that variation in a novel gene, proprotein convertase subtilisin/kexin type 9 (PCSK9), cosegregated with hypercholesterolemia.1 The gene, which encodes a putative proprotein convertase, was first identified as a gene that was upregulated in neural cells undergoing apoptosis (neural apoptosis–regulated convertase-1); thus, no underlying causative mechanism for hypercholesterolemia was apparent. Measurement of lipoprotein turnover in 2 patients heterozygous for a PCSK9 mutation suggested that apolipoprotein B (apoB) synthesis was increased.3 Also, microarray analyses of global gene expression in mice fed a high-cholesterol diet4 or overexpressing the transcription factor SREBP2 revealed that expression of this gene was regulated by sterols and that it was, therefore, likely to play some role in cholesterol homeostasis. Adenoviral-mediated expression of PCSK9 in the liver of mice resulted in reduction of hepatic low-density lipoprotein (LDL) receptor protein, with no change in the LDL receptor (LDLR) mRNA; however, there was no apparent difference between mice expressing wild-type (WT) and mutant PCSK9.7 On the other hand, genetic ablation of pcsk9 in mice resulted in increased LDLR levels and a reduced plasma cholesterol level; in the general population, heterozygosity for a null mutation in PCSK9 is associated with reductions in serum cholesterol level and the risk of coronary heart disease.9

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Thus, it rapidly became clear that PCSK9 normally regulates LDLR levels and thereby influences serum cholesterol level, and that the rare mutations found in patients with FH are gain of function. These observations implied that PCSK9 was a target for lipid-lowering therapy. With the added incentive that inhibition of PCSK9 would act in concert with statins rather than replace them, research was conducted to understand the basis of action of PCSK9.10 Briefly, it has emerged that PCSK9 is synthesized in the liver, undergoes autocleavage to produce a prosegment that remains associ-
ated with the protein, and is then secreted. Circulating PCSK9 binds to the LDLR on the cell surface and is cointernalized through clathrin-coated pits, although other autosomal recessive hypercholesterolemia (ARH)-independent mechanisms may also exist. At the acid pH of endosomes, PCSK9 binds more tightly and prevents dissociation, with the result that the LDLR cannot recycle and is degraded. Gain-of-function PCSK9 mutants bind with even higher affinity than WT PCSK9.

We showed that the D374Y mutation in PCSK9 was associated with a particularly severe clinical phenotype. This was characterized in 4 apparently unrelated families of UK origin by an earlier onset of both severe hypercholesterolemia and coronary heart disease and a poorer response to lipid-lowering therapy with statins compared with patients with FH and null mutations in LDLR; this was subsequently confirmed elsewhere. Surprisingly, LDL from these patients differed in size and composition not only from normal LDL but also from LDL from LDLR patients with FH; the LDL was smaller, and there was a lower cholesterol to protein ratio. Furthermore, PCSK9 LDL competed less well for binding to the LDLR on cultured skin fibroblasts. We also showed that cultured rat hepatocytes (McArdle RH7777) expressing human D374Y PCSK9 secreted more apoB100 than untransfected cells or cells expressing WT PCSK9. Together, these data suggested that gain-of-function PCSK9 mutations might, in addition to reducing LDLR protein, also increase apoB secretion, thereby further contributing to hypercholesterolemia. Others have reported that they failed to observe increased apoB synthesis in mice overexpressing PCSK9 with a carboxy-terminal myc2 tag and under its own genetic control (supplemental Figure I). Random integration of the BAC constructs into the genome produced 3 transgenic mouse lines, 1 expressing WT PCSK9 and 2 expressing D374Y PCSK9. All the transgenic mice showed normal fertility, and there was no significant difference in their size or weight gain compared with BAC-negative littermates.

Transgenic mice were identified by analysis of genomic DNA by PCR (supplemental Figure IIA); the mutant D374Y transgene was distinguished from WT by digestion with AluI of a 177-bp PCR product across the site of the mutation. Phosphorimager quantification of Southern blots probed with cDNA to human PCSK9 and the single copy gene daf1 (supplemental Figure IIB) suggested that there was a single copy of the WT human PCSK9 in the genome of the WT-PCSK9 transgenic mice, 1 copy of the D374Y PCSK9 in 1 of the D374Y mouse lines (D374Y low), and 5 copies in the other D374Y mouse line (D374Y high).

Measurement of Lipoprotein Secretion

Mice fasted overnight, were anesthetized with isoflurane, and then injected intravenously with 10% (vol/vol) tyloxapol in saline (500-µg/g body weight). Blood samples were collected from the tail vein at intervals after injection.

Statistics

Data are expressed as mean ± SD. Differences between multiple groups were compared by 1-way ANOVA, followed by pairwise comparisons using the Bonferroni correction. Direct pairwise comparisons with independent controls were analyzed by the 2-tailed unpaired t test (Prism and GraphPad Software).

