

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

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Abogado
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ASUNTO	Radicación	09-112535
	Trámite	11
	Evento	378
	Actuación	519
	Oficio	
	Folios	8 E 165 161

Patente de Invención ☒

Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒

Capítulo I ☒

Capítulo II ☐

No. Solicitud Internacional PCT/EP2008/054417

Fecha de Presentación Internacional 11/Abril/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 103 a 250	9/Octubre/2009
Reivindicaciones:	Folios 251 a 265	9/Octubre/2009
	Folios 297 a 307	30/Diciembre/2009
Dibujos:	Folios 267 a 273	9/Octubre/2009
Prioridad:	US 60/911,654	13/Abril/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486), y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones aceptadas

Se acepta el nuevo capítulo reivindicatorio (Fls. 297 a 307) porque no se amplía el objeto de la Solicitud.

Expediente 09-112535 1er Art 45

4. Objeto de la invención

Título de la solicitud: "Moléculas y métodos para modular la pro-proteína convertasa-subtilisina/quexina tipo 9 (PCSK9)".

El problema planteado en esta solicitud consiste en suministrar moléculas de enlace de la pro-proteína convertasa-subtilisina/quexina tipo 9 (PCSK9) para el tratamiento de enfermedades relacionadas con altos niveles de LDL circulante.

Para resolver este problema técnico la solicitud en estudio proporciona una molécula que se enlaza con un epítipo dentro del dominio catalítico de la (PCSK9).

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 16/40; C12N 9/64.

5. No es una invención (Art 15 D 486 literal (b))

El objeto de la reivindicación 47 no se considera una invención, de acuerdo con el Art citado porque hace referencia a material biológico existente en la naturaleza correspondiente a dominios de la proteína PCSK9.

6. De la Solicitud de Patente

Título

El título no corresponde con el objeto de la solicitud porque hace referencia a métodos de tratamiento, lo cual no se considera materia patentable, por lo cual se sugiere al Solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones independientes, lo cual hace difícil definir cuál es el objeto que se busca proteger.

El capítulo reivindicatorio no es claro porque reclama una molécula de enlace caracterizada por el epítipo al cual se une. Las reivindicaciones 1 – 64 utilizan las expresiones "la cual comprende una porción de enlace de antígeno de un anticuerpo que se enlaza específicamente con una PCSK9, en donde la porción de enlace de antígeno se enlaza con un epítipo dentro del dominio catalítico de la PCSK9", "donde la porción de enlace de antígeno se enlaza con un epítipo dentro del dominio rico en cisteína de la PCSK9", "donde la porción de enlace de antígeno se enlaza con un epítipo dentro del pro-dominio de la PCSK9", "donde la porción de enlace de antígeno reacciona cruzadamente con una PCSK9", "donde la porción de enlace de antígeno se enlaza con un epítipo lineal", "donde la porción de enlace de antígeno se enlaza con un epítipo no lineal", "donde la porción de enlace de antígeno se enlaza específicamente con un epítipo de la PCSK9", "donde la porción de enlace de antígeno se enlaza con PCSK 9 con una constante de disociación (K_D) igual o menor que (...)", "donde el anticuerpo es un anticuerpo humanizado (...)", "donde la porción de enlace de antígeno es una porción de enlace de antígeno de un anticuerpo (...)", "es un anticuerpo quimérico", "comprende un fragmento Fab (...)", "donde la molécula de enlace de PCSK9 inhibe el enlace de PCSK9 con un ligando de PCSK9 (...)", "donde la molécula de enlace de PCSK9 inhibe (...)", "donde la

molécula de enlace de PCSK9, cuando se pone en contacto con un hepatocito bajo condiciones en donde esté presente la PCSK9, aumenta la absorción de colesterol (...)", "donde la molécula de enlace de PCSK9 se enlaza con PCSK9", "es cuando menos 75 por ciento idéntica (...)", "donde la porción de enlace de antígeno se deriva a partir de un anticuerpo de uno de los siguientes isotipos (...)", que son resultados a alcanzar y no definen a la molécula puesto que la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se requiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto, como la secuencia de aminoácidos de las cadenas pesada y ligera, la secuencia de aminoácidos de las regiones variables de las cadenas pesada y ligera y la secuencia de aminoácidos de las regiones determinantes de complementariedad (CDR).

La reivindicación 46 reclama una composición farmacéutica que comprende la molécula de enlace de PCSK9. Se recuerda al solicitante que una composición se caracteriza por los componentes y las proporciones dentro de la misma.

Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a 15 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**:

- 1° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 166-177 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 2° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 187-202 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 3° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 206-219 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 4° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 231-246 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 5° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 277-283 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 6° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 336-349 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 7° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 368-383 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 8° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 426-439 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.
- 9° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 443-500 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.

10° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 557-590 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.

11° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 636-678 de la SEQ ID NO: 1. Reivindicaciones 1, 4 – 7, 9, 13, 17 – 46, parcialmente.

12° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 89-134 de la SEQ ID NO: 1. Reivindicaciones 3 – 8, 13, 17 – 46, parcialmente.

13° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 101-107 de la SEQ ID NO: 1. Reivindicaciones 48 – 64, parcialmente.

14° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 123-132 de la SEQ ID NO: 1. Reivindicaciones 48 – 64, parcialmente.

15° Molécula de enlace a PCSK9 que se une al epítipo, dentro o traslapándose con los aminoácidos 101-132 de la SEQ ID NO: 1. Reivindicaciones 48 – 64, parcialmente.

No se guarda un concepto inventivo común porque las moléculas de enlace no comparten una estructura común esencial para la actividad y porque anticuerpos dirigidos a PCSK9 ya eran conocidos en los documentos D1 y D2.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Abril 13 de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	<u>The Journal of Clinical Investigation, Vol. 116, Pág. 2995-3005</u>	Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice	1/Noviembre/2006
D2	<u>PNAS, Vol. 101, Pág. 7100-7105</u>	Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype	4/mayo/2004

D1 <http://www.jci.org/articles/view/29383> divulga un anticuerpo que se enlaza con los aminoácidos 165-180 del dominio catalítico de la proteína PCSK9 (Pág. 3003, columna izquierda, líneas 28-31).

D2 <http://www.pnas.org/content/101/18/7100> divulga un anticuerpo que se enlaza con los aminoácidos 583-603 del dominio rico en cisteína de la proteína PCSK9 (Pág. 7101, columna izquierda, líneas 1 y 2).

9. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La **reivindicación 1** consiste en una molécula de enlace de polipéptido de pro-
teína convertasa-subtilisina/quexina tipo 9 aislada (PCSK9), la cual comprende
una porción de enlace de antígeno de un anticuerpo que se enlaza específicamente
con una PCSK9, en donde la porción de enlace de antígeno se enlaza con un
epítipo dentro del dominio catalítico de la PCSK9 humana (SEQ ID NO:1), dentro o
traslapándose con uno de los siguientes:

- (a) aminoácidos 166-177 de la SEQ ID NO:1;
- (b) aminoácidos 187-202 de la SEQ ID NO:1;
- (c) aminoácidos 206-219 de la SEQ ID NO:1;
- (d) aminoácidos 231-246 de la SEQ ID NO:1;
- (e) aminoácidos 277-283 de la SEQ ID NO:1;
- (f) aminoácidos 336-349 de la SEQ ID NO:1;
- (g) aminoácidos 368-383 de la SEQ ID NO:1; o
- (h) aminoácidos 426-439 de la SEQ ID NO:1.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Una molécula de enlace de polipéptido de pro- teína convertasa-subtilisina/quexina tipo 9 aislada (PCSK9), la cual comprende una porción de enlace de antígeno de un anticuerpo que se enlaza específicamente con una PCSK9, en donde la porción de enlace de antígeno se enlaza con un epítipo dentro del dominio catalítico de la PCSK9 humana (SEQ ID NO:1), dentro o traslapándose con uno de los siguientes: (a) aminoácidos 166-177 de la SEQ ID NO:1; (b) aminoácidos 187-202 de la SEQ ID NO:1; (c) aminoácidos 206-219 de la SEQ ID NO:1; (d) aminoácidos 231-246 de la SEQ ID NO:1; (e) aminoácidos 277-283 de la SEQ ID NO:1; (f) aminoácidos 336-349 de la SEQ ID NO:1; (g) aminoácidos 368-383 de la SEQ ID NO:1; o (h) aminoácidos 426-439 de la SEQ ID NO:1.	Un anticuerpo que se enlaza con los aminoácidos 165-180 de la proteína PCSK9 (Pág. 3003, columna izquierda, líneas 28-31)

Las características **esenciales** de la molécula de enlace de la reivindicación 1
habían sido divulgadas previamente en D1, el cual enseña un anticuerpo que se
enlaza con los aminoácidos 165-180 del dominio catalítico de la proteína PCSK9
(Pág. 3003, columna izquierda, líneas 28-31).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el
documento D1.

La **reivindicación 2** consiste en una molécula de enlace de PCSK9 aislada, la cual
comprende una porción de enlace de antígeno de un anticuerpo que se enlaza
específicamente con una PCSK9, en donde la porción de enlace de antígeno se
enlaza con un epítipo dentro del dominio rico en cisteína de la PCSK9 humana,
dentro o traslapándose con uno de los siguientes:

- (a) aminoácidos 443-500 de la SEQ ID NO:1;
- (b) aminoácidos 557-590 de la SEQ ID NO:1; o
- (c) aminoácidos 636-678 de la SEQ ID NO:1.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D2
Una molécula de enlace de PCSK9 aislada, la cual comprende una porción de enlace de antígeno de un anticuerpo que se enlaza específicamente con una PCSK9, en donde la porción de enlace de antígeno se enlaza con un epítipo dentro del dominio rico en cisteína de la PCSK9 humana, dentro o traslapándose con uno de los siguientes: (a) aminoácidos 443-500 de la SEQ ID NO:1; (b) aminoácidos 557-590 de la SEQ ID NO:1; o (c) aminoácidos 636-678 de la SEQ ID NO:1.	Un anticuerpo que se enlaza con los aminoácidos 583-603 de la proteína PCSK9 (Pág. 7101, columna izquierda, líneas 1 y 2)

Las características **esenciales** de la molécula de enlace de la reivindicación 2 habían sido divulgadas previamente en D2, el cual enseña un anticuerpo que se enlaza con los aminoácidos 583-603 del dominio rico en cisteína de la proteína PCSK9 (Pág. 7101, columna izquierda, líneas 1 y 2).

En consecuencia, la reivindicación 2 carece de novedad, según lo divulgado en el documento D2.

Nivel Inventivo (Art18 D486)

La **reivindicación 3** consiste en una molécula de enlace de PCSK9 aislada, la cual comprende una porción de enlace de antígeno de un anticuerpo que se enlaza específicamente con una PCSK9, en donde la porción de enlace de antígeno se enlaza con un epítipo dentro del pro-dominio de la PCSK9 humana, dentro o traslapándose con los aminoácidos 89-134 de la SEQ ID NO:1.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Una molécula de enlace de PCSK9 aislada, la cual comprende una porción de enlace de antígeno de un anticuerpo que se enlaza específicamente con una PCSK9, en donde la porción de enlace de antígeno se enlaza con un epítipo dentro del pro-dominio de la PCSK9 humana, dentro o traslapándose con los aminoácidos 89-134 de la SEQ ID NO: 1.	Un anticuerpo que se enlaza con los aminoácidos 165-180 de la proteína PCSK9 (Pág. 3003, columna izquierda, líneas 28-31)	Un anticuerpo que se enlaza con los aminoácidos 583-603 de la proteína PCSK9 (Pág. 7101, columna izquierda, líneas 1 y 2)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

D1 divulga un anticuerpo que se enlaza con los aminoácidos 165-180 del dominio catalítico de la proteína PCSK9 (Pág. 3003, columna izquierda, líneas 28-31).

La diferencia entre la invención, definida en la reivindicación 3 y el anticuerpo de D1 consiste en que la molécula de la presente solicitud se enlaza con el pro-dominio de la proteína PCSK9 correspondiente a los aminoácidos 89-134 de la SEQ ID NO: 1.

D2 enseña un anticuerpo que se enlaza con los aminoácidos 583-603 del dominio rico en cisteína de la proteína PCSK9 (Pág. 7101, columna izquierda, líneas 1 y 2).

La diferencia entre la invención, definida en la reivindicación 3 y el anticuerpo de D2 consiste en que la molécula de la presente solicitud se enlaza con el pro-dominio de la proteína PCSK9 correspondiente a los aminoácidos 89-134 de la SEQ ID NO: 1.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la molécula de enlace reclamada en esta solicitud porque no parece presentar una mayor actividad moduladora de la proteína PCSK9 según lo establecido en la descripción.

El problema técnico objetivo de esta solicitud consiste en la necesidad de moléculas de enlace de PCSK9 alternativas a los ya conocidos en los documentos D1 o D2.

Teniendo en cuenta que los documentos D1 y D2 ya divulgaban anticuerpos que se enlazan con los dominios catalítico y rico en cisteína de la proteína PCSK9, y que es bien conocido en el arte que el uso de inhibidores de PCSK9, como anticuerpos es útil para reducir los niveles plasmáticos de colesterol¹, la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, identificar otras regiones con alto índice antigénico en la secuencia de la proteína PCSK9 y utilizar métodos conocidos, como los divulgados en D1 o D2, para obtener más moléculas de enlace con la misma actividad.

De manera que la solución propuesta por esta solicitud en la reivindicación 3 y en las reivindicaciones 4 – 46 y 48 – 64, es obvia y no tiene Nivel Inventivo.

10. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	<u>The Journal of Clinical Investigation, Vol.116, Pág. 2995-3005</u>	1 – 46 y 48 – 64	Novedad y Nivel Inventivo
D2	<u>PNAS, Vol.101, Pág. 7100-7105</u>	1 – 46 y 48 – 64	Novedad y Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante para dar respuesta a este requerimiento y presentarla por escrito

1 S. Rashid, *et al.* Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. PNAS, Vol.102, Pág. 5374-5379.
Expediente 09-112535 1er Art 45

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALÁZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

18 SET. 2012
Elaborado por: Juan David Moncaleano 
Revisado por: Consuelo Leguizamón L. 



Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice

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Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proteinase K subfamily of subtilases that reduces the number of LDL receptors (LDLRs) in liver through an undefined posttranscriptional mechanism. We show that purified PCSK9 added to the medium of HepG2 cells reduces the number of cell-surface LDLRs in a dose- and time-dependent manner. This activity was approximately 10-fold greater for a gain-of-function mutant, PCSK9(D374Y), that causes hypercholesterolemia. Binding and uptake of PCSK9 were largely dependent on the presence of LDLRs. Coimmunoprecipitation and ligand blotting studies indicated that PCSK9 and LDLR directly associate; both proteins colocalized to late endocytic compartments. Purified PCSK9 had no effect on cell-surface LDLRs in hepatocytes lacking autosomal recessive hypercholesterolemia (ARH), an adaptor protein required for endocytosis of the receptor. Transgenic mice overexpressing human PCSK9 in liver secreted large amounts of the protein into plasma, which increased plasma LDL cholesterol concentrations to levels similar to those of LDLR-knockout mice. To determine whether PCSK9 was active in plasma, transgenic PCSK9 mice were parabiosed with wild-type littermates. After parabiosis, secreted PCSK9 was transferred to the circulation of wild-type mice and reduced the number of hepatic LDLRs to nearly undetectable levels. We conclude that secreted PCSK9 associates with the LDLR and reduces hepatic LDLR protein levels.

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proteinase K subfamily of subtilisin-related serine endoproteases. Like other members of this family, the PCSK9 protein has a signal sequence, followed by a prodomain, and a catalytic domain that contains a conserved triad of residues (D186, H226, and S386) required for activity (1). PCSK9 is synthesized as a soluble, approximately 74-kDa precursor that undergoes intramolecular autocatalytic cleavage in the ER, generating a 14-kDa prodomain and an approximately 60-kDa catalytic fragment. The site of intramolecular cleavage (VFAQ↓SIP) differs from that of subtilisin and other family members that typically cleave after a basic residue (2, 3). The cleaved prodomain remains associated with the catalytic domain, forming a PCSK9-prodomain complex that is transported to the Golgi complex, where it undergoes sulfation prior to secretion (1, 3).

Insights into the physiological function of PCSK9 initially came from the identification of point mutations in PCSK9 that cause an autosomal dominant form of hypercholesterolemia (4). These mutations were later shown to be gain-of-function alleles that act in a dominant fashion (3, 5). PCSK9 was independently identified as a SREBP-regulated gene in liver, using microarrays hybridized with RNA from livers of mice that either overexpressed or lacked

SREBPs (6, 7). SREBPs are a family of transcription factors that increase the expression of many genes involved in cholesterol and fatty acid synthesis, as well as the *LDL receptor* (*LDLR*) gene (6). The induction of PCSK9 by SREBPs further suggested that this protein was involved in lipid metabolism.

The biological activity of PCSK9 was revealed through overexpression studies in mice. Overexpression of PCSK9 posttranscriptionally reduced the amount of LDLR protein in liver (3, 8–10). Confirmation that PCSK9 functions normally to regulate LDLR protein levels came from loss-of-function studies in humans and mice. Individuals who are heterozygous for a nonsense mutation in allele PCSK9 have significantly lower plasma LDL cholesterol levels, suggesting that a reduction in PCSK9 activity leads to an increase in LDLRs (11). These conclusions were supported by studies in PCSK9-knockout mice, which revealed that loss of PCSK9 resulted in increased numbers of LDLRs in hepatocytes, accelerated plasma LDL clearance, and significantly lower plasma cholesterol levels (12). In the most recent studies, humans heterozygous for loss-of-function mutations in PCSK9 were shown to have a significant reduction in the long-term risk of developing atherosclerotic heart disease (13).

The genetic data from humans and the *in vivo* studies in mice demonstrate that one function of PCSK9 is to reduce the number of the LDLRs and that this function is manifest in humans in the basal state. The mechanism by which PCSK9 reduces the number of LDLRs is still undetermined. For example, it is unclear whether PCSK9 acts to destroy LDLRs in the secretory pathway or whether it acts outside of the cell. In the current studies, we provide evidence that extracellular PCSK9 can be internalized by cultured liver cells and fibroblasts in a manner that is largely dependent on LDLRs. Incubation with extracellular PCSK9 led to loss of LDLRs

Nonstandard abbreviations used: ARH, autosomal recessive hypercholesterolemia; CI-MPR, cation-independent mannose-6-phosphate receptor; LDLR, LDL receptor; LRP, LDLR-related protein; MEF, mouse embryonic fibroblast; PCSK9, proprotein convertase subtilisin/kexin type 9.

Conflict of interest: J.D. Horton is a consultant for Alnylam Pharmaceuticals, Aegerion Pharmaceuticals Inc., Merabasis, Merck, and Pfizer, and is on the Speakers' Bureau of Merck, Merck/Schering-Plough Pharmaceuticals, Schering-Plough.

Citation for this article: *J. Clin. Invest.* 116:2995–3005 (2006). doi:10.1172/JCI29383.

