SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 11-37695- -6 **FECHA**: 2013-06-13 18:21:28
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 379 FASE NACIONAL INCLUIDO CAPITULO II
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 5
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 11-37695- -6
Trámite: 11
Evento: 379
Actuación: 519
Oficio: 10492
Folios: 5

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I		Capítulo II	**X**
No. Solicitud Internacional	PCT/IB2009/053990				
Fecha de Presentación Internacional	11/09/2009				

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 16 a 181	28/Marzo/2011
Reivindicaciones:	Folios 182 a 184	28/Marzo/2011
Dibujos:	Folios 232 a 246	28/Marzo/2011
Secuencias:	Folios 186 a 231	28/Marzo/2011
Prioridad:	US 61 096 716	12/Septiembre/2008

2. Análisis de la Prioridad

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

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capitulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Para la presente solicitud, se estudió el capítulo reivindicatorio completo.

Título de la solicitud: “Antagonistas de PCSK9”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar antagonistas para la actividad de PCSK9, útiles para el tratamiento de enfermedades asociadas con el colesterol en sangre.

Para resolver este problema técnico la solicitud en estudio proporciona anticuerpos que se unen específicamente a PCSK9.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/395

4. Excepción a la Patentabilidad (Art 20 D 486 literales c y d)

Teniendo en cuenta el artículo citado, las plantas y los procedimientos esencialmente biológicos para la producción de plantas hacen parte de la materia que no es patentable. El objeto de la reivindicación 20 no es patentable debido a que hace referencia a una solicitud de patente de una célula huésped transformada que no está definida en términos de línea celular.

De igual manera, los métodos para el tratamiento humano o animal hacen parte de la materia no patentable en éste país. Por lo tanto, el objeto de la reivindicación 22, por tratarse de métodos de tratamiento en humanos, no es patentable.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capitulo reivindicatorio completo carece de claridad y concisión por cuanto,

1) Las reivindicaciones no cumplen con el requisito de concisión tanto en las reivindicaciones individuales como en el conjunto de reivindicaciones. Se sugiere al solicitante reformular las reivindicaciones de manera tal que no se llegue a repeticiones en las reivindicaciones que tienen el mismo alcance y revisar la dependencia de todo el capitulo reivindicatorio. Además se le recuerda a la solicitante que una reivindicación independiente debe ser autosuficiente;

2) La reivindicación 1 reclama un antagonista de PSCK9 que está definido en términos de un resultado a alcanzar relativo y no limitativo que no permite distinguir la invención del estado de la técnica. Se sugiere reemplazarlo por las características esenciales o estructurales que contribuyen a la obtención de ese efecto;

3) Las reivindicaciones 2 y 3 reclaman un anticuerpo por la función (resultado a alcanzar) y por el método empleado para comprobar dicho efecto, sin definir al anticuerpo. Esto no permite distinguir el alcance de la invención puesto que la reivindicación incluiría no sólo la solución propuesta, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se recuerda a la solicitante que la forma correcta de reclamar un anticuerpo es definirlo a partir de su estructura

completa, esto es, de las secuencias de las regiones variables de la **cadena liviana y cadena pesada** ó secuencias **de seis regiones CDR´s** (3 de la cadena liviana y 3 de la cadena pesada);

4) las reivindicaciones 5 y 7 están definida en términos de resultados a alcanzar no limitativos que no permiten distinguir la invención del estado de la técnica. Se recuerda al solicitante que los anticuerpos no deben definirse por el epitope al cual se unen sino por las características esenciales o estructurales que contribuyen a la obtención de dicho efecto;

5) la reivindicación 8 reclama un anticuerpo que compite por el mismo epitope de cuatro anticuerpos seleccionados, sin indicar las características esenciales o estructurales que le confieren al anticuerpo reclamado la obtención de dicho efecto. Esto no permite tener claridad sobre el alcance de la invención puesto que la reivindicación incluiría no sólo la solución propuesta por la solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se recomienda definir el anticuerpo por su estructura completa;

6) La forma correcta de reclamar un anticuerpo es definirlo a partir de su estructura completa, esto es, de las secuencias de las regiones variables de la **cadena liviana y cadena pesada** ó secuencias **de seis regiones CDR´s** (3 de la cadena liviana y 3 de la cadena pesada) (reivindicación 9, 10 y 11);

7) Las sustituciones reclamadas en la reivindicación 12 no permiten tener claridad sobre los límites de la invención pues conducen a un gran número de posibilidades de variantes del anticuerpo;

8) La expresión “una cantidad terapeuticamente eficaz” no permite tener claridad sobre la composición reclamada. Además, una composición farmacéutica debe estar definida por su estructura en términos de sus elementos esenciales (compuesto activo y excipientes) y sus proporciones (Reivindicaciones 18 y 19);

9) La molécula de ácido nucleico debe ser caracterizada a partir de la secuencia nucleotídica de la misma y hacer referencia a la(s) secuencia(s) del anticuerpo completo indicando además el número de identificación SEQ ID Nº. (reivindicación 21).

6. Unidad de invención (Art 25 D 486)

La falta de claridad en la presentación y redacción de las reivindicaciones conlleva a establecer falta de unidad de invención entre las reivindicaciones. Por lo tanto, la solicitud se refiere a 5 grupos inventivos diferentes:

-Grupo inventivo 1: Un antagonista de PCSK9 que un antagonista de PCSK9 que comprende un anticuerpo aislado, un péptido o un aptámero que interacciona con PCSK9 y, cuando se administra a un sujeto, reduce el colesterol LDL en sangre de dicho sujeto (reivindicación 1).

-Grupo inventivo 2: Un anticuerpo anti-PCSK9 aislado que se une específicamente a PCSK9 y que es un antagonista completo del efecto mediado por PCSK9 en niveles del receptor LDLR medido *in vitro* en un ensayo de regulación por disminución de LDLR en células Huh7 (Reivindicaciones 2, 4 – 6, 18 – 21).

-Grupo inventivo 3: un anticuerpo aislado que antagoniza la interacción extracelular de PCSK9 con LDLR, medida por la unión de PCSK9 al LDLR *in vitro* y, cuando se administra a un sujeto, reduce el colesterol LDL en sangre (Reivindicaciones 3, 4 y 7).

-Grupo inventivo 4: Un anticuerpo aislado que comprende una región de unión a PCSK9 que se solapa con un epitope que es reconocido por los anticuerpos monoclonales 5A10, 4A5, 6F6 y 7D4 (Reivindicaciones 8, 9, 23 y 24).

-Grupo inventivo 5: Un anticuerpo aislado que comprende un CDR1 de región variable de cadena ligera que tiene la secuencia de aminoácidos SEQ ID Nº11, un CDR2 variable de cadena ligera de secuencia SEQ ID Nº12 y/o una CDR3 de región variable de cadena ligera de secuencia SEQ ID Nº 13 o una variante de las mismas que tiene una o más sustituciones conservativas en las tres o alguna de sus CDRs (Reivindicaciones 10 – 17).

Se le sugiere al solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

El solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

7. 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: 12/Septiembre/2008 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud.

Ítem	Documento	Título	Fecha de Publicación
D1[1]	WO 2008/057457	"Antagonist of PCSK9"	15/Mayo/2008
D2[2]	Grozdanov *et al.* 2006	"Expression and localization of PCSK9 in rat hepatic cells"	27/Enero/2006
D3[3]	WO 2008/063382	"Antagonists of PCSK9"	29/Mayo/2008
D4[4]	US 6503510	"Remedies for lymphocytic tumors"	07/Enero/2003
D5[5]	US 2006 0286112	"Human monoclonal antibodies that bind to very late antigen-1 for the treatment of inflammation and other disorders"	21/Diciembre/2006

El documento D1 enseña antagonistas de PCSK9 que inhiben efectivamente la función de PCSK9 y son útiles para el tratamiento de condiciones asociadas con la actividad de PCSK9. También divulga métodos para l uso de dichos antagonistas, así como ácidos nucleicos, vectores, células huésped y composiciones farmacéuticas que comprenden dichos antagonistas (Resumen).

El documento D2 enseña la expresión y localización subcelular de PCSK9 en tejidos de rata, por medio del empleo de anticuerpos comerciales contra PCSK9 (Resumen).

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1 http://www.patentlens.net/patentlens/structured.html?query=wo2008057457

2 Grozdanov, P. *etal*.2006. Expression and localization of PCSK9 in rat hepatic cells. Biochem. Cell Biol. 84: 80–92.

3 http://www.patentlens.net/patentlens/structured.html?query=WO2008063382

4 http://www.patentlens.net/patentlens/patents.html?patnums=US_6503510

5 http://www.patentlens.net/patentlens/patents.html?patnums=US_2006_0286112

El documento D3 enseña antagonistas a PCSK9 que son efectivos en la inhibición de la función de PCSK9.

El documento D4 divulga un agente terapéutico contra tumores linfáticos que comprende un anticuerpo (Resumen).

El documento D5 divulga anticuerpos dirigidos al antígeno tardío-1 VLA-1 y sus usos (Resumen).

8. **Examen de Patentabilidad** (Art 14 D486)

Novedad (Art 16 D486)

Grupo inventivo 1:

La reivindicación 1 consiste en un antagonista de PCSK9 que comprende un anticuerpo aislado, un péptido o un aptámero que interacciona con PCSK9 y, cuando se administra a un sujeto, reduce el colesterol LDL en sangre de dicho sujeto.

Características esenciales	D1
Antagonista de PCSK9	Antagonistas de PCSK9 (Pág. 15 Líneas 10-15)
que comprende un anticuerpo aislado, un péptido o un aptámero que interacciona con PCSK9	Proteínas o anticuerpos que antagonizan la función de PCSK9 (Pág. 15 Líneas 10-15)
cuando se administra a un sujeto, reduce el colesterol LDL en sangre de dicho sujeto.	Disminuyen los niveles de colesterol LDL en plasma (Pág. 15 Líneas 10-15)

La característica esencial del antagonista de la reivindicación 1 había sido divulgadas previamente en el documento D1, el cual enseña antagonistas de PCSK9 que inhiben efectivamente la función de PCSK9 y son útiles para el tratamiento de condiciones asociadas con la actividad de PCSK9. (Resumen).

En consecuencia, el grupo inventivo 1 carece de novedad, según lo divulgado en el documento D1.

Grupo inventivo 2:

La reivindicación 2 consiste en un anticuerpo anti-PCSK9 aislado que se une específicamente a PCSK9 y que es un antagonista completo del efecto mediado por PCSK9 en niveles del receptor LDLR medido *in vitro* en un ensayo de regulación por disminución de LDLR en células Huh7.