Results

Production of Transgenic Mice Expressing Single-Copy WT and D374Y PCSK9

The first aim of this study was to produce mice expressing WT or D374Y mutant PCSK9 under physiological control and at a low copy number as a model of patients heterozygous for PCSK9 gain-of-function mutations. A BAC containing the entire human PCSK9 gene as the only entire coding gene was modified by recombination to express either WT or D374Y human PCSK9 with a carboxy-terminal myc2 tag and under its own genetic control (supplemental Figure I). Random integration of the BAC constructs into the genome produced 3 transgenic mouse lines, 1 expressing WT PCSK9 and 2 expressing D374Y PCSK9. All the transgenic mice showed normal fertility, and there was no significant difference in their size or weight gain compared with BAC-negative littermates.

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Expression of WT and D374Y PCSK9 in the Liver and Small Intestine

To determine the expression of the transgenes and their possible effect on endogenous PCSK9 expression, we measured endogenous PCSK9 and transgenic PCSK9 mRNA in the livers of the 3 transgenic mouse lines and in their BAC-negative littermate controls by quantitative RT-PCR (supplemental Table I). There was no significant difference (P>0.05) in endogenous PCSK9 mRNA between the 4 groups (Figure 1), indicating that expression of the transgene at physiological levels had no effect on endogenous PCSK9 expression. The amount of PCSK9 mRNA from the transgene was not significantly different between WT-PCSK9 and D374Y-low mice and was comparable to the level of endogenous mouse PCSK9 mRNA in whole-tissue extracts of both the liver and distal small intestine, suggesting that human PCSK9 was expressed under physiological control in these mice. As expected, a significantly greater level of D374Y
transgene mRNA was detected in D374Y-high mice (P < 0.01). Analysis of mRNA with 2 alternative housekeeping genes (Ywhaz and Atp5b) gave similar results (data not shown). Endogenous pcsk9 mRNA could also be detected in the small intestine of control mice (∼BAC) and mice expressing WT or D374Y PCSK9; however, expression was more variable and at a lower level than in the liver relative to gapdh; transgene mRNA was expressed at a similar level to the endogenous mRNA in WT mice and was undetectable in control mice (supplemental Table II). Despite the presence of PCSK9 mRNA in the intestine, immunoblotting of different tissues with an antibody to the c-myc tag on the human PCSK9 transgene showed that the protein was expressed almost exclusively in the liver, although some myc-PCSK9 protein was detectable in other tissues of D374Y-high mice (supplemental Figure IIIA). We were unable to detect endogenous pcsk9 protein in any tissues using 3 commercially available antibodies. As expected, mature myc-tagged PCSK9 protein could be detected in the serum of the transgenic mice (supplemental Figure IIIC). When PCSK9 protein levels were compared in liver membrane preparations from chow-fed mice, there was no significant difference between the WT-PCSK9 and the D374Y-low mice; however, much higher levels were found in D374Y-high mice (supplemental Figure IIIB).

Expression of D374Y PCSK9 Has a Greater Effect on Serum Lipids and Lipoproteins Than WT PCSK9

To examine the effect of transgenic expression of PCSK9 on serum cholesterol in chow- or cholesterol-fed animals, we measured cholesterol levels in control and transgenic lines at different points. The presence of 1 copy of human WT PCSK9 resulted in a significant 2-fold increase in serum cholesterol from an early age (2.7 ± 0.7 versus 5.5 ± 1.3 mmol/L at the age of 4 weeks; P < 0.001; n = 15), and this difference between control and WT-PCSK9 mice remained constant with time (3.0 ± 0.7 versus 5.3 ± 1.0 at the age of 32 weeks; P < 0.001; n = 15). In contrast, serum cholesterol levels at the age of 4 weeks were initially greater in both D374Y-low mice (6.2 ± 0.9 mmol/L) and D374Y-high mice (7.3 ± 0.9 mmol/L) compared with WT-PCSK9 mice (n = 15; P = <<0.05) and continued to increase in both with increasing age (8.2 ± 1.6 and 8.4 ± 1.9 mmol/L at the age of 32 weeks) (supplemental Table III). There was no significant difference in serum cholesterol level between D374Y-high and D374-low mice that received a chow diet. Serum triglyceride levels, measured in 16- to 20-week-old mice that had fasted overnight were significantly higher in mice expressing D374Y PCSK9 at either a low copy number (3 ± 0.8 mmol/L; n = 5; P < 0.001) or a high copy number (3.5 ± 0.2 mmol/L; n = 5; P = 0.004) than in those expressing WT PCSK9 (1.9 ± 0.4 mmol/L; n = 5) or in control nontransgenic littermates (1.9 ± 0.4 mmol/L; n = 5). The expression of WT PCSK9 did not affect serum triglycerides.