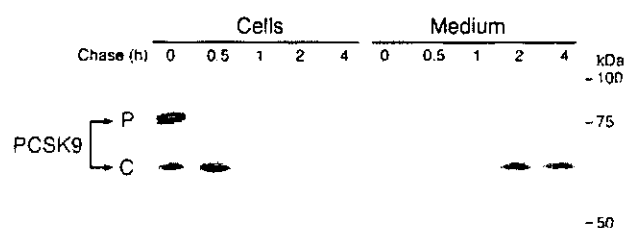


Figure 1

Pulse-chase analysis of endogenous PCSK9 secretion from HepG2 cells. Cells were cultured in sterol-depleting medium C for 18 hours prior to labeling for 30 minutes with [^{35} S]methionine/cysteine. After washing, cells were incubated in chase medium containing unlabeled methionine and cysteine for the indicated times. Cells were lysed, and PCSK9 was immunoprecipitated from the medium and cell lysates as described in Methods. Samples were subjected to 8% SDS-PAGE, and the gel was treated with Amplify fluorogenic reagent prior to drying and exposure to film. P and C denote the proprotein and cleaved forms of PCSK9, respectively. Similar results were obtained in 4 independent experiments.

from the cell surface and accelerated destruction of LDLRs in liver-derived cells. Finally, we demonstrate that increased PCSK9 levels in the circulation of mice leads to diminished liver LDLR protein and increased plasma cholesterol levels.

Results

PCSK9, like other subtilisin family proteases, is synthesized with a prodomain (1, 3). In the other subtilisin family proteases, the cleaved prodomain remains associated with the protein and acts as an inhibitor of the cognate enzyme activity. The prosegment often undergoes secondary proteolytic processing, which relieves this inhibition and unmasks enzymatic activity (14). When PCSK9 is overexpressed in cultured cells, the protein is secreted with the prodomain still attached (1, 3). This result raised the possibility that cellular PCSK9 remains inactive within the secretory pathway and that PCSK9 may act on LDLRs after secretion, either at the cell surface or after the LDLR is internalized into the endosomal/lysosomal system.

To test this possibility, we assessed the rate and extent of secretion of endogenous PCSK9 expressed at physiological levels in cultured human hepatoma HepG2 cells. This study was made possible by the development of antibodies against the catalytic domain of PCSK9. Synthesis and secretion of PCSK9

were quantified by pulse-labeling the cells with [^{35} S]methionine/cysteine, which was followed by a chase period in isotope-free medium. Radiolabeled PCSK9 was immunoprecipitated from cells and the medium at different times. Figure 1 shows results of a representative pulse-chase experiment. Two forms of the protein, corresponding to the uncleaved precursor (~74 kDa) and the cleaved mature catalytic fragment (~60 kDa), were detected in cells at 0.5 and 1 hour of chase. After 2 hours, none of the labeled uncleaved PCSK9 was detected in the cells. After 4 hours, nearly all of the radiolabeled PCSK9 was recovered in the medium, demonstrating that the cleaved form of PCSK9 is rapidly and efficiently secreted from these cells.

We recently reported that PCSK9 can be detected in human plasma by immunoprecipitation (15). To quantify the concentration and determine the physiological range of PCSK9 in human plasma, we developed an ELISA that used an anti-human PCSK9 monoclonal antibody to capture PCSK9 and a polyclonal anti-human PCSK9 antibody for detection. Plasma levels of PCSK9 were quantified in 72 volunteers. The plasma levels ranged from approximately 50 to approximately 600 ng/ml (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI29383DS1). These measurements demonstrate that considerable amounts of PCSK9 circulate in plasma and provided a range of physiologically relevant PCSK9 concentrations.

We next determined whether the secreted form of PCSK9 can reduce the number of LDLRs when added to the medium of HepG2 cells cultured in sterol-depleted medium to induce LDLR expression. Recombinant PCSK9 that contained a FLAG tag at the carboxyl terminus was purified from HEK 293S cells as described in Methods. SDS-PAGE and Coomassie blue staining analysis showed that purified FLAG-tagged PCSK9 consisted of the catalytic fragment and the cleaved prodomain that migrated with apparent molecular masses of approximately 60 kDa and approximately 17 kDa, respectively (Supplemental Figure 2A). Gel filtra-

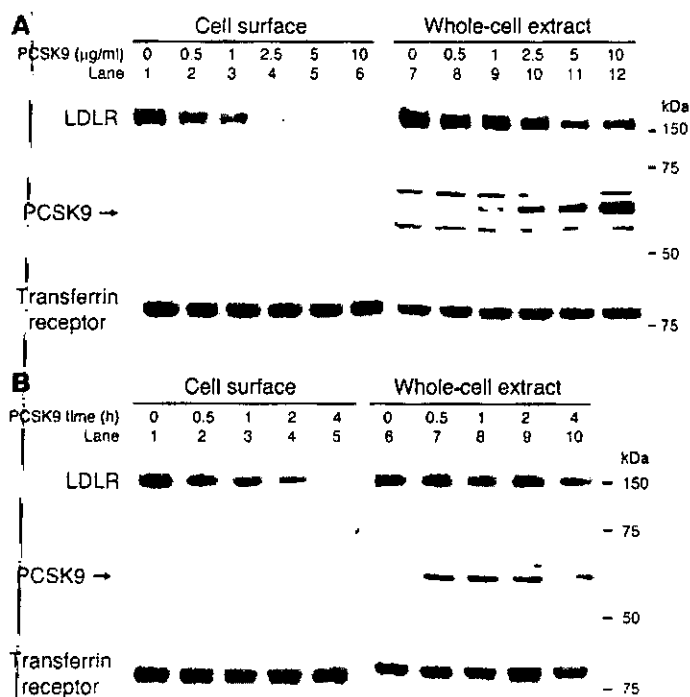


Figure 2

Reduction of endogenous LDLRs in HepG2 cells following the addition of recombinant purified PCSK9 to the culture medium. (A) Dose response of exogenous PCSK9-mediated LDLR degradation in HepG2 cells. Cells were cultured for 18 hours in medium C prior to treatment for 4 hours with the indicated amounts of purified human PCSK9. (B) Time course of exogenous PCSK9-mediated LDLR degradation. HepG2 cells cultured as described above were treated with 5 μg/ml of purified PCSK9 for the indicated times. Total lysates were prepared following cell-surface biotinylation as described in Methods. Proteins were subjected to SDS-PAGE for immunoblot analysis of the LDLR using IgG-HL1 and anti-FLAG M2 monoclonal antibody to detect purified PCSK9. The transferrin receptor protein was detected as described in Methods and used as a control for loading and nonspecific protein degradation. Similar results were obtained in 3 independent experiments.

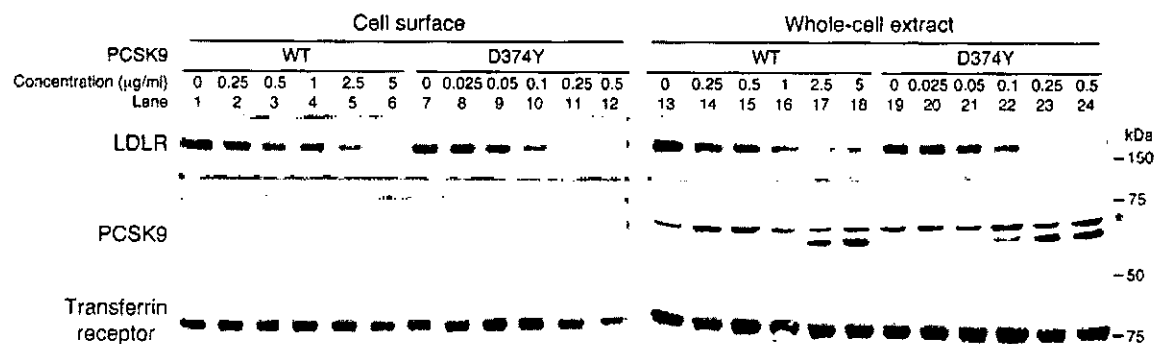


Figure 3

Increased cell association and LDLR degradation by addition of purified mutant PCSK9(D374Y) to the medium of HepG2 cells. Cells were cultured for 18 hours in medium C and then incubated for 4 hours with the indicated amounts of purified human PCSK9 or PCSK9(D374Y). Immunoblot analysis of LDLR, FLAG-tagged PCSK9, and transferrin receptor was carried out as described in the legend to Figure 2. The asterisk indicates a nonspecific band. Similar results were obtained in 3 independent experiments.

tion revealed that the protein elutes at a single peak volume corresponding to an apparent molecular mass of approximately 70 kDa (Supplemental Figure 2B). After incubation with purified PCSK9, the surface proteins of HepG2 cells were covalently modified with a cell-impermeable biotinylation reagent and isolated using streptavidin beads. The total cellular LDLRs in whole-cell extracts and cell-surface LDLRs were measured by SDS-PAGE immunoblotting using a monoclonal antibody. The total cell PCSK9 accumulation and cell-surface PCSK9 were also measured by SDS-PAGE and immunoblotting, but with anti-FLAG antibody.

As shown in Figure 2A, the number of cell-surface LDLRs declined by approximately 50% after incubation with the physiologically relevant concentration of 0.5 µg/ml PCSK9 (lane 2) and became nearly undetectable after exposure to 2.5 µg/ml PCSK9 (lane 4). Incubation of HepG2 cells for 4 hours with 5 or 10 µg/ml PCSK9 decreased whole-cell LDLR protein levels by approximately 50% (lanes 11 and 12). FLAG-tagged PCSK9 was detected in whole-cell extracts in a concentration-dependent manner (lanes 7–12), but it was not detected among the biotin-labeled cell-surface proteins (lanes 1–6), suggesting that most of the cell-associated PCSK9 had been internalized.

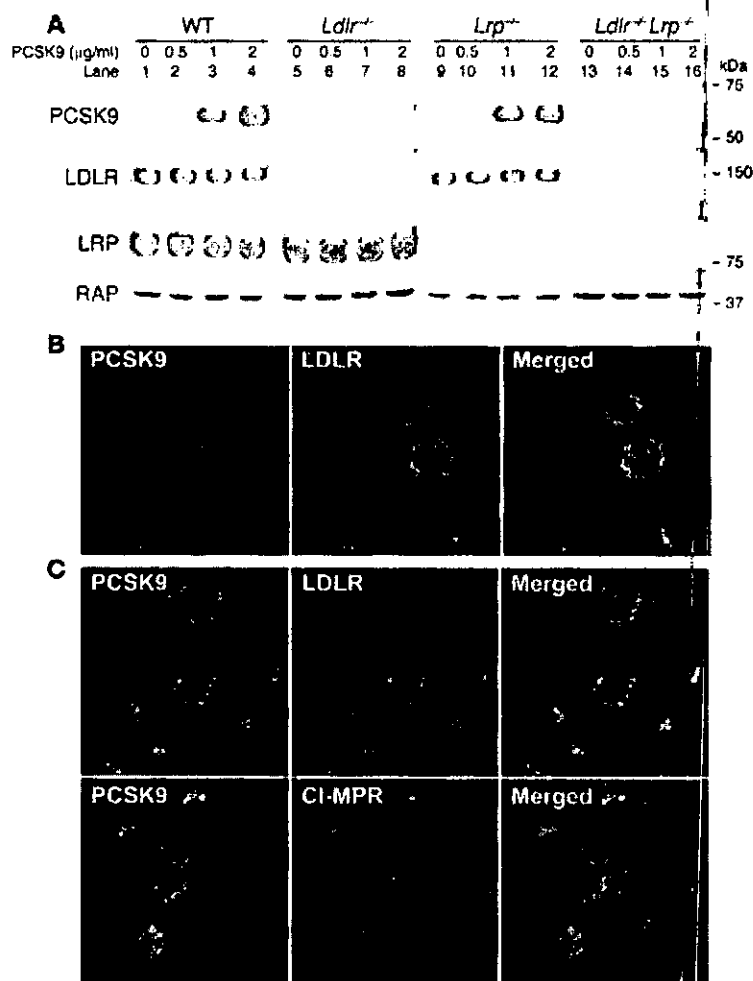
To determine the time course of PCSK9 action, HepG2 cells were incubated in the presence of 5 µg/ml PCSK9 and then harvested at different intervals over a 4-hour period (Figure 2B). The number of cell-surface LDLRs declined noticeably at 2 hours (lane 4) and was undetectable at 4 hours (lane 5). The amount of cell-associated FLAG-tagged PCSK9 was maximal after 30 minutes (lane 7) and decreased slightly at 4 hours (lane 10). PCSK9 had no effect on whole-cell or cell-surface transferrin receptors (Figure 2, A and B).

As discussed in the Introduction, certain point mutations in PCSK9 cause hypercholesterolemia. To determine whether one such mutation increases the activity of PCSK9 in a cell-based assay, varying amounts of wild-type PCSK9 and the PCSK9 mutant D374Y (4) were added to HepG2 cells, after which LDLR protein levels were measured (Figure 3). The D374Y mutation was chosen for study because individuals who harbor this mutation have been shown to manifest severe hypercholesterolemia (16). PCSK9(D374Y) was at least 10-fold more active than wild-type PCSK9 in reducing cell surface LDLRs. Thus, PCSK9(D374Y) at 0.25 µg/ml was at least as effective as 2.5 µg/ml of wild-type PCSK9 (compare lanes 5 and 11). After incubation with wild-type PCSK9,

the number of LDLRs was significantly reduced in whole-cell extracts, and similar results were obtained with 10-fold lower concentrations of PCSK9(D374Y) (lanes 13–24). Despite the different concentrations employed, the amounts of wild-type and mutant PCSK9 measured in the cell extracts were similar, indicating that the mutant protein was taken up by the cell approximately 10-fold more efficiently than the wild-type protein.

To determine whether cellular association/uptake of PCSK9 is dependent upon LDLRs, we turned to mouse embryonic fibroblasts (MEFs), to exploit the availability of cells from gene knock-out mice that lack the LDLR and the closely related receptor, the LDLR-related protein (LRP). MEFs derived from wild-type, *Ldlr*^{−/−}, *Lrp*^{−/−}, and *Ldlr*^{−/−}*Lrp*^{−/−} mice were incubated with varying amounts of PCSK9 (Figure 4A). Immunoblots revealed that abundant PCSK9 was associated with the wild-type (lanes 1–4) and *Lrp*^{−/−} cells (lanes 9–12). PCSK9 cellular association was markedly reduced in *Ldlr*^{−/−} MEFs (lanes 5–8); however, a small amount of PCSK9 was detectable in cells incubated with the highest concentration of PCSK9 (lane 8). This small amount of association was abolished in cells that lacked both LDLR and LRP (lane 16). These data suggest that exogenous PCSK9 associates with MEFs in a manner that is almost totally dependent upon the LDLR and that LRP may play a small role in uptake at high concentrations of PCSK9.

The uptake of PCSK9 was characterized morphologically using indirect immunofluorescence in MEFs. Double immunofluorescence labeling of cells incubated with PCSK9 for 4 hours showed diffuse localization of the protein in small punctate structures, possibly representing endocytic vesicles, which partially overlapped with the LDLR staining pattern (Figure 4B). To determine whether these structures were endosomal and whether PCSK9 colocalized intracellularly with the LDLR, we incubated MEFs in the presence of chloroquine, which raises the pH of acidic cellular compartments and inhibits lysosomal hydrolases. In the presence of chloroquine, the cation-independent mannose-6-phosphate receptor (CI-MPR) accumulates in late endosomes and was used as a marker of these structures (17). As shown in Figure 4C, PCSK9 became concentrated in large perinuclear vacuoles of MEFs in the presence of chloroquine. These vacuoles were also labeled with anti-LDLR and anti-CI-MPR antibodies. Considered together, the data indicate that exogenous PCSK9 is taken up by fibroblasts in a manner that is dependent upon the LDLR and

**Figure 4**

LDLR-dependent endocytosis of PCSK9 in MEFs. (A) Immunoblot analysis of PCSK9 association with MEFs. Immortalized MEFs derived from wild-type, *Ldlr*^{-/-}, *Lrp*^{-/-}, and *Ldlr*^{-/-}*Lrp*^{-/-} mice were cultured for 18 hours in medium F prior to treatment with purified human PCSK9 for 4 hours. Cell lysates (30 μg) were subjected to SDS-PAGE and immunoblot analysis using IgG-15A6 to detect PCSK9 or a polyclonal antiserum (Ab 3143) that recognizes the LDLR, as described in Methods. Immunoblots of receptor-associated protein (RAP) were used as a loading control. (B) Indirect immunofluorescence localization of PCSK9 and the LDLR in wild-type MEFs. MEFs were incubated for 4 hours with 5 μg/ml purified human PCSK9 and processed for double immunofluorescence of PCSK9 (green) and the LDLR (red) as described in Methods. (C) Indirect immunofluorescence localization of PCSK9, LDLR, and the late-endosomal marker CI-MPR in MEFs cultured in the presence of chloroquine. Wild-type MEFs were incubated for 4 hours with 5 μg/ml purified human PCSK9 in the presence of 0.1 mM chloroquine and processed for double immunofluorescence of PCSK9 (green) and the LDLR (red) or CI-MPR (red) as described in Methods. The merged image shows areas of colocalization of PCSK9 with the LDLR and CI-MPR. Magnification, ×630. Similar results were obtained in 3 independent experiments.

that the PCSK9 travels with the LDLR to the endosome/lysosome. Despite this uptake, PCSK9 does not destroy the LDLR in fibroblasts at the concentrations tested, either in the absence or presence of chloroquine. A similar lack of destruction was observed earlier when PCSK9 was overexpressed in fibroblasts and Chinese hamster ovary cells by transfection (9).

The requirement of LDLRs for PCSK9 internalization suggests that PCSK9 may bind to the LDLR. Moreover, PCSK9(D374Y) was taken up with higher efficiency than wild-type PCSK9, suggesting that the mutant protein may bind to the LDLR more avidly than wild-type PCSK9. To test this possibility, coimmunoprecipitation studies with wild-type PCSK9 or mutant PCSK9(D374Y) and the LDLR were carried out in HepG2 cells. The cells were incubated with wild-type and mutant PCSK9 proteins containing FLAG epitope tags. After a 1-hour incubation in the presence of chloroquine, cells were solubilized, and exogenously added PCSK9 was precipitated with an anti-FLAG antibody. As shown in Figure 5A (lanes 1 and 5), the FLAG antibody efficiently pulled down the wild-type and mutant PCSK9 from cell extracts, and the immunoprecipitation was reduced in the presence of competing FLAG octapeptide (lanes 2 and 6). In cells that were incubated with wild-type PCSK9, no detectable LDLR was coimmunoprecipitated with PCSK9. In contrast, a significant amount of LDLR was pulled down from cells incubated with PCSK9(D374Y) (lanes 1 and 5). When the LDLR antibody was used for the immunoprecipitation,

a small, but consistently detectable amount of wild-type PCSK9 was coimmunoprecipitated. The LDLR antibody pulled down much larger amounts of PCSK9(D374Y) (lanes 3 and 7).