Características esenciales	D1
Anticuerpo anti-PCSK9 aislado se une específicamente a PCSK9	anticuerpo anti-PCSK9 aislado que se une específicamente a PCSK9 (Pág. 15 Líneas 10-15)
Y es un antagonista completo del efecto mediado por PCSK9 en niveles del receptor LDLR	Antagoniza el efecto de PCSK9 incluido el del receptor LDLR (Pág 31 Líneas 12-14)
Efecto medido en un ensayo in vitro de regulación por disminución de LDLR en células Huh7	Cualquier tipo de célula que contenga el receptor LDL puede ser empleado en el ensayo in vitro (Pág. 38 Línea 25)

Las características esenciales del anticuerpo de la reivindicación 2 habían sido divulgadas previamente en el documento D1, el cual enseña antagonistas de PCSK9 que inhiben efectivamente la función de PCSK9 y son útiles para el tratamiento de condiciones asociadas con la actividad de PCSK9. También divulga métodos para el uso de dichos

antagonistas, así como ácidos nucleicos, vectores, células huésped y composiciones farmacéuticas que comprenden dichos antagonistas (Resumen).

De este modo, la reivindicación 2 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 4 - 6, 18 – 21 no mencionan alguna característica que haga nueva al anticuerpo de la reivindicación 2, por lo cual se considera que no son nuevas.

En conclusión, el grupo inventivo 2 carece de novedad.

Grupo inventivo 3:

La reivindicación 3 consiste en un anticuerpo aislado que antagoniza la interacción extracelular de PCSK9 con LDLR, medida por la unión de PCSK9 al LDLR *in vitro* y, cuando se administra a un sujeto, reduce el colesterol LDL en sangre.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D1**
Anticuerpo aislado	Anticuerpo aislado
que antagoniza la interacción extracelular de PCSK9 con LDLR medida por la unión de PCSK9 al LDLR in vitro y	Antagoniza el efecto de PCSK9 con del receptor LDLR in vitro (Pág 31 Líneas 12-14)
cuando se administra a un sujeto, reduce el colesterol LDL en sangre.	Disminuyen los niveles de colesterol LDL en plasma (Pág. 15 Líneas 10-15)

Las características esenciales del anticuerpo de las reivindicación 3 habían sido divulgadas previamente en el documento D1, el cual enseña antagonistas de PCSK9 que inhiben efectivamente la función de PCSK9 y son útiles para el tratamiento de condiciones asociadas con la actividad de PCSK9 (Resumen).

En consecuencia, la reivindicación 3 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 4 y 7 no mencionan alguna característica que haga nueva al anticuerpo, por lo cual se considera que no son nuevas.

Nivel inventivo (*Art 18 D486*)

Grupo inventivo 4:

Este grupo inventivo hace referencia a un anticuerpo aislado que comprende una región de unión a PCSK9 que se solapa con un epitope que es reconocido por los anticuerpos monoclonales 5A10, 4A5, 6F6 y 7D4 que comprende una CDR1 de región variable de cadena pesada que tiene la secuencia de aminoácidos SEQ ID Nº59 u 8, un CDR2 de región variable de cadena pesada de secuencia SEQ ID Nº61 o 9 y/o una CDR3 de región variable de cadena pesada de secuencia SEQ ID Nº 10 o una variante de las mismas que tiene una o más sustituciones conservativas en las tres o alguna de sus CDRs y/o una CDR1 de región variable de cadena ligera que tiene la secuencia de aminoácidos SEQ ID Nº11, un CDR2 variable de cadena ligera de secuencia SEQ ID Nº12 y/o una CDR3 de región variable de cadena ligera de secuencia SEQ ID Nº 13 o una variante de las mismas que tiene una o más sustituciones conservativas en las tres o alguna de sus CDRs. Así como una línea celular que produzca de forma recombinante dicho anticuerpo.

En el estado del arte son conocidos los anticuerpos anti-PCSK9. Por ejemplo, el

documento D2, enseña diferentes anticuerpos que se unen específicamente a PCSK9 en diferentes epitopes y que han sido producidos por diferentes casas comerciales (Pág. 82 Tabla 1); y el documento D3, el cual presenta también anticuerpos anti-PCSK9 así como las líneas celulares para su producción (Rsumen).

La diferencia que existe entre los anticuerpos reclamados en la presente solicitud y los anticuerpos de las anterioridades, es que se trata de anticuerpos con una estructura diferente.

Dicha diferencia no involucra un efecto sorprendente o inesperado, puesto que los anticuerpos aquí divulgados tienen especificidad de unión a PCSK9, al igual que los anticuerpos divulgados en los documentos D1 y D2.

El problema técnico objetivo de la presente solicitud es, por lo tanto, la construcción de nuevos anticuerpos para PCSK9, alternativos a los ya existentes con mayor afinidad a PCSK9.

Sin embargo, el documento D3 ya presentaba anticuerpos con alta afinidad a PCSK9 con valores de K_D menores a 5nM (Pág. 45 Tabla 3), mejores a los obtenidos para los anticuerpos de la presente solicitud (Fl. 170 Tabla 4).

Por lo tanto, la falta de un efecto mejorado, en comparación a los anticuerpos ya existentes, no involucra nivel inventivo para la presente solicitud, más aún, cuando en el estado de la técnica son conocidos los procedimientos de ingeniería genética para la producción de nuevos anticuerpos a partir de modificación de sus secuencias.

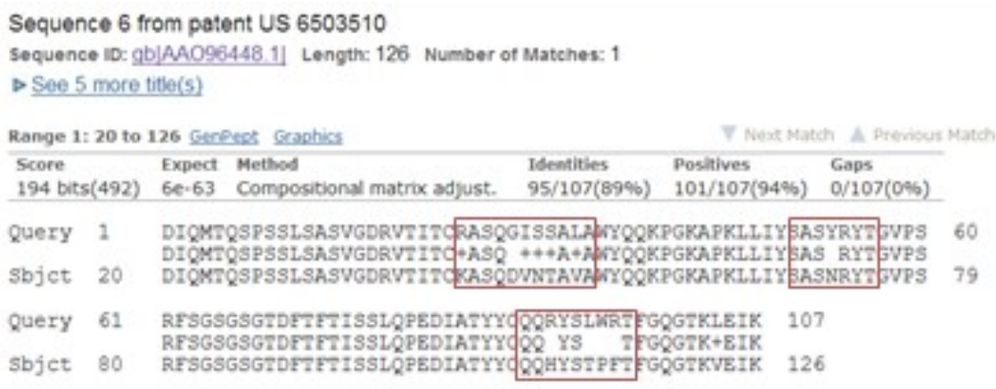
Por consiguiente, el grupo inventivo 4 carece de nivel inventivo y a su vez, las reivindicaciones 8, 9, 23 y 24.

Grupo inventivo 5:

La reivindicación 10 declara un anticuerpo aislado que comprende un CDR1 de región variable de cadena ligera que tiene la secuencia de aminoácidos SEQ ID Nº11, un CDR2 variable de cadena ligera de secuencia SEQ ID Nº12 y/o una CDR3 de región variable de cadena ligera de secuencia SEQ ID Nº 13 o una variante de las mismas que tiene una o más sustituciones conservativas en las tres o alguna de sus CDRs.

La reivindicación 11 reclama además una CDR1 de región variable de cadena pesada que tiene la secuencia de aminoácidos SEQ ID Nº59 u 8, un CDR2 de región variable de cadena pesada de secuencia SEQ ID Nº61 o 9 y/o una CDR3 de región variable de cadena pesada de secuencia SEQ ID Nº 10 o una variante de las mismas que tiene una o más sustituciones conservativas en las tres o alguna de sus CDRs.

El documento D4 divulga previamente una secuencia de aminoácidos (SEQ Nº 6) que codifica para un anticuerpo anti-HM1.24, útil en el tratamiento de tumores linfáticos (). Dicha secuencia se encontró 89% idéntica a la SEQ ID Nº 53 (región VL) de la presente solicitud, la cual contiene las CDRs reclamadas en la reivindicación 10 (ver figura 1).


Sequence 6 from patent US 6503510

Sequence ID: gb|AAO96448.1| Length: 126 Number of Matches: 1

See 5 more title(s)

Range 1: 20 to 126 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
194 bits(492)	6e-63	Compositional matrix adjust.	95/107(89%)	101/107(94%)	0/107(0%)

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Query  1    DIQMTQSPSSLSASVGDRVTITCRASQGISSALAWYQQKPGKAPKLLIYSASYRYTGVPS  60
            DIQMTQSPSSLSASVGDRVTITC+ASQ  +++A+AWYQQKPGKAPKLLIYSAS RYTGVPS
Sbjct  20   DIQMTQSPSSLSASVGDRVTITCKASQDVNTAVAWYQQKPGKAPKLLIYSASNRYTGVPS  79

Query  61   RFSGSGSGTDFTFTISSLQPEDIATYYCQQRYSLWRTFGQGTKLEIK  107
            RFSGSGSGTDFTFTISSLQPEDIATYYCQQ YS   TFGQGTK+EIK
Sbjct  80   RFSGSGSGTDFTFTISSLQPEDIATYYCQQHYSTPFTFGQGTKVEIK  126
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Figura 1. Alineamiento de proteínas (Blastp) que muestra porcentaje de identidad del 89% entre la SEQ Nº53 de la presente solicitud (Sbjct) y la SEQ Nº 6 del D4 (Query).

Por otro lado, el documento D5 divulga previamente una secuencia de aminoácidos (SEQ Nº 218) que codifica para un anticuerpo que se une al antígeno tardio 1, útil en el tratamiento de de la inflamación (Pág. 103). Dicha secuencia se encontró 83% idéntica a la SEQ ID Nº 54 (región VH) de la presente solicitud, la cual contiene las CDRs reclamadas en la reivindicación 11 (ver figura 2).

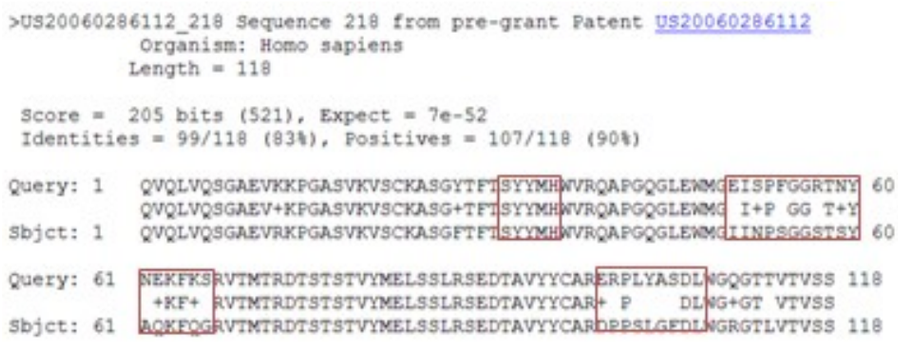

Figura 2. Alineamiento de proteínas (Blastp) elaborado en NCBI que muestra porcentaje de identidad del 83% entre la SEQ Nº54 de la presente solicitud (Sbjct) y la SEQ Nº 218 del D5 (Query).