When the mice were fed a chow diet supplemented with 1% cholesterol (HCHOL) for 4 weeks (Table), serum cholesterol levels increased approximately 2-fold in WT-PCSK9 mice, 3-fold in D374Y-low mice, and 4-fold in D374Y-high mice; however, there was no significant increase in the serum cholesterol level of BAC-negative controls. As in chow-fed animals, serum cholesterol levels in HCHOL-fed WT-PCSK9 mice then remained constant at 9.6 ± 1.6 mmol/L during the next 15 weeks; both D374Y lines showed an increase in serum cholesterol levels during that period, reaching 23.1 ± 7.0 mmol/L in D374Y-low and 32.6 ± 7.4 mmol/L in D374Y-high mice.

Feeding a High-Cholesterol Diet Reduces PCSK9 Expression

To determine whether feeding a cholesterol-rich diet affected transgene expression, we first measured liver cholesterol levels in mice receiving a chow or HCHOL diet. Cholesterol feeding increased liver total cholesterol level in all mice (supplemental Figure IV), although the increase did not reach statistical significance in D374Y-low mice, probably because liver cholesterol levels were already increased in chow-fed animals. We then measured PCSK9 mRNA levels. Both endogenous and transgene mRNA levels were slightly, but significantly, reduced in all animals fed the HCHOL diet (supplemental Table IV); the reduction was greatest in D374Y mice (2- to 3-fold), with a reduction of 1.5- to 2.0-fold in WT and D374Y-low mice. We then compared myc-PCSK9 protein levels in the livers of chow- and cholesterol-fed mice. The results showed that there was no significant effect of the HCHOL diet on the amount of myc-tagged PCSK9 protein in the livers of any of the mice lines (supplemental Figure V).

D374Y PCSK9 Reduces, but Does Not Completely Eradicate, LDLR Protein

Earlier studies have implicated a decrease in LDLR protein levels in the mechanism of action of PCSK9, and adenoviral-
mediated overexpression of PCSK9 in mice resulted in undetectable LDLRs.6 To determine whether this was the case in our transgenic mice expressing PCSK9 at more physiological levels, we assayed hepatic LDLR protein by semiquantitative immunoblotting of membrane fractions. WT-PCSK9 and D374Y-low mice fed a chow diet contained less hepatic LDLR protein than controls (Figure 2), with lower levels present in D374Y-high mice. However, LDLR protein was still clearly detectable, even in D374Y-high mice. Feeding the HCHOL diet slightly increased hepatic LDLR protein, as previously shown in cholesterol-fed rats.19

The Cholesterol Diet Induced a More Atherogenic Lipid Profile in D374Y Mice

When fed a chow diet, the 3 transgenic mouse lines showed virtually identical lipid profiles, with a slight increase in total cholesterol in the apoB-containing LDL fraction compared with their BAC-negative littermates (Figure 3A).

When fed an HCHOL diet, the lipid profile for control mice remained unchanged, as expected36; there was an approximately 2-fold increase in the LDL peak of WT-PCSK9 mice (Figure 3B). However, the HCHOL diet induced a more atherogenic lipid profile in both D374Y lines, with large increases in apoB- and apoE-containing lipoproteins. Total cholesterol appeared to be equally distributed between the fractions containing large particles of the size of very-low-density lipoprotein and those containing LDL in the D374Y-low mice; however, the total cholesterol level was predominantly associated with the large fraction in the D374Y-high mice. The cholesterol level in the HDL fraction remained unchanged in all 4 lines.

Increased Secretion of ApoB-Containing Lipoproteins in D374Y Mice

Immunoblotting of whole serum showed that serum apoB levels were slightly increased in WT-PCSK9 mice compared with control mice; however, these levels were significantly increased in D374Y mice (Figure 4A). To determine whether changes in the secretion of apoB-containing lipoprotein play a role in hypercholesterolemia in PCSK9 transgenic mice, we

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time Receiving Only a Chow Diet</th>
<th>Time Receiving a Chow Diet Plus a 1% Cholesterol Diet</th>
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<tbody>
<tr>
<td></td>
<td>4 wk</td>
<td>8 wk</td>
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<tr>
<td>Control†</td>
<td>2.4±0.6</td>
<td>2.7±0.9</td>
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<tr>
<td>WT</td>
<td>4.4±0.9</td>
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<td>D374Y PCSK9</td>
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PCSK9 indicates proprotein convertase subtilisin/kexin type 9; WT, wild type.