To determine whether the interaction of PCSK9 and the LDLR was direct, ligand blotting was performed using purified extracellular domain LDLR protein and PCSK9. First, we determined the migration of the purified LDLR under reduced and nonreduced conditions (Figure 5B). Next, we determined whether PCSK9 could bind to the extracellular domain of the LDLR in a specific manner. The reduced and nonreduced LDLR protein was resolved by SDS-PAGE and transferred to nitrocellulose. The filters were then incubated with 5 μg/ml of purified PCSK9 protein, and bound PCSK9 was visualized using the monoclonal antibody IgG-15A6. As shown in Figure 5C, PCSK9 bound to nonreduced LDLR protein but not to LDLRs that had been reduced. The binding of wild-type PCSK9 and PCSK9(D374Y) to increasing amounts of the LDLR protein was then determined by ligand blotting (Figure 5D). Both forms of purified PCSK9 bound to the extracellular domain of the LDLR protein in a concentration-dependent manner. Consistent with the coimmunoprecipitation, the PCSK9(D374Y) mutant appeared to bind to the LDLR protein with a greater affinity. Combined, the results of these studies indicate that PCSK9(D374Y) binds to LDLRs with higher affinity than does wild-type PCSK9, a finding that correlates with the enhanced ability of the mutant PCSK9 to destroy LDLRs.

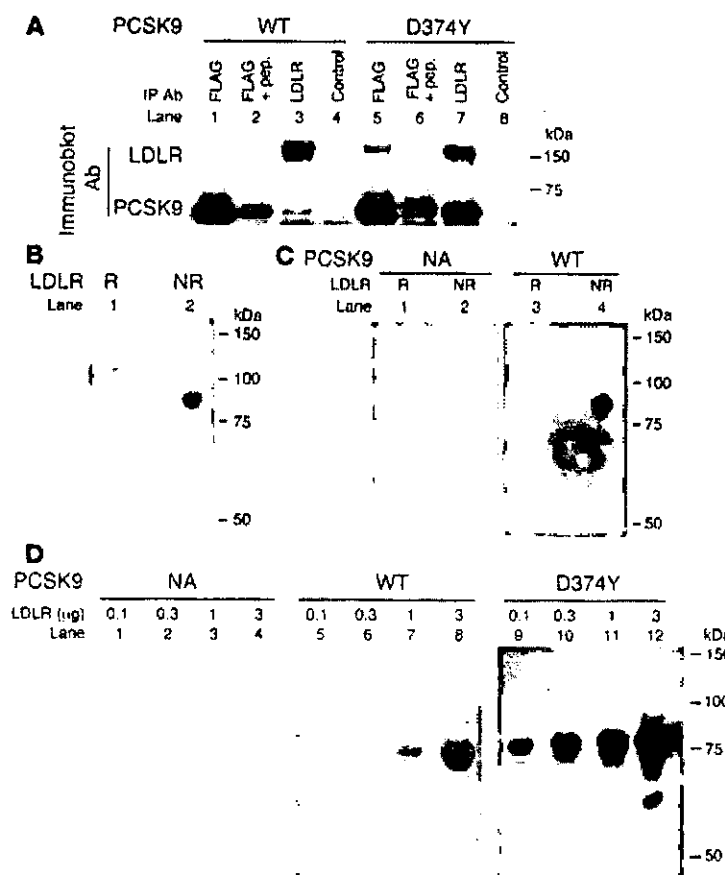


Figure 5

Association of PCSK9 and PCSK9(D374Y) with the LDLR. (A) Coimmunoprecipitation of the LDLR and exogenously added wild-type PCSK9 or PCSK9(D374Y) protein. HepG2 cells were cultured for 18 hours in medium C prior to treatment for 1 hour in the presence of 0.1 mM chloroquine with 20 μg/ml or 2 μg/ml of purified human PCSK9 or PCSK9(D374Y), respectively. Cells were harvested and lysed and proteins immunoprecipitated with the indicated antibodies. Pellets of the immunoprecipitation were subjected to SDS-PAGE and immunoblot analysis. Polyclonal antiserum (Ab 3143) was used to detect LDLR, and IgG-15A6 was used to detect PCSK9. (B) Reduced (R) or nonreduced (NR) LDLR extracellular domain protein (2 μg) was resolved by SDS-PAGE. LDLR protein was detected with Coomassie brilliant blue R-250 stain. (C) Binding of PCSK9 to LDLR on ligand blots. LDLR protein (2 μg) was transferred to nitrocellulose and incubated with 5 μg/ml of purified PCSK9 or no addition (NA). Bound PCSK9 was detected by immunoblot analysis as described in Methods. (D) The indicated amounts of purified LDLR were subjected to nonreduced SDS-PAGE, transferred to nitrocellulose, and incubated with 5 μg/ml of purified wild-type PCSK9, PCSK9(D374Y), or buffer control (no addition). Bound PCSK9 was detected by immunoblot analysis as described in Methods. pep., FLAG octapeptide.

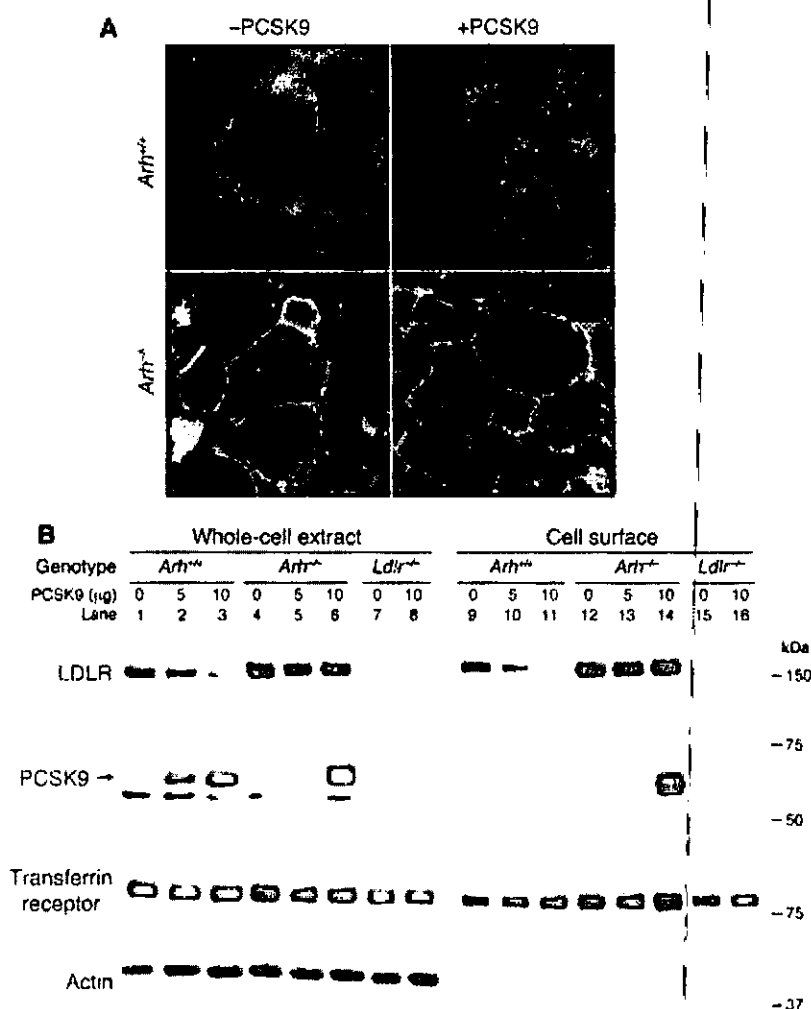
In hepatocytes, the internalization of LDLRs is dependent upon functional autosomal recessive hypercholesterolemia (ARH), an adaptor protein that binds to the intracellular domain of the LDLR and to clathrin, thereby clustering the receptor in coated vesicles (18, 19). To determine whether ARH was similarly required for removal of LDLR from the cell surface, PCSK9 was added to the medium of primary hepatocytes isolated from 2 types of genetically engineered mice: those that express only the human LDLR (*LDLR^hArh^{-/-}*) (20) and those that express the human LDLR and lack ARH (*LDLR^hArh^{-/-}*) (21). In *LDLR^hArh^{-/-}* hepatocytes, LDLRs were visualized on the cell surface and in intracellular vesicles concentrated in the cell periphery (Figure 6A, upper left). Incubation with PCSK9 eliminated the surface LDLRs, and the remaining LDLRs were concentrated in perinuclear vesicles (Figure 6A, upper right). In *LDLR^hArh^{-/-}* hepatocytes, the LDLRs were visualized almost entirely on the cell surface (Figure 6A, lower left), and PCSK9 did not reduce the amount of surface LDLRs in these cells (Figure 6A, lower right).

To confirm that PCSK9 still associated with the cell in the absence of ARH, total lysates and cell-surface proteins were isolated for immunoblot analysis from *LDLR^hArh^{-/-}* and *LDLR^hArh^{-/-}* primary hepatocytes incubated with increasing concentrations of PCSK9 (Figure 6B). The level of LDLR protein was reduced by exogenously added PCSK9 in a dose-dependent manner in both whole-cell extracts and on the cell surface in hepatocytes expressing ARH (lanes 1–3 and 9–11). In contrast, incubation of *LDLR^hArh^{-/-}* hepatocytes with PCSK9 did not alter LDLR protein levels in either fraction (lanes 4–6 and 12–14), despite similar lev-

els of PCSK9 cell association (lanes 1–3 and 4–6). In the absence of ARH, significantly more PCSK9 was detected at the cell surface (lanes 13–14). No PCSK9 was detected in hepatocytes isolated from *Ldlr^{-/-}* mice (lanes 7–8 and 15–16). PCSK9 had no effect on whole-cell or cell-surface transferrin receptors in hepatocytes, and cellular integrity was maintained during cell-surface biotinylation, as indicated by a lack of detection of intracellular actin among biotin-labeled proteins. These results demonstrate that PCSK9 can associate with the cell in the absence of ARH but that internalization is required for PCSK9 to reduce the cell-surface expression of LDLR protein. They also show that PCSK9 binding is largely dependent upon LDLR expression in mouse hepatocytes.

To test whether PCSK9 could function as a secreted protein in vivo, transgenic mice that express human PCSK9 in liver (TgPCSK9 mice) were produced. The transgene was under the control of an apoE promoter with a liver-specific enhancer (22). As shown in Figure 7A, transgenic overexpression of human PCSK9 eliminated LDLR protein expression in liver and caused a marked increase in plasma LDL cholesterol levels (Figure 7B). The increase in plasma LDL cholesterol was similar to that measured in *Ldlr^{-/-}* mice that lacked LDLRs in all tissues. The effects of transgenic PCSK9 overexpression are consistent with the results of previously published experiments in which adenoviral vectors were used to overexpress PCSK9 in mice (3, 8–10). The concentration of human PCSK9 in plasma from the transgenic mice as measured using the ELISA assay ranged from approximately 146 to 440 μg/ml (Table 1, animals 9a, 10a, 11a, and 12a).

Although an apoE promoter fragment and a liver-specific enhancer were used to express the human PCSK9 cDNA in mice,

**Figure 6**

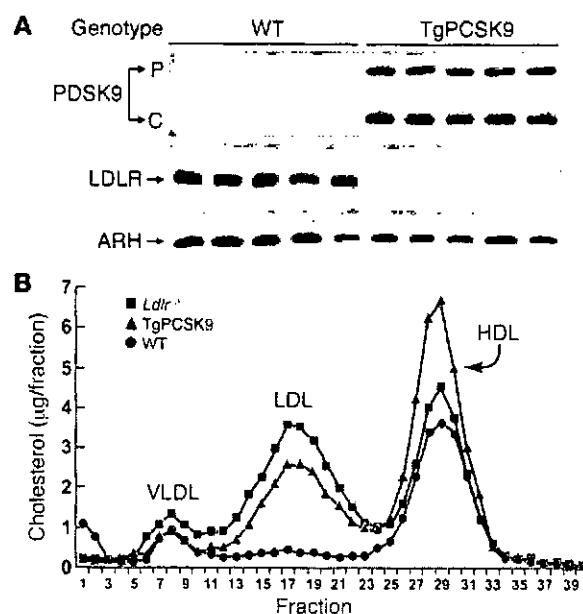
PCSK9-mediated degradation of the LDLR is dependent on ARH. Immunofluorescence (**A**) and immunoblot (**B**) analyses of the PCSK9-mediated changes of LDLR protein in mouse primary hepatocytes derived from *LDLR^{fl/fl}Arh^{+/+}* (*Arh^{+/+}*) or *LDLR^{fl/fl}Arh^{-/-}* (*Arh^{-/-}*) mice. (**A**) Primary hepatocytes were incubated with no PCSK9 (-PCSK9) or with 5 μg/ml purified PCSK9 (+PCSK9) for 4 hours and processed for indirect immunofluorescence of LDLR using IgG-C7 and Alexa Fluor 563-conjugated secondary antibody, as described in Methods. Images were taken using a 63 × 1.3 objective using a confocal microscope (Leica TCS SP). Similar results were obtained in 2 independent experiments. (**B**) Immunoblot analysis of LDLR, FLAG-tagged PCSK9, transferrin receptor, and actin proteins in primary hepatocytes from mice of the indicated genotype incubated with varying amounts of PCSK9.

the transgene mRNA was detected at low levels in other tissues, such as the adrenal gland (data not shown). This expression compromised analysis of the effect of plasma PCSK9 on LDLR protein expression in these extrahepatic tissues. Therefore, TgPCSK9 mice were parabiosed with wild-type mice to determine whether secreted PCSK9 derived from the transgenic mouse was able to decrease LDLR protein levels in liver when transferred via the shared circulation to the recipient wild-type mouse. For these studies, a liver biopsy was performed 1 week prior to parabiosis to obtain a liver sample for quantifying basal levels of LDLR protein expression. The mice were allowed to recover from the partial hepatectomy and then were parabiosed for 2 weeks to permit the development of shared circulation. To control for potential changes induced by surgery, wild-type mice were parabiosed with wild-type mice. TgPCSK9 mice manifest elevated plasma LDL cholesterol levels that could independently alter LDLR protein expression in the recipient wild-type mouse. To control for this effect, wild-type mice were parabiosed with *Ldlr*^{-/-} mice, which have lipoprotein profiles similar to those of TgPCSK9 mice (Figure 7B).

As shown in Table 1, parabiosis itself slightly increased plasma total cholesterol levels in most mouse pairs. Plasma cholesterol concentrations in wild-type mice parabiosed with *Ldlr*^{-/-} mice (pairs 5–8) were not significantly higher than those measured in

the wild-type mouse parabiotic pairs (pairs 1–4). Concentrations of human PCSK9 in parabiotic TgPCSK9:wild-type pairs ranged from approximately 17 to 250 μg/ml (pairs 9–12), suggesting that the protein was transferred from the plasma of transgenic mice into the plasma of the parabiosed wild-type mouse with varying efficiencies. There also appeared to be a correlation between the plasma PCSK9 concentration and the degree of plasma cholesterol elevation of the recipient wild-type mouse. The wild-type mouse with the highest PCSK9 concentration (mouse 9b) had an approximately 10-fold increase in plasma total cholesterol, whereas the wild-type mouse with the lowest PCSK9 level (mouse 12b) had an approximately 2-fold increase in plasma cholesterol.

To confirm that the observed plasma cholesterol changes were a result of alterations in LDLR protein levels, immunoblots of liver protein were performed in all parabiotic pairs before and 2 weeks after parabiosis (Figure 8). LDLR protein levels in livers of wild-type mice parabiosed with wild-type mice were not different before and after parabiosis (Figure 8A). Similarly, the LDLR protein levels in wild-type mice parabiosed with *Ldlr*^{-/-} mice were not different after parabiosis, suggesting that the increased level of LDL cholesterol present in the knockout mouse did not alter LDLR expression in the wild-type mouse liver (Figure 8B). In contrast, the LDLR protein was essentially undetectable in livers of wild-type

**Figure 7**

Decreased amounts of hepatic LDLR protein and increased levels of plasma LDL cholesterol in transgenic PCSK9 mice. (A) Whole-cell lysates (30 μg) from livers of 5 wild-type and 5 TgPCSK9 mice were subjected to SDS-PAGE and immunoblot analyses of human PCSK9, mouse LDLR, and mouse ARH. (B) Plasma from the mice of each genotype described in A and from 5 age-matched male *Ldlr*^{-/-} mice was pooled and subjected to gel filtration by fast performance liquid chromatography. The concentration of total cholesterol in each fraction was measured as described in Methods.

mice after they were parabiosed with TgPCSK9 mice (Figure 8C), indicating that PCSK9 was active in mouse plasma.

To ensure that the reduction in LDLR protein levels measured in livers of the recipient wild-type mice parabiosed to the TgPCSK9 mice was due to posttranscriptional changes, mRNA levels of the LDLR and SREBP-2 were measured using real-time RT-PCR (Supplemental Table 1). Although individual mRNAs varied in amount from mouse to mouse, no consistent changes in the mRNAs encoding these genes were measured before and after parabiosis. Combined, the data suggested that secreted PCSK9 in plasma of mice reduced hepatic LDLR protein levels independent of changes in mRNA levels.

Discussion

In the current report, we demonstrate that endogenous PCSK9 is rapidly secreted from cells and that secreted PCSK9 destroys LDLRs when added to the medium of cultured HepG2 cells and mouse primary hepatocytes. The effective concentration of PCSK9 required to reduce the number of LDLRs in cultured cells was within the range of plasma concentrations measured in human plasma. The cell association and uptake of PCSK9 occurred via binding to the LDLR, and both proteins colocalized to a late endocytic/lysosomal compartment. The internalization of PCSK9 with the LDLR into an endosomal/lysosomal compartment was required for PCSK9 to reduce LDLR protein levels, since this activity was blocked in the absence of ARH. Finally, we show that PCSK9 was present in plasma of transgenic mice and that the secreted protein was active in destroying hepatic LDLRs.