Dado que las secuencias SEQ ID No. 53 y 54 enseñan las regiones variables de cadena liviana y pesada del anticuerpo reclamado en la presente solicitud, y tiene una identidad de secuencia superior al 80% con la materia previamente divulgada en los documentos D4 y D5, las sustituciones en las CDRs responsables de dicho 80% de identidad, podrían considerarse como las variantes reclamadas en las reivindicaciones 10 y 11, por lo tanto, la persona del oficio normalmente versada en la materia a partir de las cadenas livianas y pesadas de anticuerpo divulgado en las anterioridades lograría realizar el anticuerpo de la solicitud con dichas variantes o modificaciones.

Así, el anticuerpo reclamado en las revindicaciones 10 y 11 carece de nivel inventivo frente a las enseñanzas de los documentos D4 y D5 e incumplen el artículo 18 de la D486.

Las reivindicaciones dependientes 12 – 17 no aportan características inventivas por lo que también incumplen con el requisito del artículo 18.

En consecuencia, el grupo inventivo 5 carece de nivel inventivo.

9. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO 2008/057457	1 – 7 y 18 – 21	Novedad
D2	Grozdanov *et al.* 2006	8, 9, 23, 24	Nivel inventivo
D3	WO 2008/063382	8, 9, 23, 24	Nivel inventivo
D4	US 6503510	10-17 parcialmente	Nivel inventivo
D5	US 2006 0286112	11-17 parcialmente	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 de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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 SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-06-18

Elaboró: Edna Carolina Bonilla Leon
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Expression and localization of PCSK9 in rat hepatic cells

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Abstract: Proprotein convertase subtilisin/kexin type 9 (PCSK9), recently cloned in several laboratories, including ours, causes a third form of autosomal dominant hypercholesterolemia. Its mechanism of action remains unclear. We studied the expression and subcellular localization of PCSK9 in fetal and adult rat tissues associated with cholesterol homeostasis using quantitative reverse transcriptase - PCR, Western blot analysis, subcellular fractionation, and confocal immunofluorescent microscopy. PCSK9 mRNA is most abundant in yolk sac and fetal liver, but the highest expression of the protein was found in differentiated hepatoma FAO-1 cell line, which also shows the highest expression of LDLR. In FAO-1 cells PCSK9 expression is downregulated by cholesterol and 25-hydroxycholesterol and upregulated in the absence of sterols following the same pattern of expression as HMG-CoA reductase, synthase, and LDLR. Subcellular fractionation, combined with Western blotting, showed that PCSK9 is localized in the ER and intermediate vesicular compartment of the cell but not in Golgi cisternae. The mature enzyme is secreted from the liver and hepatoma cells. Double labeling with antibodies to PCSK9 and LDLR or clathrin revealed some colocalization of PCSK9 with clathrin-coated vesicles and LDLR. In conclusion, our results show that PCSK9 is processed in the ER, and the mature convertase is secreted in the plasma.

Key words: PCSK9 expression, PCSK9 localization, hepatic cells.

Résumé : La PCSK9, clonée récemment dans plusieurs laboratoires, incluant le nôtre, cause une troisième forme d'hypercholestérolémie autosomique dominante. Son mécanisme d'action demeure nébuleux. Nous avons étudié l'expression et la localisation sub-cellulaire de PCSK9 dans des tissus fœtaux et adultes associés à l'homéostasie du cholestérol, par RT-PCR quantitatif, immunobuvardage, fractionnement sub-cellulaire et immunofluorescence analysée en microscopie confocale. L'ARNm de PCSK9 était très abondant dans le sac vitellin et le foie fœtal, mais l'expression la plus forte de la protéine a été trouvée dans la lignée différenciée d'hépatome FAO-1, qui présente aussi les niveaux les plus élevés de LDLR. Dans les cellules FAO-1, l'expression de PCSK9 est régulée négativement par le cholestérol et le 25-hydroxycholestérol alors qu'elle est régulée positivement en absence de stérols, suivant ainsi le même patron d'expression que la HMG-CoA réductase, la HMG-CoA synthase et le LDLR. Un fractionnement sub-cellulaire, combiné à l'immunobuvardage a montré que PCSK9 est localisé dans le RE et les compartiments vésiculaires intermédiaires de la cellule, mais pas dans les citernes du Golgi. L'enzyme mature est secrétée par le foie et les cellules d'hépatome. Un double marquage avec des anticorps dirigés contre PCSK9 et le LDLR ou la clathrine a révélé une co-localisation de PCSK-9 avec les vésicules recouvertes de clathrines et le LDLR. En conclusion, nos résultats montrent que PCSK9 est transformé dans le RE et que la convertase mature est secrétée dans le plasma.

Mots clés : expression de PCSK9, localisation de PCSK9, cellules hépatiques.

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Introduction

Using suppression subtractive hybridization we previously identified cDNA clones overexpressed in 13 d fetal compared with adult liver. One of these clones (2G8) was found to encode a novel putative serine protease–subtilase (Petkov

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Abbreviations: ADH, autosomal dominant hypercholesterolemia; APOB, apolipoprotein B100; ED, embryonic day; ER, endoplasmic reticulum; ERGIC, ER/Golgi intermediate compartment; H89, isoquinolinesilfonamide; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HMGCS, 3-hydroxy-3-methylglutaryl-coenzyme A synthase; IDL, intermediate-density lipoprotein; IF, immunofluorescence; IP, immunoprecipitation; LDL, low-density lipoprotein; LDLR, LDL receptor; NARC-1, neuronal apoptosis related convertase1; 25-OH, 25-hydroxycholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; PVDF, polyvinylidene fluoride; RT, reverse transcriptase; SCAP, SREBP cleavage-activating protein; SREBP, sterol regulatory element binding protein; VLDL, very low-density lipoprotein.

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et al. 2000). The rat, human, and mouse cDNAs of this serine protease were cloned by Millennium Pharmaceuticals (Cambridge, Mass.) and by the Mammalian Gene Collection Team and given the name NARC-1 and PCSK9 (Chiang 2001; Strausberg at al. 2002). PCSK9/NARC-1 is a secretory K-like subtilase with a signal sequence for release from the ER (Seidah et al. 2003) with unknown function. The enzyme undergoes autocatalytic intramolecular processing, resulting in the cleavage of its prosegment and the generation of an active proteinase (Seidah et al. 2003; Naureckiene et al. 2003).

Mutations in PCSK9 were found to be the cause of a third form of ADH (Abifadel et al. 2003; Timms et al. 2004; Leren 2004; Shioji et al. 2004). Increased production of APOB, VLDL, IDL, and LDL was shown in human subjects carrying a mutation in PCSK9 (Ouguerram et al. 2004). Using Affymetrix microarrays, Horton and colleagues (2003) found that NARC-1/PCSK9 was upregulated in SREBP-1a and SREBP-2 transgenics and downregulated in SCAP-deficient mice. In a similar study PCSK9 was also identified among downregulated genes in the livers of mice subjected to a high cholesterol diet and upregulated in transgenic mice overexpressing truncated SREBP-1a and SREBP-2 (Maxwell et al. 2003).

It has been shown that the expression of PCSK9 mRNA was induced in cholesterol-depleted and statin-treated cells (Dubuc et al. 2004). Adenoviral-mediated overexpression of PCSK9 in mice caused an increase in plasma LDL-cholesterol and depletion of the LDL receptor from the liver, suggesting that PCSK9 regulates LDLR expression and the LDL-cholesterol level by a novel mechanism (Maxwell and Breslow 2004; Benjannet et al. 2004; Park et al. 2004). How is this regulation achieved? Is it through increased turnover of the receptor (Maxwell et al. 2005) or its retention in the ER (Park et al. 2004), through its degradation in the post-endoplasmic reticulum (Maxwell et al. 2005) or some other mechanism? Whether LDLR depletion is the only cause of elevated LDL cholesterol (Maxwell and Breslow 2004; Park et al. 2004) or whether PCSK9 also affects APOB production and lipoprotein secretion (Benjannet et al. 2004) remain open questions. Nothing is known about the function of PCSK9 in fetal liver or whether it is related only to cholesterol homeostasis.

To understand the function of PCSK9 in fetal rat liver, we searched for a cell line in which the expression of this gene is regulated. We found that the differentiated rat hepatoma FAO-1 cell line expresses PCSK9, and its expression is regulated by cholesterol. Using custom made antibodies against this enzyme and immunofluorescent techniques, we found that it resides in the ER and ER/Golgi intermediate compartments and that it is secreted in the rat plasma.

Material and methods

Animals, blood and serum collection

Pregnant female and normal male Sprague–Dawley rats were purchased from Taconic Farms (German Town, N.Y.). All studies with animals were conducted under the protocol approved by the Animal Care Institute of the Albert Einstein College of Medicine.

Antibodies

Rabbit polyclonal antibodies specific for rat PCSK9 were raised against 3 polypeptides: (*i*) close to the N-terminus (ATATFRRCSKGA; N-Ab), which detects only the proenzyme; (*ii*) internal peptide (CKALNAFGGEGVYAVAR; I-Ab); and (*iii*) close to the C-terminus (CRSRPSAKAS; C-Ab). I-Ab and C-Ab recognize both forms of the enzyme. The antibodies were produced and affinity-purified by Covance (Denver, Penn.). The primary antibodies used are listed in Table 1. Secondary antibodies for IF microscopy made in donkeys were purchased from Jackson Immunoresearch Laboratories (West Grove, Penn.) and used at a dilution of 1:100. Horseradish peroxidase (HRP)-conjugated anti-chicken IgG antibody was from Pierce Biotechnology Inc. (Rockford, Ill.) and used at a dilution of 1:50 000. All other secondary antibodies for Western blots were purchased from Amersham Biosciences (Piscataway, N.J.) and used at the same dilution.

Cloning of PCSK9

The clone 2G8, previously identified by us, comprised 240 bp (Petkov et al. 2000). The full length cDNA of this clone was obtained from fetal liver RNA using GeneRacer Kit (Invitrogen Corp., Carlsbad, Calif.), primers specific for the original clone, and RNA oligonucleotides (oligos) included in the GeneRacer Kit, following the procedure recommended by the supplier. For the transfection experiments Pcsk9 cDNA was cloned into pcDNA3.1 B (Invitrogen) as a fusion protein with c-myc tag and the obtained construct named pcDNA/Pcsk9/c-myc.