*Data are given as mean±SD serum cholesterol level (measured in mmol/L) in mice fed only a chow diet for the first 8 weeks of life, followed by mice fed a chow diet plus a 1% cholesterol diet for an additional 4 to 15 weeks.
†Non–bacterial artificial chromosome littermates.
‡P<0.05.
§P<0.01 vs non-BAC littermate controls.
¶P<0.01 for D374Y mice vs WT.
||P<0.05 for D374Y mice (low vs high).
measured the rate of triglyceride secretion in chow-fed mice that fasted, in which the lipase inhibitor, tyloxapol (Triton WR1339), was injected. There was no significant difference in secretion rate between WT-PCSK9 mice and BAC-negative littermates; however, there was a significantly increased rate of secretion of triglyceride seen in the D374Y-low mice (Figure 4B). There was also no significant difference in the secretion rate between ldlr-/-/H11002/mice, WT-PCSK9, or control mice, suggesting that the increased secretion in D374Y-low mice was not solely the result of lack of LDLR activity. As shown in Figure 4A, the basal levels of apoB48 and apoB100 were so high in the D374Y mice that it was not possible to determine by semiquantitative immunoblotting whether apoB secretion was also increased. Our license did not permit use of radioactive tracers. It has been shown that antisense-mediated downregulation of PCSK9 induces apobec-1 expression.21 Because there was an apparent slight increase in the ratio of apoB100 to apoB48 in mice expressing mutant PCSK9, we measured hepatic mRNA levels of apobec-1 and apobec-1 complementation factor (A1cf), an intrinsic component of the apoB mRNA editing complex22; we found no significant or consistent differences in mice fed a chow or an HCHOL diet (supplemental Table V).

Extensive Atherosclerotic Plaques Are Found Only in D374Y Mice

To investigate the consequences of the atherogenic lipid profile in the transgenic mice, aortas from HCHOL-fed mice were perfused with the lipid stain, Sudan IV. Neither control nor WT-PCSK9 mice showed any evidence of lesion formation in their descending aortas or aortic branches after 15 weeks on an HCHOL diet, whereas all D374Y-high mice showed many lesions throughout their aortas. D374Y-low mice also showed extensive lesion formation in their aortic branches but varied in the amount of lesions seen in the descending aorta (Figure 5). Lesions could be detected in the D374Y-low and the D374Y-high mice after 10 weeks of an HCHOL diet; these lesions were detected to a lesser extent and were mainly situated in the main aortic arch and branches (data not shown). Small lesions were also detectable in the aortic arches of chow-fed D374Y mice, but not in other mice, after 1 year (data not shown). Thus, expression of a single copy of WT PCSK9 did not induce atherosclerosis, even in cholestrol-fed animals, whereas expression of a single copy of D374Y PCSK9 was sufficient to induce an atherogenic phenotype that resulted in extensive plaque formation after 15 weeks of the high-cholesterol diet or a chow diet in older mice.

Discussion

In the present study, we produced transgenic mice that express human WT PCSK9 or the D374Y gain-of-function mutant from a BAC that should contain all endogenous regulatory sequences. Indeed, the WT-PCSK9 and D374Y-low mice, both of which had a single additional copy of PCSK9 compared with BAC-negative littermate controls,
expressed PCSK9 mRNA at levels comparable to that of endogenous pcsk9 and with similar tissue distribution, suggesting that expression was under endogenous control. As expected, D374Y-high mice, with approximately 5 copies of PCSK9, expressed significantly more PCSK9 mRNA than either WT-PCSK9 or D374Y-low mice.

Both the pro- and mature PCSK9 protein could be detected in the livers of all 3 transgenic lines, with only the mature protein secreted into the bloodstream (supplemental Figure I). We were unable to detect significant amounts of human PCSK9 protein in any other tissues, confirming previous reports that most circulating PCSK9 is derived from the liver, although pcsk9 mRNA could also be detected in the small intestine, as previously shown. All 3 transgenic lines displayed a modest increase in serum cholesterol level and a decrease in LDLR protein level compared with controls. The changes were more marked in D374Y-high mice; however, even in these mice, detectable levels of LDLR protein remained. Two previous transgenic mice that express human PCSK9 have been described; expression was not driven by the endogenous promoter in either case. In one case, gene expression was under the control of the albumin promoter and resulted in high levels of expression in the kidney; in the other case, expression was under the control of the human apoE promoter and resulted in marked overexpression in the liver, apparently comparable to that seen in mice expressing adenoviral constructs of PCSK9. In both models, and in contrast to our observations, the researchers reported that LDLR protein was essentially abolished in the livers of mice expressing WT PCSK9.

The lipoprotein phenotype in our mice expressing WT or mutant PCSK9 was relatively mild when the mice were fed a chow diet; however, when this diet was supplemented with 1% cholesterol, hypercholesterolemia in the D374Y-low and the D374Y-high mice became more severe. Thus, as is the case with the ldlr 


mice, the phenotype reflected that seen in patients heterozygous for gain-of-function PCSK9 mutations only when the mice were fed a high-cholesterol diet.

PCSK9 mRNA is regulated by sterols because feeding mice or rats a cholesterol-rich diet resulted in reduction of hepatic pcsk9 mRNA. We observed a moderate reduction in the expression of liver pcsk9 mRNA, both endogenous and transgene, after 15 weeks of a diet containing 1% cholesterol.

