Universidad de Oviedo

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Programa de Doctorado “Investigación en Cáncer”

Análisis funcional y patológico de adamalisinas en modelos celulares y animales

Ángela Moncada Pazos
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Ángela Moncada Pazos
Autora

Carlos López Otín
Director/tutor

Santiago Cal Miguel
Director

ABREVIATURAS Y SIGLAS

ADN	ácido desoxirribonucleico
ANOVA	análisis de varianza
ARN	ácido ribonucleico
CAPS	ácido 3-ciclohexilamino-1-propanosulfónico
EDTA	ácido etilendiaminotetraacético
h	horas
kb	kilobase
kD	kilodalton
MDCK	Madin–Darby Canine Kidney
min	minutos
pb	pares de bases
PBS	tampón fosfato salino
SSC	tampón citrato sódico
TAE	tampón Tris/acetato con EDTA
TBE	tampón Tris/borato con EDTA
TE	tampón Tris con EDTA
Tris	tris(hidroximetil)-aminometano

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INTRODUCCIÓN

Las proteasas son enzimas con capacidad para hidrolizar enlaces peptídicos. Evolutivamente, la proteólisis habría surgido en una etapa temprana ligada a los procesos esenciales de catabolismo necesarios en todos los seres vivos. Así, las proteasas han sido consideradas tradicionalmente como meros enzimas degradativos encargados de la digestión de proteínas procedentes de la dieta. No obstante, hoy sabemos que estas funciones de hidrólisis generalizada suponen sólo una tarea minoritaria de entre el vasto conjunto de acciones desempeñadas por los enzimas proteolíticos. A lo largo de la evolución, lo mismo que ante nuestros ojos en los últimos años, las proteasas habrían ido diversificándose y ejecutando cortes proteicos exclusivos y rigurosamente controlados que se denominan procesamientos proteolíticos. Estas modificaciones irreversibles funcionan en la célula como parte de las estrategias post-traduccionales que expanden el repertorio proteico escrito en el genoma, genuinamente más limitado. Como resultado, las proteasas intervienen en la localización, la actividad o las interacciones de multitud de proteínas, procesos cuya universalidad implica a estos enzimas virtualmente en cualquier función biológica de un organismo vivo. De hecho, la abundante información acumulada en décadas recientes así lo atestigua: las proteasas participan en fenómenos tales como la división y diferenciación celular, la construcción y remodelación de tejidos, la angiogénesis, la ovulación, la fertilización, la inflamación, la coagulación sanguínea o la cicatrización de heridas, entre muchos otros (López-Otín y Bond, 2008).

La relevancia funcional de los enzimas proteolíticos en los seres vivos se traduce también en términos de diversidad. Hasta el momento, se han descrito en el genoma humano 578 genes que codifican proteasas. Este número es aun más elevado en el caso de los genomas de rata y ratón que incluyen al menos 652 y 664 genes de proteasas, respectivamente (http://degradome.uniovi.es). En todos estos casos, el número de genes invertidos en la producción de proteasas supone más de un 2% del total genómico. Estas cifras representan apropiadamente la magnitud del universo proteolítico, a la vez que exigen un esfuerzo de síntesis para su mejor comprensión y manejo. Para hacer posible el abordaje de estudios globales, el conjunto de proteasas de cualquier organismo, denominado degradoma, se ha organizado en grupos que a su vez

son extensibles a las diferentes especies. El primer nivel de clasificación está basado en el mecanismo de catálisis, distinguiéndose 6 clases: las aspartato, las glutamato y las metaloproteasas, y las cisteín-, serín- y treonín-proteasas. Si bien la ruptura del enlace peptídico se produce en todos los casos mediante un ataque nucleofílico, en las tres primeras clases el nucleófilo es una molécula de agua activada, mientras que en las tres últimas son residuos de cisteína, serina o treonina, respectivamente, los responsables de la reacción. Muy recientemente, se ha descrito una séptima clase catalítica de enzimas proteolíticos, en las que el residuo responsable de la ruptura peptídica es una asparagina. No obstante, los enzimas de esta nueva clase no catalizan reacciones de hidrólisis, sino que son liasas peptídicas que rompen las proteínas generando un anillo en su estructura (Rawlings y col., 2011). Cada una de estas clases catalíticas de proteasas se organiza a su vez en familias y clanes en base a las similitudes en su secuencia y estructura. El resultado es un conjunto inmenso y dispar, tanto en términos de complejidad estructural como de especificidad (Figura 1). De esta manera, el degradoma alberga desde enzimas con exclusividad por un corte único, como el enzima convertidor de angiotensina, a otras mucho más promiscuas, como la proteinasa K, y desde sencillas formas limitadas a un pequeño dominio catalítico como las matrilisinas, a laboriosas maquinarias que implican a múltiples enzimas, como el proteasoma. Adicionalmente, la acción de las proteasas en los sistemas vivos depende también de cofactores, inhibidores y elementos moduladores que, junto con los sustratos, establecen el concepto funcional de sistemas proteolíticos, los cuales a su vez forman parte de redes más amplias que también son objetivo del estudio degradómico (López-Otín y Overall, 2002; Mason y Joyce, 2011).

Hoy en día nuestro conocimiento del degradoma supera con creces la limitada visión de hace poco más de un siglo, cuando P. A. Levene inició el estudio de las proteasas (López-Otín y Bond, 2008). El espectacular avance en este campo ha tenido lugar en gran parte, gracias a las herramientas para el análisis de genomas completos. Nuestro laboratorio, dedicado desde su formación a la investigación del mundo proteolítico, ha participado activamente en estos análisis globales mediante el empleo de la bioinformática. La

combinación de la información procedente de los proyectos de secuenciación, junto con las secuencias existentes de proteasas y el desarrollo de modelos predictivos ha permitido describir nuevas proteasas y homólogos de las mismas (Puente y col., 2003). Estas estrategias han servido para detallar el inventario de proteasas de numerosas especies, incluyendo el de humano, ratón, rata, chimpancé, orangután, ornitorrinco y pinzón cebra (Puente y col., 2003; Gibbs y col., 2004; Consortium, 2005; Warren y col., 2008; Warren y col., 2010; Locke y col., 2011).

La comparación entre especies ha hecho posible deducir nuevos datos sobre las funciones de las proteasas. Recíprocamente, la caracterización de diferentes enzimas proteolíticos ha contribuido a comprender mejor las distancias genómicas y proteómicas que nos separan. En fechas futuras y a medida que la tecnología ayude a superar los límites actuales, el número de especies cuyo degradoma haya sido descifrado seguirá creciendo. Lo mismo harán, presumiblemente, los degradomas individuales tal y como viene ocurriendo en los últimos años con el descubrimiento de nuevas proteasas hasta ahora desconocidas. En paralelo al crecimiento de estos censos de proteasas, el estudio del universo proteolítico se ha ocupado también de la caracterización funcional de muchos de los enzimas anotados. Esta labor implica conocer, en primer lugar, los sustratos naturales de todos ellos. Las interacciones que se establecen entre las proteasas y sus sustratos habitualmente no son relaciones unidireccionales, abundando las situaciones de inespecificidad, en las que una misma proteasa es capaz de procesar múltiples sustratos, y de redundancia, existiendo proteínas que son simultáneamente dianas de diversas proteasas. El resultado de esta situación es que los sistemas proteolíticos esconden más información que la simple suma de sus componentes individuales de modo que cada contexto fisiológico o patológico sólo puede ser entendido como un todo. Por este motivo, en la búsqueda de los sustratos naturales de los enzimas proteolíticos ha de prevalecer también un enfoque global. En este sentido, el análisis del degradoma funcional de un organismo se puede abordar de manera general a través de tecnologías como los *chips*, tanto a nivel transcriptómico (por ejemplo mediante *microarrays* de expresión) como proteómico (con plataformas

capaces de medir abundancia o actividad) (López-Otín y Overall, 2002). Estos estudios integrales se complementan con los de carácter individual que permiten la identificación de la gama de sustratos de una proteasa concreta mediante, entre otros, bibliotecas de péptidos y sistemas como el de doble híbrido en levadura (Schilling y Overall, 2008). Muchas de estas tecnologías se hallan hoy en día en ciernes y su perfeccionamiento, junto con el desarrollo de estrategias nuevas y creativas, permitirá asignar sustratos a las que aún son proteasas huérfanas.

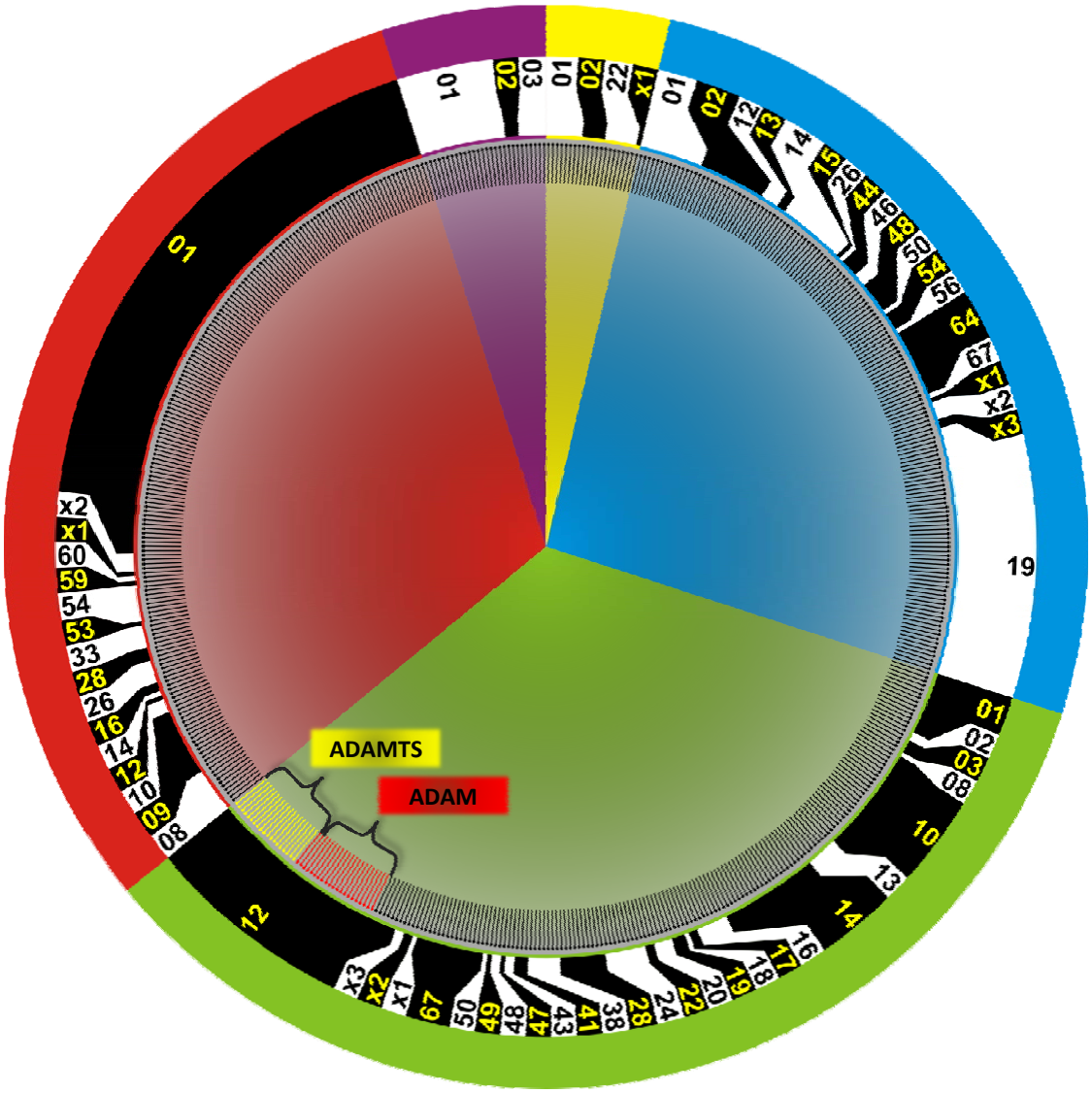

Figura 1. Degradoma humano. Cada una de las líneas de la rueda representa una proteasa humana. Los sectores de colores corresponden a cada clase catalítica: en rojo serín-proteasas, en morado treonín-proteasas, en amarillo aspartil-proteasas, en azul cisteín-proteasas y en verde metaloproteasas. Las familias se indican en los bloques blancos y negros. Se destacan dentro de las metaloproteasas y de la familia 12 los miembros de los grupos ADAM y ADAMTS.