Cells, cell lines, and maintenance of the cells

Fetal hepatoblasts were isolated from ED14–ED17 fetuses and cultured as described previously (Dabeva et al. 2000; Oertel et al. 2003).

Human hepatoma HepG2 (HB-8065), rat hepatoma Reuber H-35 (CRL-1600), and SV-40 transformed simian fibroblasts COS-7 (CRL-1651) were purchased from the American Type Culture Collection (Manassas, Va.). The differentiated hepatoma cell line FAO-1 was described previously (Deschatrette and Weiss 1974). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Invitrogen Corp., Grand Island, N.Y.) containing 10% fetal calf serum (FCS), 100 U penicillin/mL, and 100 µg streptomycin/mL.

For experiments with cholesterol, FAO-1 and HepG2 cells were plated in 6-well dishes at a concentration of 2.5–3.0 × 10^5 cells per well in DMEM medium supplemented with 10% FCS. Twenty four h later the cells were washed, 2 of the wells received regular medium (control), and the other 4 received DMEM supplemented with 10% lipoprotein-deficient bovine calf serum (Biomedical Technologies Inc., Stoughton, Mass.). Loading of the cells (2 wells) with cholesterol was done 24 h later by adding 50 µg of cholesterol/mL and 5 µg of 25-OH/mL (both from Sigma-Aldrich, St. Louis, Mo.) in 0.05% ethanol (final concentration). After 24 h RNA and protein were isolated as described below.

For brefeldin A and H89 (Sigma-Aldrich) treatment FAO-1 cells were grown on cover slips and treated with 10 µg of brefeldin A/mL for 40 min alone or with 10 µg of brefeldin A/mL followed by 10 min of incubation with 100 µmol of H89/L or with H89 alone for 30 min.

Table 1. Antibodies used in this study.

Antibody (clone name)	Antigen (species)	Working dilution		Company	Isotype	Catalog No.
		Western blot	IF			
c-myc (clone 9B11)	410-419* (human)	1:1000	—	Stressgene (San Diego, Calif.)	Mouse IgG2a	2276
LDL receptor	29-205* (human)	1:5000	1:250	Abcam (Cambridge, Mass.)	Chicken IgY	ab14056
Clathrin heavy chain (clone 23)	4-171* (rat)	1:1000	1:250	BD biosciences (San Diego, Calif.)	Mouse IgG1	610499
Rab4 (clone 7)	97-213* (human)	1:1000	—	BD biosciences (San Diego, Calif.)	Mouse IgG1	610888
Rab7 (clone H-50)	158-207* (human)	1:1000	—	Santa Cruz (Santa Cruz, Calif.)	Rabbit IgG	sc-10767
Protein disulfite isomerase	499-509* (rat)	1:1000	1:200	Sterssgen (San Diego, Calif.)	Mouse IgG1	SPA-891
Golgi/58K (clone 9)	whole protein (rat)	1:1000	1:50	Sigma-Aldrich (St. Louis, Mo.)	Mouse IgG1	G 2404
GM130 (clone 35)	869-962* (rat)	1:250	—	BD biosciences (San Diego, Calif.)	Mouse IgG1	610823
COPII	1-18* (rat)	1:1000	1:200	Affinity BioReagents (Golden, Colo.)	Rabbit IgG	PA1-069
Alpha-fetoprotein	whole protein (rat)	—	1:200	Gift from Dr. S. Sell	Goat IgG	—

*Amino acids.

Isolation of RNA, RT-PCR, and quantitative RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen). RT reaction was carried out with Super Script II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Rat-specific primers were chosen with the Primer3 program (Rozen and Skaletsky 2000). National Centre for Biotechnology Information gene accession numbers, primer sequences, and amplicon sizes are given in Table 2.

Real-time PCR reaction was carried out with SYBR Green PCR Master Mix on ABI Prism sequence detection system 7000 (Applied Biosystems, Warrington, UK). The relative ratio and standard deviation were calculated using comparative C_T method ($\Delta\Delta C_T$ value) as recommended by the manufacturer. The data were normalized to the expression level of glyceraldehydes-3 phosphate dehydrogenase.

Protein isolation, immunoprecipitation and Western blots

Cells were lysed with 1 mL of IP lysis buffer: in mmol/L: 20 Hepes (pH 7.4), 2 EGTA, 2 EDTA, 150 NaCl, 1% Triton X-100, 5% glycerol supplemented with protease inhibitors Complete Mini (Roche Diagnostics, Branchburg, N.J.), and 1 phenylmethanesulfonyl fluoride (PMSF) (Sigma). Cell lysates were centrifuged at 10 000*g* for 10 min, and the amount of protein was measured with a bicinchoninic acid kit (Pierce).

IP experiments were performed with Seize Primary Mammalian Immunoprecipitation Kit (Pierce). The antibodies to Pcsk9 and c-myc were immobilized on the beads and processed according to the supplier's manual. The immunoprecipitated protein was washed and boiled in loading buffer (Bio-Rad, Hercules, Calif.) with 5% 2-mercaptoethanol (Sigma) before loading.

For determination of PCSK9 in the plasma, blood was drawn in the presence of EDTA, PMSF was added to a final concentration of 1 mmol/L, and the blood was centrifuged at low speed for 30 min at 4 °C. Plasma was filtered through a 45 µm filter, diluted 10 fold with 0.15 mol NaCl·L^{-1} / 50 mmol Tris–HCl·L^{-1}, pH 7.4 / 1% Triton X-100 and subjected to immunoprecipitation overnight. Commercial available rat serum from Sigma (cat. No. R9759) was also used as a control.

For Western blotting 20–50 µg of total cell protein was resolved in 7.5% or 12% SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The membranes were blocked with 5% nonfat milk and incubated consecutively with primary and secondary antibodies, and the signal from HRP was developed with Super Signal West Femto Maximum Sensitive Substrate (Pierce). The chemiluminescence was visualized on Kodak X-Omat or Fuji Super RX films (Fisher Scientific, Pittsburgh, Penn.).

Immunofluorescent microscopy

FAO-1 cells or hepatoblasts, grown on cover slips, were fixed in either acetone or 4% paraformaldehyde for 10 min at 4 °C, permeabilized with 0.1% Triton X-100, treated with sodium borohydrate, and blocked with 5% donkey serum / 1% BSA. The primary antibodies were applied for 1 h at room temperature and the secondary, fluorescent conjugated antibodies for 40 min. Pictures were taken with a Leica AOBS confocal microscope or Olympus IX70 inverted microscope.

Separation of the subcellular fractions and cell surface protein biotinylation

Different cellular fractions were obtained by linear sucrose gradient as described elsewhere (Bostrom et al. 1986). Briefly, eight 75 mm flasks with 80%–90% confluent FAO-1 cells were washed, scraped in PBS, washed in 0.25 mol sucrose·L^{-1} / 3 mmol imidazol·L^{-1}, pH 7.4, and broken in a Dounce homogenizer at 0 °C. The postnuclear supernatant obtained after 15 min of centrifugation at 1300*g* was adjusted to 520 µL with 0.25 mol sucrose·L^{-1} / 3 mmol imidazol·L^{-1}, pH 7.4, and layered on top of a 3 mL sucrose gradient (22%–50% *w/w*) / 3 mmol imidazol·L^{-1}, pH 7.4, over a 0.48 mL cushion of 50% (*w/w*) sucrose. After centrifugation for 15 h at 33 400 rpm at 4 °C in a Beckman SW 60 rotor, 28 fractions of 140 µL each were collected from the bottom of the tubes. The fractions were supplemented with sample buffer and 5% 2-mercapto ethanol, and boiled before loading.

Cell surface proteins were biotinylated with EZ-link sulfo-

Table 2. Sequence of primers used in real-time RT-PCR with SYBR green.

mRNA	Accession No.	Forward primer (5′→3′)	Reverse Primer (5′→3′)	Amplicon size (bp)
PCSK9	AY847775	gcactggagaaccacacagg	tggctgcatgacattgcttctc	101
SREBF2	XM_216989	cacctgtggagcagtctcaa	tgccagagtgttgtcctcag	104
LDLR	NM_175762	cctcagctaccagcacctct	agccatgtcaccttggactt	97
HMGCS1	NM_017268	tcgcaagtatcgagagttctgt	gcctggcaatggactctg	92
HMGCR	NM_013134	agttgcgcctcttgtgactt	ccagcatcaacactcaatgc	93
GAPDH	NM_017008	ggcattgctctcaatgacaa	atgtaggccatgaggtccac	94

NHS-biotin reagents (Pierce). Briefly, 80%–90% confluent FAO-1 cells were washed and incubated with 10 mmol EZ-link sulfo-NHS-biotin reagents/L for 30 min at 4 °C. After completion of the reaction, cells were washed with PBS (pH 7.0) / 100 mmol glycine·L^{-1}. Biotinylated proteins were recovered with avidin-linked agarose beads (Pierce). One-third to one-half of the biotinylated proteins and 50 µg of nonbiotinylated proteins were fractionated on SDS-PAGE.

Results

Rat PCSK9 cDNA

The full-length rat PCSK9 cDNA was cloned as described in Materials and methods, sequenced, and deposited in GenBank under accession number AY847775 (protein accession No. AAW31850). PCSK9 cDNA is 3350 nucleotides in length and codes for a protein of 691 amino acids. Two nucleotide substitutions between our clone and the previous sequence deposited in GenBank (AX207690 and NM_199253) were identified: at position 69 T instead of A and at position 71 T instead of C, which results in a protein that is 55 amino acids shorter than that given in AX207690 and NM_199253. The correctness of these nucleotides was confirmed by sequencing genomic DNA from Sprague–Dawley rats. The N-terminal sequence of the protein reported by us (deduced from nucleotide sequence) is consistent with the beginning of human and mouse PCSK9. Rat PCSK9 has the same length as the mouse protein.

Expression of PCSK9 mRNA in rat hepatic cells, fetal liver, and yolk sac

The expression of PCSK9 mRNA was studied in several tissues that maintain cholesterol homeostasis during embryogenesis and in adult rats: fetal and adult liver, yolk sac, and intestine. We also analyzed its expression level in the differentiated rat hepatoma cell line FAO-1, which could be a good in vitro model for studying PCSK9 function. The highest expression of PCSK9 mRNA was observed in fetal liver and yolk sac, and the lowest in the intestine (Fig. 1). The highest expression of APOB was also found in the yolk sac, the principal producer of LDL during early and mid-fetal life (Terasawa et al. 1999; Farese et al. 1996). In contrast, ApoB mRNA expression in the fetal liver was barely detectable compared with that of the other 2 tissues: adult liver and intestine. The same reverse pattern of expression was found for SREBP-2, the cholesterol-dependent activator of transcription of PCSK9. The abundance of LDLR mRNA in adult liver and yolk sac was much higher than that in fetal liver, indicating high uptake of exogenous cholesterol by these organs. These data suggest that high expression level of PCSK9 in fetal liver does not correlate with high production of LDL or LDLR, and that the expression in fetal liver may not be dependent merely on SREBP-2.