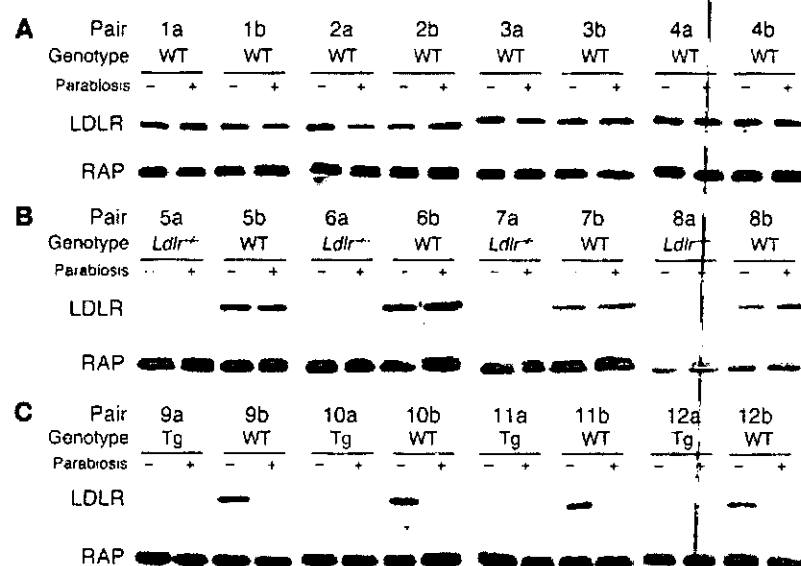
Insights into the mechanism of secreted PCSK9's action were derived from studies in MEFs and mouse hepatocytes, which showed that LDLRs were required for the majority of PCSK9 to associate with the cell surface (Figure 4A and Figure 6B). These studies suggested that the LDLR and PCSK9 may directly interact, which was confirmed by coimmunoprecipitation and ligand blotting studies with the LDLR and PCSK9 (Figure 5). The coimmunoprecipitation studies with PCSK9(D374Y) and the LDLR suggested

that the interaction of the mutant protein with the LDLR was much more avid than that of the wild-type PCSK9 (Figure 5A). In addition, PCSK9(D374Y) was approximately 10-fold more efficient in destroying cell-surface LDLRs when added exogenously to HepG2 cells (Figure 3). Previous overexpression studies in McArdle-7777 rat hepatoma cells with PCSK9(D374Y) suggested that the mutant protein increased plasma LDL cholesterol levels through the overproduction of apoB (5). Similar conclusions were drawn from in vivo kinetic studies in humans that carry a different point mutation in PCSK9 (S127R) (23). The current studies with the D374Y mutant suggest an alternative mechanism that involves enhanced LDLR association and degradation. Whether the S127R and F216L point mutations in PCSK9 behave in a manner similar to PCSK9(D374Y) will be tested in future studies. Our observations that purified PCSK9(D374Y) added to cells reduced the number of LDLRs extend the results from a publication that appeared during the preparation of this manuscript demonstrat-

Table 1

Total plasma cholesterol and PCSK9 concentrations in pre- and post-parabiotic pairs of wild-type, *Ldlr*^{-/-}, and TgPCSK9 (Tg) mice

Parabiotic pair	Genotype	Before parabiosis Cholesterol (mg/dl)	After parabiosis Cholesterol (mg/dl)	hPCSK9 (μg/ml)
1a	WT	54	79	0
1b	WT	60	85	0
2a	WT	67	91	0
2b	WT	67	60	0
3a	WT	54	85	0
3b	WT	42	63	0
4a	WT	51	81	0
4b	WT	60	71	0
5a	<i>Ldlr</i> ^{-/-}	178	288	0
5b	WT	56	76	0
6a	<i>Ldlr</i> ^{-/-}	182	270	0
6b	WT	57	64	0
7a	<i>Ldlr</i> ^{-/-}	175	278	0
7b	WT	53	82	0
8a	<i>Ldlr</i> ^{-/-}	179	145	0
8b	WT	68	101	0
9a	Tg	142	365	440
9b	WT	70	775	246
10a	Tg	132	350	198
10b	WT	66	364	148
11a	Tg	180	278	146
11b	WT	61	233	80
12a	Tg	140	171	184
12b	WT	61	131	17

**Figure 8**

Amounts of LDLR protein in livers of parabiosed mice. Three combinations of mice were parabiosed for study: wild-type:wild-type (A); *Ldlr*^{-/-}:wild-type (B); and TgPCSK9:wild-type (C). Prior to parabiosis, a liver sample (<100 mg) via a partial hepatectomy and approximately 50 μ l of blood via the tail vein were obtained from each mouse. After a 7-day recovery period, 12- to 20-week-old female mice of the indicated genotype were parabiosed as described in Methods. After 2 weeks of parabiosis, the animals were killed, and blood and liver samples were collected for analysis. Liver membrane protein (40 μ g) obtained from each mouse before (-) and after (+) parabiosis was subjected to SDS-PAGE for immunoblot analyses of LDLR and RAP as a loading control (9).

ing that conditioned medium containing PCSK9(D374Y) reduced the uptake of LDL (24).

A small amount of residual PCSK9 binding to LRP was detected in LDLR-deficient MEFs (Figure 4B). We have previously shown that hepatic LRP protein levels are not altered by the adenoviral overexpression of PCSK9 in mice (9), and LRP protein levels were not significantly different in livers of wild-type mice parabiosed with the TgPCSK9 mice (data not shown). Thus, although a small amount of PCSK9 may associate with LRP *in vitro*, it does not appear to significantly regulate LRP protein levels. The residual LRP-dependent cellular association of PCSK9 might be due to the sequence similarity between LRP and the LDLR. LRP contains 4 copies of the LDLR extracellular domain with similar charge distributions and may sufficiently resemble LDLR to support PCSK9 binding (25).

Recent studies by Maxwell et al. (26) showed that overexpression of PCSK9 in HepG2 cells induced the degradation of LDLR intracellularly in a post-ER compartment. These experiments also suggested that PCSK9-mediated reduction in the number of LDLRs was not attributable to the proteasome or lysosomal cysteine proteases. In addition, our previous studies using adenoviral overexpression of PCSK9 in mice demonstrated that high levels of PCSK9 expression in liver eliminated LDLR protein expression in wild-type and *ApoB*^{-/-} mice equally, indicating that functional ARH was not required for PCSK9 activity (9). This result differs from the data presented in Figure 6, which show that functional ARH is required for PCSK9 to destroy LDLRs when added exogenously to primary hepatocytes. Therefore, it is possible that PCSK9 overexpression may result in the association of PCSK9 with the LDLR within the cell and that this binding leads to intracellular LDLR protein degradation through the pathway described by Maxwell et al., whereas PCSK9's action at the cell surface is mediated by a distinct mechanism that is dependent upon ARH-mediated endocytosis.

Considered together, the available data now suggest that PCSK9 can function both extra- and intracellularly, but we do not know which pathway predominates under normal and/or pathologic conditions. Currently, all studies suggesting that the protein functions intracellularly have been performed using PCSK9 overexpression via a strong CMV promoter. Overexpression may permit association of PCSK9 and the LDLR in an intracellular compartment

that does not occur physiologically. In the current studies, we were able to demonstrate that physiologically relevant concentrations of PCSK9 could significantly reduce the number of cell-surface LDLRs when added to HepG2 cells (Figures 2 and 3). Additional studies to determine the relative contribution of extracellular PCSK9 in the regulation of LDLR protein levels will require determining whether the infusion of PCSK9 into the circulation of PCSK9-knockout mice will decrease the number of LDLRs in liver and raise plasma cholesterol levels.

The results of the parabiosis studies shown in Figure 8 provide evidence that PCSK9 can function in plasma to destroy LDLRs in liver. The liver is the principal site of PCSK9 expression, and PCSK9 is transcriptionally regulated by SREBP-2 (6). SREBP-2 regulates all genes required for cholesterol synthesis, and its activity is modulated by cellular sterol levels (6, 27). If the concentration of PCSK9 in plasma is primarily determined by the liver, then plasma PCSK9 levels may be a reflection of hepatic cholesterol stores, since PCSK9 transcription is reduced under conditions where SREBP-2 is suppressed, i.e., high cellular cholesterol concentrations. Such a regulatory event also raises the possibility that the liver indirectly regulates the amount of LDLRs in peripheral tissues by determining the amount of PCSK9 secreted into plasma. It is currently not known whether PCSK9 in plasma is capable of destroying LDLRs in tissues other than liver. This could not be directly addressed in the current studies, owing to the detection limits of the anti-LDLR antibody in tissues other than liver.

The genetic data from humans with loss-of-function mutations in PCSK9 combined with the studies in knockout mice that lack PCSK9 clearly indicate that inhibitors of the protease would be of therapeutic benefit for the treatment of hypercholesterolemia. Inasmuch as overexpression of the catalytically inactive form of PCSK9 in mice did not alter LDLR protein levels (9), an inhibitor of PCSK9's protease activity in the ER should be sufficient to block its ability to reduce LDLR protein levels. If PCSK9 functions as a secreted factor as suggested by the current data, then additional approaches to neutralize its activity, including the development of antibodies to block its interaction with the LDLR or inhibitors to block its action in plasma, can be explored for the treatment of hypercholesterolemia.

Methods

Cultured cell experiments. HepG2, HEK 293 and MEF cells were cultured as described in Supplemental Methods.

Tissue culture medium. Medium A contained DMEM (cellgro; Mediatech Inc.) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 1 g/l glucose. Medium B contained medium A supplemented with 10% (vol/vol) FCS. Medium C contained medium A with 5% (vol/vol) newborn calf lipoprotein-deficient serum (NCLPDS), 10 µM sodium compactin, and 50 µM sodium mevalonate. Medium D contained DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin sulfate, and 4.3 g/l glucose. Medium E contained medium D supplemented with 10% (vol/vol) FCS. Medium F contained medium D supplemented with 5% (vol/vol) NCLPDS, 10 µM sodium compactin, and 50 µM sodium mevalonate.

Purification of human wild-type and PCSK9(D374Y)-FLAG fusion proteins. HEK 293S cells stably expressing FLAG-tagged human PCSK9 or PCSK9(D374Y) were cultured in suspension without CO₂ in IS GRO serum-free medium (Irvine Scientific) supplemented with 10% FCS, 10 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. PCSK9 was purified by anti-FLAG M2 affinity gel chromatography from 500 ml of medium per the manufacturer's instructions, followed by size-exclusion chromatography on a Tricorn Superose 6 10/300 fast-performance liquid chromatography column (Amersham Biosciences). Fractions containing PCSK9 were concentrated approximately 5-fold using a Centricon filter (10 kDa-MW cut-off; Millipore). Protein purity was monitored by SDS-PAGE and Coomassie Brilliant Blue R-250 staining (Bio-Rad).

Antibodies. For the anti-human PCSK9 polyclonal antibody, the human PCSK9 amino acid sequence was analyzed using Protean software (Lasergene; DNASTar) for immunogenic regions. Amino acids 165–180 (RYRAIDEYQPPDGGSLV) and 220–240 (ASKCDSHGTHLAGVVSGRDAG) were synthesized, conjugated to keyhole-limpet hemocyanin using the Inject Maleimide Activated mKHLH kit (Pierce), and rabbits were injected with a mixture of the peptides (20 µg each) as described previously (28). IgG fractions from sera were purified using the ImmunoPure (A/G) IgG purification kit (Pierce).

Monoclonal antibodies that recognize human PCSK9 were generated by fusion of Sp2/mIL-6 (CRL-2016; ATCC) mouse myeloma cells with splenic B lymphocytes derived from a female BALB/c mouse injected with purified human PCSK9 protein (29). The resulting antibodies, 13D3 and 15A6, belong to IgG subclass 1 and recognize epitopes in the catalytic domain and the C-terminal region of PCSK9, respectively. Additional antibodies used are described in Supplemental Methods.

Immunoblot analysis. Whole-cell lysate protein extracts were subjected to SDS-PAGE for immunoblot analysis as previously described (9). Biotinylation of cell-surface proteins of HepG2 cells was performed as previously described (30). Following biotinylation, whole-cell lysates were prepared, and protein concentration was determined. An aliquot of the whole-cell lysate was reserved, and the remainder was transferred to a fresh tube containing 50 µl of Ultralink NeutrAvidin Protein Plus (Pierce) (1:1 slurry). After 18 hours of incubation with rotation at 4°C, beads were collected by centrifugation at 300 g for 5 minutes and washed 3 times with lysis buffer. Proteins were eluted from the beads by boiling in SDS-PAGE sample buffer and subjected to 8% SDS-PAGE and immunoblot analysis (9).

PCSK9 "sandwich" ELISA. LumiNunc Maxisorp white assay plates (Nunc) were coated with anti-human PCSK9 monoclonal antibody (IgG-13D3) diluted to 5 µg/ml in 100 µl of buffer A (20 mM sodium phosphate pH 7.5, 100 mM sodium chloride) by incubation overnight at 4°C. The assay plates were then washed with 350 µl of PBS with Tween-20, pH 7.4 (PBST) and blocked with 150 µl of 0.5% BSA in buffer A for 1 hour at room temperature with shaking.

Sample preparation was carried out in a separate polypropylene microtiter sample plate. Standards were prepared in duplicate using dilutions of

purified PCSK9 protein in buffer A plus 0.5% BSA. Plasma samples were prepared in triplicate by diluting 6 µl of plasma in 84 µl of buffer A plus 0.5% BSA. Thirty microliters of buffer B (buffer A, 0.5% BSA, 7.2 M urea, and 0.68% Tween-20) was then added to each well, and the sample plate was incubated at 46°C for 30 minutes. Aliquots (100 µl) were transferred to the assay plate and incubated for 2 hours at room temperature with shaking. The plate was then washed with PBST and incubated for 1 hour at room temperature with 100 µl of rabbit anti-human PCSK9 polyclonal antibody (7.5 µg/ml in buffer A plus 0.5% BSA). After washing with PBST, 100 µl of donkey anti-rabbit IgG plus HRP (GE Healthcare) diluted 1:10,000 was added for 1 hour at room temperature. After a final wash with PBST, 100 µl of SuperSignal ELISA Femto Substrate (Pierce) was added for 1 minute and luminescence quantified using a Dynex MLX microtiter plate luminometer (Dynex Technologies). To determine plasma PCSK9 concentrations, luminescence of the sample was compared with that generated in the standard curve with purified PCSK9. Assay validation experiments were conducted in accordance with current bioanalytical recommendations (31).

Human plasma was obtained from 72 volunteers (35 men and 37 women) after an overnight fast. Informed consent and blood samples were obtained for all participating subjects by institutional review board-approved protocols. The age of the individuals ranged from 21 to 56, and none were taking lipid-altering medications.

Pulse-chase analysis of PCSK9. HepG2 cells were cultured in medium C, and the pulse-chase was carried out as previously described (12). Immunoprecipitation of cell and medium extracts was performed using the polyclonal anti-PCSK9 antibody (32). Immunoprecipitates were eluted in SDS sample buffer and subjected to 8% SDS-PAGE. The gels were fixed and then immersed for 15 minutes in Amplify fluorogenic reagent (Amersham Biosciences), dried under vacuum, and exposed to film.

Coimmunoprecipitation of PCSK9 and the LDLR. HepG2 cells were cultured for 18 hours in medium C prior to treatment with 20 µg/ml or 2 µg/ml of purified PCSK9 or PCSK9(D374Y) for 1 hour in the presence of 0.1 mM chloroquine. Pooled cell pellets from 6 dishes of cells were lysed in 2 ml of buffer C (20 mM HEPES-KOH at pH 7.4, 150 mM NaCl, 1 mM MgCl₂, and protease inhibitors) containing 1% (wt/vol) digitonin. Cell lysates were incubated with 50 µl anti-FLAG M2 agarose (Sigma-Aldrich) to immunoprecipitate FLAG-tagged PCSK9 (for competition assays, 0.1 mg/ml of FLAG octapeptide was included); IgG-HL1 to immunoprecipitate LDLR; or IgG-2001, a mouse monoclonal antibody to an irrelevant antigen (33). Immunoprecipitates were washed 3 times with 500 µl buffer C containing 0.1% (wt/vol) digitonin, eluted in SDS sample buffer by boiling for 5 minutes, and subjected to 8% SDS-PAGE.

Ligand blotting. Purified human LDLR extracellular domain (amino acids 1–699) was kindly provided by J. Deisenhofer (University of Texas Southwestern Medical Center) and was described previously (34). LDLR protein was resolved on nonreducing SDS-PAGE and transferred to nitrocellulose as described (35). LDLR protein was heated for 5 minutes at 96°C in sample buffer containing 2.5% (vol/vol) 2-mercaptoethanol for reducing conditions. Blots were blocked for 30 minutes in buffer D (50 mM Tris-Cl at pH 7.4, 90 mM NaCl, 2 mM CaCl₂, 5% [wt/vol] BSA) and incubated sequentially for 60 minutes with gentle agitation in buffer D containing purified PCSK9 (5 µg/ml), the monoclonal anti-PCSK9 antibody IgG-15A6 (5 µg/ml), and HRP-conjugated goat anti-mouse IgG. PCSK9 and antibody incubations were followed by washes in buffer E (50 mM Tris-Cl at pH 7.4, 90 mM NaCl, 2 mM CaCl₂, 0.5% [wt/vol] BSA). Blots were visualized by immunoblotting as described above.

Immunofluorescence microscopy. Indirect immunofluorescence using antibodies that detect the LDLR, PCSK9, and CI-MPR in MEFs was performed as follows. MEFs cultured on glass coverslips to 50% confluence were fixed in 4% (wt/vol) paraformaldehyde in buffer F (PBS containing 2 mM MgCl₂),

quenched with 5 mM NH₄Cl in buffer F, permeabilized in buffer F containing 0.05% (wt/vol) Triton X-100 (Pierce) for 10 minutes at -20°C, and blocked for 30 minutes in buffer G (buffer F containing 1% [wt/vol] BSA). For double-label staining, cells were incubated sequentially with the monoclonal anti-PCSK9 antibody IgG-15A6 (2.5 µg/ml), Alexa Fluor 488-conjugated goat anti-mouse IgG (0.6 µg/ml), polyclonal LDLR antiserum Ab 4548 (1:1,000) or polyclonal CI-MPR IgG (2 µg/ml), and Alexa Fluor 555-conjugated goat anti-rabbit IgG (0.6 µg/ml). Primary (16 hours at 4°C) and secondary (1 hour at 20°C) antibody incubations were followed by three 20-minute washes with buffer G. Coverslips were mounted in VECTASHIELD Mounting Medium (Vector Laboratories), and images were taken on a Zeiss confocal microscope (model LSM510-META). Indirect immunofluorescence for the LDLR in primary hepatocytes derived from *LDLR⁺Arb⁻* and *LDLR⁺Arb⁻* mice was carried out as previously described (12, 21).

TgPCSK9 mice. A cDNA encoding human PCSK9 was inserted into a pLiv-11 vector that contains the constitutive human apolipoprotein promoter and its hepatic control region (a gift from J. Taylor, Gladstone Institute of Cardiovascular Disease, San Francisco, California, USA) (22). Transgenic mice were generated by injecting linearized pLiv-11-hPCSK9 into the fertilized eggs as described previously (28).

Liver biopsy and parabiosis. One week prior to parabiosis, a liver biopsy was performed to obtain a liver sample for protein analysis. Mice were anesthetized with pentobarbital, hair over the abdomen shaved, and skin cleaned with chlorhexidine. A 0.5-cm transverse incision was made in the upper abdomen, and a small lobe of the liver (~50–100 mg) was tied off with a 5-0 silk suture and excised. The liver sample was placed in liquid nitrogen for storage. The incision was closed in 2 layers using a 5-0 silk suture and wound clips. During sedation, 50 µl of blood was removed from the tail vein for plasma cholesterol measurements. The mice were allowed to recover from this surgery before the parabiosis was performed.

For parabiosis, mice were surgically joined following a previously published protocol (36), with slight modifications. Matching skin incisions were made from the shoulder to the hip joint of each mouse, and a 10-mm incision was made in the muscles of the abdominal wall. The muscular incisions were joined using a 5-0 silk suture. The scapulae

were attached by a 2-0 silk suture, and the skin was approximated using wound clips and 5-0 silk suture.