La información proporcionada por los estudios comparativos y la identificación de sustratos establecen la base para comprender mejor muchas patologías humanas y para idear alternativas terapéuticas frente a las mismas. La universalidad de los eventos proteolíticos explica cómo la alteración en las funciones normales de muchas proteasas constituye una causa de enfermedad. En algunos casos los cambios se producen directamente en las proteasas, ya sea a través de mutaciones que modifican sus propiedades o de alteraciones en sus patrones de expresión o de actividad en el tiempo o en el espacio. En otros casos, los afectados son distintos componentes de los sistemas proteolíticos, como sustratos o inhibidores. La lista de patologías con esta etiología es amplia e incluye enfermedades neurodegenerativas, cardiovasculares, inflamatorias y diversos tipos de cáncer. Por ejemplo, se conoce desde hace años que defectos en la actividad de la plasmina provocan trombofilia, mientras que la ganancia de función de las presenilinas se asocia a formas hereditarias de la enfermedad de Alzheimer (Aoki y col., 1978; Levy-Lahad y col., 1995; Sherrington y col., 1995). En ocasiones, proteasas próximas pertenecientes a una misma familia son responsables de patologías muy diferentes. Así sucede con las serín-proteasas TMPRSS3, mutada en un tipo de sordera, y TMPRSS6, cuyas mutaciones causan anemia (Scott y col., 2001; Folgueras y col., 2008).

En lo que se refiere al escenario tumoral, las proteasas se han considerado tradicionalmente como promotoras del desarrollo neoplásico y metastásico. Según esta visión histórica, que ya fue postulada por Fischer en 1946, los enzimas proteolíticos del compartimento extracelular favorecerían la progresión del tumor al degradar los componentes de la matriz, permitiendo su crecimiento y la diseminación de células malignas (Fischer, 1946). Esta idea se vio reforzada con la evidencia de que muchas proteasas estaban sobreexpresadas en gran variedad de tumores y tras el hallazgo de que los inhibidores de metaloproteasas eran capaces de reducir la capacidad de invasión *in vitro* y de metástasis *in vivo* (Folgueras y col., 2004). Un ejemplo paradigmático de esta versión clásica lo encontramos en la metaloproteasa MMP-13, enzima sobreexpresada en numerosos tipos de cáncer y asociada a invasión y a mal pronóstico clínico de la enfermedad (Pendás y col., 2000). Las

metaloproteasas, y en general las proteasas del entorno extracelular y pericelular, emergieron hace una década como dianas prometedoras en las terapias contra el cáncer. Como resultado, florecieron los ensayos clínicos basados en inhibidores de estas moléculas. Sorprendentemente, la mayoría de estos ensayos no produjeron el esperado efecto terapéutico, e incluso en ocasiones sirvieron para acelerar el desarrollo del tumor (López-Otín y Matrisian, 2007). Hoy sabemos que existen proteasas extracelulares e intracelulares que participan en todos los estadios tumorales a través del control de una gran variedad de procesos biológicos. Así, estos enzimas desarrollan diversos roles en la progresión tumoral, y pueden incluso comportarse como supresores tumorales. Estos descubrimientos ayudan a explicar el fracaso de los inhibidores de proteasas de amplio espectro como tratamientos anti-tumorales. Uno de los ejemplos más estudiados es el de MMP-8, un enzima con actividad colagenasa pero que paradójicamente actúa como un agente anti-cancerígeno. Trabajos previos de nuestro laboratorio han podido demostrar que este enzima modula la respuesta inflamatoria, afectando a la quimiotaxis y a la apoptosis de neutrófilos (Balbín y col., 2003). Además, MMP-8 incrementa la adherencia a la matriz extracelular y disminuye la capacidad invasiva de las células tumorales (Gutiérrez-Fernández y col., 2008). Dichas acciones se deben al procesamiento de moléculas distintas del colágeno, como por ejemplo ciertas citoquinas u otros componentes de la matriz extracelular. En conjunto, los nuevos hallazgos destacan la relevancia y el carácter multifacético de los procesos proteolíticos en la enfermedad y muestran la necesidad de replantear, desde un abordaje no simplista, el diseño racional de terapias adecuadas.

Los recursos genómicos y proteómicos y la información que se deriva de ellos son de extraordinario valor para el estudio de los problemas biológicos. El volumen de dicha información es tal que incluso excede nuestra capacidad actual de asimilación y análisis. Hoy en día, la descripción de los sistemas y de las redes proteolíticos es una tarea incompleta. Además, estos conocimientos teóricos son parciales y al trasladarse a los organismos vivos pueden resultar incongruentes. En este sentido, los modelos celulares y animales facilitan nuestro acercamiento a unas condiciones más semejantes a las que acontecen

en la fisiología normal o en las patologías de los humanos. Por esta razón, mientras ahondamos en el conocimiento del degradoma completo de cada especie o de la gama de sustratos de cada proteasa, el empleo de estos modelos nos ayuda a acometer cuestiones imperativas como la búsqueda de terapias a las patologías humanas. En las últimas décadas, el desarrollo de la ingeniería genética con la generación de seres vivos modificados genéticamente se ha convertido en una de las estrategias más poderosas para comprender la función de las proteasas *in vivo*. Las nuevas técnicas permiten generar células o animales con ganancias (transgénicos) o pérdidas de función (*knock-out*) de potencialmente cualquier gen (Fanjul-Fernández y col., 2010). De entre las numerosas especies modificadas genéticamente y empleadas en la investigación, los modelos generados en ratones son los más aplicados al estudio de las enfermedades humanas.

La utilización de las metodologías globales genómicas y proteómicas y la generación de modelos celulares y murinos han sido las principales estrategias que nuestro laboratorio ha seguido para profundizar en el conocimiento del degradoma. En tanto que la biografía y los hábitos completos de los enzimas proteolíticos nos sigan siendo parcial o totalmente desconocidos, esta meta continúa vigente. Dentro de este marco, en la presente Tesis Doctoral se ha abordado un amplio estudio de algunas proteasas presentes en los degradomas humano y murino, a través de la búsqueda y descripción de sus funciones mediante distintas aproximaciones.

Adamalisinas: estructura y función

Las adamalisinas son una familia de proteasas que incluye a los grupos ADAM y ADAMTS, siglas en inglés de "*A Disintegrin And Metalloprotease*" y "*A Disintegrin And Metalloprotease with ThromboSpondin domains*", respectivamente. Miembros de estos dos grupos se hallan ampliamente distribuidos en mamíferos y en otros eucariotas como *Xenopus laevis*, *Drosophila melanogaster* o *Caenorhabditis elegans*, pero no se han encontrado en plantas ni en bacterias. Estructural y funcionalmente, las adamalisinas se relacionan estrechamente con la familia de metaloproteasas de venenos de

serpientes, las cuales son responsables de los efectos hemorrágicos y daños tisulares desencadenados por las mordeduras de estos animales. Su principal característica común es la coexistencia en una misma proteína de una región de adhesión y otra proteolítico. El dominio proteasa es muy similar al de otros miembros de la superfamilia de las metzinquinas, como por ejemplo las MMPs. Este dominio común se caracteriza por la presencia en su centro activo de un átomo de zinc y por la existencia en su región carboxilo terminal de un residuo conservado de metionina que forma el característico “giro de metionina” (Gomis-Ruth, 2003).

En lo que se refiere a las funciones de adhesión, las adamalisinas contienen un dominio desintegrina y un dominio rico en cisteínas. Además, las ADAMs poseen en su estructura un dominio EGF y las ADAMTSs un número variable de repeticiones trombospondina tipo-1 (TSP-1). Funcionalmente, las adamalisinas están implicadas en procesos fundamentales como las interacciones celulares o la señalización intracelular. Así, los primeros miembros de la familia identificados en mamíferos fueron las fertilinas α y β de cobaya (posteriormente denominadas ADAM-1 y ADAM-2), que permiten la fusión entre óvulo y espermatozoide durante la fertilización (Primakoff y col., 1987; Blobel y col., 1990). Desde entonces, se han catalogado nuevos miembros del grupo, incluyendo algunos que, si bien conservan todas las características estructurales de las adamalisinas, muestran un dominio catalítico inactivo, pudiendo actuar funcionalmente como proteínas reguladoras a través de su porción adhesiva. Finalmente, en 1997 Kuno y colaboradores identificaron en células de carcinoma de colon una proteína estrechamente relacionada con las ADAMs a la que denominaron ADAMTS-1 (Kuno y col., 1997). En la Figura 2 se muestran la disposición de dominios de ADAMs, ADAMTSs y otras metaloproteasas relacionadas. En la actualidad, el grupo de adamalisinas incluye 38 ADAMs, 22 de las cuales están presentes en humanos, y 19 ADAMTSs. Esta diversidad es indicio de la relevancia funcional de estos enzimas, y así lo refleja también el creciente interés por su estudio, que las ha situado como piezas clave en numerosos procesos biológicos y patológicos.

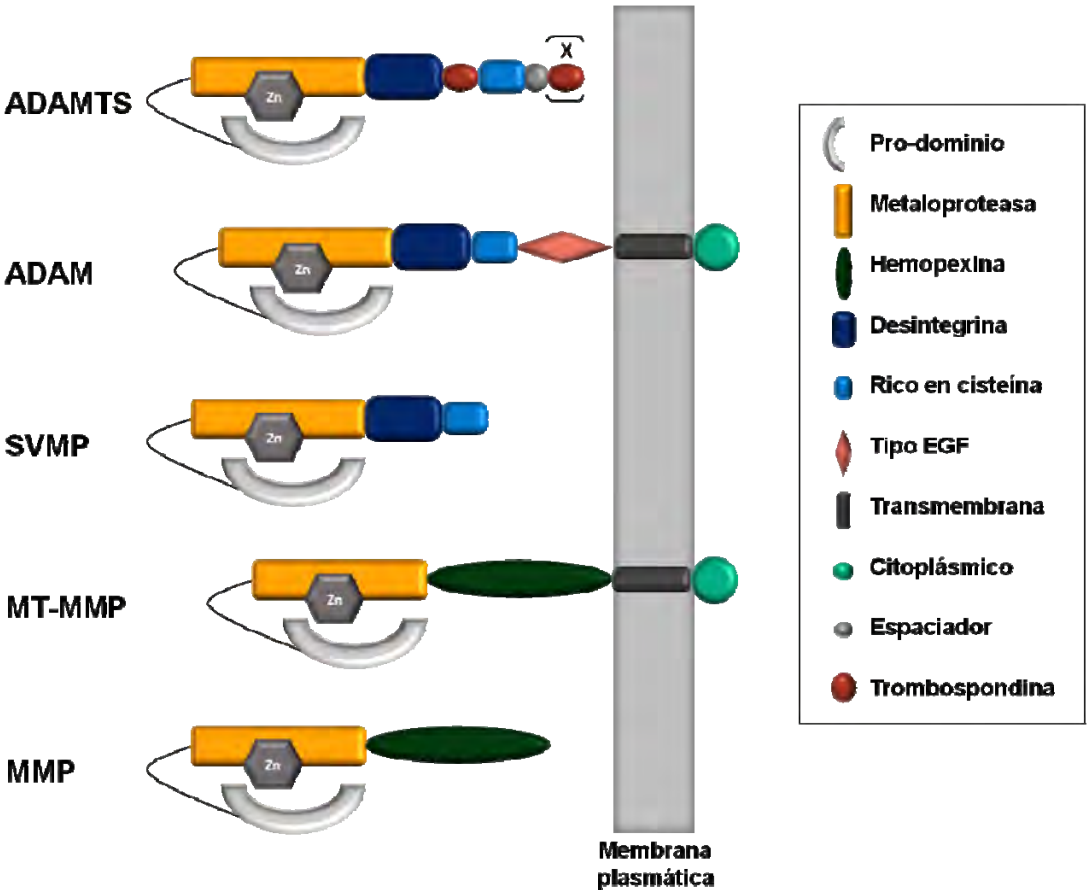

Figura 2. Estructura de las metzinquinas. Esquema de la organización en dominios de MMPs, MMPs ancladas a membrana (MT-MMPs), metaloproteasas de veneno de serpientes (SVMPs), ADAMs y ADAMTSs.