Fig. 1. Expression of PCSK9 mRNA in rat fetal and adult tissues. Quantitative RT-PCR for PCSK9, APOB, LDLR, and SREBP-2 mRNAs in ED 14 fetal liver (Fetal), adult liver (Adult), FAO-1 cell line (FAO-1), ileum, and yolk sac. mRNA levels were normalized to GAPDH mRNA. mRNA abundance in fetal liver is taken as 1.

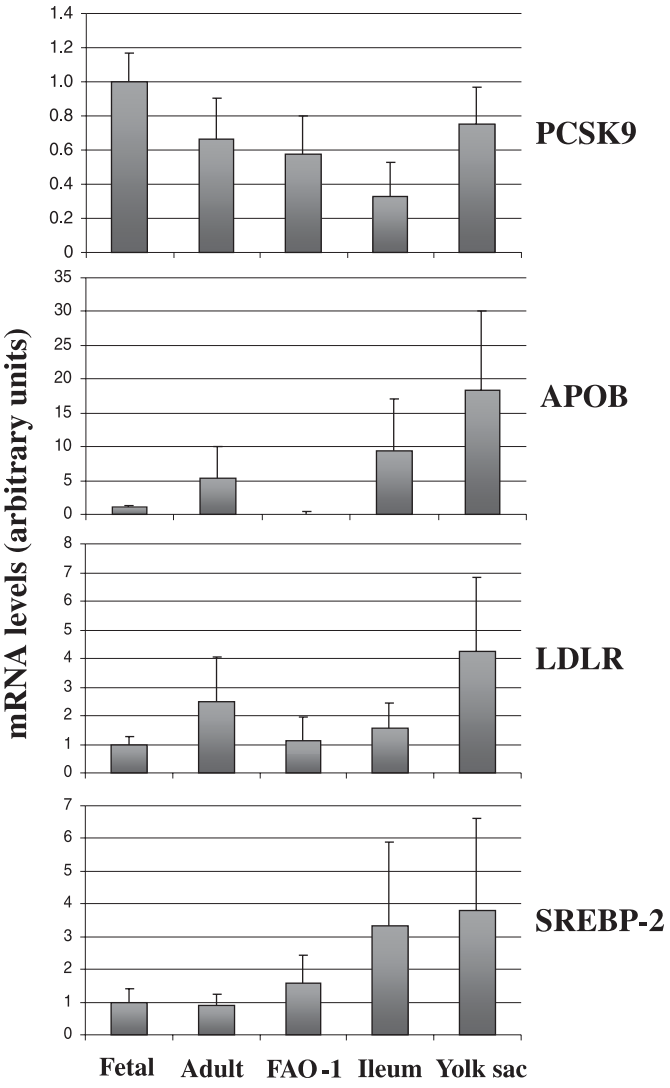
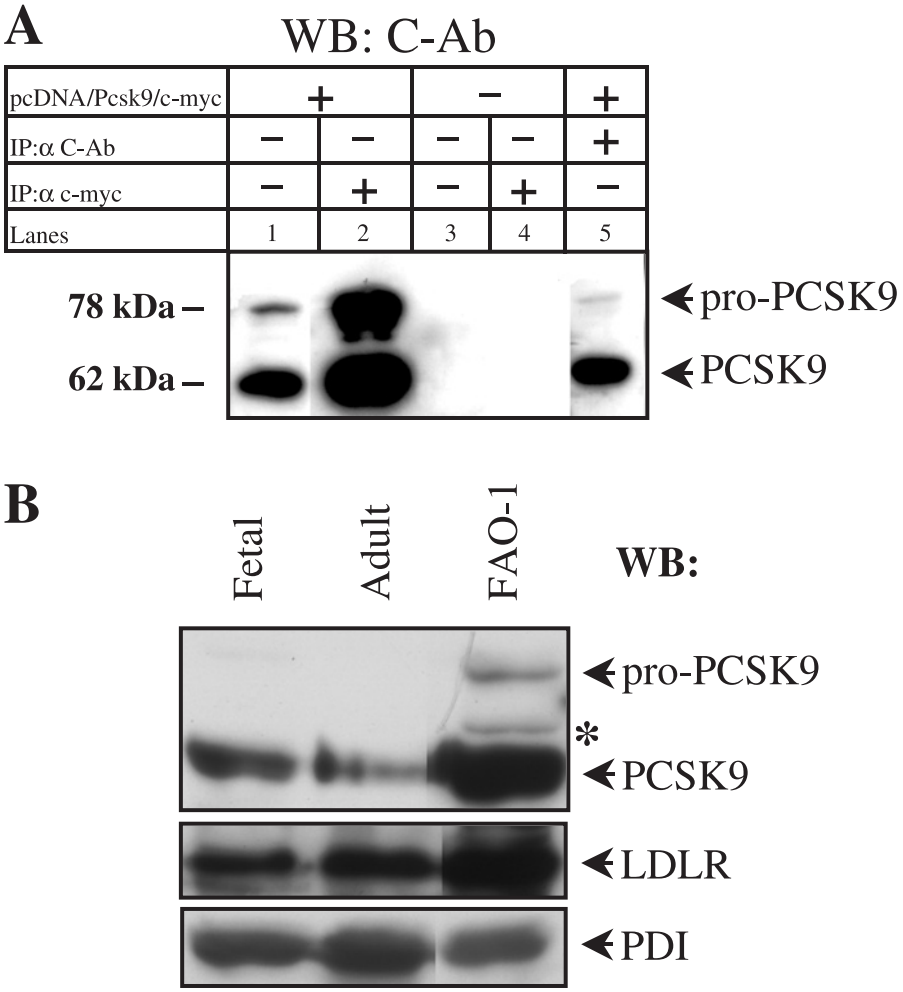


Fig. 2. (A) Specificity of PCSK9 C-Ab. Cos-7 cells were transfected with pcDNA (lanes 3 and 4) or with rat pcDNA/Pcsk9/c-myc (lanes 1, 2 and 5). Total cell lysate (lanes 1 and 3) and IP were carried out with C-Ab (lane 5) or c-myc antibodies (lanes 2 and 4), fractionated on SDS-PAGE, and blotted as described in Materials and methods. The PVDF membrane was probed with C-Ab antibodies. Molecular mass of processed (PCSK9) and unprocessed PCSK9 (pro-PCSK9) is given in kDa on the left. (B) Expression of PCSK9 in fetal, adult liver, and differentiated hepatoma FAO-1 cell line. Total cell lysates of fetal, adult liver, and FAO-1 cell line were fractionated on SDS/PAGE and blotted on PVDF membrane, and the convertase (PCSK9), LDLR, and PDI (protein disulfide isomerase) were detected with C-Ab, chicken anti-LDLR antibody, and rabbit anti-PDI, respectively. PDI is used as loading control. *, unspecific band recognized by anti-PCSK9 (C-Ab) antibodies.

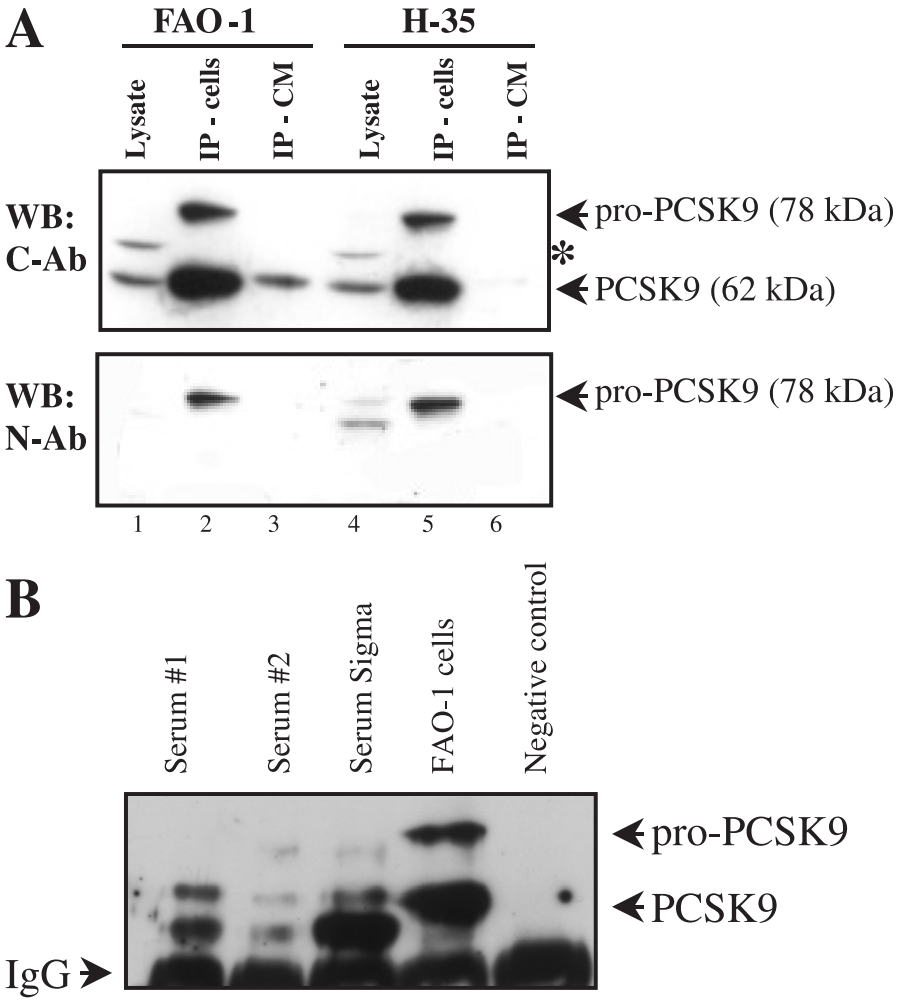


Immunochemical detection of rat PCSK9

To study the expression of PCSK9 in liver cells, we prepared custom antibodies as described in Materials and methods. These antibodies were tested on Western blots from total cell lysates or after IP and in IF experiments. In order to assess their specificity, we transiently transfected COS-7 cells with rat PCSK9 cDNA clone, tagged with the c-myc epitope (pcDNA/PCSK9/c-myc). Whole cell lysate or immunoprecipitated lysate with anti-c-myc antibody proteins from mock transfected cells did not react with C-Ab (Fig. 2A, lanes 3 and 4), showing that this antibody is specific for rat PCSK9. Total protein lysates and IP proteins with anti-c-myc from COS-7 cells transfected with pcDNA/PCSK9/c-myc reacted with the C-Ab. A light band of approximately 78 kDa and a strong band, corresponding to 62 kDa, revealed the 2 forms of rat PCSK9: the proconvertase (pro-PCSK9) and mature convertase (PCSK9) (Fig. 2A lanes 1 and 2). When IP was carried out with C-Ab, the Western blot of precipitated proteins displayed the same 2 forms of PCSK9 (Fig. 2A, lane 5). Re-probing the membrane with N-Ab revealed only the upper light band with molecular mass of 78 kDa (data not shown). These results proved the specificity of our custom-made antibodies.