Three genetically distinct groups of female mice were studied: wild-type mice joined with wild-type mice; *Ldlr^{-/-}* mice joined with wild-type mice; and TgPCSK9 mice joined with wild-type mice. Shared circulation was confirmed by injecting Evans blue into one of the parabiotic pairs prior to study. Mice were killed by isoflurane overdose 2 weeks after parabiosis, and blood and liver were harvested for analysis. Animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

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Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype

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Proprotein convertase subtilisin kexin 9 (Pcsk9) is a subtilisin serine protease with a putative role in cholesterol metabolism. Pcsk9 expression is down-regulated by dietary cholesterol, and mutations in Pcsk9 have been associated with a form of autosomal dominant hypercholesterolemia. To study the function of Pcsk9 in mice, an adenovirus constitutively expressing murine Pcsk9 (Pcsk9-Ad) was used. Pcsk9 overexpression in wild-type mice caused a 2-fold increase in plasma total cholesterol and a 5-fold increase in non-high-density lipoprotein (HDL) cholesterol, with no increase in HDL cholesterol, as compared with mice infected with a control adenovirus. Fast protein liquid chromatography analysis showed that the increase in non-HDL cholesterol was due to an increase in low-density lipoprotein (LDL) cholesterol. This effect appeared to depend on the LDL receptor (LDLR) because LDLR knockout mice infected with Pcsk9-Ad had no change in plasma cholesterol levels as compared with knockout mice infected with a control adenovirus. Furthermore, whereas overexpression of Pcsk9 had no effect on LDLR mRNA levels, there was a near absence of LDLR protein in animals overexpressing Pcsk9. These results were confirmed *in vitro* by the demonstration that transfection of Pcsk9 in McA-RH7777 cells caused a reduction in LDLR protein and LDL binding. In summary, these results indicate that overexpression of Pcsk9 interferes with LDLR-mediated LDL cholesterol uptake. Because Pcsk9 and LDLR are coordinately regulated by cholesterol, Pcsk9 may be involved in a novel mechanism to modulate LDLR function by an alternative pathway than classic cholesterol inhibition of sterol regulatory element binding protein-mediated transcription.

Dietary cholesterol raises plasma low-density lipoprotein (LDL) cholesterol levels, but the response varies considerably among individuals, presumably on a genetic basis (1–3). In a study (4) designed to identify genes involved in dietary-cholesterol responsiveness, mice were fed low versus high-cholesterol diets and liver gene-expression profiling was performed. We found that 37 genes were consistently down-regulated by dietary cholesterol and that 32 genes were up-regulated. The down-regulated genes included many genes that were involved in cholesterol biosynthesis and transport as well as genes of unknown function. One of the latter genes was proprotein convertase subtilisin kexin 9 (Pcsk9), a member of the subtilisin serine protease family (5). This gene had also been cloned by Seidah *et al.* (6) as neural apoptosis-regulated convertase 1 (Narc-1) and was hypothesized originally to play a role in liver regeneration and neuronal differentiation.

The mammalian subtilisin family contains nine members, including Pcsk9. Seven members are proprotein convertases, whose protease activities activate a wide variety of serum proteins, prohormones, receptors, and zymogens (7, 8). These proteins have the highest similarity to the kexin subfamily of bacterial/yeast subtilisin proteases and cleave after basic residues in the consensus (K,R)(X)_n(K,R)↓, where *n* = 0, 2, 4, or 6 (9). The eighth member of the subtilisin family is the site-1 protease (S1P), whose protease activity activates sterol regulatory element binding proteins (SREBPs) and brain-derived neurotrophic factor (10, 11). S1P shows highest sim-

ilarity to the pyrolysins subfamily of bacterial subtilisin proteases (6) and cleaves after nonbasic residues in the consensus (K,R)(X)₂(L,T)↓ (9). Pcsk9, whose function is unknown, is distinct from these family members because it is most similar to the proteinase K subfamily of bacterial subtilisin proteases. As for S1P, Pcsk9 does not cleave after basic residues (12), but its consensus cleavage site has not been defined. Pcsk9 requires activation by intramolecular self-cleavage (12), and two different intramolecular cleavage sites have been reported: (Y,I)VV(V,L)(L,M)↓ (6) and LVFAQ↓ (12). The natural substrates of Pcsk9, other than itself, are currently unknown.

Regulation of Pcsk9 by dietary cholesterol suggests that it may play an important role in cholesterol metabolism. This idea is supported by the observation that missense mutations in Pcsk9 are associated with a form of autosomal dominant hypercholesterolemia (ADH) referred to as Hchola3 (OMIM 603776; <http://www.ncbi.nlm.nih.gov/omim>) (13, 14). ADH is characterized clinically by highly elevated total and LDL cholesterol levels and is accompanied by cholesterol deposition in tissues and premature atherosclerosis (15). The most common form of ADH is familial hypercholesterolemia and is caused by mutations in the LDL receptor (LDLR) (16), which is responsible for clearing LDL from plasma. Another form of ADH is familial defective apolipoprotein B-100 (apoB), which is caused by structural mutations in apoB that preclude LDL binding to the LDLR (17). Hchola3 is a disease found in ADH families not linked to the *LDLR* or *apoB* loci (18, 19). Hchola3 has been linked to Pcsk9 missense mutations in two French families and one Utah kindred (S127R, F216L, and D374Y, respectively) (13, 14), and it has been suggested that they act as either gain-of-function or dominant-negative mutations. However, neither the precise mechanisms of these mutations nor the role of Pcsk9 in cholesterol metabolism is currently understood.

To begin to elucidate the role of Pcsk9 in cholesterol metabolism, we performed *in vivo* studies by using an adenovirus constitutively expressing the C57BL/6 mouse form of Pcsk9. These studies demonstrate an elevation of plasma LDL cholesterol levels coincident with the disappearance of hepatic LDLR protein, mimicking the phenotype observed in LDLR-deficient mice. Thus, Pcsk9 appears to play a major role in the regulation of LDLR-mediated LDL removal from plasma.

Methods

Materials. To create antibodies specific for Pcsk9, the murine Pcsk9 primary amino acid sequence was analyzed with PROTEAN software (DNASar, Madison, WI) to identify regions with high antigenic index. The amino acid sequences of two positions,

Abbreviations: ADH, autosomal dominant hypercholesterolemia; DIL, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; FPLC, fast protein liquid chromatography; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, LDL receptor; pfu, plaque-forming unit; Pcsk9, proprotein convertase subtilisin kexin 9; SREBP, sterol regulatory element binding protein.

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amino acids 500–520 and 583–603 of the C57BL/6 mouse form of Pcsk9 (GenBank accession no. AY273821), were submitted to Bethyl Laboratories (Montgomery, TX), where corresponding peptides were made and used to immunize two rabbits each. The resulting affinity purified antibodies were tested extensively in cells overexpressing Pcsk9 by transient transfection or infection. The antibodies consistently recognized two bands at ~76 kDa and 62 kDa, corresponding to the precursor and processed forms of Pcsk9; binding could be blocked by coinubation of the antibodies with the immunogenic peptides (data not shown). Antibodies to the murine LDLR cytoplasmic tail (Ab 3143) were a generous gift from Joachim Herz (University of Texas Southwestern Medical Center, Dallas). Antibodies to γ -tubulin were obtained from Sigma. McA-RH7777 cells were obtained from the American Type Culture Collection (CRL-1601), and standard cell culture reagents were obtained from Gibco. Human LDL labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-LDL) was obtained from Biomedical Technologies (Stoughton, MA). Unlabeled human LDL was obtained from Calbiochem, and lipoprotein-deficient serum was made by ultracentrifugation by standard techniques.

Construction of Adenovirus and Plasmids Expressing Pcsk9. The Pcsk9 sequence used in this study (GenBank accession no. AY273821) was cloned by RT-PCR from C57BL/6 mouse liver as described (4) and aligns exactly to the public mouse genome. It contains the Asp-202–His-242–Ser-402 catalytic triad and the Asn-333 oxyanion hole characteristic of subtilisin proteases. The Pcsk9 ORF was inserted into the pAd5-CMV-NpA shuttling vector. Recombinant adenoviral particles containing the Pcsk9 ORF under the control of a constitutive CMV promoter were created by Viraquest (North Liberty, IA). These viruses are referred to as Pcsk9-Ad. Upon infection of multiple different cell lines, Pcsk9-Ad produced the expected 76-kDa precursor and 62-kDa processed forms by Western blotting (data not shown). A control adenovirus, referred to as “Ad empty,” was obtained from Viraquest. For transfection experiments, the Pcsk9 ORF was cloned into the expression vector pcDNA3 by standard techniques (Pcsk9-pcDNA) and the empty vector was used as a control. The Pcsk9 catalytic mutant, Pcsk9(S402A), was made by site-directed mutagenesis using the QuikChange kit (Stratagene). All constructs were sequence verified.

Animals and in Vivo Delivery of Recombinant Adenovirus. Wild-type C57BL/6 male mice (stock no. 00664) and LDLR knockout mice on the C57BL/6 background (B6.129S7-Ldlr^{tm1Her}, stock number 002207) were purchased from The Jackson Laboratory. Animals were housed in a humidity- and temperature-controlled room with a 12:12 h dark/light cycle and maintained on standard laboratory chow. For adenovirus injections, animals were anesthetized with Nembutal and injected through the tail vein with 1×10^8 plaque-forming units (pfu), 5×10^8 pfu, 1×10^9 pfu, or 3×10^9 pfu of adenovirus in PBS per mouse. At 4 days after injection, animals were fasted for 5 h and anesthetized with ketamine and xylazine before blood and tissue collection. All animal protocols were approved by The Rockefeller University Animal Care and Use Committee.

Plasma, Liver, and Gallbladder Bile Analysis. Blood was collected by left ventricular puncture and plasma isolated by centrifugation. To separate high-density lipoprotein (HDL) and non-HDL plasma fractions, plasma was mixed 1:1 with KBr at a density of 1.12 g/ml and centrifuged at 40,000 rpm for 18 h. To obtain lipoprotein profiles, plasma was subjected to fast protein liquid chromatography (FPLC). Cholesterol in total plasma and fractions was measured by enzymatic assay (Roche). Triglycerides in total plasma and fractions were measured by enzymatic assay (Roche). Liver tissue was excised and frozen immediately in

liquid nitrogen. Liver total, free, and esterified cholesterol (mg/g liver) were measured by gas chromatography with coprostanol as an internal standard as described (20). Gallbladder bile was isolated and analyzed for cholesterol, phospholipids, and bile acids by enzymatic assay (Roche, Sigma).

Quantitative RT-PCR. Quantitative RT-PCR was performed as described (4). Briefly, liver tissue in RNAlater was homogenized in TRIzol reagent (Invitrogen), and total RNA was isolated according to the manufacturer's instructions. Total RNA was then subjected to RNeasy clean-up (Qiagen, Valencia, CA) and treated with DNase I (Ambion, Austin, TX), and 5 μ g of total RNA was reverse-transcribed by using Superscript II (Invitrogen) and a mixture of oligo(dT) and random hexamer primers. Each sample was amplified in duplicate for the genes of interest and a housekeeping gene, *cyclophilin A*, on an 7700 Sequence Detection System (Applied Biosystems) by using the quencher dye TAMRA as a passive reference. Primers and probes for quantitative RT-PCR were obtained from Applied Biosystems, and the sequences were as follows: LDLR, 5'GGATGGCTATACCTACCCCTCAA (forward) and 5'CGGCTCTCCCGGCTG (reverse); and probe, 6FAM-TCAGCCTGGAGGACGATGTGGCA-TAMRA. Pcsk9 and cyclophilin A sequences have been reported (4, 21). The threshold was set in the linear range of normalized fluorescence, and a threshold cycle (C_t) was measured in each well; data were analyzed as in ref. 21.

Western Blotting. Proteins were isolated from cells or mouse liver by homogenization in RIPA buffer (50 mM Tris, pH 7.4/150 mM NaCl/1 mM EDTA/0.1% SDS/1% Triton X-100/1% deoxycholate). Crude extracts were centrifuged at 16,000 $\times$ g for 10 min to pellet cellular debris and nuclei. Protein concentration was measured by the BCA assay (Pierce), and 10–40 μ g of protein was electrophoresed for Western blotting. The following dilutions were used: anti-Pcsk9–583, 1:5,000; anti-LDLR, 1:1,000; and anti- γ -tubulin, 1:10,000. Band intensities were measured by using IMAGE PRO PLUS.

LDL Binding Studies. McA-RH7777 cells were plated at a density of 2×10^5 cells and transfected 6–8 h later with 100 ng of plasmid DNA in Lipofectamine Plus (Invitrogen). After 36 h, cells were washed two times in ice-cold PBS and then treated with 10 μ g/ml DiI-LDL without or with 200 μ g/ml unlabeled LDL in medium containing lipoprotein-deficient serum for 2 h at 4°C. Cells were then washed three times in rapid succession in Tris-buffered saline (TBS)/2 mg/ml BSA, one time in TBS/BSA for 2 min, and finally two times in TBS. Cell lysates were made in RIPA buffer, and cell-associated fluorescence was measured in a microplate reader. DiI-LDL fluorescence was corrected for cellular protein. Specific binding was calculated by subtracting the normalized DiI-LDL fluorescence in the presence of excess unlabeled LDL from fluorescence in its absence.

Results

Cloning of Pcsk9 and Construction of Adenovirus. The mouse C57BL/6 Pcsk9 ORF sequence identical to that found in the public genome database was used to create adenovirus particles expressing Pcsk9. The Pcsk9 adenovirus (Pcsk9-Ad) was injected into C57BL/6 male mice through the tail vein at concentrations of 1×10^8 to 3×10^9 pfu per mouse and compared with mice injected with PBS alone or 1×10^9 pfu of a control empty adenovirus (Ad empty). As shown in Fig. 1A, the livers of mice injected with PBS or Ad empty have no immunoreactive Pcsk9. However, injection of increasing concentrations of Pcsk9-Ad produced increasing amounts of both the pro and processed forms of Pcsk9. Because processing of Pcsk9 is an intramolecular event (12), the presence of the processed form in these mice indicates that the adenovirus expresses catalytically active Pcsk9.

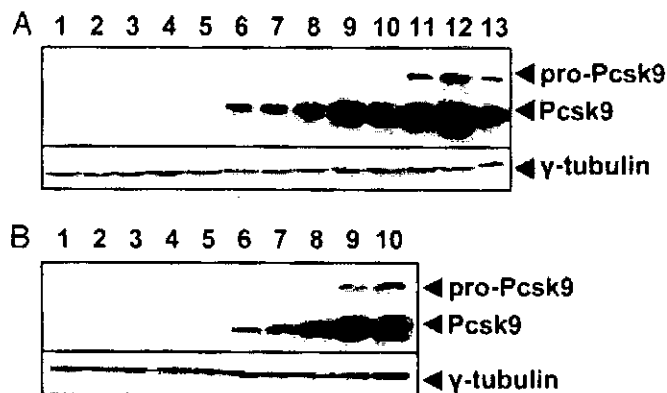


Fig. 1. Adenoviral-mediated expression of Pcsk9. (A) Male C57BL/6 mice of >10 weeks of age that were fed a chow diet were injected through the tail vein with PBS (lanes 1 and 2), Ad empty (lanes 3 and 4), or an adenovirus constitutively expressing the murine C57BL/6 Pcsk9 ORF under the control of a CMV promoter (Pcsk9-Ad, lanes 5–12). Pcsk9-Ad was injected at increasing concentrations from 1×10^8 pfu per mouse to 3×10^9 pfu per mouse (lanes 5–12). As a control, McA-RH7777 cells were infected with Pcsk9-Ad at an moi of 2,000 (lane 13). (B) Male LDLR knockout mice on a C57BL/6 background of >10 weeks of age that were fed a chow diet were injected through the tail vein with Ad empty (lanes 1–5) or increasing concentrations of Pcsk9-Ad (lanes 6–10). In both cases, liver homogenates were immunoblotted with an antibody to Pcsk9 and γ -tubulin.

Adenoviral-Mediated Expression of Pcsk9 Increases Plasma LDL Cholesterol Levels. The effect of adenoviral-mediated Pcsk9 expression on plasma cholesterol levels was determined as shown in Fig. 2A. Injection of Pcsk9-Ad into C57BL/6 mice resulted in elevated plasma total cholesterol levels compared with injection of Ad empty (147 ± 17 mg/dl vs. 72 ± 6 mg/dl, $P = 0.0003$). This change was due to an increase in plasma non-HDL cholesterol levels (86 ± 14 mg/dl vs. 16 ± 3 mg/dl, $P = 0.0002$), with no change in HDL cholesterol (56 ± 2 mg/dl vs. 51 ± 2 mg/dl, no significant difference). There was also an increase in plasma triglycerides (91 ± 25 mg/dl vs. 47 ± 2 mg/dl, $P = 0.03$). The distribution of cholesterol in FPLC-separated lipoprotein fractions is shown in Fig. 2B. The increase in non-HDL cholesterol in Pcsk9-Ad-injected mice was shown to be due to a specific increase in LDL cholesterol, with little or no change in cholesterol in very-low-density lipoprotein or chylomicrons. The increase in triglycerides was also found to be in the LDL fractions (data not shown).

The Pcsk9-Ad-Induced Increase in Plasma LDL Cholesterol Depends on the LDLR. Similar to what was observed in C57BL/6 mice, the livers of LDLR knockout mice injected with Ad empty had no immunoreactive Pcsk9, and injection of increasing concentrations of Pcsk9-Ad produced increasing amounts of both the pro and processed forms of Pcsk9 (Fig. 1B). Although LDLR knockout mice had higher levels of total cholesterol, non-HDL cholesterol and triglycerides than wild-type mice, no significant differences in these lipid levels were observed between Pcsk9-Ad and Ad empty-injected LDLR knockout mice (202 ± 26 mg/dl vs. 205 ± 27 mg/dl, no significant difference; 135 ± 19 mg/dl vs. 145 ± 23 mg/dl, no significant difference; 96 ± 20 mg/dl vs. 89 ± 25 mg/dl, no significant difference) (Fig. 2C). The distribution of cholesterol in FPLC separated lipoprotein fractions is shown in Fig. 2D. The increases in non-HDL cholesterol in Pcsk9-Ad-injected, Ad empty-injected, and uninjected LDLR knockout mice were all due to a specific increase in LDL cholesterol. The increases in triglycerides were also found to be in the LDL fractions (data not shown).

Pcsk9-Ad Injection Results in the Absence of Liver LDLR Protein with Normal mRNA Levels. The effects of Pcsk9 overexpression on liver LDLR mRNA and protein levels were examined. As shown in Fig. 3A, there was no significant change in LDLR mRNA levels despite a large increase in Pcsk9 mRNA levels in Pcsk9-Ad vs. Ad empty-injected mice. Effects on LDLR protein levels were determined by Western blotting with an antibody to the LDLR cytoplasmic tail as shown in Fig. 3B. Wildtype C57BL/6 mice have a band at ≈ 160 kDa, which is absent in LDLR knockout mice. This band is virtually absent in Pcsk9-Ad-injected mice, whereas it is unaffected in Ad empty-injected mice.

Pcsk9-Ad Injection Does Not Alter Hepatic Cholesterol Levels or Gallbladder Bile Composition. The effects of Pcsk9-Ad on other physiological measures related to cholesterol metabolism were studied. As shown in Table 1, there were no differences in hepatic total, free, or esterified cholesterol between Pcsk9-Ad and Ad empty-injected mice. There were also no differences in gallbladder bile concentrations of cholesterol, bile acids, or phospholipids.