ADAMs

Las ADAMs fueron los primeros miembros caracterizados dentro de la familia de las adamalisinas. Se trata de proteínas con un dominio transmembrana y por lo tanto integradas en la superficie celular. Existen también formas solubles generadas mediante proteólisis o como consecuencia de *splicings* alternativos (Gilpin y col., 1998; Hotoda y col., 2002; Klein y Bischoff, 2011). Evolutivamente, miembros de la subfamilia ADAM están presentes en todos los mamíferos además de en algunos cordados no vertebrados y en ciertos eucariotas primitivos. Incluso entre especies cercanas de mamíferos, la variabilidad de los genes de este grupo es notable, estando sometidos a frecuentes fenómenos de pseudogenización y de eliminación selectiva de genes en algunas especies. La variabilidad en estos genes se debe probablemente al hecho de que muchas de estas proteínas están

implicadas en la fertilización y en la reproducción, de modo que sus rápidas tasas de evolución responderían a estrategias de especiación (Edwards y col., 2008). Estructuralmente, todas las ADAMs exhiben una estructura modular en la que se distinguen los siguientes dominios, desde el extremo amino al carboxilo-terminal:

- **Péptido señal**: secuencia de unos 20 aminoácidos que dirige al enzima hacia la ruta secretora de la célula.
- **Prodominio**: región de unos 200 residuos que actúa como un chaperón intramolecular hasta la activación de la proteína por proteasas tipo furina o por autoproteólisis. Funciona a través de un mecanismo tipo "interruptor de cisteína", manteniendo el átomo de zinc y el dominio proteasa en un estado latente.
- **Dominio metaloproteasa**: incluye el motivo consenso HExxHxxGxxH que coordina el átomo de zinc. Las 12 ADAMs humanas que conservan esta secuencia completa son ADAM-1, -8, -9, -10, -12, -15, -17, -19, -20, -28, -30 y -33. Este dominio es capaz de degradar componentes de la matriz extracelular como colágeno o fibronectina, aunque la relevancia *in vivo* de estas acciones no ha podido ser demostrada. La importancia fisiológica de este dominio sí se ha probado, en cambio, en el procesamiento y liberación de componentes activos a partir de sus proformas ancladas a membrana, como ocurre con el enzima convertidor de TNF-α (TACE o ADAM-17).
- **Dominio desintegrina**: su presencia en las ADAMs es una característica distintiva de entre las proteínas de superficie celular. Este dominio afecta la adhesión celular y las interacciones con otras células a través de su unión con integrinas. Si bien esta asociación ha sido ampliamente caracterizada, la relevancia biológica de la misma no se conoce con exactitud. Actualmente, el único proceso fisiológico para el que se ha documentado la necesidad de interacción del dominio desintegrina con integrinas es el de fertilización que implica a ADAM-1 y ADAM-2. Otras ADAMs para las que se ha probado su capacidad de unión a integrinas son ADAM-9, -12, -15 y -23 (Zhang y col., 1998; Iba y col., 1999; Cal y col., 2000; Nath y col., 2000).

- **Dominio rico en cisteínas**: esta región proporciona posibilidades de adhesión adicionales a las del dominio desintegrina. En concreto, se ha descrito su capacidad para interaccionar con proteoglicanos del tipo sindecano y con componentes de la matriz como la fibronectina. Además en algunas ADAMs el dominio rico en cisteínas comparte ciertas características estructurales con proteínas de fusión virales, existiendo la posibilidad de que estos motivos intervengan en fenómenos de fusión de membranas.
- **Dominio EGF**: esta estructura, de entre 30 y 40 residuos de longitud, es común a muchas proteínas de la superficie celular y está implicada también en fenómenos de interacción proteica.
- **Dominio transmembrana**: dispuesto a continuación del dominio rico en cisteínas, es el responsable de la localización de membrana de todas las ADAMs. Las distingue además del otro grupo de adamalisinas, las ADAMTSs.
- **Región citoplasmática**: de longitud variable (entre 40 y 250 aminoácidos), contiene en la mayoría de ADAMs el motivo PxxP que interviene en la unión a proteínas con dominios SH3 (*Src-homology 3*). A través de este tipo de interacciones, las regiones intracelulares de estas proteasas son capaces de afectar a su localización al asociarse a componentes del citoesqueleto, así como de desencadenar rutas de señalización en el citoplasma. Algunas ADAMs contienen además residuos susceptibles de fosforilación por quinasas, que podrían regular la actividad del resto de la proteína.

Funcionalmente, los miembros de la subfamilia ADAM están implicados en una gran variedad de procesos biológicos. La mayoría de evidencias experimentales al respecto se refieren a su actividad metaloproteasa aunque si consideramos su compleja estructura, es fácil anticipar que sus efectos van a ir más allá de la proteólisis. De hecho, tan sólo 12 del total de 22 ADAMs humanas conservan todas las características para ser catalíticamente activas, poniendo de manifiesto la relevancia de sus otros dominios para el ejercicio de sus funciones (van Goor y col., 2009; Klein y Bischoff, 2011). Se conoce escasamente el papel de estas ADAMs inactivas, aunque podrían estar

involucradas en interacciones célula-célula, en la modulación de la migración o adhesión celular o en el control de la señalización al interior de la célula. Entre las funciones relacionadas con la actividad metaloproteasa destaca la liberación de proteínas de la superficie celular, incluyendo factores de crecimiento, citoquinas, receptores o moléculas de adhesión. ADAM-10, por ejemplo, es responsable del procesamiento *in vivo* de diversas proteínas de membrana como Notch, CD23, Fas-L o RAGE (Klein y Bischoff, 2011). La acción de ADAM-10 sobre Notch supone uno de los tres eventos proteolíticos que debe sufrir esta proteína para iniciar su participación en rutas de señalización intracelular. En concreto, ADAM-10 efectúa el segundo procesamiento para la activación de Notch. Pese a que otras proteínas son capaces de ejecutar este paso (como ADAM-17), en ausencia de ADAM-10 no se produce una compensación suficiente, y la deficiente activación de la ruta Notch desencadena muerte embrionaria por fallos en el desarrollo de los sistemas nervioso y vascular en ratones mutantes (Hartmann y col., 2002). En relación al papel de ADAM-10 en el procesamiento de otras proteínas, se ha podido determinar que se trata de la principal proteasa encargada del procesamiento del receptor de IgE de baja afinidad CD23 (Weskamp y col., 2006). Esta proteína, presente en la superficie celular, es la reguladora esencial de la síntesis de IgE en la célula, exacerbada durante los procesos alérgicos, y por lo tanto ADAM-10 ha emergido como una diana terapéutica potencial en estas disfunciones. Complementariamente, se ha demostrado que la acción de ADAM-10 tiene efectos protectores en procesos inflamatorios a través de RAGE y que está implicada en los fenómenos de apoptosis mediante la digestión de Fas-L. Finalmente, por su actividad α-secretasa podría desarrollar un papel significativo en la enfermedad de Alzheimer (Klein y Bischoff, 2011).

ADAM-17 es probablemente el miembro más estudiado de la familia de las ADAMs y una de las proteasas más relevantes en el procesamiento de proteínas de membrana. Este enzima es el principal responsable de la solubilización de TNF-α desde la superficie de la célula, un acontecimiento clave en la respuesta inflamatoria. Sin embargo, ADAM-17 no es el único enzima capaz de llevar a cabo este procesamiento, ya que otros miembros de la misma familia, como ADAM-10, también catalizan esta reacción (Le Gall y

col., 2009). Además de TNF-α, ADAM-17 es capaz de procesar otros sustratos como proTGF-α, Notch, numerosas citoquinas y receptores de citoquinas y también presenta actividad α-secretasa (Gooz, 2010).

La función adhesiva de las ADAMs permite su participación en muchos otros procesos biológicos. Algunos de los ejemplos más ilustrativos son los relacionados con la ADAM-15 y las meltrinas α y β (ADAM-12 y -19). En lo que respecta a ADAM-15, su dominio desintegrina presenta ciertas particularidades con respecto al del resto de miembros del grupo. En concreto, es la única ADAM que posee la secuencia consenso RGD, la cual permite la interacción de ADAM-15 con una mayor variedad de integrinas. En general, ADAM-15 promueve la adhesión e inhibe la migración. Funcionalmente, este enzima está involucrado en la cicatrización de heridas y en la inflamación, y desarrolla un papel protector en enfermedades degenerativas como la osteoartritis. En alteraciones cardiovasculares, no obstante, su acción puede resultar dañina habiéndose implicado en patologías trombóticas (Langer y col., 2005). En cuanto a las meltrinas α y β, ambas desarrollan un papel relevante en el desarrollo de células mesenquimales a través de la interacción con integrinas, participando en la formación del músculo y en su regeneración tras lesión (Kronqvist y col., 2002; Kurisaki y col., 2003).

En conjunto, con la descripción de la primera ADAM comenzó a extenderse el abanico de funciones en las que estos enzimas toman parte y es presumible que continúe haciéndolo en los próximos años. En tanto que eso ocurre, es necesario incrementar los esfuerzos para conocer en profundidad sus mecanismos de funcionamiento, su conformación en condiciones nativas y sus mecanismos de regulación.

ADAMTSs

La complejidad de la familia de adamalisinas se incrementó considerablemente con la identificación de la primera ADAMTS (Kuno y col., 1997). Esta nueva proteína, denominada ADAMTS-1, se parecía notablemente a las ADAMs pero poseía varias repeticiones TSP-1 en su región carboxilo terminal y carecía del dominio transmembrana. Se trataba por lo tanto de un

enzima secretado y de estructura modular más compleja que las ADAMs. Hasta el momento, se han identificado un total de 19 ADAMTSs en mamíferos, además de otras 6 proteínas relacionadas con ellas (*ADAMTS-like*) pero carentes de los dominios metaloproteasa y desintegrina, por lo que no se clasifican como componentes del degradoma. En los animales no mamíferos, las ADAMTSs son mucho más infrecuentes, habiéndose identificado 5 miembros en *C. elegans* y 3 en *D. melanogaster*. De manera análoga a lo que ocurre con las ADAMs, no se han detectado ADAMTSs en plantas ni levaduras (Porter y col., 2005). Estructuralmente, además de la ausencia de un dominio de membrana y la presencia de varios dominios TSP-1, existen algunos motivos en ciertas ADAMTSs que no aparecen en las ADAMs y que se esquematizan en la Figura 2. A continuación, se resumen las características diferenciales o exclusivas del diseño de las ADAMTSs:

- **Dominio metaloproteasa:** muy semejante al de las ADAMs, aunque en las ADAMTSs este dominio se caracteriza además por la presencia de un residuo de aspártico al final del motivo consenso (HExxHxxGxxHD). Son todavía escasas las ADAMTSs a las que se han asociado sustratos diana, siendo la mayoría de los identificados componentes de la matriz extracelular de distintos tejidos.
- **Dominio desintegrina:** a diferencia de las ADAMs, ninguna ADAMTS conserva la secuencia consenso RGD en su dominio desintegrina y no se ha probado todavía su capacidad para interaccionar con integrinas.
- **Dominios trombospondina (TSP-1)**: este tipo de dominios se describieron inicialmente en las trombospondinas 1 y 2, proteínas secretadas con funciones antiangiogénicas. Las ADAMTSs disponen de un dominio TSP-1 central, localizado entre el dominio desintegrina y el rico en cisteínas, y un número variable en el extremo carboxilo terminal (desde ninguno en el caso de ADAMTS-4 a 19 en ADAMTS-20). El dominio central es el más conservado, mientras que los dominios terminales muestran una mayor variabilidad.
- **Dominio espaciador**: se extiende desde el dominio rico en cisteínas hasta las repeticiones TSP-1 finales, con una longitud variable y cuya

secuencia y características estructurales no se han podido adscribir a otros dominios conocidos.