The abundance of rat PCSK9 was determined in fetal and adult rat liver and FAO-1 cells, which expressed high levels of PCSK9 mRNA. Western blots of total cell lysates from fetal liver revealed a high level of processed convertase and a very small amount of proconvertase. In adult liver, expression of PCSK9 compared with fetal liver was low, and the proconvertase was not detectable. On the other hand, the expression of pro- and processed convertases was very high in FAO-1 cells (Fig. 2B). In contrast, the expression of LDLR in fetal liver was much lower than in adult liver; highest expression was observed in FAO-1 cells (Fig. 2B). The sub-

Fig. 3. (A) Expression of PCSK9 in FAO-1 and Reuber H-35 cell lines. Total cell lysate (lysate), and immunoprecipitated protein with C-Ab from total cell lysate (IP-cells) and from the medium (IP-M) were fractionated and blotted to PVDF membrane. The same membrane was consequently probed with C-Ab (WB: C-Ab), stripped, and then re-probed with N-Ab (WB: N-Ab). *, nonspecific band recognized by anti-PCSK9 (C-Ab) antibodies. (B) Immunoprecipitation of serum. A half millilitre of serum from 2 different animals (Serum nos. 1 and 2) and regular serum from Sigma (Serum Sigma) was immunoprecipitated as described in Materials and methods. Cell lysate from FAO-1 cells (FAO-1) was used as a positive control. Membrane was probed consequently with C-Ab and I-Ab. Western blot with I-Ab is shown. Pro-PCSK9 (pro-PCSK9), mature forms of the PCSK9 (PCSK9), and IgG heavy chain (IgG) are designated.



stantial difference between mRNA and protein expression in the 3 different hepatic tissues implies that PCSK9 could be post-translationally regulated.

Secretion of PCSK9 from hepatic cells

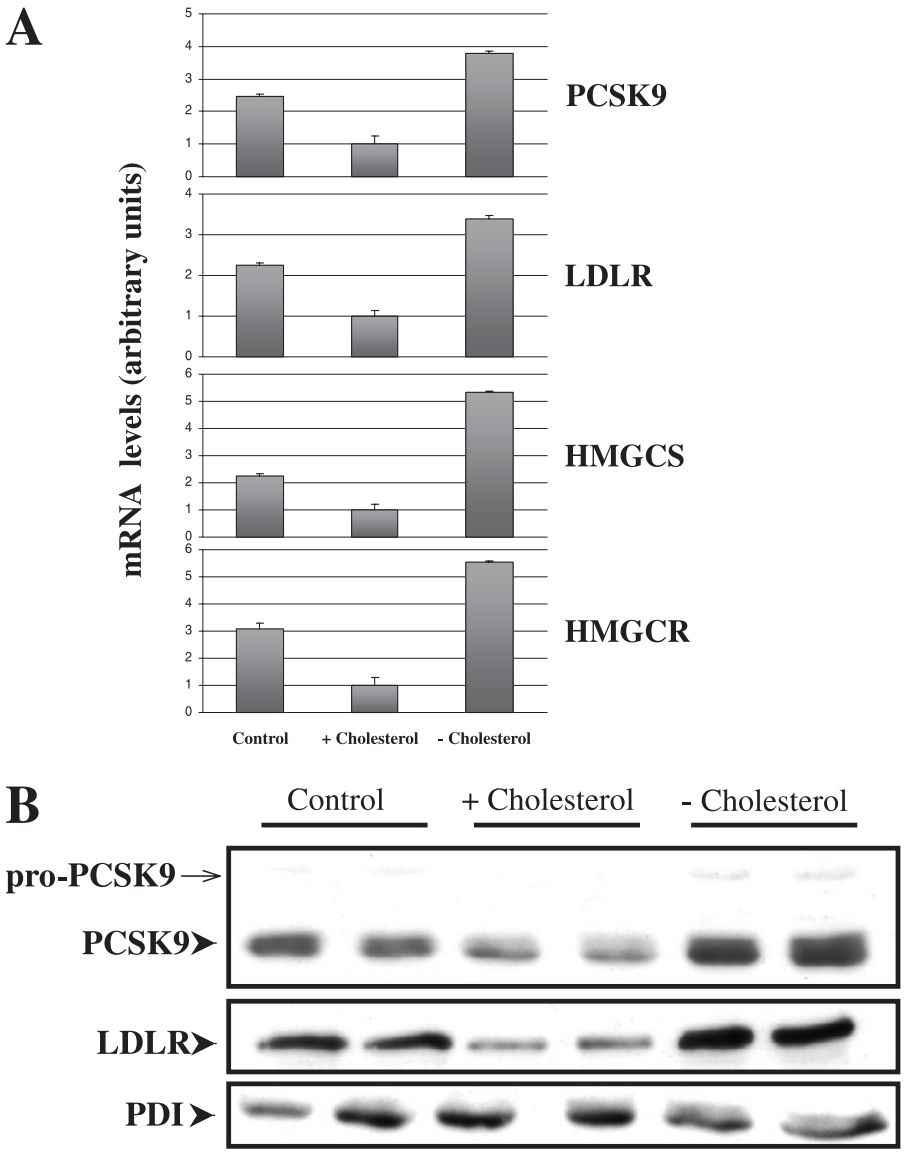
It was reported previously that PCSK9 is secreted from HepG2 cells (Seidah et al. 2003; Naureckiene et al. 2003; Benjannet et al. 2004). To determine whether secretion of PCSK9 from hepatic cells is of physiological importance we tested 2 cell lines: Reuber H-35 and FAO-1 cells. Secreted PCSK9 was detected in the medium after IP with C-Ab. In the control lanes (2 and 5, Fig. 3A) after IP with C-Ab and visualization with either C-Ab or N-Ab, 2 bands corresponding to the proenzyme and mature enzyme were detected. The amount of secreted protein from FAO-1 cells was clearly detectable, while that secreted from Reuber H-35 was negligible (Fig. 3A, upper panel). Immunodetection with N-Ab did not produce a band corresponding to the proconvertase (Fig. 3A, lower panel). These results show that the secretion of PCSK9 from different hepatoma cell lines differs and that only the mature convertase is secreted from the cells.

IP of PCSK9 from normal rat plasma and serum revealed that its secretion from the liver is a normal process (Fig. 3B). These results were obtained with the plasma from several animals and also with normal rat serum (Sigma). Persistently, only the mature form of PCSK9 was found when a third antibody against the mature form of the PCSK9 (I-Ab) was used (Fig. 3B).

Regulation of PCSK9 expression by cholesterol

To prove directly that the expression of PCSK9 is dependent on cellular cholesterol, FAO-1 cells and HepG2 cells (not shown) were grown in 3 different media: cholesterol depleted, cholesterol/25-OH loaded, and control medium. In both cells lines, cholesterol depletion caused significant upregulation of mRNA for PCSK9 and also for HMGCR, HMGCS, and LDLR. Conversely, loading the cells with cholesterol/25-OH led to a significant reduction of mRNA abundance for all 3

Fig. 4. Regulation of PCSK9 expression in FAO-1 cell line by cholesterol. (A) Quantitation of mRNA was performed with Q-RT/PCR as described in Materials and methods, in cholesterol supplemented (+cholesterol), cholesterol depleted (–cholesterol), and untreated (control) FAO-1 cells. mRNA level of PCSK9, LDLR, HMGCS, and HMGCR are given in arbitrary units. (B) Changes in PCSK9 protein and LDLR in untreated (control), cholesterol supplemented (+cholesterol), and cholesterol depleted (–cholesterol) FAO-1 cells. Protein disulfide isomerase (PDI) is used as loading control. The results from 2 independent experiments are presented.



enzymes and LDLR (Fig. 4A). The changes in PCSK9 and LDLR expression followed the changes in mRNA expression for these proteins (Fig. 4B). These data demonstrate directly that PCSK9 is involved in maintenance of cellular cholesterol homeostasis and that FAO-1 cells are a good rat hepatoma model for studying regulation of PCSK9 expression.

Localization of PCSK9 revealed by IF

The cellular distribution of PCSK9 in FAO-1 cells was analyzed by double IF confocal microscopy (see Materials and methods). PCSK9 was detected with C-Ab or N-Ab. Cis-Golgi cisternae were revealed with monoclonal antibody against p58K protein. The results presented in Fig. 5A show a narrow distribution of the proconvertase (N-Ab), delineating the nucleus, reminiscent with its ER localization and more diffuse vesicular distribution of the mature form (C-Ab). This suggests that PCSK9 may be also located in transport vesicles budding from the ER. Very light staining for mature PCSK9 was detected in cis-Golgi, which could be attributed to background staining; the proprotein was not detected (Fig. 5A).

These data strongly suggest that (*i*) the proconvertase is in the ER, where it is processed and (*ii*) mature PCSK9 is not localized in the Golgi but, rather, in the ER and possibly in transport vesicles. In primary culture of ED 14 hepatoblasts, alpha-fetoprotein was abundant in the Golgi cisternae, but PCSK9 was again absent from Golgi (Fig. 5B). Further experiments on PCSK9 subcellular distribution were performed using anti-clathrin heavy chain and anti-LDLR antibodies in colabeling experiments. We could expect colocalization of

clathrin and PCSK9 if the enzyme was secreted and taken by endocytosis in early endosomes (Fig. 5C). Double labeling experiments revealed that some of the convertase was colocalized with clathrin. Double immunolabeling of PCSK9 and LDLR also showed some vesicular colocalization of the 2 proteins. The nature of these vesicles will be the subject of future analyses.