Overexpression of Pcsk9 in Rat Hepatoma Cells Leads to a Decrease in LDLR Protein and a Corresponding Decrease in LDL Binding. To investigate the effects of overexpression of Pcsk9 *in vitro*, McA-RH7777 rat hepatoma cells were transiently transfected with empty vector, wild-type Pcsk9, and a catalytically inactive mutant Pcsk9(S402A). As shown in Fig. 4A, the catalytic mutant shows only the pro form of Pcsk9, as expected (12). Overexpression of wild-type Pcsk9 caused a 72% decrease in LDLR protein, whereas overexpression of catalytically inactive Pcsk9 caused a 15% decrease in LDLR protein (Fig. 4A). Furthermore, overexpression of wild-type Pcsk9 caused a 52% decrease in the binding of DiI-LDL, whereas overexpression of catalytically inactive Pcsk9 caused a 15% decrease in the binding of DiI-LDL. These observations support the hypothesis that overexpression of Pcsk9 increases plasma cholesterol levels in mice by interfering with LDLR-mediated LDL uptake.

Discussion

To determine how Pcsk9 affects cholesterol metabolism, we injected an adenovirus constitutively expressing murine Pcsk9 into C57BL/6 male mice. Injection of Pcsk9-Ad in mice resulted in an increase in total cholesterol because of a specific increase in LDL cholesterol. Injections into LDLR knockout mice revealed that the Pcsk9-induced increase in LDL cholesterol depended on the LDLR. Furthermore, wild-type mice injected with Pcsk9 had a near absence of liver LDLR protein with normal levels of mRNA. Transient transfection of Pcsk9 into McA-RH7777 rat hepatoma cells resulted in decreased LDL binding proportional to decreased LDLR protein, supporting the *in vivo* results.

The phenotype of mice injected with the Pcsk9 adenovirus is nearly identical to that of LDLR knockout mice. Homozygous LDLR knockout mice fed a normal chow diet, with no detectable LDLR protein, have elevated plasma total cholesterol levels with a major increase in LDL cholesterol and a minor increase in very-low-density lipoprotein (22), as is seen in Pcsk9-Ad-injected mice. It is also noteworthy that the increase in plasma triglycerides in both LDLR knockout and Pcsk9-Ad-injected mice is in the LDL fractions. Other characteristics of LDLR knockout mice include normal hepatic cholesterol levels (23) and gallbladder bile composition (E. Sehayek and J.L.B., unpublished data) and these characteristics are seen also in Pcsk9-Ad-injected mice. Thus, Pcsk9 appears to affect LDLR function specifically rather than have a global effect on hepatic cholesterol metabolism.

The demonstration that Pcsk9-Ad-mediated overexpression results in the disappearance of LDLR protein without evidence of accumulation of immature forms and without alteration of steady-state mRNA levels indicates a defect in LDLR synthesis

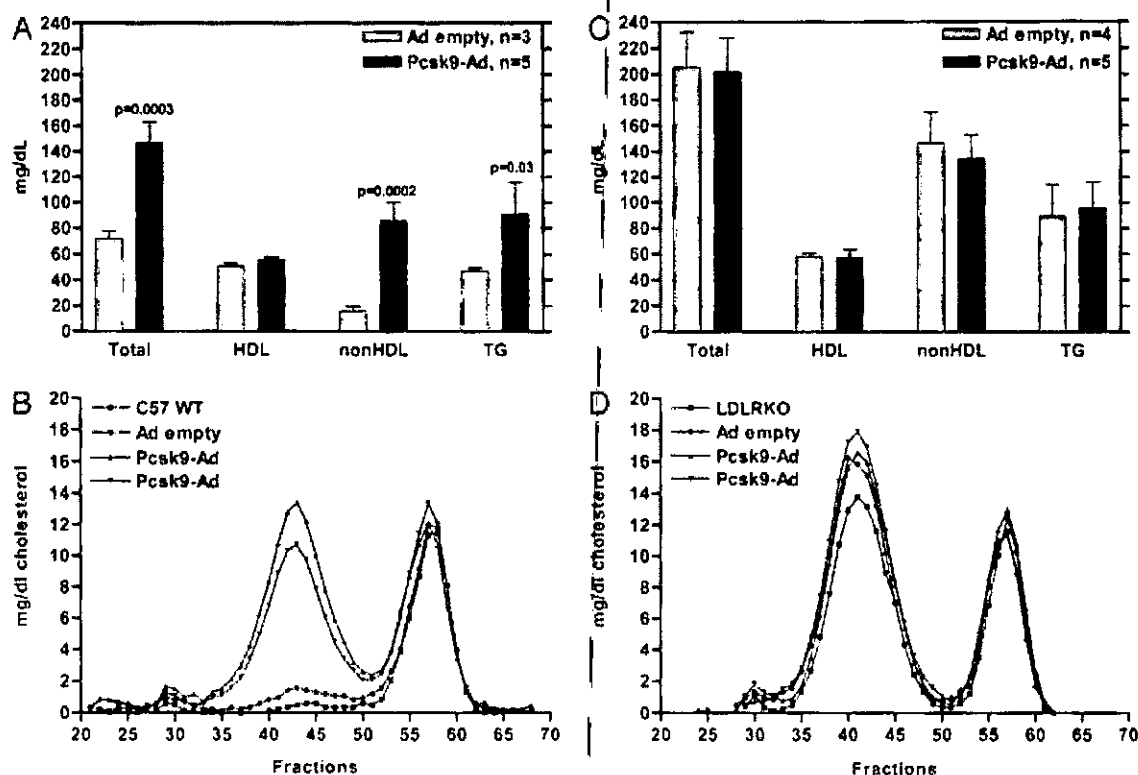


Fig. 2. Overexpression of Pcsk9 increases LDL cholesterol levels in an LDLR-dependent manner. (A) Plasma was isolated from wild-type male C57BL/6 mice injected with Ad empty and Pcsk9-Ad, and HDL and non-HDL plasma fractions were separated by ultracentrifugation. Cholesterol levels in these fractions were measured by enzymatic assay. Overexpression of Pcsk9 caused an increase in total and non-HDL cholesterol with no change in HDL cholesterol levels. (B) Plasma was pooled from at least two wild-type animals (C57 WT), animals injected with Ad empty, and animals injected with Pcsk9-Ad; plasma was fractionated by FPLC, and cholesterol was measured by enzymatic assay. Two runs of Pcsk9-Ad-injected mice are shown to demonstrate reproducibility. Wild-type mice and mice injected with Ad empty had mainly HDL-derived cholesterol (fractions 52–62). Mice injected with Pcsk9-Ad had similar levels of HDL-derived and very-low-density lipoprotein-derived cholesterol, but they had high levels of LDL-derived cholesterol (fractions 35–50). (C) LDLR knockout mice were injected with Ad empty and Pcsk9-Ad. LDLR knockout mice injected with Ad empty had elevated total cholesterol and non-HDL cholesterol with no change in HDL cholesterol levels. Overexpression of Pcsk9 did not cause a further increase in total or non-HDL cholesterol levels in LDLR knockout mice. (D) FPLC analysis demonstrated that the lipoprotein profile of LDLR knockout mice injected with Pcsk9-Ad was indistinguishable from that of uninjected LDLR knockout mice (LDLRKO) and LDLR knockout mice injected with Ad empty.

or degradation rather than a defect in transcription, glycosylation, or trafficking. Very little is known about the posttranscriptional regulation of LDLR synthesis, but some literature exists about the regulation of LDLR degradation. Pulse labeling of human fibroblasts indicated that the half-life of LDLRs was greatly decreased by cycloheximide, suggesting protein-mediated LDLR degradation (24). The site and mechanism of degradation was not determined. Treatment of human fibroblasts with lysosomal inhibitors did not alter LDLR degradation in the absence of LDL, but paradoxically, it increased degradation in the presence of LDL (25, 26). Incubation of CHO cells with proteasome inhibitors did not affect degradation of wild-type LDLRs, but it prevented degradation of class II LDLR mutants (27). A mechanism of LDLR degradation was reported recently by Begg *et al.* (28), who showed that phorbol ester [4 β -phorbol 12-myristate 13-acetate (PMA)] treatment of

HepG2 cells caused the release of a soluble 140-kDa LDLR into the medium. Proteolytic release of soluble counter parts of receptors is a common phenomenon (29) and has been shown for other members of the LDLR family, including LRP1, VLDLR, and apoER2 (30, 31). It is intriguing to speculate that Pcsk9 may proteolytically cleave the LDLR, releasing soluble LDLR and preventing LDLR-mediated LDL uptake. Alternatively, Pcsk9 protease or another activity may degrade the LDLR by another mechanism. It is important to note that any effects of Pcsk9 could be made directly on the LDLR or on proteins that interact with the LDLR.

Both missense Pcsk9 mutations in the human disease Hchola3 and Pcsk9-Ad-mediated overexpression in mice result in elevated LDL cholesterol levels. Several explanations are possible for this paradox. In Hchola3, if the missense mutations result in a gain of function, this hypothesis would be supported by our

Table 1. Liver cholesterol and gallbladder bile composition in mice overexpressing Pcsk9

Virus	Liver			Gallbladder bile		
	Total cholesterol	Free cholesterol	Cholesterol ester	Cholesterol, mg/dl	Bile acids, mM	Phospholipids, mg/dl
Ad empty	3.11 $\pm$ 0.72	2.49 $\pm$ 0.66	0.81 $\pm$ 0.31	62.3 $\pm$ 20.4	130.9 $\pm$ 54.1	1,430 $\pm$ 406
Pcsk9-Ad	3.12 $\pm$ 0.16	2.42 $\pm$ 0.19	0.74 $\pm$ 0.17	53.8 $\pm$ 10.9	140.7 $\pm$ 21.9	1,254 $\pm$ 182

Liver cholesterol is given as milligrams of cholesterol per gram of liver weight.

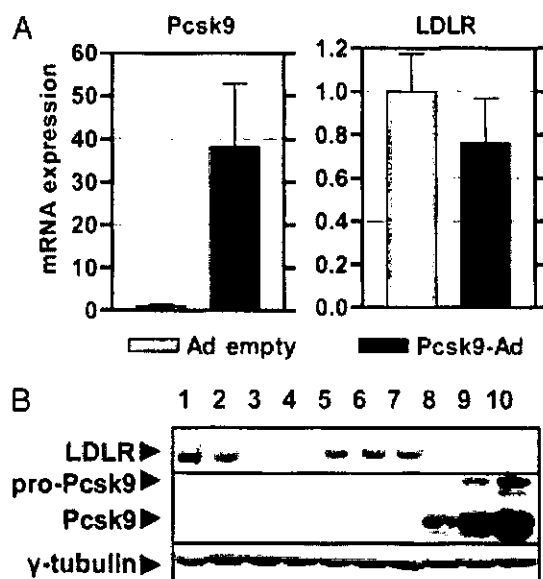


Fig. 3. Overexpression of Pcsk9 results in an absence of LDLR protein with normal LDLR mRNA levels. (A) The levels of LDLR mRNA in livers of mice injected with Ad empty and Pcsk9-Ad were measured by quantitative RT-PCR. Overexpression of Pcsk9 did not change the levels of LDLR mRNA. (B) The levels of LDLR protein in livers of mice injected with Ad empty and Pcsk9-Ad were detected by immunoblotting with an antibody specific to the murine LDLR cytoplasmic tail. Wild-type C57BL/6 mice (lanes 1 and 2) had a predominant LDLR band at ~160 kDa; this band was absent in LDLR knockout mice (lanes 3 and 4). Mice injected with Ad empty had normal levels of LDLR protein (lanes 5–7), whereas mice injected with Pcsk9-Ad had a complete absence of LDLR protein (lanes 8–10).

results. All of the missense mutations are of residues conserved between human and mouse, suggesting functional importance. However, because none of the missense mutations are at the prodomain cleavage site or the catalytic triad (13, 14), it is not obvious how they might affect function either positively or negatively. Second, it is possible that a loss-of-function mutation and overexpression of Pcsk9 could result in the same phenotype. This phenomenon could occur if LDLR function depends on an optimal amount of Pcsk9 and either deficiency or excess inhibits function, as is seen with the LDLR-related protein (LRP) and the receptor-associated protein (RAP). In RAP knockouts, hepatic LRP protein is diminished and shows decreased physiological function (32, 33), whereas adenoviral-mediated RAP overexpression also results in decreased physiological function of LRP (34, 35). Third, it is possible that human Pcsk9 (hPcsk9) and mouse Pcsk9 (mPcsk9) have opposite effects on LDLR function. In this regard, human and mouse Pcsk9 are only 77% identical at the amino acid level. Some of these differences may be functionally important. (i) hPcsk9 has a lysine at the processing site reported in Seidah *et al.* (6), whereas mPcsk9 has a methionine; (ii) hPcsk9 has the sequence RGE after the catalytic site where mPcsk9 has the potential integrin binding site, RGD (36); (iii) hPcsk9 contains a domain similar to the prokaryotic lipoprotein lipid attachment site whereas mPcsk9 does not (37); and (iv) hPcsk9 and mPcsk9 have multiple differences in putative PKC, CK2, and cAMP phosphorylation sites (37).

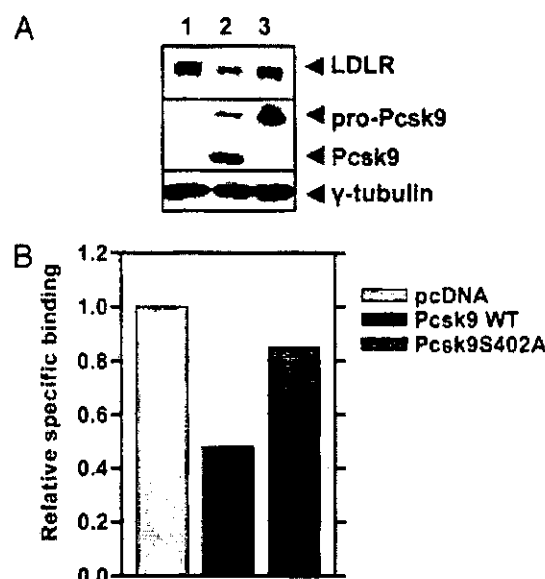


Fig. 4. Overexpression of Pcsk9 *in vitro* results in a decrease in LDLR protein and LDL binding. (A) McA-RH7777 cells were transiently transfected with pcDNA empty vector (lane 1), wild-type Pcsk9-pcDNA (lane 2), and the mutant Pcsk9(S402A)-pcDNA (lane 3). Overexpression of wild-type Pcsk9 resulted in a 72% decrease in LDLR protein, whereas overexpression of catalytically inactive Pcsk9 caused a 15% decrease in LDLR protein. (B) Specific binding of DII-LDL was measured in transiently transfected McA-RH7777 cells. Overexpression of wild-type Pcsk9 resulted in a 52% decrease in DII-LDL binding, whereas overexpression of catalytically inactive Pcsk9 resulted in a 15% decrease in DII-LDL binding. Data shown are representative of three independent experiments.

If Pcsk9 is a physiological inhibitor of LDLR activity, then it is peculiar that both genes are coordinately regulated by cholesterol at the transcriptional level through the SREBPs (4, 38). However, it is possible that the function of Pcsk9 is to modulate LDLR activity by a mechanism that is more rapid than termination of SREBP-mediated transcription of LDLR. This process might be analogous to the regulation of HMG CoA reductase (HMGCR), in which termination of activity is achieved by both proteasomal degradation and termination of SREBP-mediated transcription (38). Interestingly, Insig-1, which mediates the proteasomal degradation of HMGCR (39), is regulated coordinately with HMGCR by means of the SREBPs (40).

In conclusion, overexpression of Pcsk9 diminishes LDLR protein and function with no change in LDLR mRNA levels, causing an LDLR knockout phenotype in mice. Thus, Pcsk9 may induce LDLR degradation and modulate LDLR activity in a more rapid manner than is achieved by sterol inhibition of SREBP-mediated transcription. The molecular mechanism of Pcsk9 action on the LDLR and the relationship of the observations reported herein to Hchola3 remain to be determined.

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Decreased plasma cholesterol and hypersensitivity to statins in mice lacking *Pcsk9*

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PCSK9 encodes proprotein convertase subtilisin/kexin type 9a (PCSK9), a member of the proteinase K subfamily of subtilases. Missense mutations in *PCSK9* cause an autosomal dominant form of hypercholesterolemia in humans, likely due to a gain-of-function mechanism because overexpression of either WT or mutant PCSK9 reduces hepatic LDL receptor protein (LDLR) in mice. Here, we show that livers of knockout mice lacking PCSK9 manifest increased LDLR protein but not mRNA. Increased LDLR protein led to increased clearance of circulating lipoproteins and decreased plasma cholesterol levels (46 mg/dl in *Pcsk9*^{-/-} mice versus 96 mg/dl in WT mice). Statins, a class of drugs that inhibit cholesterol synthesis, increase expression of sterol regulatory element-binding protein-2 (SREBP-2), a transcription factor that activates both the *Ldlr* and *Pcsk9* genes. Statin administration to *Pcsk9*^{-/-} mice produced an exaggerated increase in LDLRs in liver and enhanced LDL clearance from plasma. These data demonstrate that PCSK9 regulates the amount of LDLR protein in liver and suggest that inhibitors of PCSK9 may act synergistically with statins to enhance LDLRs and reduce plasma cholesterol.

low-density lipoprotein receptor | lipoproteins | proteinase | sterol regulatory element-binding protein

The activity of the low-density lipoprotein receptor (LDLR) in liver is the major determinant of plasma LDL cholesterol concentrations (1, 2). Transcription of the LDLR is regulated by sterol regulatory element-binding protein-2 (SREBP-2), one of three SREBP family members that regulate the expression of many enzymes involved in cholesterol and fatty acid synthesis (3, 4). When hepatocellular sterols are low, SREBP-2 is activated, which restores cholesterol to normal levels by simultaneously activating enzymes required for *de novo* cholesterol synthesis and by increasing cholesterol uptake from the plasma through enhanced expression of the LDLR (3).

Recent studies suggest that hepatic LDLRs also may be post-transcriptionally regulated by proprotein convertase subtilisin/kexin type 9a (PCSK9) (5–7). PCSK9 belongs to the proteinase K subfamily of subtilases, which are proteinases synthesized as soluble zymogens that subsequently undergo autocatalytic cleavage to active enzymes (8). *Pcsk9* was identified as an SREBP-regulated gene in liver by using oligonucleotide arrays hybridized with RNA from livers of mice that either overexpressed or lacked SREBPs (4, 9). *Pcsk9* was regulated in a manner similar to other SREBP-responsive genes involved in lipid homeostasis, suggesting that PCSK9 might also participate in lipid metabolism.

This suggestion was confirmed by the finding that missense mutations in *PCSK9* are associated with an autosomal dominant form of hypercholesterolemia (10–12). The clinical phenotype of these subjects is indistinguishable from two other autosomal dominant forms of hypercholesterolemia, both of which are caused by defective receptor-mediated clearance of LDL: (i) familial hypercholesterolemia, which is caused by mutations in the LDLR; and (ii) familial defective apolipoprotein B (apoB), caused by mutations in the ligand for the LDLR (13). This similarity raised the possibility that PCSK9 somehow lowers the amount or activity of LDLRs in liver.