- **Dominio tipo mucina**: interrumpe los dominios TSP-1 del final de ADAMTS-7 y ADAMTS-12, y se encuentra altamente glicosilado.
- **Motivo proteasa y lacunina (PLaC)**: se trata de un péptido de pequeña longitud (30-40 aminoácidos) que se identificó por primera vez en la proteína lacunina de la polilla (*Manduca sexta*). Está presente en el extremo carboxilo de numerosas ADAMTSs.
- **Motivo gon-1**: se sitúa en el extremo carboxilo terminal de ADAMTS-9 y ADAMTS-20, y recibió su nombre de la ADAMTS de *C. elegans* Gon-1, la cual participa en la morfogénesis gonadal de esta especie.
- **Motivo cubilina (Cub)**: es exclusivo de ADAMTS-13, que muestra dos dominios de este tipo en su región carboxilo terminal. Es típico también de la familia de las astacinas y constituye un módulo funcionalmente independiente implicado en procesos de desarrollo.

La región carboxilo terminal de las ADAMTSs es por lo tanto extraordinariamente variable y confiere a estos enzimas muchas de sus propiedades exclusivas. Los dominios presentes en esta zona, en su mayoría con adhesivos y de anclaje, afectan profundamente a la manera en la que la proteína se relaciona con sus sustratos y con el entorno celular. Además, esta región es diana de frecuentes modificaciones mediante proteólisis, las cuales en ocasiones dan lugar a drásticos efectos en las cualidades de estas proteínas. Tal es el caso de ADAMTS-1: este enzima se muestra en dos formas activas y maduras distintas producto de dos eventos consecutivos de procesamiento proteolítico. El primero de ellos, mediado por furina, elimina de la estructura el pro-dominio generando una proteína funcional de 87 KDa. A continuación, un segundo procesamiento que pueden llevar a cabo metaloproteasas como MMP-2, -8 y -15 provoca una rotura en la región espaciadora, liberando los dos dominios TSP-1 finales y produciendo una nueva forma de 65 KDa. Ambas moléculas coexisten en la célula y son activas, aunque sus propiedades son marcadamente diferentes. La proteína de 87 KDa muestra mayor afinidad por heparina y permanece localizada en la superficie celular y asociada a la matriz extracelular. La segunda forma, en cambio, es

soluble y presenta menor capacidad de unión a heparina y al factor de crecimiento del endotelio vascular (VEGF) (Rodríguez-Manzaneque y col., 2000; Luque y col., 2003). Este tipo de modificaciones post-traduccionales se ha documentado en otras ADAMTSs, como ADAMTS-4, -8, -9 y -12 (Vazquez y col., 1999; Cal y col., 2001; Somerville y col., 2003; Kashiwagi y col., 2004).

Los dominios TSP-1 y las variables regiones carboxilo, definen una unidad funcional de carácter preferentemente adhesivo que, junto con el dominio metaloproteasa y de manera análoga a lo que ocurre con las ADAMs, determina sus funciones en las células y tejidos. El aspecto catalítico de las ADAMTSs ha sido menos explorado que el de las ADAMs, y aún son muchos los miembros de la familia para los que no se ha identificado sustrato alguno (Porter y col., 2005). Los datos de los que se dispone muestran una preferencia por sustratos que son componentes de la matriz y del entorno celular de los distintos tejidos, a diferencia de las ADAMs, que se encargan preferencialmente del procesamiento de proteínas de membrana. Una de las funciones catalíticas mejor caracterizada de las ADAMTSs es su actividad agrecanasa. El agrecano es un proteoglicano de alto peso molecular presente en el cartílago. De hecho, agrecano y colágeno tipo II constituyen las dos proteínas mayoritarias del cartílago. La degradación proteolítica del agrecano, necesaria para la remodelación normal del tejido, es también un acontecimiento clave en patologías articulares como la artritis y la artrosis. Hasta el momento, se han identificado 6 ADAMTS con capacidad para hidrolizar el agrecano: ADAMTS-1, -4, -5, -9. -12 y -15. Si bien algunas MMPs disponen también de actividad agrecanasa, el corte característico de una y otra familia es diferente, y se considera en general que son las ADAMTSs las más implicadas en los procesos patológicos. De hecho, son dos ADAMTSs las consideradas como principales agrecanasas: ADAMTS-4/agrecanasa-1 y ADAMTS-5/agrecanasa-2 (Glasson y col., 2005; Verma y Dalal, 2011). Otras ADAMTSs para las que se han identificado sustratos son ADAMTS-2, -3 y -14, capaces de procesar distintas formas de pro-colágenos, eliminando sus propéptidos y participando en la maduración de estos componentes de la matriz. Finalmente, uno de los tándem ADAMTS-sustrato más estudiado es el de ADAMTS-13 y el factor de von Willebrand (vWF), otro proteoglicano de elevado peso molecular que

participa en la agregación de plaquetas al establecer interacciones entre la superficie de éstas y componentes de la pared vascular. ADAMTS-13 es capaz de degradar las cadenas multiméricas de vWF, contribuyendo a la adecuada homeostasis en la formación de trombos (Dong y col., 2002). En lo que respecta a las funciones no relacionadas con la actividad catalítica, el protagonismo recae sobre los dominios trombospondina y los distintivos de los distintos miembros del grupo. Así, los motivos TSP-1 son capaces de inhibir la angiogénesis mediante el secuestro de factores pro-angiogénicos como VEGF (Vazquez y col., 1999). Otras regiones de la molécula, como el dominio CUB de ADAMTS-13, son también necesarios para el ejercicio de su función (Tao y col., 2005; Zhang y col., 2007).

Las adamalisinas son, por lo tanto, una familia de proteínas numerosa y polifacética. Si bien definimos este grupo como proteasas, la realidad es que confluyen en una misma molécula una proteasa y una unidad adhesiva. La conjunción de ambas responde probablemente a la necesidad de cubrir un nicho funcional en la célula que aún no comprendemos en detalle.

Adamalisinas en cáncer y otras patologías

Las adamalisinas, a través de sus acciones catalíticas y no catalíticas ejercen un papel crucial como moduladores de procesos fisiológicos. Las alteraciones en su actividad normal conducen con frecuencia a la aparición de enfermedades. De esta manera, su relevancia clínica se evidencia con su implicación, por pérdida o ganancia de función, en diversos procesos patológicos, incluyendo el cáncer y diversas enfermedades inflamatorias, cardiovasculares y degenerativas. En algunos casos, el cometido que llevan a cabo es tan primordial que la falta del mismo desencadena una patología. En otros casos, la proximidad de miembros de la familia y los fenómenos de compensación enmascaran las contribuciones individuales. A continuación se recogen algunos de los ejemplos más relevantes de adamalisinas asociadas a enfermedades humanas, y que contribuyen a ilustrar el carácter multifuncional de estas proteasas.

Enfermedades monogénicas

Síndrome de Ehlers-Danlos tipo VIIC

Se trata de una enfermedad con herencia autosómica recesiva, caracterizada por una extrema fragilidad de la piel, hiperlaxitud de las articulaciones y estatura reducida, además de por una fisonomía facial característica. La causa de este síndrome deriva de mutaciones en el gen *ADAMTS2*, que provocan la pérdida de su función normal de modo que no se produce la correcta maduración de los pro-colágenos I, II y III (Colige y col., 2004).

Síndrome de Weill-Marchesani

Es también una enfermedad autosómica recesiva, causada por mutaciones en los genes *ADAMTS10* y *ADAMTS17*. Alternativamente, mutaciones en el gen de la fibrilina-1 (*FBN-1*) son responsables de una variante dominante de la enfermedad. Los individuos afectados muestran corta estatura, braquidactilia, anquilosamiento progresivo de las articulaciones y defectos oculares y cardiacos (Morales y col., 2009). Si bien no se conoce el mecanismo exacto que conduce a la patología, recientemente se ha probado que ADAMTS-10 interactúa con la fibrilina-1, posiblemente participando en su maduración (Kutz y col., 2011).

Púrpura trombocitopénica trombótica

Esta patología vascular se desencadena por pérdida de función de ADAMTS-13, el enzima procesador del vWF, debido a mutaciones en el gen que la codifica o a la presencia de anticuerpos autoinmunes contra la proteína. Los síntomas característicos son la formación frecuente de trombos por acumulación de plaquetas y formas ultra-largas del vWF. Además, los enfermos sufren anemia, fallo renal y alteraciones neurológicas (Levy y col., 2001).

Enfermedades degenerativas

Enfermedad de Alzheimer

La enfermedad de Alzheimer es una forma de neurodegeneración que provoca demencia. Su principal causa es la acumulación en el cerebro del péptido β amiloide, formándose placas que son la responsables del daño neurológico. El péptido β amiloide se genera a partir de la proteína precursora amiloide, que experimenta dos procesamientos consecutivos llevados a cabo por β- y γ-secretasas (Zhang y col., 2011). Un tercer tipo de proteasas, las α-secretasas, producen un péptido soluble no amiloidogénico que se considera neuroprotector. Al menos tres miembros de la familia de adamalisinas desarrollan actividad α-secretasa: ADAM-9, ADAM-10 y ADAM-17, y en concreto ADAM-10 se considera como la α-secretasa constitutiva (Lammich y col., 1999; Allinson y col., 2003).

Distrofia muscular de Duchenne

Las distrofias musculares son un conjunto de miopatías caracterizadas por la debilidad del músculo estriado. La forma más común es la distrofia muscular de Duchenne, una enfermedad recesiva ligada al cromosoma X y producida por mutaciones en el gen que codifica la distrofina, una proteína estructural de las fibras musculares (Kanagawa y Toda, 2006). Un miembro de la familia ADAM, meltrina-α, parece jugar un papel relevante en esta enfermedad. Este enzima, como ya hemos indicado, participa en el desarrollo y en la regeneración del músculo. Además, mediante el empleo de modelos murinos para la distrofia muscular de Duchenne, se ha observado que la sobreexpresión de *Adam12* mejora los síntomas en los primeros estadios de la enfermedad. Sin embargo, estudios posteriores han revelado que en edad avanzada, esta sobreexpresión se asocia a un incremento en la degeneración muscular y a un agravamiento de la enfermedad (Jorgensen y col., 2007). Estos efectos de ADAM-12 en el músculo son mediados, probablemente, a través de la regulación de componentes estructurales de este tejido.

Enfermedades inflamatorias

Asma

El asma es una enfermedad inflamatoria crónica causada por la hiper-reactividad de las vías aéreas a determinados antígenos. Así, en algunos individuos la exposición a ciertos alergenos provoca una reacción inflamatoria en los pulmones, caracterizada por una primera fase con participación de mastocitos y macrófagos, seguida de una fase tardía protagonizada por eosinófilos, linfocitos T CD4, basófilos y neutrófilos. Cuando la exposición a los antígenos que han promovido la respuesta se hace recurrente, se desarrolla una situación crónica caracterizada por cambios morfológicos en el pulmón, alteraciones en la composición de la matriz extracelular e incremento en la mucosidad y la angiogénesis (Paulissen y col., 2009).

La etiología del asma es compleja y multifactorial, pero se ha apuntado a algunas adamalisinas como implicadas en su aparición. En el año 2002, se describió por primera vez una asociación entre polimorfismos en el gen *ADAM33* y asma (Van Eerdewegh y col., 2002). Desde entonces, la información derivada de otros análisis genéticos poblacionales y del uso de modelos murinos condujo a conclusiones en ocasiones contradictorias sobre el papel de ADAM-33 en asma, si bien los últimos estudios parecen confirmar su relevancia en esta patología (Awasthi y col., 2011; Chi y col., 2011). ADAM-8 también se ha relacionado ampliamente con el asma, tras su identificación como un gen candidato asociado a la enfermedad y la detección de su sobreexpresión en pacientes asmáticos (King y col., 2004; Tremblay y col., 2008). Estos resultados han quedado reforzados por estudios en modelos animales, dado que los ratones deficientes en *Adam8* se muestran protegidos frente al desarrollo de esta enfermedad (Naus y col., 2010; Paulissen y col., 2011). El mecanismo molecular subyacente a esta función de ADAM-8 está presumiblemente ligado a su actividad en la liberación de CD23 desde la superficie celular. Otras adamalisinas también se han implicado directa o indirectamente en el asma. Así ADAM-9, -10, -12, -17 y -28, además de ADAMTS-4, y -9, se hallan sobreexpresadas en muestras de pacientes o de ratones con asma (Paulissen y col., 2006; Di Valentin y col., 2009), mientras

que ADAMTS-12 se ha identificado como posible gen candidato de susceptibilidad a asma (Kurz y col., 2006).