Subcellular localization of PCSK9 revealed by gradient centrifugation

To determine whether PCSK9 is localized only in the ER or is also present in transport vesicles in the ERGIC of the cells, we performed subcellular fractionation of FAO-1 cells using discontinued sucrose gradients (see Materials and methods). The individual fractions were subjected to Western blot analysis with antibodies to PCSK9 and antibodies specific for cell organelles (Fig. 6A). Although PCSK9 showed a broad distribution throughout the gradient, it was not present in the preparation of pure nuclei or in the cytosol (data not shown). The proconvertase was present only in fraction 10–15 corresponding to the rough ER (Bostrom et al. 1986), revealing the site of its synthesis and processing. Electron microscopy analysis of these fractions revealed that they are composed exclusively of rough ER (data not shown). Most of the mature form was also present in this compartment (fraction 10–15). The rest of the convertase was distributed in fraction 16 through 21. It should be noted that the fractions containing the largest amount of PCSK9 were also rich in coatomer protein complex II (COPII) vesicles (fraction 11–14 and 17–23), showing that PCSK9 is also localized in the ERGIC. The light PCSK9 fractions (fraction 20–21) also overlapped with LDLR and Rab4 and partially with clathrin, suggesting that there could be small amounts of PCSK9 in early endosomes. In accordance with the immunofluorescent study above, PCSK9 did not cosediment with the Golgi apparatus (GM130 or p58K) or with late endosomes (Rab7).

To determine whether some of the convertase is localized on the plasma membrane, we labeled cell surface proteins with biotin and isolated the biotin-labeled fraction with avidin beads (see Materials and methods). As seen in Fig. 6B, a small amount of mature PCSK9 could be detected on the plasma membrane of FAO-1 cells. It has to be noted, however, that this amount is very small compared with that of total cell lysates. As could be expected, the glycosylated form of LDLR was also present on the cell membrane (Fig. 6B).

Localization of PCSK9 after treatment with brefeldin A and H89

To obtain independent proof that PCSK9 is localized in ERGIC, we treated FAO-1 cells with brefeldin A and then incubated them with H89 or H89 alone. PCSK9 was revealed with C-Ab, cis-Golgi with p58K antibody, and COPII with antibody produced in rabbit (data not shown). Brefeldin A is known to inhibit ARF1-dependent coat protein binding to Golgi membranes (Donaldson et al. 1992; Jackson and Casanova 2000), preventing formation and transport of COPI and clathrin-coated vesicles, which results in fusion of the cisternae with ER (Scales et al. 2000; Kreis et al. 1995). While COPII vesicles are not initially affected, addition of a second drug, isoquinoline H89, which inhibits the small GTPase Sar1, prevents COPII polymerization and blocks ER to Golgi vesicle transport, causing immediate collapse of Golgi (Aridor and Balch 2000; Lee and Linstedt 2000). When brefeldin A was added to FAO-1 cells, there were no noticeable differences in the distribution of PCSK9 between treated and untreated cells (Fig. 7). Brefeldin A treatment followed by 10 min of incubation with H89 caused collapse of Golgi body and reduction of PCSK9 (Fig. 7). The same changes occurred when the cells were treated for 30 min with H89 alone: the label for PCSK9 was confined to the ER (Fig. 7). These data clearly showed that PCSK9 is localized in the ERGIC of the cells.

Discussion

This work describes our initial studies on a novel convertase, PCSK9, and provides evidence regarding its subcellular localization and secretion from hepatic tissues and cells. We have shown that PCSK9 is not only expressed in fetal and adult rat liver but is also highly expressed in FAO-1 cells and in the yolk sac of rat fetuses. While these studies were in progress, Dubuc and colleagues (2004) reported that PCSK9 mRNA expression in HepG2 cells is regulated by cholesterol. Our work extends this conclusion for the differentiated rat hepatoma cell line FAO-1, for which we showed that PCSK9 together with other cholesterol dependent enzymes, HMGCR, HMGCS, and LDLR, is downregulated upon addition of cholesterol and upregulated in cholesterol-depleted medium. We conclude that this differentiated hepatoma cell line is a good model for studying endogenous PCSK9 expression and PCSK9-dependent cholesterol homeostasis.

Our results show further that there is a reverse relation between PCSK9 and LDLR expression in adult and fetal liver, which suggests that the high level of convertase in fetal liver could maintain the low level of LDLR (Fig. 2B). However, such a relation could not be observed for FAO-1 cells, in which both proteins, PCSK9 and LDLR, are highly expressed, indicating that this convertase may have additional function in rapidly growing cells.

It is noteworthy that organs with high production of APOB highly express PCSK9. In early and mid-gestation in mice the yolk sac is a major producer of APOB, VLDL, and LDL; towards the end of fetal life, the liver gradually becomes the principal organ of lipoprotein synthesis and secretion (Terasawa et al. 1999; Farese et al.1996). Here we showed that PCSK9 is also highly expressed in the yolk sac. In adult animals, the highest expression of PCSK9 is found in the liver and then in the intestine (Seidah et al. 2003) (Fig. 1). These results suggest that there is a direct link between PCSK9 expression and APOB production. Such an assumption does not disagree with the data of other investigators showing that overexpression of PCSK9 causes downregulation of LDLR in mouse liver and HepG2 cells (Maxwell and Breslow 2004; Benjannet et al. 2004; Park et al. 2004; Maxwell et al. 2005). It has been reported that LDLR mediates APOB degradation in the presecretory pathway (Twisk et al. 2000; Gillian-Daniel et al. 2002; Larsson et al. 2004). We can hypothesize that PCSK9 (or its putative substrate) is the factor that triggers release of APOB from LDLR and subsequent degradation of the receptor. If this were the case, in the $LDLR^{-/-}$ mouse overproduction of PCSK9 would not result in increased secretion of LDL,

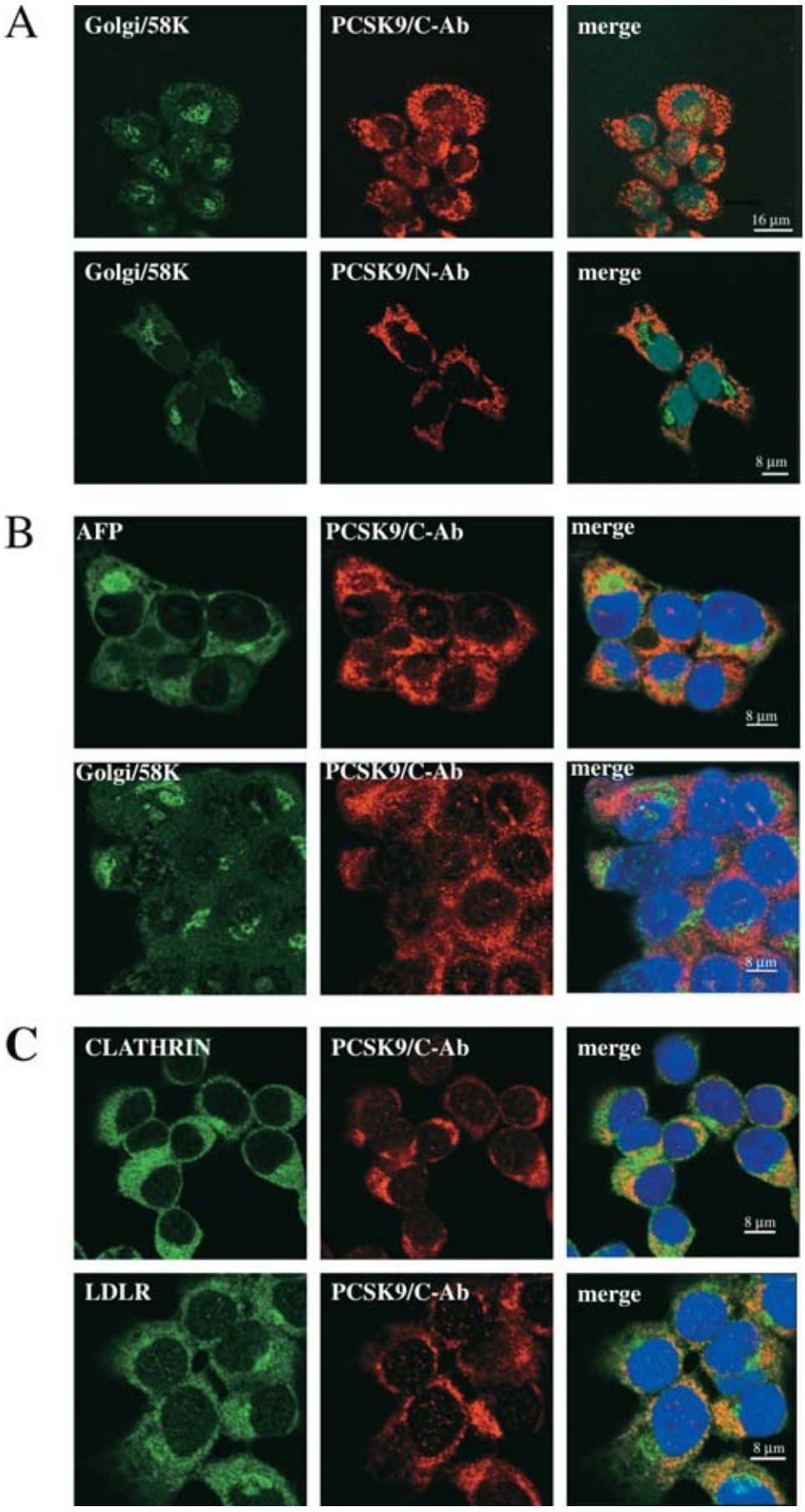


Fig. 5. Confocal immunofluorescence localization of PCSK9 in FAO-1 cells and rat hepatoblasts. (A) FAO-1 cells were grown on cover slips, acetone fixed, and incubated with antibodies against PCSK9 (C-Ab or N-Ab) and anti-Golgi/58K, followed by anti-rabbit Cy3- (red) and anti-mouse Cy2 (green). Nuclei were contrastained with DAPI. Bars: 16 and 8 μm, respectively. (B) Hepatoblasts were isolated as described in Materials and methods, grown on cover slips, acetone fixed, and incubated with antibodies against PCSK9 (C-Ab) and anti-α-fetoprotein or anti-Golgi/58K, followed by anti-rabbit Cy3 (red) and anti-goat FITS (green) or anti-rabbit Cy3 (red) and anti-mouse Cy2 (green), respectively. Nuclei were contrastained with DAPI. Bar = 8 μm. (C) FAO-1 cells were acetone fixed and incubated with antibodies against PCSK9 (C-Ab) and anti-clathrin heavy chain or anti-LDLR antibody followed by anti-rabbit Cy3 (red) and anti-mouse Cy2 (green) or anti-chicken FITS (green), respectively. Nuclei were contrastained with DAPI. Bar = 8 μm.