The main question we wanted to address with these mice was whether the hypercholesterolemic effects of D374Y PCSK9 were mediated entirely through reduction in LDLR protein levels. Our data suggest that there is some additional mechanism involved. First, we noted that fasting serum triglyceride levels in chow-fed animals were increased in transgenic mice expressing the mutant but not WT PCSK9. Two recent studies have indicated that human serum triglyceride levels are correlated with circulating PCSK9 protein. We also found that patients with FH heterozygous for PCSK9 D374Y had significantly higher fasting serum triglycerides than patients with FH and LDLR defects. Second, secretion of triglyceride-rich lipoproteins was also increased in these mice compared with mice expressing WT PCSK9 and, more important, compared with ldlr 


mice. These data suggest that expression of D374Y PCSK9 at physiological levels results in increased secretion of triglyceride-rich lipoproteins from the liver, which is in agreement with previous findings of increased apoB secretion in isolated hepatocytes expressing D374Y, but not WT PCSK9. There also appeared to be an increase in the proportion of apoB100 to apoB48 in mice expressing D374Y PCSK9. However, this mechanism did not involve changes in the expression of the apoB mRNA editing enzyme, apobec 1, although ablation of PCSK9 expression previously resulted in increased expression of this enzyme. Interestingly, it has been reported that PCSK9-knockout mice show reduced postprandial lipemia associated with reduced secretion of apoB from the intestine, whereas isolated hepatocytes from PCSK9-null mice secrete less apoB100.

In conclusion, we produced an animal model of FH caused by the dominant D374Y mutation in PCSK9 that appears to mimic the human disorder caused by heterozygous inheritance of D374Y PCSK9 more accurately than others previously described. The D374Y-transgenic mice should prove useful for testing whether new therapies are effective in reducing atherosclerosis.

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Disclosures
None.

References


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Supplementary Information

Supplementary Methods

Generation of PCSK9 transgenic mice

Human wild type and D374Y myc2-tagged PCSK9 cDNA was introduced by homologous recombination\textsuperscript{17} into the exon1-intron1 region of PCSK9 in a bacterial artificial chromosome containing human PCSK9 (BAC RP11-894E21 in pBACe3.6). Full-length cDNA for human PCSK9, either wild-type or carrying the D374Y mutation, was constructed to contain a carboxy-terminal myc\textsubscript{2} tag. The 3’ untranslated region (UTR) of the cDNA was replaced with that of bovine growth hormone (BGH) to prevent incorrect recombination between the 3’UTR in human PCSK9 cDNA and the same region in the BAC. Before insertion of the PCSK9 cDNA, the BAC was first modified by homologous recombination to remove a second coding gene (USP24).

Linearised constructs were injected into B6/CBA pronuclei. Transgenic founders and their transgenic offspring were identified by amplification of a 1400 bp fragment of the human PCSK9 cDNA sequence from genomic DNA with a forward primer located in exon 7 of PCSK9 and reverse primer in BGH 3’UTR.
Mice were maintained on a 12-h light/dark cycle and fed RM1 chow pellets or RM1+1% cholesterol (HChol) (Lillico, UK) ad libitum. The mice were fed HChol diet for up to fifteen weeks beginning at eight weeks of age. All animal experiments were performed under Home Office Licenses 70/6014 and 70/6906. Ldlr-/- mice were generously provided by M. Botto and T. Malik Imperial College London).

**Copy Number Analysis by Southern Blotting**

Genomic DNA was analysed by Southern blotting as described previously with a full length cDNA probe to PCSK9 and a cDNA probe to dafI, kindly provided by M. Botto and T. Malik. The blots were quantified with a Phosphorimager (STORM 820, Amersham Pharmacia Biotech.).