Artritis

La degradación excesiva del cartílago es el síntoma principal de las patologías artríticas, fundamentalmente artrosis y artritis reumatoide. Mientras que la artrosis u osteoartritis es una enfermedad asociada a la edad o a lesiones previas, la artritis reumatoide tiene una etiología autoinmune y es esencialmente inflamatoria. Como consecuencia de la inflamación en la articulación, se desencadena una destrucción del cartílago y del hueso (Goldring y Marcu, 2009). Tal y como se ha descrito previamente, ciertas MMPs y ADAMTSs son las principales encargadas de los eventos catabólicos en el cartílago a través de la degradación del colágeno tipo II (MMPs) y del agrecano (MMPs y ADAMTSs). En la degradación patológica del agrecano, son las ADAMTSs las que parecen desarrollar un papel preferente. Así, mientras que modelos de ratón deficientes en las MMPs con actividad agrecanolítica muestran incluso un exceso en la degradación de este sustrato (Zhou y col., 2000; Colnot y col., 2003), los ratones que carecen de *Adamts5* están protegidos frente a la pérdida de cartílago (Stanton y col., 2005).

Choque séptico

En ocasiones, una infección grave puede dar lugar a una reacción inflamatoria sistémica, especialmente en individuos inmunodeprimidos. El TNF-α funciona en estos casos como una potente citoquina inflamatoria. Aunque la deleción de *Adam17* en ratones resulta letal, los ensayos funcionales llevados a cabo con ratones mutantes condicionales, en los que se elimina esta proteasa temporalmente en el adulto o selectivamente en la línea mieloide, han mostrado que la ausencia de Adam-17 protege considerablemente de los daños tras la inducción de sepsis (Horiuchi y col., 2007). ADAM-17 sería, por tanto, el principal enzima que procesa TNF-α tras la inducción por endotoxinas, y representa una diana terapéutica prometedora en el tratamiento de las diversas enfermedades inflamatorias mediadas por esta citoquina.

Aterosclerosis

La aterosclerosis se produce cuando se depositan sustancias lipídicas en la pared de los vasos sanguíneos de calibre intermedio y grueso, a la vez que se desencadena una reacción inflamatoria, estrechándose la luz del tubo hasta el punto de dificultarse la circulación. Esta enfermedad constituye la causa mayoritaria de infarto y supone uno de los principales motivos de mortalidad en el mundo desarrollado. En su etiología se unen factores ambientales, como la dieta y modo de vida, y factores genéticos (Libby y col., 2011). Los datos experimentales recogidos en los últimos años implican a varias adamalisinas en la patogenia de esta enfermedad. Así, se ha detectado la presencia de ADAM-15 y de Adam-17 en las placas ateroscleróticas de pacientes y ratones afectados, respectivamente (Shiomi y col., 2010). Otros estudios han mostrado la presencia en estas lesiones de ADAMTS-1, -4 -5 y -8. Concretamente, ADAMTS-4, -5 y -8 están producidas por macrófagos, mientras que serían las células endoteliales y las del músculo liso de la pared vascular las encargadas de liberar ADAMTS-1. En el caso de esta última, el cruce de ratones transgénicos para esta proteasa con ratones deficientes en apolipoproteína-E (un modelo de aterosclerosis) provoca una mayor afectación de las arterias del animal (Jonsson-Rylander y col., 2005). El papel de las ADAMTSs en este proceso patológico posiblemente vaya ligado a su capacidad para digerir versicano, un proteoglicano semejante al agrecano, pero especialmente abundante en la matriz de los vasos sanguíneos. En condiciones normales, el versicano proporciona estabilidad estructural mediante su interacción con hialuronano, y en situaciones patológicas actuaría favoreciendo la migración de células de músculo liso tras la aparición de la lesión, así como reteniendo células inflamatorias o incluso lipoproteínas de bajo peso molecular (Salter y col., 2010). Las ADAMTSs, por su capacidad para digerir versicano podrían ser relevantes en estos fenómenos.

Cáncer

Las adamalisinas, como mediadores esenciales de procesos de remodelación de la matriz, señalización celular o angiogénesis están también implicadas en la aparición y progresión de tumores y en el desarrollo de

metástasis. Entre las ADAMs, un ejemplo bien documentado es el de ADAM-10, para el que abundantes trabajos describen un papel pro-tumoral y pro-metastásico. Así, *ADAM10* se sobreexpresa en tumores y metástasis de distinto origen, como cánceres orales, gástricos o melanoma (Ko y col., 2007; Lee y col., 2010a; Wang y col., 2011b); además, ensayos *in vitro* e *in vivo* han demostrado que su silenciamiento permite disminuir la proliferación y la invasión de las células tumorales (Gaida y col., 2010; Xu y col., 2010). El mecanismo por el que ADAM-10 interviene favoreciendo la progresión del tumor no se ha dilucidado aún, pero su capacidad para liberar múltiples citoquinas de las células o para degradar el colágeno tipo IV representan explicaciones plausibles. ADAM-9 se ha revelado también como un enzima promotor de metástasis, aunque en este caso resulta determinante la regulación por *splicing*, pues mientras que la variante soluble tiene un comportamiento pro-metastásico, la forma anclada a membrana actúa inhibiendo la migración celular (Mazzocca y col., 2005; Fry y Toker, 2010). Por el contrario, otro miembro de la misma familia, ADAM-23, se comporta como un supresor tumoral cuyo gen resulta frecuentemente silenciado mediante hipermetilación de su promotor en tumores de diverso origen (Takada y col., 2005; Choi y col., 2009). En este caso, ADAM-23, catalíticamente inactiva, actuaría probablemente a través de su dominio desintegrina, que es capaz de mediar la interacción de las células tumorales con integrinas disminuyendo la migración (Verbisck y col., 2009).

Múltiples ADAMTSs se han relacionado también con el cáncer, frecuentemente a través de la acción de sus dominios trombospondina. El caso más estudiado es el de ADAMTS-1, que modula los procesos de angiogénesis y metástasis, pudiendo su estado proteolítico determinar el efecto pro- o anti-tumoral de la proteína, como se ha mencionado previamente (Iruela-Arispe y col., 2003; Liu y col., 2006b). Más recientemente, se ha implicado indirectamente a esta proteasa en el tropismo de las células invasivas. En concreto, se ha observado en ratones que las células tumorales inyectadas se dirigían diferencialmente hacia el hígado o hacia el pulmón en función del estado de procesamiento de trombospondina-1 en uno y otro tejido. Este procesamiento depende de ADAMTS-1 y es el distinto nivel de actividad de

ADAMTS-1 entre pulmón e hígado el que determina, en último término, el establecimiento de las células cancerígenas (Lee y col., 2010b). Otras ADAMTSs, como ADAMTS-2 y ADAMTS-9 también inhiben la angiogénesis, y podrían funcionar como supresores tumorales *in vivo* (Dubail y col., 2010; Lo y col., 2010). Así, se han identificado frecuentes mecanismos de inactivación de genes de *ADAMTSs* en tumores humanos, tanto mediante mutación como mediante silenciamiento epigenético. Cabe destacar el ejemplo de *ADAMTS18*, diana frecuente de alteraciones genéticas en melanoma (Wei y col., 2010) y de hipermetilación en múltiples tipos de tumor (Dunn y col., 2004; Jin y col., 2007).

A lo largo de los últimos años, nuestra visión de las adamalisinas y de las proteasas en general ha variado sustancialmente. Así, hoy sabemos que las adamalisinas gestionan multitud de acontecimientos fisiológicos, influenciando los ritmos de vida y muerte de las células. Además, numerosos estudios han demostrado que la desregulación de sus funciones conduce con frecuencia al desarrollo de enfermedades. Esta nueva perspectiva ha sido posible gracias a las evidencias proporcionadas por tecnologías como las aproximaciones genéticas y epigenéticas y los estudios funcionales basados en modelos celulares y animales. Sin embargo, aún quedan muchos retos por superar para poder traducir los conocimientos acumulados en beneficios clínicos, y estos pasarán necesariamente por estas mismas estrategias así como por las de nuevo desarrollo que se adopten en el camino. Dentro de este marco se ha propuesto la presente Tesis Doctoral.

OBJETIVOS

La relevancia de las proteasas ha crecido excepcionalmente en los últimos años. En este sentido, nuestro laboratorio se ha dedicado intensamente desde su inicio al estudio de los sistemas proteolíticos, incluyendo a la familia de adamalisinas. De hecho, muchos de sus miembros han sido identificados en trabajos previos de nuestro grupo. Desde entonces, los análisis funcionales han permitido describir el papel de algunas de estas proteasas en el organismo, si bien otras permanecen como enzimas "huérfanos" sin función conocida. En esta Tesis Doctoral nos hemos propuesto, en continuidad con las investigaciones previas, dilucidar el sentido biológico de algunas adamalisinas, estableciendo nuevos nexos con el cáncer y otras patologías, y explorar diversas posibilidades terapéuticas en torno a ellas.

Los objetivos concretos de este trabajo fueron los siguientes:

- Estudio de la implicación en cáncer de miembros de las ADAMs y ADAMTSs mediante análisis genéticos y epigenéticos y el empleo de modelos celulares.

- Generación y caracterización de un modelo murino deficiente en ADAMTS-12.

- Búsqueda de compuestos naturales con capacidad para inhibir ADAMTSs.

MATERIAL Y MÉTODOS

Material

Enzimas de restricción

Los enzimas de restricción, así como otros enzimas utilizados en la clonación molecular (polinucleótido quinasa de T4, ADN ligasa de T4, Taq ADN polimerasa, etc.) se adquirieron de New-England Biolabs, Roche Molecular Biochemicals, Amersham Biosciences, Invitrogen Life Technologies y TAKARA BIO Inc.

Reactivos de electroforesis

La separación de ADN en geles de agarosa se realizó utilizando agarosa de grado Biología Molecular de Roche Molecular Biochemicals. Para electroforesis de proteínas se empleó acrilamida/N,N'-metilenbisacrilamida, N,N,N',N'-tetrametiletilendiamina (TEMED) y persulfato amónico, todos ellos de BioRad.

Anticuerpos

Los anticuerpos empleados fueron los siguientes: anti-actina, anti-ERK, anti-VEGF, anti-vimentin y BC-3 de Abcam; anti-ADAMTS-12 H-142, anti-ADAMTS-15 H-135, anti-calgranulina y anti-hemopexina de Santa Cruz Biotechnology; anti-Akt, anti-fosfo Akt ser473 y anti-p44/42 MAPK de Cell Signaling; anti-CD31, anti-colágeno tipo IV, anti-FLAG M2 y anti-NG2 de Sigma Aldrich; anti-actina α de músculo liso (anti-αSMA) y anti-keratina de Dako; anti-S100A8 y anti-S100A9 de R&D Systems; anti-fosfo MET de Biosource International; anti-His-TAG de GE Healthcare; anti-MET DL-21 de Upstate; anti-mieloperoxidasa de Thermo Fisher Scientific; BC-14 de MD Biosciences; anti-E-cadherina, suministrado por la Dra. A. Cano, Instituto de Investigaciones Biomédicas, Madrid y anti-MT1-MMP, proporcionado por la Dra. A.G. Arroyo, Centro Nacional de Investigaciones Cardiovasculares, Madrid.

Reactivos para ensayos enzimáticos

El agrecano, la condroitinasa, la endo-β-galactosidasa y la transferrina se adquirieron en Sigma Aldrich. El ilomastat (GM6001), la curcumina, el resveratrol, la z-guggulsterona, el piceatanol, la luteolina y la genisteína fueron de Calbiochem. Los enzimas recombinantes MMP-2, MMP-13, ADAMTS-4 y ADAMTS-5, además de la IL-1α, la IL-1β y el péptido fluorogénico Mca-K-P-L-G-LDpa-A-R-NH_2 fueron comprados en R&D Systems. El kit SensoLyte® 520 Aggrecanase-1 fue adquirido en AnaSpec y el péptido fluorogénico Dabcyl-K-E-L-A-E-L-R-E-S-T-S-Glu en JPT Peptide Technologies.