This hypothesis was supported by the finding that overexpression of mutant forms of PCSK9 in mice significantly reduced LDLR protein in liver and raised plasma LDL (5–7). Overexpression of WT PCSK9 reduced hepatic LDLRs to a similar extent as expression of PCSK9 mutant forms (7). These studies suggested that PCSK9 might function normally to reduce LDLR expression levels in liver. If this hypothesis is correct, then the elimination of PCSK9 through targeted disruption of its gene should lead to an increase in LDLRs and a decrease in plasma LDL. To test this hypothesis, we deleted *Pcsk9* in mice and characterized the effects on cholesterol metabolism.

Materials and Methods

DNA manipulations were performed by using standard molecular biology techniques (14). Cholesterol and triglyceride concentrations in plasma and liver were measured as described (15). Plasma lipoprotein fractions were separated by FPLC gel filtration by using a Superose 6 column. Measurements of cholesterol concentrations eluted from the FPLC fractions and Coomassie staining of plasma lipoproteins were performed as described (16). Protein concentrations were determined by using the BCA Protein Assay Reagent (Pierce). Other reagents were obtained from Sigma-Aldrich.

Construction of Targeting Vector for Disruption of *Pcsk9*. Mouse *Pcsk9* was disrupted by using a gene-replacement vector that deleted the 3' half of exon 2 through intron 4. Details of the gene-targeting vector construction are available upon request.

ES Cell Culture for Disruption of *Pcsk9*. Passage 11 SM-1 ES cells were electroporated with the *Pcsk9* targeting vector as described (17). Recombined clones were identified by PCR using primers P1 (5'-GCT TCT GAG GCG GAA AGA ACC AGC-3') from the 5' coding region of the *neo* gene and P2 (5'-TCA TCA TCC AAT GGG TGG GCC TGA AG-3') from the promoter of *Pcsk9* located outside of the targeting vector. The targeted allele produced a 1.1-kb PCR product. Targeted clones were confirmed by Southern blot analysis using a 0.35-kb DNA probe from the *Pcsk9* promoter region (Fig. 8, which is published as supporting information on the PNAS web site).

Generation of *Pcsk9* Knockout Mice. Two targeted ES clones with a disrupted *Pcsk9* allele were injected separately into C57BL/6J blastocysts, yielding chimeric males whose coat color (*agouti*) indicated a contribution of ES cells from 75% to 100%. Three chimeric males derived from each clone subsequently produced offspring that harbored the disrupted *Pcsk9* allele. Mice carrying

Abbreviations: ARH, autosomal recessive hypercholesterolemia; Apo, apolipoprotein; LDLR, low-density lipoprotein receptor; LRP, LDLR-related protein; PCSK9, proprotein convertase subtilisin/kexin type 9a; SREBP, sterol regulatory element-binding protein; RAP, receptor-associated protein; VLDL, very low-density lipoprotein; HDL, high-density lipoprotein.

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the disrupted allele were identified by Southern blotting or by PCR (Fig. 8).

Mice were housed in colony cages and maintained on a 12-h light/12-h dark cycle, fed Teklad Mouse/Rat Diet 7002 from Harlan Teklad Premier Laboratory Diets, and killed at the end of the dark cycle. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

Real-Time RT-PCR. Total RNA was prepared from mouse livers by using an RNA STAT-60 kit (Tel-Test, Friendswood, TX). The primers for real-time PCR and details of PCR conditions were as described (17–19).

Antibodies and Immunoblot Analysis. The murine PCSK9 amino acid sequence was analyzed by using PROTEAN software (DNASTAR, Madison, WI), and two segments (amino acids 163–188 and amino acids 220–240) predicted to be immunogenic were synthesized by the Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center. Peptides were conjugated to KLH by using the Inject Maleimide Activated mKLH Kit (Pierce), and rabbits were immunized with a mixture of the peptides (20 μ g each) as described (15). IgG fractions from preimmune and immune sera were purified by using the Immunopure (A/G) IgG purification kit (Pierce). The resulting antibody detected two proteins corresponding to proprotein ($\approx$ 76 kDa) and cleaved ($\approx$ 62 kDa) forms of PCSK9. Polyclonal antibodies directed against the mouse LDLR, LDL receptor-related protein (LRP), receptor-associated protein (RAP), autosomal recessive hypercholesterolemia (ARH), SREBP-1, and SREBP-2 were as described (7).

For immunoblot analysis, membranes ($10^5 \times$ g pellet) and nuclear extracts were prepared from mouse liver as described (15, 19). For whole-cell lysates, equal portions of liver were homogenized in buffer A (20 mM Tris-HCl, pH 7.4/0.25 M sucrose/50 mM KCl/2 mM $MgCl_2$ /1 mM sodium EDTA/supplemented with 1 mM phenylmethanesulfonyl fluoride/1 mM 1,10-phenanthroline/50 μ g/ml leupeptin/1 μ g/ml pepstatin A/0.5 μ g/ml aprotinin/25 μ g/ml N-acetyllecucylleucylnorleucinal). The homogenate was centrifuged at $10^5 \times$ g for 15 s to pellet cellular debris. Protein concentrations were measured in the supernatant, and equal aliquots of protein were mixed with an equal volume of buffer B (62.5 mM Tris-HCl, pH 6.8/8 M urea/15% SDS (wt/vol)/10% glycerol (vol/vol)/100 mM DTT) and heated for 5 min at 95°C.

Samples were subjected to SDS/PAGE as described (15, 19). Immunoblot analyses were performed by using either a horseradish peroxidase-conjugated anti-rabbit secondary antibody from donkey (Amersham Pharmacia Biosciences) and SuperSignal West Pico Chemiluminescent Substrate System (Pierce), or a ^{125}I -labeled secondary anti-rabbit antibody from donkey (Amersham Pharmacia Biosciences) (15).

Immunohistochemistry. Indirect immunofluorescence using antibodies against LDLR in livers of WT, *Ldlr*^{-/-}, and *Pcsk9*^{-/-} mice was carried out as described (20).

Plasma Clearance of [^{125}I]LDL. Mouse LDL (density 1.019–1.063 g/ml) was prepared from the pooled plasma of 70 *Ldlr*^{-/-} mice by sequential ultracentrifugation and radiolabeled with sodium ^{125}I (16). Clearance of ^{125}I -labeled apoB (apoB48 plus apoB100) from plasma of WT and *Pcsk9*^{-/-} mice was measured at the indicated times by isopropanol precipitation (16, 17).

ApoB Secretion from Primary Hepatocytes. Hepatocytes were isolated as described (7) and cultured in Methionine/Cysteine-free DMEM (Sigma) supplemented with 5% lipoprotein-deficient newborn calf serum, 4 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μ g/ml streptomycin sulfate (medium A) for 2 h.

Table 1. Phenotypic comparison of wild-type and *Pcsk9*^{-/-} mice

Parameter	WT	<i>Pcsk9</i> ^{-/-}
No. of mice	4	4
Body weight, g	25.5 $\pm$ 0.6	30.0 $\pm$ 1.6
Liver cholesterol, mg/g	2.20 $\pm$ 0.16	2.00 $\pm$ 0.02
Liver TG, mg/g	9.2 $\pm$ 0.6	7.2 $\pm$ 0.7
Plasma cholesterol, mg/dl	95.7 $\pm$ 9.4	46.3 $\pm$ 1.9*
Plasma TG, mg/dl	70.0 $\pm$ 11	85.8 $\pm$ 7.5

Male mice (12–13 weeks of age) were fed a rodent chow and killed nonfasted. Each value represents the mean $\pm$ SEM. *, A level of statistical significance (Student's *t* test) of *P* < 0.01 between WT and *Pcsk9*^{-/-} mice. TG, triglycerides. Similar results were obtained from four independent experiments.

The hepatocytes were pulse-labeled with 250 μ Ci/ml (1 Ci = 37 GBq) [^{35}S]Methionine/Cysteine (Redivue PRO-MIX [^{35}S], Amersham Pharmacia Biosciences) in medium A without serum for 30 min. Thereafter, hepatocytes were washed twice with PBS, and the medium was changed to DMEM supplemented with 10 mM L-methionine, 10 mM L-cysteine, 5% lipoprotein-deficient newborn calf serum, 100 units/ml penicillin G sodium, and 100 μ g/ml streptomycin sulfate. The cells were incubated for the indicated times, after which the medium and cells were harvested as described (7).

ApoB48 and apoB100 were immunoprecipitated and separated on a 4% SDS/PAGE gel (7). The gels were dried and exposed to a PhosphorImager plate, and the resulting signals were quantified by using a PhosphorImager Molecular Dynamics Storm 820 system (Amersham Pharmacia Biosciences). ApoB secreted from the hepatocytes was calculated as the percentage of apoB initially synthesized during the pulse-labeling period.

Diet Studies. Mice were fed ad libitum a cereal-based powdered diet (Teklad Mouse/Rat Diet 7001) or the powdered diet supplemented with 0.2% (wt/wt) lovastatin (Merck & Co.) for 7 days.

Results

The strategy used to disrupt *Pcsk9* in mice is shown in Fig. 8. A targeting vector containing the neomycin-resistance gene was used to replace the C-terminal half of exon 2, exon 3, and exon 4. Northern blotting and immunoblot analysis confirmed that this deletion eliminated detectable PCSK9 mRNA and protein (Fig. 8).

Matings between *Pcsk9*^{-/-} mice produced offspring in the expected Mendelian 1:2:1 ratio. *Pcsk9*^{-/-} mice were normal in appearance. Body and liver weights were not significantly different from littermate WT mice (Table 1). No significant differences were found when the hepatic cholesterol and triglyceride concentrations were compared with WT littermates. Plasma cholesterol levels in the *Pcsk9*^{-/-} mice were 48% lower than those of WT mice, despite similar levels of plasma triglycerides.

Lipoproteins from pooled plasma of 16 WT and 16 *Pcsk9*^{-/-} male mice were isolated by ultracentrifugation and fractionated by FPLC (Fig. 1A). Normal mouse plasma contains very little apoB-containing lipoproteins [very low-density lipoprotein (VLDL) or LDL]; the majority of circulating cholesterol in mice is associated with high-density lipoprotein (HDL). In plasma from *Pcsk9*^{-/-} mice, the level of LDL cholesterol was even further reduced so that it was nearly undetectable. The level of HDL cholesterol was reduced by $\approx$ 30%. Mouse HDL contains apoE, which allows it to bind to LDLRs. Fig. 1B shows the protein composition of the lipoprotein fractions resolved by FPLC. As expected from the reduction in LDL cholesterol, the plasma level of apoB100 was clearly lower in the *Pcsk9*^{-/-} mice and the level of apoB48 was also diminished. There also was a marked reduction in the apoE content of VLDL and HDL fractions.

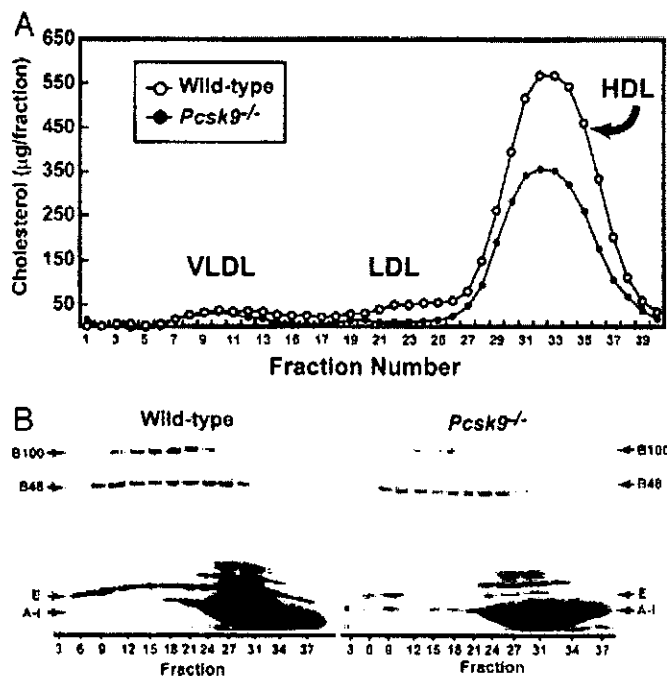


Fig. 1. FPLC profiles and SDS/PAGE of plasma apolipoproteins from WT and *Pcsk9*^{-/-} mice. (A) Plasma from 16 WT and 16 *Pcsk9*^{-/-} male mice was pooled (6.5 ml for each genotype) and subjected to ultracentrifugation at $d = 1.215$ g/ml. The lipoprotein fractions were separated by FPLC gel filtration, and the cholesterol content of each fraction was measured (16). (B) SDS/PAGE of plasma apolipoproteins from WT and *Pcsk9*^{-/-} mice. Equal aliquots (0.5 ml) from three consecutive FPLC fractions were pooled and delipidated, and apoproteins were precipitated (16). Apoproteins were subjected to 3–15% gradient SDS/PAGE and stained with Coomassie blue. The positions of migration of apoB100, apoB48, apoE, and apoA-I are indicated.

The reduction in apoE- and apoB-containing lipoproteins suggested that less VLDL was secreted from liver or else these particles were cleared faster from the circulation in the *Pcsk9*^{-/-} mice. The major route of clearance of apoE- and apoB-containing lipoproteins is by means of LDLR-mediated endocytosis in the liver (2). Fig. 2 shows the levels of LDLR and other proteins in pooled livers from four *Pcsk9*^{-/-} and four WT littermate controls as determined by immunoblotting. As expected, no PCSK9 protein was detected in the livers of *Pcsk9*^{-/-} mice when a polyclonal antibody directed against the mouse protein was used for immunoblotting (Fig. 2A). The level of hepatic LDLR protein was ≈ 2.8 -fold higher in the *Pcsk9*^{-/-} mice than in the WT mice as determined by densitometric scanning of the autoradiogram. No changes were found in the amount of LRP, a member of the LDLR family (21), and ARH, an adaptor protein involved in hepatic LDLR internalization (20), or in SREBP-1 or SREBP-2, the transcriptional regulators of the *Ldlr* and lipid biosynthetic enzymes (Fig. 2B). Similarly, no changes in protein levels were measured for the VLDL receptor, another member of the LDLR family, or scavenger receptor class B type I (SR-BI), a receptor involved in HDL clearance (data not shown).

To determine whether the increase in LDLR was secondary to an increase in the level of mRNA encoding the LDLR, we measured the level by quantitative RT-PCR. We found no changes in the mRNAs encoding the LDLR or several other proteins involved in cholesterol and fatty acid biosynthesis (see Table 3, which is published as supporting information on the PNAS web site).

Indirect immunofluorescence confocal microscopy was used to confirm the increase in LDLRs on the surface of the hepatocytes from *Pcsk9*^{-/-} mice (Fig. 3). The specificity of the polyclonal anti-LDLR antibody was confirmed by the absence of staining in a

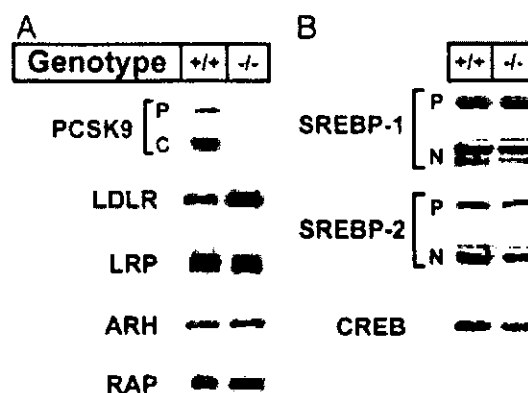


Fig. 2. Levels of proteins in livers of WT and *Pcsk9*^{-/-} mice. Livers from mice described in Table 1 were pooled, and aliquots of membrane protein (40 μ g), whole cell lysate (30 μ g), or nuclear protein (30 μ g) were subjected to SDS/PAGE (7). (A) Immunoblot analyses of PCSK9, ARH (whole cell lysate), LDLR, LRP, and RAP (membrane fraction). P and C for PCSK9 denote the precursor and cleaved forms of PCSK9, respectively. (B) Immunoblot analysis of SREBP-1, SREBP-2 (membrane and nuclear fractions), and cAMP response element binding protein (CREB) (nuclear fraction). P and N denote the precursor and cleaved nuclear forms of SREBP-1 and SREBP-2. Similar results were obtained in four independent experiments.

liver of an *Ldlr*^{-/-} mouse (Fig. 3A) (22). A marked increase in staining for the LDLR was observed in hepatocytes from *Pcsk9*^{-/-} mice compared with those from WT mice (compare Fig. 3B and C).

To measure the rate of LDL clearance from plasma, we isolated LDL from the blood of *Ldlr*^{-/-} mice and labeled its apoB with ¹²⁵I. Radiolabeled LDL was injected into two different groups of WT and *Pcsk9*^{-/-} mice (six mice in each group). The first group was used to measure the rate of disappearance of ¹²⁵I-labeled LDL during the first 30 min after injection (Fig. 4A), and the second group was used to measure the rate of LDL clearance at longer time points (Fig. 4B). The time required for clearance of $\approx 50\%$ of the ¹²⁵I-LDL was 12 min in *Pcsk9*^{-/-} mice and >60 min in WT mice. We attribute this 5-fold increase in ¹²⁵I-LDL clearance to the ≈ 3 -fold increase in hepatic LDLRs in *Pcsk9*^{-/-} mice.

We next determined whether the absence of *Pcsk9* altered the rate of apoB secretion from primary hepatocytes derived from WT and *Pcsk9*^{-/-} mice. For this purpose, freshly isolated hepatocytes were incubated with [³⁵S]methionine/cysteine. After a 30-min labeling period, the medium was switched to medium containing cold methionine/cysteine. ApoB100 and apoB48 were immunoprecipitated from cells and medium after 1 or 2 h, and the amount of labeled apoB was quantified by SDS/PAGE autoradiography. The deletion of *Pcsk9* was associated with a slight reduction in the secretion of apoB48 (Fig. 5). ApoB100 secretion was not significantly different in hepatocytes from WT or *Pcsk9*^{-/-} mice. Whether this reduction is due to a presecretory mechanism that degrades apoB or to recapture of secreted apoB owing to higher levels of LDLR protein expression in hepatocytes from *Pcsk9*^{-/-} mice could not be determined in these experiments.

The SREBP-mediated up-regulation of PCSK9 creates a potential problem for therapy with 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors like lovastatin and other statins. These inhibitors block cholesterol synthesis and thereby cause an increase in cleaved SREBP-2. Nuclear SREBP-2 enhances the transcription of the *LDLR* gene, leading to increased LDLRs and a fall in plasma LDL. The elevated nuclear SREBP-2 also increases the mRNA for PCSK9, and this result would lead to a reduction in LDLR protein. If this scenario is correct, lovastatin should increase LDLRs to a greater extent in *Pcsk9*^{-/-} mice than in WT mice.