**Immunoblotting**

Harvested tissues were frozen immediately on dry ice. For preparation of lysates, tissues were homogenized in 50mM Tris pH8, 4mM EDTA, 150mM NaCl, 2% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS. For membrane preparation, tissues were homogenized in 20mM Tris-HCl pH8, 0.15M NaCl, 1mM CaCl$_2$.2H$_2$O, 1mM PMSF, pelleted by centrifugation at 240,000g for 1hr and solubilised in 200 µl of 50mM HEPES pH7.4, 100mM NaCl, 1mM PMSF, 10mM EDTA, 10mM EGTA, 10mM N-ethylmaleimide, 2.2% (v/v) DMSO, 1% (v/v) triton X100 and 0.5mM leupeptin as described by Van Driel. Protein content was assayed with the Bio-Rad DC Protein Assay system with bovine serum albumin as standard. Samples (1µl of serum or 5µl of FPLC fractions) and protein extracts (30µg of protein) were subjected to SDS-PAGE on reduced polyacrylamide gels (non-reduced for detection of the LDLR protein) and transferred to nitrocellulose membranes. Blots were incubated at ambient temperature with primary antibodies at the following dilutions: rabbit anti–c-myc (Santa Cruz
Biotechnology Inc.) 1:3000, 1h; mouse anti–c-myc (Cell Signalling Technology) 1:2000, 1h; goat anti-mouse LDLR (R&D Systems) 1:1000, 1h; goat anti-ApoB (Santa Cruz Biotechnology Inc.) 1:500, 2 hours. As indicated in the figure legends, blots were then incubated for 1h at ambient temperature with the appropriate secondary antibodies. Bound horseradish peroxidase–conjugated secondary antibodies (Dako) were visualized by chemiluminescence (Amersham Biosciences), whilst bound fluorescently-labeled second antibodies (680nm conjugated antibodies, Invitrogen; 800nm conjugated antibodies, LI-COR Biosciences), were visualized using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). To control for loading, blots of membrane proteins were re-probed with anti-protein disulphide isomerase (PDI; Santa Cruz Biotechnology Inc.).

**RT-PCR and real-time quantitative PCR**

Mouse tissues were homogenized in liquid nitrogen and total RNA was isolated with RNA-Bee (Ambio) according to the manufacturer’s instructions and quantitated by A260 (Nanodrop); DNase-treated RNA was reverse transcribed (High-Capacity cDNA Reverse Transcription kit, Applied Biosystems).

Real-time quantitative PCR was performed on an ABI 7500 Fast Real-Time PCR detection system with TaqMan® Universal PCR Master Mix (Applied Biosystems), with primer-probe sets specific for either mouse or human PCSK9 (Applied Biosystems), and mouse housekeeping genes *Gapdh*, *Ywhaz* and *Atp5b* (GeNorm) according to GeNorm protocols (Supplementary Table I). Ct values were compared to a standard curve and normalized to *Gapdh*, *Ywhaz* or *Atp5b*.

**FPLC and analysis of lipoprotein distribution**
Serum (50µl) was fractionated by fast-performance liquid chromatography (FPLC; Pharmacia Smart System®) on a Superose 6 PC 3.2/30 column (GE Healthcare)\textsuperscript{19} with a constant flow of 30 µl/min; 35µl fractions were collected and total cholesterol was assayed in 5µl aliquots (Infinity Cholesterol kit, Microgenics).

**Measurement of hepatic cholesterol levels**

Lipids from ~30mg liver tissue were extracted with chloroform:isopropanol:triton X-100 (7:11:0.1 by vol), solubilised in 200µl of reaction buffer from the Cholesterol/Cholesteryl Ester Quantification Kit (Calbiochem) and assayed for total, free and esterified cholesterol, all according to the manufacturer’s instructions.

**Analysis of atherosclerotic lesions**

Aortic atherosclerosis was assessed as described\textsuperscript{21}. Briefly, hearts were perfused sequentially with PBS, 2% (v/v) formalin (Sigma) and Sudan IV solution (0.5% w/v Sudan IV in 35% ethanol, 50% acetone, Sigma). Aortae were sectioned longitudinally for *en face* imaging (Leica DC 500 imaging system). Lesions were expressed as a percentage of the surface area of the aorta (ImagePro software, Media Cybernetics, Bethesda, Md).
**Supplementary References**


Supplementary Figures

Supplementary Figure I. Diagram showing the construction of the modified BAC expressing human PCSK9.

The wide grey boxes represent human genes present in the BAC, with arrows indicating direction of the coding sequence, the narrow grey box indicated intergenic human DNA and the black lines, the BAC ends; PCSK9 and USP24 were full length genes; exon 1 was absent from BSND. Recombination events in EL350 cells were selected with spectinomycin (Sp) and the Sp^r gene was subsequently excised by activation of CRE recombinase.

Supplementary Figure II. Detection of the transgene by PCR and Southern blotting of genomic DNA from transgenic mice.

A. Genomic DNA from different female mice as indicated below the figure was amplified with primers located in the BGH 3’-untranslated region and exon 7 of human PCSK9. B. Genomic DNA from mouse liver (11 µg) was digested with Nhe1 and analysed by Southern blotting. Blots were probed sequentially with a 1.1kb ^32P-labelled cDNA probe encompassing exons 1-11 of human PCSK9, and with a 501bp ^32P-labelled cDNA probe to the single-copy gene Decay Accelerating Factor (Daf), and developed with a Phosphorimager. Transgene copy number was calculated as the ratio of PCSK9/Daf, taking into account probe size.

Supplementary Figure III. Detection of transgenic PCSK9 protein in tissue lysates.