Líneas celulares

Las líneas celulares de cáncer de colon, así como las líneas MDCK, 293-EBNA, A549 y CCD-18Co se adquirieron de la colección American Type Culture Collection (ATCC). La línea celular BAE-1 se obtuvo de la European Collection of Cell Cultures (ECACC). Los condrocitos ATDC5 fueron donados por el Dr. Oreste Gualillo, del Complejo Hospitalario Universitario de Santiago de Compostela. Las células PVDA procedían del laboratorio de la Dra. Agnès Nöel (*Laboratoire de Biologie des Tumeurs et du Développment*) en la Universidad de Lieja, Bélgica.

Muestras humanas

Las muestras de ADN y ARN de colon se obtuvieron del Institut Català d'Oncología, con la excepción de las muestras de ADN copia (ADNc) de colon normal y tumoral, adquiridos a Clontech. Las muestras de ADN genómico de melanoma procedieron del Surgery Branch of the National Cancer Insitute (EEUU). Los tejidos de colon empleados en inmunohistoquímica e inmunofluorescencia se obtuvieron del Banco de Tumores del Hospital Universitario Central de Asturias. Los neutrófilos se extrajeron a partir de sangre de donantes voluntarios sanos.

Animales de experimentación

Los ratones deficientes en *Adamts12* se generaron en nuestro laboratorio mediante la técnica de recombinación homóloga en células pluripotenciales embrionarias de ratón. Los ratones heterocigotos obtenidos se retrocruzaron durante al menos 10 generaciones con animales C57Bl/6J (Charles River), hasta obtener un fondo puro. Para los ensayos de tumorigenicidad *in vivo*, se emplearon ratones con inmunodeficiencia combinada severa (SCID, C.B-17/IcrCrl-scid-BR), también de Charles River. En los ensayos de angiogénesis con explantes aórticos, se utilizaron ratas Wistar (Charles River). Los animales de experimentación se criaron y mantuvieron en las instalaciones especializadas del animalario de la Universidad de Oviedo y todos los procedimientos se realizaron siguiendo los protocolos aprobados por el Comité de Experimentación Animal de dicha entidad. Finalmente, los animales se sacrificaron mediante asfixia por CO_2 o mediante dislocación cervical bajo anestesia y sus tejidos se extrajeron y se fijaron o se congelaron a -80 ºC hasta su uso.

Otro material

Las disoluciones tampón utilizadas (TE, SSC, TBE, TAE, solución Denhardt) se prepararon como se ha descrito anteriormente (Sambrook y Russel, 2001). El resto de material empleado en este trabajo se detalla en los siguientes apartados.

Técnicas de biología molecular

Técnicas básicas de biología molecular

Las técnicas básicas de biología molecular utilizadas en este trabajo que no se detallan (digestiones con enzimas de restricción, ligación de fragmentos de ADN, electroforesis en geles de agarosa, transferencia *Southern blot*, etc.) se realizaron según se describe en la bibliografía o siguiendo las instrucciones de las casas comerciales distribuidoras de los

distintos enzimas. Los oligonucleótidos utilizados fueron suministrados por Sigma-Aldrich.

Purificación de fragmentos de ADN

Los fragmentos de ADN, obtenidos por PCR y/o digestión con enzimas de restricción de los diferentes vectores recombinantes, se separaron por electroforesis en geles de agarosa en tampón TBE y se purificaron utilizando el kit NucleSpin Extract II, de Machery-Nagel.

Preparación de sondas radiactivas

Los fragmentos de ADN bicatenario utilizados como sondas se marcaron con [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/mL) mediante síntesis a partir de cebadores aleatorios utilizando el kit comercial Rediprime (GE Healthcare). A continuación, la mezcla de marcaje resultante se centrifugó a través de una columna de Sephadex G-25 (GE Healthcare) para retirar los nucleótidos no incorporados a la sonda.

Reacción en cadena de la polimerasa (PCR)

Los experimentos de PCR se efectuaron en termocicladores de Perkin Elmer (modelo 9700) y Applied Biosystems (modelo Verity), indistintamente. Para las reacciones, se utilizó la ADN polimerasa Taq-Platinum de Invitrogen, siguiendo las instrucciones del fabricante y ajustando las cantidades de reactivos para un volumen final de 20 μL. Los oligonucleótidos específicos para la amplificación de un ADN particular se diseñaron de acuerdo con la secuencia a amplificar. Los productos obtenidos se analizaron mediante electroforesis en geles de agarosa en TBE.

Transcripción reversa de ARN acoplada a PCR (RT-PCR)

Las RT-PCR semicuantitativas se llevaron a cabo mediante el kit de Invitrogen Thermoscript-RT-PCR, siguiendo las instrucciones indicadas y empleando hexámeros aleatorios como oligonucleótidos para la síntesis. Como molde y salvo indicación contraria, se empleó 1 μg de ARN total. El ADNc resultante se amplificó por PCR utilizando oligonucleótidos específicos.

Como control de carga e integridad se amplificó GAPDH bajo las mismas condiciones y los fragmentos generados se analizaron mediante electroforesis en geles de agarosa en TBE.

Análisis transcripcional mediante RT-PCR cuantitativa

El ARN se obtuvo de tejido congelado y de células en cultivo utilizando GIT-fenol-cloroformo y el kit comercial RNeasy Mini (QIAGEN). La RT-PCR se llevó a cabo como se ha descrito. Los análisis de expresión por RT-PCR cuantitativa de los genes seleccionados se realizaron usando ensayos de expresión Taqman de Applied Biosystems, en los sistemas de detección Applied Biosystems 7500HT Fast Real-Time PCR y Applied Biosystems 7300 Real-Time PCR System, siguiendo las instrucciones del fabricante. Los resultados se analizaron mediante cuantificación relativa frente al control endógeno β-actina empleando el Software SDS 1.4 y 2.1 de Applied Biosystems.

Secuenciación de ácidos nucleicos

Todas las reacciones de secuenciación capilar, tanto de productos de PCR como de plásmidos, se llevaron a cabo usando el kit DR terminator *Taq*FS y el secuenciador automático de ADN ABI-PRISM 310 (Applied Biosystems), con oligonucleótidos específicos como cebadores.

Extracción de proteínas

Los cultivos celulares se lavaron con PBS y se homogenizaron mediante la adición del volumen adecuado de un tampón Tris-HCl 20 mM, pH 7,4, NaCl 150 mM, Triton X-100 1%, EDTA 10 mM, que contenía cóctel completo de inhibidores de proteasas (Complete protease inhibitor cocktail, Roche Molecular Biochemicals) y los inhibidores de fosfatasas ortovanadato sódico 200 μM y β-glicerofosfato 1 mM. Los tejidos frescos o congelados de ratones se homogenizaron directamente mediante un homogenizador Ultraturrax T-8 (IKA) en volumen apropiado del tampón anterior. Los lisados celulares o de tejidos se sometieron a centrifugación a 12.000 rcf a 4 ºC para eliminar las partículas insolubles y se recolectaron los sobrenadantes para su

análisis. La concentración de proteínas de los sobrenadantes se determinó mediante la técnica del ácido bicinconínico (Pierce BCA Protein Assay Kit).

Electroforesis en geles de poliacrilamida-SDS (SDS-PAGE)

Las muestras proteicas se mezclaron con tampón de disociación (Tris-HCl 62,5 mM, pH 6,8, SDS 2%, glicerol 10% y azul de bromofenol 0,0005%) y se calentaron a 95 ºC durante 5 min antes de cargarlas en el gel. La electroforesis se realizó en tampón Tris-HCl 24,8 mM, glicina 192 mM, SDS 0,1%, pH 8,8 y a un voltaje constante de 200 V, hasta que el azul de bromofenol alcanzó el final del gel. Las proteínas se fijaron con ácido tricloroacético al 20% durante 5 min a 55 ºC y se tiñeron con azul de Coomassie al 0,25% en metanol:ácido acético:agua (45:10:45) durante 10 min a 55 ºC. Los geles se destiñeron con metanol:ácido acético:agua (10:7,5:82,5) a 55 ºC.

Análisis Western

Las muestras a analizar se separaron mediante electroforesis en un gel SDS-PAGE del porcentaje adecuado al tamaño de las proteínas a detectar (8-13%). El gel se incubó en tampón de transferencia (CAPS 10 mM, NaOH 4 mM, metanol 20%) durante 30 min. La transferencia a membranas de nitrocelulosa (Hybond ECL, Amersham Biosciences) o polifluoruro de vinilideno (PVDF) (Immobilon-P, 0,45 μM, BioRad) se realizó en el mismo tampón de transferencia durante 60 min a 50 V en un soporte Miniprotean II (BioRad). Salvo indicación expresa, las membranas se bloquearon en PT (PBS 1x, Tween-20 0,1%) con leche en polvo desnatada al 5% durante 1 h, tras lo cual la membrana se incubó de 1 a 12 h con el anticuerpo primario diluido en leche en polvo desnatada o albúmina bovina al 3% en PT. La membrana se lavó 3 veces durante 10 min con PT y, por último, se incubó 1 h con el anticuerpo secundario diluido en leche en polvo desnatada al 1,5% en PT. Tras lavar de nuevo con PT, se llevó a cabo la detección de los complejos antígeno-anticuerpo mediante generación de quimio-luminiscencia con un sustrato comercial (Millipore) y visualización en el LAS3000 mini (Fujifilm).

DiGE, digestión con tripsina y análisis MALDI-ToF

Para la electroforesis diferencial en gel (DiGE), el colon de los ratones se homogenizó manualmente en tampón TUCT (urea 7M, tiourea 2M, CHAPS 4% y tampón Tris-HCl 30 mM, pH 8,5). A continuación, 50 µg de cada muestra se marcaron con 400 pmol del fluoróforo específico (GE Healthcare): CyDye3 (muestra silvestre), CyDye5 (muestra mutante) y CyDye2 (mezcla equimolecular de ambos extractos). Tras añadir un volumen equivalente de tampón UCDA (urea 8 M, CHAPS 4%, DTT 130 mM y tampón IEF 2%), completando hasta 450 µL con tampón UCda (urea 8 M, CHAPS 4%, DTT 13 mM y tampón IEF 1%), la mezcla se cargó en el sarcófago de cerámica y se extendió por toda su superficie, sobre la que se colocó una tira de 24 cm de pH 3-11 no lineal (IPG strip, GE Healthcare). La separación de las proteínas se llevó a cabo aplicando un voltaje no constante durante 18 h a 20 °C. Posteriormente, las tiras de pH se equilibraron en SES-DTT durante 15 min (urea 6 M, glicerol al 30%, SDS al 2%, tampón Tris-HCl 75 mM pH 6,8, DTT al 0,5% y azul de bromofenol) y otros 15 min en SES-IA (SES con iodoacetamida al 4,5%) para facilitar el paso de las proteínas al gel de poliacrilamida-SDS al 13%, donde se separaron a voltaje constante y a 18 °C. Seguidamente, los geles se escanearon (Typhoon 9400; GE Healthcare) y las imágenes digitalizadas se analizaron con Progenesis SameSpots (Nonlinear Dynamics). Tras teñir los geles con SyproRuby (Molecular Probes), los puntos diferenciales se recortaron y digirieron con tripsina (12 ng/µL, Promega) durante 1 h a 60 °C. Los péptidos trípticos generados en cada muestra se purificaron a 18 °C con Zip-Tip C18 (Millipore) y se eluyeron con CHCA (ácido α-ciano-4-hidroxicinnámico) 10 mg/mL en acetonitrilo/TFA al 0,1% (50/50 v/v) sobre una placa de teflón para MALDI-ToF (Applied Biosystems). El análisis por espectrometría de masas se realizó utilizando ionización-desorción láser asistida por matriz (MALDI) en un equipo de espectrometría de masas de tiempo de vuelo (ToF) con una fuente láser de nitrógeno (Voyager-DE STR; Applied Biosystems). Para cada espectro se realizaron entre 50 y 200 disparos láser (error estándar ± 20 ppm), y los datos se analizaron con el programa Data explorer versión 4.0.0.0 de Applied Biosystems.