Fig. 6. (A) Subcellular localization of PCSK9 in FAO-1 cells. Subcellular fractions of FAO-1 cells were obtained by ultracentrifugation as described in Materials and methods. The membranes with blotted protein were probed with different antibodies as shown. PNS, post nuclear supernatant; Pro-PCSK9 (pro-PCSK9), mature forms of the PCSK9 (PCSK9). Longer exposure of the heavy fractions, which reveals the proconvertase, is also given. Markers: Golgi, 58K and GM130; early endosomes, Rab 4; late endosomes, Rab 7; ERGIC, Ub-COPII (ubiquitinated COPII) molecules; clathrin-coated vesicles. The figure is representative of 3 independent fractionations. (B) Cell surface localization of PCSK9. Cell surface proteins were biotinylated as described in Materials and methods. Western blots were carried out with antibodies against PCSK9 (C-Ab) and anti LDLR. The membrane was consequently probed with the 2 antibodies. *, denotes unspecific band recognized by anti-PCSK9 (C-Ab) antibody (in both A and B).

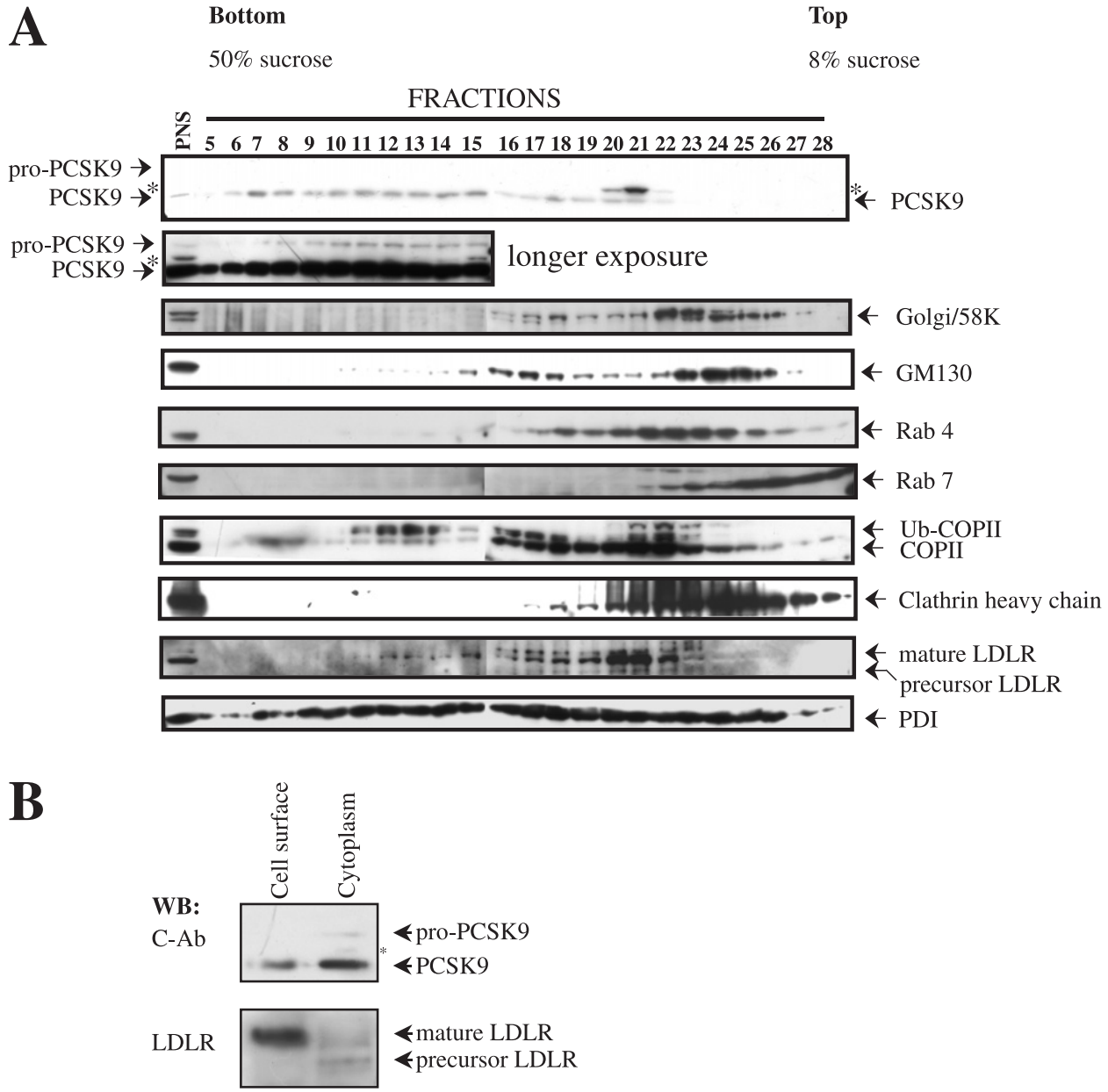
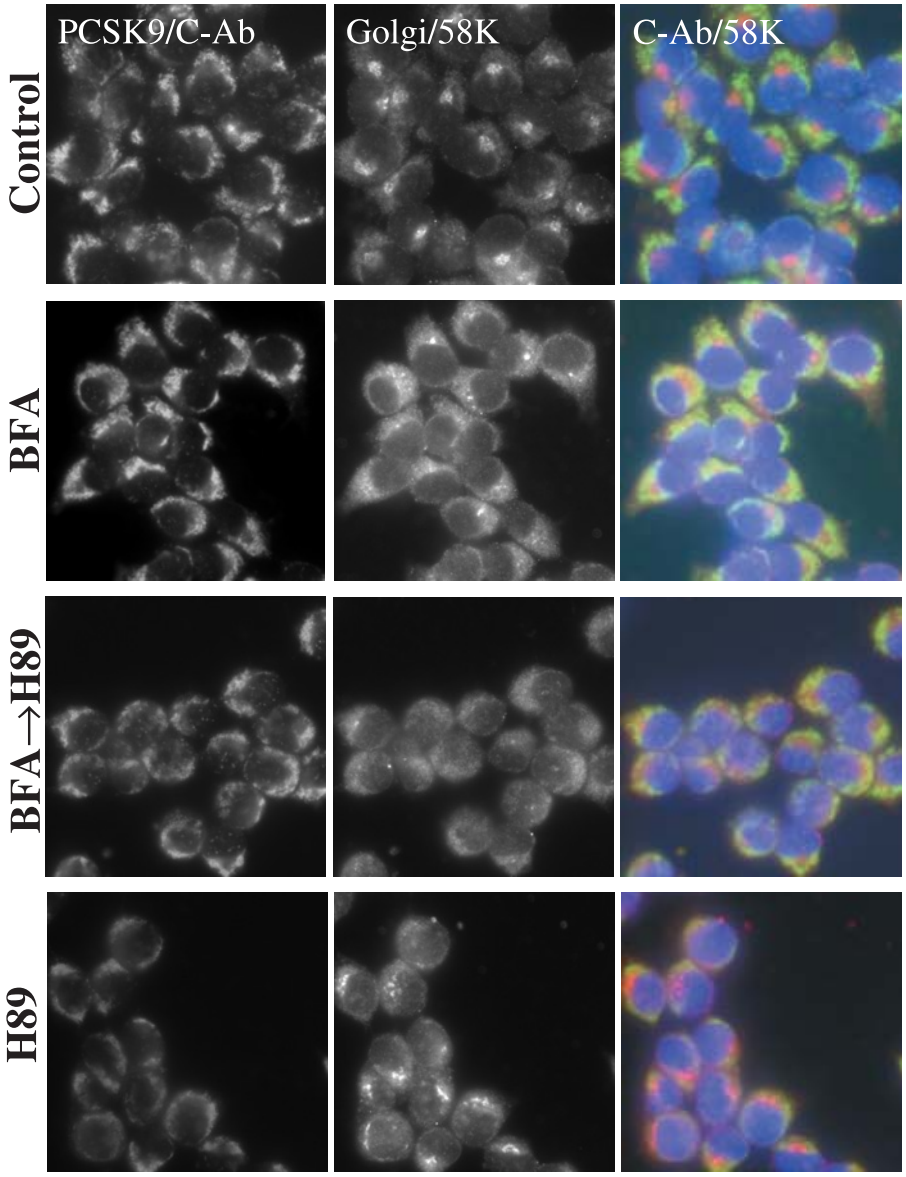


Fig. 7. Expression of PCSK9 after brefeldin A and H89 treatment. FAO-1 cells were grown on cover slips, treated with brefeldin A (BFA) for 40 min followed by H89 for 10 min (BFA → H89) or with H89 for 30 min (H89) as described in Materials and methods, acetone fixed, and incubated with antibodies against PCSK9 (C-Ab) followed by anti-rabbit Cy2-conjugated (green) and monoclonal anti-Golgi/58K followed by anti-mouse Cy3-conjugated (red). The nuclei were contrastained with DAPI. Original magnification: × 600.



which is exactly what was observed in these studies (Maxwell and Breslow 2004; Park et al. 2004).

Our work showed further that the suggested N-linked glycosylation of PCSK9 (15) occurs in the ER, as we did not detect the protein in the Golgi. Confocal immunofluorescent microscopy combined with ultracentrifugal subcellular fractionation and Western blot analysis revealed that the bulk of PCSK9 is localized in the ER: (*i*) the pro-enzyme shows diffuse distribution and it sediments with the ER membranes and (*ii*) most of the mature form also sediments with the ER. The remainder of the convertase is localized in transport vesicles in the ERGIC of the cell: (*i*) PCSK9 also appears in the lighter fractions of the gradient where COPII transport vesicles cosediment and (*ii*) when the cells are treated with brefeldin A and H89 the immunofluorescent signal for the convertase retreats into the ER. Some of PCSK9 is present on the cell surface and is secreted: (*i*) experiments with biotinylation of the cell surface proteins demonstrate that small amounts of the convertase (about 10% of the total) reside on the cell surface and (*ii*) secreted convertase from FAO-1 cells was immunoprecipitated from the medium and from rat plasma–serum. We also found that some PCSK9 is in clathrin-coated vesicles, and this opens the possibility that some of the secreted PCSK9 is taken back into the cells by endocytosis.

In conclusion, our data show that PCSK9 is predominantly expressed in cells producing APOB, and its expression is regulated by cholesterol. PCSK9 is localized in the ER, where it is processed, and in the ERGIC of the cell but not in the Golgi cisternae. The convertase is secreted in the plasma. Our results also suggest that PCSK9 may upregulate APOB production in these compartments. Further functional

analyses of PCSK9 are of great importance for clarification of how this enzyme regulates LDL stability and APOB production and hence the plasma cholesterol level in fetal and adult liver.

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