A. Protein tissue lysates (as indicated above the gel) from control or transgenic mice (as indicated on the right), were separated on a 10% reducing gel and immunoblotted with anti-myc to detect the transgenic myc-tagged PCSK9 protein, followed by HRP
conjugated anti-mouse IgG secondary antibody. All gels were analysed together, except for D374Y-low mice. B. Liver membrane samples from chow-fed D374Y-low, WT and D374Y-high mice were immunoblotted with mouse anti-myc (for tagged human PCSK9), detected with 680nm conjugated anti-mouse IgG, and then rabbit anti-PDI, detected with 800nm conjugated anti-rabbit IgG and the Licor system, which provided a linear response for all samples. C. Whole serum (1µl) from control or transgenic mice, as indicated above the blot, was immunoblotted with anti-myc to detect PCSK9 protein. (+HEK293), lysate of HEK293 cells transfected with human PCSK9 cDNA; P, proprotein; M, mature PCSK9; PDI, protein disulphide isomerase as a loading control.

Supplementary Figure IV. Effect of the high cholesterol diet on hepatic cholesterol content.

Total cholesterol was measured in the livers of control and transgenic mice fed chow or HCHOL diet for 15 weeks. Lipids from liver tissue were extracted and assayed for total cholesterol as described in the method. The values are mean ± standard deviation and three mice for each group (P<0.05:HCHOL vs chow in control, D374Y mice; P<0.05: WT mice compared to controls).

Supplementary Figure V. Comparison of PCSK9 protein in the livers of chow and high cholesterol fed transgenic PCSK9 mice.

Liver membranes from chow- and cholesterol-fed transgenic mice as indicated on the left were immunoblotted for myc-tagged PCSK9 protein and PDI as described in the legend to Supplementary Figure 3. At least two independent experiments were carried out with mice on each diet, with n = 3-5 mice for each. P, proprotein, M, mature PCSK9
**Supplementary Table I. Sequences of oligonucleotide primers**

<table>
<thead>
<tr>
<th>Use</th>
<th>Primer or Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR of human <em>PCSK9</em> (BAC)*</td>
<td>Forward</td>
<td>5’-CTAGTTGCCAGCCATCTGTGTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GGCACCTTCCAGGGTCAAG</td>
</tr>
<tr>
<td></td>
<td>FAM-labelled probe</td>
<td>5’-TGCCCCCTCCCCCGTGCCT</td>
</tr>
<tr>
<td>Mouse <em>pcsk9</em></td>
<td>Forward</td>
<td>5’-ATTGCAGGACGGTGTCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CTGTGGAAGCTGTCCCAT</td>
</tr>
<tr>
<td></td>
<td>FAM-labelled probe</td>
<td>5’-TACCCGACTTCAACGATGTGCCGG</td>
</tr>
<tr>
<td>Genomic PCR to detect transgene</td>
<td>Forward</td>
<td>5’-GCTCCCGAGGTCATCACAGTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-gggccgcgcCATGCCTGCTATTGTCTT</td>
</tr>
</tbody>
</table>

*Primers and probes designed with PE Applied Bio Systems software and purchased from the same company. Primer/probe sets for mouse *apobec1* (Mm00482894) and *Alcf* (Mm 011948) were purchased from PE Applied Bio Systems Ltd.

**Supplementary Table II. Endogenous and transgene PCSK9 intestinal mRNA**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Endogenous <em>pcsk9</em></th>
<th>Transgene PCSK9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (-BAC) (n= 5)</td>
<td>0.68± 0.37</td>
<td>n.d.</td>
</tr>
<tr>
<td>WT (n= 5)</td>
<td>0.79± 0.31</td>
<td>0.51± 0.59</td>
</tr>
<tr>
<td>D374Y-high (n= 3)</td>
<td>0.79± 0.18</td>
<td>0.91± 0.45</td>
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</table>

Endogenous *Pcsk9* or transgenic *PCSK9* mRNA was measured by qRT-PCR in small intestine of mice fed a chow diet, and expressed relative to the housekeeping gene *Gapdh*.) Values are mean ± standard deviation; n.d. not detectable. Intestinal mRNA was not available from D374Y-low mice.
**Supplementary Table III. Serum cholesterol levels in mice fed a chow diet.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Serum cholesterol (mmol/l) at different ages (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>2.7±0.7</td>
</tr>
<tr>
<td>WT</td>
<td>5.5±1.3</td>
</tr>
<tr>
<td>Low D374Y</td>
<td>6.2±0.9</td>
</tr>
<tr>
<td>High D374Y</td>
<td>7.3±0.8*</td>
</tr>
</tbody>
</table>

Mice were weaned at three weeks of age and then fed a standard chow diet *ad libitum*. Serum cholesterol levels (mmol/l) were measured at four weeks of age and then monthly. WT, mice expressing wild type PCSK9; Low PCSK9 and High PCSK9, mice expressing D374Y PCSK9 at high or low copy number; control, non-BAC littermates. Values shown are mean ± standard deviation, n=15;