Ensayos enzimáticos

Para los ensayos de digestión de transferrina carboximetilada (Tf-Cm), la transferrina se carboximetiló con ácido iodoacético no radiactivo (Nagase, 1995). La Tf-Cm se empleó a una concentración de 60 µM en una solución tamponada para agrecanasas, compuesta por Tris-HCl 50 mM pH 8,5, NaCl 150 Mm, $CaCl_2$ 10 mM, NaN_3 0,02% y Brij 35 0,05%. Los enzimas se utilizaron a 50 nM, excepto MMP-7 que se empleó a 40 nM. La mezcla de reacción, en un volumen final de 15 µL, se incubó durante 16 h a 37 ºC. Para detener la digestión, se añadió una solución reductora para SDS-PAGE con EDTA 20 mM. Para los ensayos de actividad agrecanasa, se emplearon 20 µg de agrecano. El sustrato se incubó en solución para agrecanasas y con los distintos enzimas en un volumen final de 100 µL, durante 16 h y a 37 ºC. Para detener la reacción, se añadió a la mezcla un volumen idéntico de tampón para desglicosilación 2X (acetato sódico 200 mM, Tris-HCl 50 mM pH 6,8 y EDTA 100 mM). A continuación, las muestras se desglicosilaron con 0,01 U de condroitinasa y 0,01 U de keratanasa por cada 10 µg de agrecano, durante 16 h a 37 ºC. Las muestras se concentraron en una Speed-Vac y se resuspendieron en 20 µL de solución para SDS-PAGE. Los ensayos con los péptidos fluorogénicos se realizaron a una concentración de los enzimas de 10 µM y de 50 µM para el péptido Dabcyl-K-E-L-A-E-L-R-E-S-T-S-Glu y 10 µM para el Mca-K-P-L-G-LDpa-A-R-NH_2. La incubación se llevó a cabo a 37 ºC y a distintos tiempos. Las mediciones se realizaron en un espectrofotómetro LS55, de Perkin Elmer Life. La actividad de ADAMTS-4 se calculó mediante el kit para ensayos de actividad agrecanasa Sensolyte® 520, y siguiendo las instrucciones del fabricante.

Análisis mutacionales

Para el análisis mutacional en tejido normal y tumoral de colon, se secuenciaron todos los exones codificantes de *ADAMTS15* en 50 pacientes, siguiendo el protocolo previamente descrito (Sjoblom y col., 2006). Las secuencias obtenidas se analizaron con el programa Mutation Surveyor (SoftGenetics) y el efecto potencial de las mutaciones encontradas se predijo *in silico* empleando el algoritmo SIFT ("*Sorting Intolerant From Tolerant*") (Ng

y Henikoff, 2003). Para validar los cambios identificados, se resecuenciaron las muestras tumorales y sanas además de ADN de sangre del paciente. Para los estudios en melanoma, se secuenciaron los exones correspondientes a todos los genes *ADAM* en tejido sano y tumoral de un total de 31 pacientes. El protocolo empleado fue el mismo indicado anteriormente. En la primera fase del estudio se analizaron las secuencias utilizando las herramientas Consed, Polyphred 6.11 y DIPDetector (Gordon y col., 1998; Bhangale y col., 2006). Los genes en los que se encontraron mutaciones, se secuenciaron en tejido normal y tumoral de 48 pacientes adicionales, y los datos se analizaron mediante Mutation Surveyor.

Análisis del estado de metilación

La búsqueda de islas CpG y el diseño de oligonucleótidos para las PCR específicas de metilación y para la secuenciación de bisulfito, se realizaron con ayuda del programa Methyl Primer Express 1.0 (https://produtcs.appliedbiosystems.com), analizándose una región de unas 2 Kb en sentido 5' y 0,5 Kb en dirección 3' desde el sitio de inicio de la transcripción. La conversión del ADN genómico con bisulfito se llevó a cabo con el kit EpiTect Bisulfite de Qiagen. Para la secuenciación de bisulfito, los productos de PCR obtenidos se purificaron y se clonaron para su posterior secuenciación. Complementariamente, el nivel de metilación del promotor de *ADAMTS12* en un panel de tejidos normales y líneas tumorales se analizó mediante el chip de metilación Goldengate Methylation Array, de Illumina. Se emplearon para ello dos sondas localizadas a 250 pb por encima del inicio de la transcripción y 52 pb en sentido contrario. El nivel de metilación se calculó entre 0 y 1 a partir de la cantidad total de ADN metilado que hibridaba con cada sonda.

Análisis de expresión con microarrays

Para el análisis de la expresión génica en colon de ratón, se extrajo el ARN total utilizando TRIzol (Invitrogen) y siguiendo las instrucciones del fabricante. El ARN extraído se purificó con el kit RNeasy Mini, de Qiagen, y se calculó su concentración y calidad en un 2100 Bioanalyzer, de Agilent. Las

muestras de mejor calidad se seleccionaron para hibridar con el GeneChip Mouse Gene 1.0 ST (Affymetrix). Para analizar la calidad de los datos resultantes se utilizó el programa Expression Console, también de Affymetrix.

Técnicas de biología celular

Cultivos celulares y transfecciones

Salvo indicación contraria, todas las líneas celulares se cultivaron en medio DMEM suplementado con 10% de suero fetal bovino (FBS) y 1% de penicilina/estreptomicina/glutamina, todos ellos de Gibco. Las transfecciones se llevaron a cabo utilizando el sistema Lipofectamine de Invitrogen.

Aislamiento y cultivo de neutrófilos

Los neutrófilos humanos se purificaron a partir de sangre periférica de donantes sanos. Para ello, se extrajeron 4 mL de sangre en tubos con EDTA, de BD Vacutainer. Inmediatamente después, la sangre se mezcló en condiciones estériles con un volumen idéntico de solución salina. La sangre diluida se añadió a un tubo con 4 mL de Lymphoprep™ y se centrifugó durante 20 min a 800 rcf. Tras descartar el sobrenadante, el precipitado resultante se resuspendió en 12 mL de solución de lisis de eritrocitos (NH_4Cl 150 mM, $KHCO_3$ 10 mM, EDTA 0,1 mM). Al cabo de 10 min a temperatura ambiente, la mezcla se centrifugó durante 5 min a 400 rcf. Se eliminó el sobrenadante y las células se lavaron con 800 µL de solución de lisis de eritrocitos. El precipitado resultante del lavado, constituido esencialmente por neutrófilos, se resuspendió en medio RPMI. El aislamiento de neutrófilos murinos se llevó a cabo a partir de lavado peritoneal tras inducción con caseína. Para ello, se inyectó intraperitonealmente 1 mL de sal sódica de caseína bovina (Sigma Aldrich) al 9% en PBS. A la mañana siguiente, se realizó una segunda inyección con una dosis idéntica y 3 h después se sacrificaron los animales. A continuación, se llevó a cabo un lavado peritoneal con 8 mL de PBS. Las muestras obtenidas se centrifugaron y se

lavaron con PBS. El precipitado resultante se empleó para medir los niveles de anexina mediante citometría de flujo.

Ensayos de adhesión y migración

La capacidad de adhesión a distintos sustratos de la matriz extracelular se analizó con el kit colorimétrico ECM Cell Adhesion Array de EMD Biosciences, siguiendo las instrucciones indicadas. Para analizar la migración de las diferentes líneas y clones, 10^5 células se sembraron en pocillos con poros de 8 µm (BD Biosciences) y con medio sin suero. En la parte inferior del pocillo se añadió medio con FBS. Tras 24 h de incubación, las células que habían migrado se tiñeron y se contaron en el microscopio como se ha descrito en el apartado anterior.

Ensayos de invasión

Para estudiar la capacidad de invasión *in vitro*, se emplearon las cámaras de invasión adaptadas a placas de 24 pocillos, con 8 µm de poro y cubiertas con Matrigel de BD Biosciences. En cada ensayo se sembraron entre $5 \cdot 10^4$ y 10^5 células en medio sin suero. En la parte inferior de la cámara se añadió medio con 2% de FBS. Tras una incubación de 96 h, las células que habían alcanzado la superficie inferior de la cámara se tiñeron y contaron en el microscopio como se ha descrito previamente.

Ensayos de tubulogénesis en geles tridimensionales

Los ensayos de tubulogénesis se llevaron a cabo mediante el sistema de cultivo celular en geles tridimensionales de colágeno (Chemicon International) y siguiendo las instrucciones detalladas por el fabricante. Concretamente, $1{,}5 \cdot 10^3$ células MDCK se mezclaron con 200 µL de la mezcla de colágeno fría. A continuación, el gel se polimerizó a 37 ºC en placas de 24 pocillos. Se cubrió la mezcla con medio de cultivo enriquecido con 3% de FBS y 30 ng/mL de factor de crecimiento de hepatocitos (HGF). Se renovó el medio cada 2-3 días, y al cabo de 7 días se realizaron las fotografías microscópicas. En el caso de las células BAE-1, se utilizaron

$5x10^3$ células y VEGF como inductor y las fotografías se tomaron 2 días después del inicio del ensayo.

Co-cultivos celulares

En los co-cultivos entre fibroblastos CCD-18Co y líneas de cáncer de colon, se sembraron los fibroblastos a una confluencia de aproximadamente el 60-70% en placas de 6 pocillos. Sobre ellos, se colocaron insertos de 8 µm de poro (BD Biosciences) con las líneas de cáncer de colon también semi-confluentes. Se incubaron conjuntamente durante 36 h en medio de cultivo enriquecido con un 0,25% de FBS. Para el co-cultivo entre neutrófilos humanos y células 293-EBNA, los neutrófilos se añadieron en medio RPMI con un 10% de FBS sobre las células tumorales confluentes en placas de 66 pocillos, y se cultivaron conjuntamente durante 16 h.

Citometría de flujo

Para determinar el porcentaje de células vivas y de células apoptóticas se utilizó el kit Anexin V-FITC/IP de Sigma Aldrich. Para distinguir las distintas poblaciones celulares en sangre de ratón se empleó una combinación de anticuerpos específicos para citometría de flujo, todos ellos de BD Biosciences: anti-Ly-6G, anti-CD45, anti-CD45R, anti-CD3e, anti-Ly-6G y Ly-6C y anti F4/80. El citómetro empleado fue un Cytomics FC500, de Beckman Coulter.

Ensayos con animales de experimentación

Generación de tumores subcutáneos

Para la generación de tumores subcutáneos se emplearon animales SCID anestesiados con una mezcla de ketamina (50 mg/kg peso corporal) y xilacina (10 mg/kg peso corporal). Dependiendo del ensayo, se inyectaron bajo la piel de ambos flancos $6x10^6$ células A549 ó $7x10^6$ células SW620. A lo largo de los experimentos, se hizo un seguimiento rutinario para evaluar la apariencia y el estado de salud de los animales. Asimsimo, se midió el

tamaño de los tumores con un calibre, empleando como aproximación para el cálculo del volumen del tumor la fórmula $V=0,4 \cdot A \cdot B^2$, donde A y B son, respectivamente, la mayor y la menor de las medidas. El sacrificio de los animales se llevó a cabo cuando los tumores de mayor tamaño alcanzaron un volumen de aproximadamente 1 cm^3, o al cabo de un máximo de 8 semanas. Los tumores fueron extraídos y fijados inmediatamente después del sacrificio.

Extracción de explantes de cartílago

La acción de las agrecanasas se evaluó mediante la determinación de los niveles de glicosaminoglicanos liberados a partir de cartílago murino. Para ello, se extrajeron los cartilagos de la cabeza del fémur de ratones de 4 semanas de edad recién sacrificados. Los explantes obtenidos se cultivaron en medio DMEM enriquecido con 10% FBS. Dos días después, se sustituyó por medio sin suero y en presencia, en los casos indicados, de IL1α (10ng/mL) o ácido retinoico (10µM).

Evaluación de la angiogénesis

Para evaluar la capacidad de angiogénesis en los ratones deficientes en *Adamts12*, se emplearon 3 modelos distintos, todos ellos realizados en colaboración con el grupo de la Dra. Agnès Noël (Universidad de Lieja, Bélgica): trasplante de células PDVA, inyección subcutánea de matrigel y cultivo de explantes aórticos.