To test this hypothesis, WT and knockout mice were fed normal chow or chow supplemented with 0.2% lovastatin. Table 2 contains

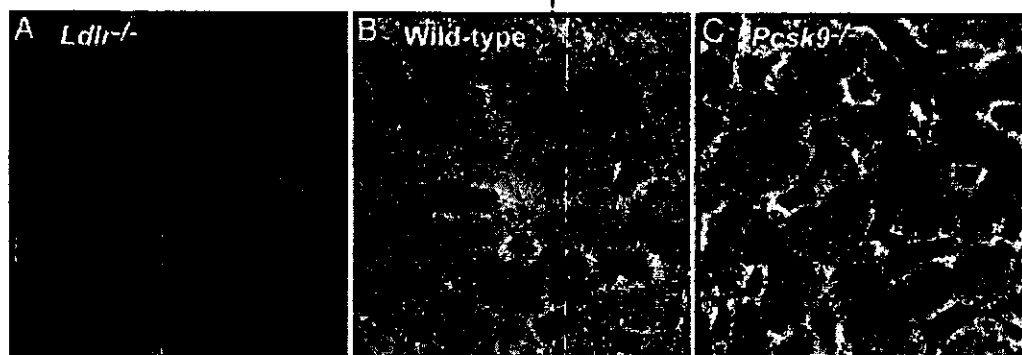


Fig. 3. Indirect immunofluorescence in liver using antibodies against LDLR. Frozen sections of liver from *Ldlr*^{-/-} (A), WT (B), and *Pcsk9*^{-/-} (C) mice were incubated with a polyclonal antibody against the LDLR. Bound IgG was detected with 20 μ g/ml Alexa Fluor 488-labeled goat anti-rabbit IgG (20).

pooled data from three independent experiments. Addition of 0.2% lovastatin to chow did not significantly reduce the mean plasma cholesterol in WT mice (71 mg/dl versus 81 mg/dl). As observed previously, the *Pcsk9*^{-/-} mice maintained on chow had a plasma cholesterol level that was significantly lower than WT mice (51 mg/dl versus 81 mg/dl). Addition of lovastatin to the diet of knockout mice resulted in the plasma cholesterol level falling to 41 mg/dl; this value was significantly less than those measured in *Pcsk9*^{-/-} mice fed chow. Plasma triglycerides were significantly reduced in mice from both genotypes fed lovastatin.

Analysis of hepatic gene expression revealed that feeding the lovastatin-containing diet to WT and *Pcsk9*^{-/-} mice resulted in increases in SREBP-2 mRNA levels and a corresponding increase in all measured SREBP target gene mRNA levels, including those encoding 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, PCSK9, and LDLR (Table 4, which is published as supporting information on the PNAS web site). The fold-increase of cholesterol biosynthetic gene mRNAs in livers of lovastatin-treated *Pcsk9*^{-/-} mice was similar to that measured in WT mice fed the same diet.

Lovastatin administration to WT mice led to an increase in PCSK9 protein in liver (Fig. 6A). Lovastatin also resulted in the predicted increase in nuclear SREBP-2 protein levels in WT and *Pcsk9*^{-/-} mice. To more accurately quantify the changes in levels of LDLR and RAP, a ¹²⁵I-labeled secondary antibody was used to generate the data shown in Fig. 6B. LDLR protein levels in livers of WT mice fed lovastatin were consistently lower than chow-fed mice despite the measured increase in LDLR mRNA expression. The expression of the LDLR was 2.8-fold higher in livers of

chow-fed *Pcsk9*^{-/-} mice compared with the WT mice and was further increased after administration of lovastatin to a level 4.6-fold higher than chow-fed WT mice. Fig. 6C shows the relative amount of LDLR protein in livers of WT and *Pcsk9*^{-/-} mice administered lovastatin from three independent experiments. In all experiments, lovastatin administration resulted in a significantly greater increase in LDLR expression in the absence of PCSK9.

To determine whether lovastatin increased LDL clearance, radiolabeled LDL was injected into WT and *Pcsk9*^{-/-} mice fed chow or lovastatin (eight mice per group) (Fig. 7). Lovastatin administration to WT mice did not significantly alter LDL clearance from the plasma after only 30 min. *Pcsk9*^{-/-} mice again displayed a marked increase in the rate of LDL clearance from plasma compared with that of WT mice. Lovastatin administration to *Pcsk9*^{-/-} mice resulted in an additional $\approx$ 2-fold increase in the rate of labeled LDL clearance from the plasma compared with chow-fed *Pcsk9*^{-/-} mice. This change was similar in magnitude to the change measured in LDLR protein levels in chow versus lovastatin-fed *Pcsk9*^{-/-} mice (Fig. 6B).

Discussion

The current study demonstrates that the inactivation of *Pcsk9* reduces plasma cholesterol levels primarily by increasing LDLR protein expression in liver and accelerating the clearance of circulating cholesterol. These results, together with the previous observation that overexpression of WT PCSK9 in mice leads to elevated plasma LDL cholesterol levels by reducing the LDLR protein in liver (7), indicate that one function of PCSK9 is to negatively regulate LDLR protein levels. No changes were detected in

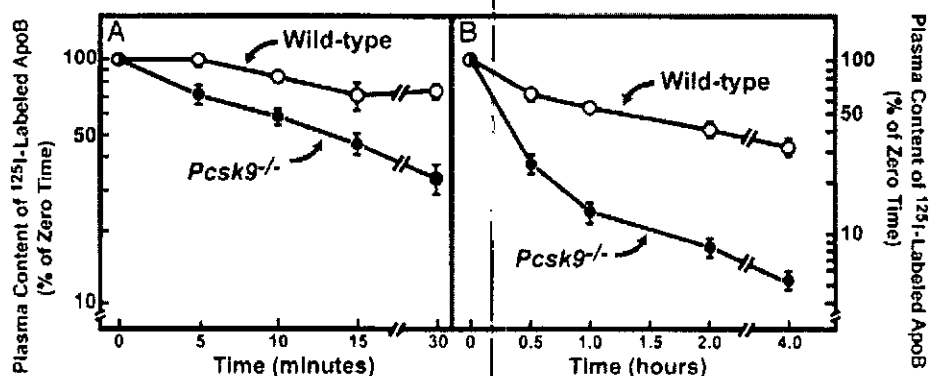


Fig. 4. Plasma clearance of ¹²⁵I-labeled mouse LDL in WT and *Pcsk9*^{-/-} mice. (A) Six male mice (10–12 weeks of age) of the indicated genotype were injected i.v. with ¹²⁵I-labeled LDL (30 μ g of protein, 294 cpm/ng apoB protein). Blood was obtained at 30 s (time 0) and at 5, 10, 15, and 30 min for quantification of plasma content of ¹²⁵I-labeled total apoB (17). (B) Six male mice (10–12 weeks of age) of the indicated genotype were injected i.v. with the same ¹²⁵I-labeled LDL used in A. Blood was obtained at 30 s (time 0) and at 0.5, 1, 2, and 4 h for quantification of plasma content of ¹²⁵I-labeled total apoB. Data are plotted as the percentage of zero time value. Each value represents mean $\pm$ SEM of six mice.

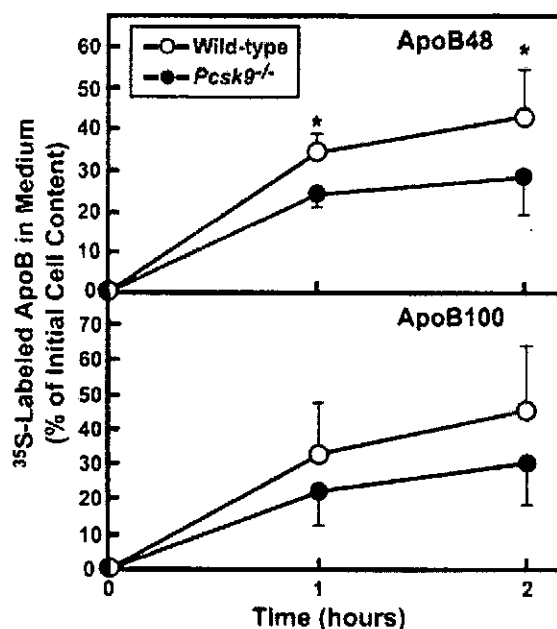


Fig. 5. Rates of apoB secretion by primary hepatocytes from WT mice and *Pcsk9*^{-/-} mice. Hepatocytes were prepared from mice of the indicated genotype, and apoB48 and apoB100 were immunoprecipitated and separated by SDS/PAGE gel electrophoresis as described under *Materials and Methods*. The data are expressed as the apoB content in the medium as a percentage of the ³⁵S-labeled apoB in the cells at zero time. Each value is mean $\pm$ SEM of duplicate incubations from eight WT and eight *Pcsk9*^{-/-} mice. *, Statistical difference of $P < 0.05$ (Student's *t* test).

SREBP-2 protein or in the mRNAs of genes encoding cholesterol synthesis enzymes in livers of *Pcsk9*^{-/-} mice, which suggests that the reduction in plasma cholesterol levels was not due to decreased cholesterol synthesis. A small reduction in apoB48 secretion was detected in primary hepatocytes derived from *Pcsk9*^{-/-} mice; however, the marked increase in plasma LDL clearance measured in the *Pcsk9*^{-/-} mice suggests that the primary mechanism responsible for lower plasma cholesterol levels is increased LDLR expression.

3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors function by reducing cellular cholesterol levels, which activates SREBP-2, leading to the transcriptional activation of the LDLR. Inasmuch as SREBP-2 also activates *Pcsk9*, the increase in PCSK9 expression may normally attenuate the increase in LDLR expression in animals administered statins. The data of Fig. 6 are consistent with this interpretation. The administration of lovastatin to WT mice increased nuclear SREBP-2 protein and the mRNAs of both the LDLR and PCSK9 (Table 4). Despite the transcriptional activation of the

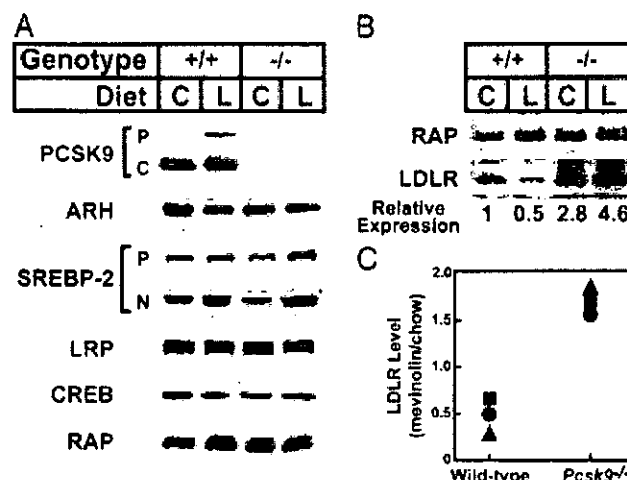


Fig. 6. Levels of proteins in livers of WT and *Pcsk9*^{-/-} mice fed chow (C) or chow supplemented with 0.2% lovastatin (L). Livers from four male mice in the groups of Table 2 were pooled, and aliquots of membrane protein (40 μ g), whole cell lysate (30 μ g), or nuclear protein (30 μ g) were subjected to SDS/PAGE. (A) Immunoblot analysis of PCSK9, ARH (whole cell lysate), SREBP-2 (membrane and nuclear fractions), cAMP response element binding protein (CREB) (nuclear fraction), LRP, and RAP (membrane fraction). P and C for PCSK9 denote the proprotein and cleaved forms of PCSK9. For SREBP-2, P and N denote the precursor and cleaved nuclear forms. (B) Immunoblot analyses of LDLR and RAP. A ¹²⁵I-labeled secondary anti-rabbit antibody from donkey was used for the LDLR and RAP to quantify the expression of the LDLR by using a PhosphorImager. The relative expression of the LDLR protein is normalized to the amount of LDLR expressed in livers of WT mice fed chow. (C) Relative amount of hepatic LDLR protein in WT and *Pcsk9*^{-/-} mice fed 0.2% lovastatin versus chow. Each symbol represents an independent experiment with four mice per group.

Ldlr, total hepatic LDLR protein was slightly lower than WT mice fed chow, and the plasma cholesterol levels were not statistically reduced (Table 2 and Fig. 6). One reason for the apparent paradoxical response seems to be the simultaneous induction of PCSK9, which posttranscriptionally reduces LDLR protein levels. This interpretation is supported by the findings that *Pcsk9*^{-/-} mice administered lovastatin had higher LDLR protein levels, increased LDL clearance from plasma, and lower plasma cholesterol levels than *Pcsk9*^{-/-} mice fed chow. These results suggest that inhibitors of PCSK9 may have beneficial effects on plasma cholesterol levels, especially when combined with statins.

Our findings in mice are consistent with those of Ness *et al.* (23), who previously showed that statins administered to rats increased the LDLR mRNA levels but not LDLR protein levels in liver. The lack of an increase in LDLR protein was attributed to increased LDLR degradation in these studies. It is likely that

Table 2. Phenotypic comparison of wild-type and *Pcsk9*^{-/-} mice fed 0.2% lovastatin

Parameter	WT		<i>Pcsk9</i> ^{-/-}	
	Chow	0.2% lov.	Chow	0.2% lov.
No. of mice	12	12	12	12
Body weight, g	24.4 $\pm$ 1.0	26.4 $\pm$ 1.2	25.8 $\pm$ 1.0	24.7 $\pm$ 0.7
Plasma cholesterol, mg/dl	80.9 $\pm$ 5.1	71.1 $\pm$ 3.0	51.4 $\pm$ 2.3*	40.8 $\pm$ 1.8***
Plasma TG, mg/dl	94.4 $\pm$ 8.7	64.5 $\pm$ 4.6*	100 $\pm$ 5.9	66.2 $\pm$ 4.2***

Mean values of pooled data from three independent experiments. The eight male and four female mice (12–14 weeks of age) in each group were fed a standard rodent chow or the standard chow supplemented with 0.2% (wt/wt) lovastatin (lov.) for 7 days. The mice were killed nonfasted. Each value represents the mean $\pm$ SEM. *, A statistical difference of $P < 0.01$ (Student's *t* test) between the indicated group and WT mice fed chow. **, A statistical difference of $P < 0.01$ (Student's *t* test) between *Pcsk9*^{-/-} mice fed 0.2% lovastatin and chow.

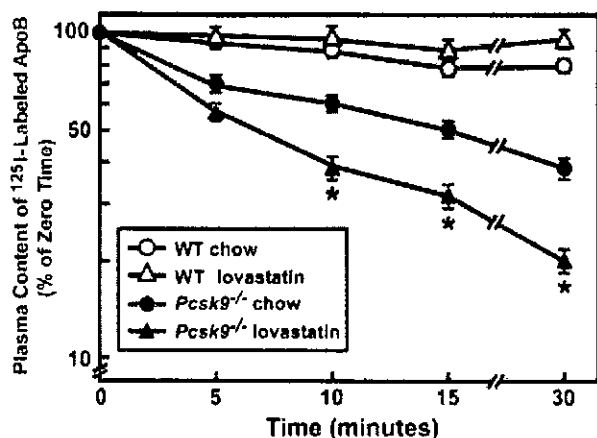


Fig. 7. Plasma clearance of ^{125}I -labeled mouse LDL in WT and *Pcsk9*^{-/-} mice fed chow or lovastatin. Eight male mice (10–12 weeks of age) of the indicated genotype were fed chow or chow supplemented with 0.2% lovastatin for 7 days. Plasma clearance of ^{125}I -labeled total apoB was determined as described in the legend of Fig. 4. *, Statistical difference of $P < 0.05$ (Student's *t* test) between *Pcsk9*^{-/-} mice fed chow or lovastatin.

a statin-induced increase in PCSK9 expression also was responsible for their results. Lovastatin administration did reduce plasma triglycerides in WT mice in the absence of a detectable increase in LDLR protein or LDL clearance from plasma. A reduction in VLDL production by statins probably accounts for this observation.

Studies investigating the role of PCSK9 in regulating apoB secretion in liver have resulted in conflicting results. Two studies showed that apoB secretion is unaltered by PCSK9 overexpression (5, 7), whereas another reported that PCSK9 overexpression increases apoB secretion from the livers of mice (6). In the absence of PCSK9, it seems that there is reduced apoB48 secretion from mouse primary hepatocytes. The increase in LDLR protein expression resulting from the deletion of *Pcsk9* could explain this observation by means of the mechanism described by Twisk *et al.* (24), who previously demonstrated that the LDLR protein can mediate posttranslational presecretory degradation of apoB in hepatocytes. A second possibility is that increased LDLR expression on the cell surface mediates immediate VLDL recapture, thus reducing total apoB secretion into the medium. Additional studies will be required to clarify the operative mechanism.

The current study confirms that the LDLR is regulated both at the transcriptional and posttranscriptional levels. SREBP-2 transcriptionally activates *Pcsk9* and *Ldlr* and thus promotes both the synthesis and destruction of the LDLR. The biological

reason why SREBP-2 up-regulates both *Ldlr* and *Pcsk9* still cannot be easily reconciled with the current information. However, it is possible that the simultaneous transcriptional activation of *Pcsk9* and *Ldlr* by SREBP-2 may provide a posttranscriptional mechanism to degrade the LDLR and shorten the protein's half-life, which could protect the cell from excessive LDL uptake and cholesterol accumulation (7).

Several important questions regarding the function of PCSK9 remain unanswered. First, the substrate(s) of PCSK9 has not been defined, and whether this protease directly cleaves the LDLR, or acts indirectly by destroying another protein, remains to be determined. Second, although recent studies indicate that PCSK9 mediates the degradation of the LDLR in a post-ER compartment (25), it is not known whether this degradation occurs intracellularly or after the LDLR reaches the cell surface. Third, the importance of PCSK9-mediated regulation of LDLR expression in tissues other than liver remains to be determined. PCSK9 is expressed at low levels in all tissues tested (8). The liver normally accounts for 65–70% of all LDL-cholesterol clearance in mice (2). Most tissues other than liver and the adrenal gland have low LDLR activity and presumably low LDLR protein levels (2). In experiments not shown, levels of LDLR protein in the adrenal gland were higher in *Pcsk9*^{-/-} mice, suggesting that PCSK9 also may regulate the expression of LDLR in extrahepatic tissues. Further delineation of each tissue's contribution to plasma LDL-cholesterol clearance in *Pcsk9*^{-/-} mice will require more quantitative LDL uptake studies.

The results reported here are consistent with the findings of Cohen *et al.* (26), who found that individuals heterozygous for a nonsense mutation in *PCSK9* had a 40% reduction in LDL cholesterol levels. Individuals with loss-of-function mutations in *PCSK9* were heterozygous for the mutant allele. Heterozygous *Pcsk9*^{-/-} mice had a phenotype that was intermediate between homozygous *Pcsk9*^{-/-} mice and WT mice in terms of plasma cholesterol concentrations and LDL clearance, which is consistent with a gene-dosage effect. The phenotypes observed in humans with mutations in *PCSK9*, combined with results of the current studies in mice, suggest that variations in the levels of PCSK9 significantly affect plasma cholesterol levels and that inhibitors of PCSK9 may be useful for the treatment of hypercholesterolemia.

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