At each time point, all three transgenic mouse lines had significantly raised serum cholesterol levels (P<0.001) compared to the control group. As confirmed by linear regression analysis, this increase was stable and maintained in WT mice ($r^2=0.041$, P=0.63) but increased with age in D374Y-low ($r^2=0.863$, P<0.001) and D374Y-high ($r^2=0.0052$, P<0.01) mice. Serum cholesterol levels were significantly increased in D374Y-high at all time points and in D374Y-low mice from 12 weeks of age onwards in comparison to the WT mice (P<0.05).
**Supplementary Table IV. Comparison of endogenous and transgene PCSK9 mRNA in chow and high cholesterol fed transgenic mouse livers.**

<table>
<thead>
<tr>
<th></th>
<th>mRNA level normalised to Gapdh</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endogenous Pcsk9</td>
<td>PCSK9 transgene</td>
<td></td>
<td></td>
<td></td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>Chow</td>
<td>HCHOL</td>
<td>P*</td>
<td>Chow</td>
<td>HCHOL</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.77±0.18</td>
<td>0.96±0.32</td>
<td>0.04</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
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<tr>
<td>WT</td>
<td>1.66±0.38</td>
<td>0.94±0.22</td>
<td>0.009</td>
<td>1.72±0.57</td>
<td>0.71±0.21</td>
<td>0.0005</td>
</tr>
<tr>
<td>D374Y-low</td>
<td>1.74±0.12</td>
<td>0.92±0.19</td>
<td>0.001</td>
<td>0.86±0.27</td>
<td>0.52±0.22</td>
<td>0.044</td>
</tr>
<tr>
<td>D374Y-high</td>
<td>2.18±0.02</td>
<td>0.75±0.02</td>
<td>0.0001</td>
<td>4.95±1.53</td>
<td>2.33±1.66</td>
<td>0.017</td>
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</table>

Endogenous *Pcsk9* or transgenic *PCSK9* mRNA was measured by qRT-PCR in livers of mice fed either chow or the HCHOL diet, and expressed relative to the housekeeping gene *Gapdh*). Values are mean ± standard deviation; n.d. not detectable. P, significance HCHOL vs chow diet.
Supplementary Table V. Apobec1 and A1cf expression in chow and high cholesterol fed transgenic mouse livers.

<table>
<thead>
<tr>
<th></th>
<th>mRNA level normalised to Gapdh</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apobec 1</td>
<td>A1cf</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Chow</td>
<td>HCHOL</td>
<td>Chow</td>
<td>HCHOL</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.37±0.06</td>
<td>0.68±0.34</td>
<td>0.99±0.28</td>
<td>1.68±1.23</td>
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</tr>
<tr>
<td>WT</td>
<td>1.48±0.13</td>
<td>0.97±0.30</td>
<td>0.95±0.17</td>
<td>1.15±0.85</td>
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</tr>
<tr>
<td>D374Y-low</td>
<td>1.22±0.03</td>
<td>0.60±0.55</td>
<td>2.39±0.42</td>
<td>1.56±0.72</td>
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</tr>
<tr>
<td>D374Y-high</td>
<td>1.21±0.02</td>
<td>1.14±0.19</td>
<td>1.35±0.49</td>
<td>1.12±0.29</td>
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</table>

Endogenous *apobec1* or *A1cf* mRNA was measured by qRT-PCR in livers of mice fed either chow or HCHOL diet, and expressed relative to the housekeeping gene *Gapdh*. Values are mean ± standard deviation, n=4 or 5.
Supplementary Figure I

189Kb

BSND (3’ end)  PCSK9  USP24

homologous recombination in EL350 cells (1) to excise upstream gene

83.3 Kb  3’  5’

BSND (3’ end)  PCSK9

homologous recombination (2) to insert myc-tagged PCSK9 cDNA (WT or D374Y) in exon 1

3’ homology (intron 1/exon 1)  WT PCSK9 cDNA  5’ homology (promoter/exon 1)

3’- untranslated region bovine growth hormone  2x myc.STOP

Sp(Flanked by Lox D sites)

Cre-dependent excision of Sp gene

WT/D374Y PCSK9 cDNA under physiological control
Supplementary Figure II

(A)

(B)

- VE  WT  D374Y Low  D374Y High

1  1  5

PCSK9 11.4kb
DAF 6.6kb

~ Copy No.
Supplementary Figure IV

- **Chow**
- **High Cholesterol**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Cholesterol (mg/g)</th>
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<tbody>
<tr>
<td>Control</td>
<td>2.5</td>
</tr>
<tr>
<td>WT</td>
<td>3.1</td>
</tr>
<tr>
<td>D374Y-low</td>
<td>5.0</td>
</tr>
<tr>
<td>D374Y-high</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Significance levels:
- P<0.04
- P<0.05
- NS
- P<0.03
Supplementary Figure V