Trasplante de células PDVA

La línea celular PDVA procede de keratinocitos murinos transformados como consecuencia de un tratamiento carcinogénico (Fusenig y col., 1983). Para cada trasplante se emplearon $2 \cdot 10^5$ células, que fueron pre-cultivadas en una mezcla de colágeno tipo I de rata (aislado a partir de tendones de la cola y a una concentración de 4 mg/mL) y cubiertas por cámaras de silicona de Renner. A continuación, el conjunto se implantó en el tejido conjuntivo del músculo dorsal de cada ratón, donde permaneció 3 semanas hasta el sacrificio.

Inyección subcutánea de Matrigel

Para este ensayo se inyectaron en la zona abdominal de los ratones geles de 500 µL de Matrigel con heparina (10 U/mL) y factor de crecimiento de fibroblastos básico (bFGF, 250 ng/mL). El sacrificio de los ratones y la extracción de los implantes se efectuó una semana después. Tras disgregar los geles extraídos, la cuantificación de la angiogénesis se llevó a cabo disolviendo en solución de Drapkin (Sigma Aldrich) y midiendo la absorbancia a 560 nm en un espectrofotómetro.

Cultivo de secciones de aorta en tres dimensiones

Se sacrificaron los animales (ratones o ratas) y se extrajeron las aortas, que se cortaron en secciones de 1 mm de longitud con forma de anillo. Estos explantes se incluyeron en un gel de colágeno intersticial de cola de rata a 1,5 mg/mL, preparado mezclando 7,5 volúmenes de colágeno 2 mg/mL (Serva), 1 volumen de 10x MEM (Minimun Essential Medium, Life Technologies), 1,5 volúmenes de $NaHCO_3$ 15,6 mg/mL y 0,1 volúmenes de NaOH 1 M para ajustar el pH a 7,4. Los geles de colágeno conteniendo los anillos de aorta se polimerizaron en placas de cultivo de 6 cm de diámetro a 37 ^{o}C, y se añadieron 5 mL de medio MCDB-131 (Life Technologies) suplementado con $NaHCO_3$ 25 mM, suero de ratón al 2,5% y penicilina/estreptomicina/glutamina. Los anillos se mantuvieron en cultivo durante una semana, observándose el crecimiento de capilares en el microscopio cada 2 días.

Modelo de colitis ulcerosa

La inducción de colitis aguda se llevó a cabo mediante administración de dextransulfato sódico (DSS) al 2% en el agua de bebida durante 7 días, seguidos de un día de recuperación en el que se suministró a los animales agua normal. En el protocolo de colitis crónica, se administró DSS al 5% durante 10 días, seguidos de 15 días de recuperación con agua normal. A lo largo de todo el ensayo, se monitorizó el estado general de los animales y la progresión de la enfermedad, asignándose una puntuación en función de los síntomas específicos: 0 para heces normales, 1 para heces blandas, 2 para heces muy blandas con trazas de sangre y 3 para los casos de diarrea grave

y hemorragia rectal. Al final de cada protocolo se sacrificaron los animales y su colon fue extraído para análisis posteriores.

Modelo de choque séptico

En este protocolo, se inyectó lipopolisacárido bacteriano (LPS, Sigma Aldrich) en solución salina, intraperitonealmente y a una concentración de 8,5 µg/kg de peso corporal. A los controles se les inyectó solución salina únicamente. Se evaluó diariamente el peso corporal y la apariencia general de los animales. En los días 2 y 8 después del tratamiento, parte de los animales fueron anestesiados con ketamina/xilacina para extraer muestras de sangre mediante punción cardíaca. Las muestras sanguíneas se emplearon para determinar las proporciones de leucocitos mediante citometría de flujo. A continuación, los animales fueron sacrificados y se les extrajo el hígado y bazo para análisis posteriores.

Modelo de pancreatitis aguda

Para la inducción de pancreatitis se inyectó intraperitoneamente a los animales ceruleína (Sigma Aldrich) disuelta en solución salina y a una concentración de 50 µg/kg peso corporal. Los animales recibieron una inyección cada hora durante un total de 10 h. Los animales control fueron inyectados con solución salina. Una hora después de la última administración se anestesió a los animales con ketamina/xilacina para extracción de sangre por punción cardíaca. Las muestras de sangre se emplearon en la valoración enzimática de los niveles de lipasa y amilasa. A continuación, se procedió al sacrificio y extracción del páncreas.

Ensayo de cicatrización de heridas

La capacidad de cicatrización de los animales se valoró midiendo el cierre de heridas cutáneas. Para ello, los animales se anestesiaron mediante inhalación de isofluorano y se les afeitó y desinfectó la región dorsal. A continuación se llevaron a cabo dos incisiones en la piel de ambos flancos con ayuda de un sacabocados de 8 mm (Acu-punch de Acuderm). Se hicieron fotos de las heridas en los días 0, 1, 3, 5, 7 y 9, con una regla como

referencia. A partir de las fotos y con ayuda del programa ImageJ se calculó para cada animal y día el porcentaje de herida abierta respecto del área inicial. Finalmente, lo animales se sacrificaron por grupos para recoger a distintos tiempos las muestras de piel con las heridas, que se emplearon en análisis histológicos.

Estudios histológicos

Inmediatamente tras la extracción, todas las muestras de tejidos se fijaron mediante inmersión en formaldehido al 4% en solución salina y durante 24 h. Tras la fijación, las muestras se lavaron con etanol 70% y se incluyeron en bloques de parafina. Las secciones obtenidas a partir de los bloques se tiñeron con hematoxilina/eosina y el análisis de la histología de los distintos tejidos corrió a cargo de un patólogo experto.

Análisis inmunohistoquímicos

Los tejidos en los que se llevó a cabo una detección inmunohistoquímica se fijaron en formaldehido al 4% disuelto en PBS y se embebieron en parafina. Los bloques de parafina se cortaron en secciones de 5 µm y las secciones se desparafinaron tras un paso sucesivo por alcoholes de menor graduación hasta llegar a agua. Dichas secciones se incubaron con tampón de bloqueo de la peroxidasa de Dako y albúmina sérica bovina (BSA) al 1%. A continuación, las muestras se incubaron toda la noche a 4 ºC con los correspondientes anticuerpos primarios diluidos en PBS. Seguidamente, los anticuerpos primarios se incubaron con los anticuerpos secundarios (EnVision, Dako) durante 30 min a temperatura ambiente, se lavaron y se revelaron con el sustrato diaminobenzidina. Las muestras se contratiñeron con hematoxilina de Mayer y se cubrieron en presencia de medio Entellan, también de Dako. Alternativamente, las tinciones inmunohistoquímicas se llevaron a cabo con el sistema automatizado Discovery XT System, de Ventana, y los reactivos diseñados para el mismo.

Análisis inmunofluorescentes

Los tejidos y células empleados directamente se fijaron con formaldehido 4% y se permeabilizaron con Triton X-100 0,5% durante 5 min. Las muestras previamente incluidas en parafina, se desparafinaron como se ha descrito y se trataron con Triton X-100 0,1% para permeabilizar y con glicina 0,1 M para disminuir la autofluorescencia de la muestra. Las condiciones de bloqueo y de incubación con los anticuerpos se optimizaron para cada una de las tinciones llevadas a cabo, y se detallan en el apartado de Resultados.

Análisis bioinformático y estadístico

Los datos obtenidos del análisis de expresión génica se analizaron con ayuda de la plataforma Babelomics (http://babelomics.bioinfo.cipf.es). En concreto, se empleó esta herramienta para determinar posibles enriquecimientos en las muestras evaluadas de genes con función relacionada. Para la cuantificación del cierre de heridas en el modelo de cicatrización, así como para la valoración de algunos parámetros histológicos a partir de fotos microscópicas, se empleó el programa ImageJ. Los análisis estadísticos se realizaron con el programa Prism (GraphPad). Para la comparación de medias entre muestras con distribución normal se utilizó el test *t* de Student. Cuando las poblaciones no se ajustaban a una distribución gaussiana se empleó la prueba *U* de *Mann-Whitney*. En los estudios en los que se consideró más de una variable se realizó un ANOVA. Finalmente, para comparar frecuencias entre clases se empleó la prueba χ^2. Los p-valores inferiores a 0,05 se consideraron estadísticamente significativos.

RESULTADOS

I. Revisión sobre la metaloproteasa ADAMTS-12

El interés por el estudio del mundo proteolítico ha generado una intensa actividad científica, dando lugar a una inmensa cantidad de trabajos en las últimas décadas. De hecho, si exploramos la literatura científica existen más de 380.000 artículos con referencias a proteasas, datando los primeros de hace casi un siglo. Para facilitar el estudio del degradoma, el *Handbook of Proteolytic Enzymes*, editado por Alan J. Barret, J. Fred Woessner y Neil D. Rawlings, es una obra de referencia que funciona como un manual completo y exhaustivo con información actualizada de todos los grupos de enzimas proteolíticos descritos. Juzgando su relevancia y utilidad, nos pareció interesante participar en este proyecto escribiendo el capítulo correspondiente a la metaloproteasa ADAMTS-12, un enzima ligado estrechamente a nuestro laboratorio. Para ello, hemos elaborado una revisión basándonos en toda la información disponible sobre este enzima desde su identificación y clonación en 2001.

Artículo 1: **Ángela Moncada-Pazos**, Carlos López-Otín y Santiago Cal. “ADAMTS-12”.

Handbook of Proteolytic Enzymes, 3ra edición. Editores: A. J. Barrett, N. D. Rawlings and J. F. Woessner. Editorial Academic Press (Londres); 2011. En prensa.

Aportación personal al trabajo

Para la realización de este trabajo compartí, junto con el Dr. Santiago Cal, la tarea de recopilar y organizar toda la información disponible acerca de la metaloproteasa ADAMTS-12. Además, participé en la elaboración del manuscrito bajo la supervisión del Dr. Carlos López-Otín.

ADAMTS-12

Angela Moncada-Pazos, Carlos López-Otín and Santiago Cal

Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología, IUOPA. Universidad de Oviedo. 33006-Oviedo, Spain

Corresponding author: Carlos López-Otín. Departamento de Bioquímica y Biología Molecular. Universidad de Oviedo. C/ Fernando Bongera s/n. 33006-Oviedo, Spain. Telephone: +34 98 5104201. Fax: +34 98 5103564. E-mail: clo@uniovi.es

Name and History

ADAMTS-12 belongs to the *A Disintegrin And Metalloprotease Domains with ThromboSpondin motifs* (ADAMTS) family, which consists of 19 secreted zinc metalloproteinases which have been implicated in a broad range of biological functions related to extracelullar matrix (ECM) assembly [1, 2]. Thus, different members have been associated with processes such as tissue remodelling, ovulation, angiogenesis or blood clotting. *ADAMTS12* cDNA was originally cloned from a human fetal lung cDNA library and its expression was also detected in human fetal fibroblasts following treatment with TGF-β [3]. It was also found that this metalloprotease was attached to the extracellular matrix upon secretion. *ADAMTS12* was also found widely expressed in gastrointestinal and pancreatic carcinomas but not in the paired normal tissues, suggesting that this enzyme could also participate in the development and/or progression of tumors from different origins [3].

Activity and specifity

Different studies have suggested a role for ADAMTS-12 in tissue remodelling processes during physiological conditions, and in cell adhesion or migration. However, little is known regarding its substrate specificity. Thus, ADAMTS-12 shows aggrecan-degrading activity, similarly to aggrecanases ADAMTS-4 and ADAMTS-5, and to other members of the family such as ADAMTS-1, -9, -15, -16 and -18 [4]. Nevertheless, the ability of ADAMTS-12 to degrade aggrecan is reduced and it has only been reported to occur *in vitro* [5]. It has also been shown how ADAMTS-12 displays cartilage oligomeric matrix protein (COMP)-degrading activity [6, 7], which could relate ADAMTS-12 with arthritic diseases. In relation to potential endogenous inhibitors, it has been described that α_2–macroglobulin and the granulin-epithelin precursor, a growth factor highly expressed in chondrocytes, can interact with ADAMTS12, leading to an inhibition of its COMP-degrading activity [8].

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Structural chemistry

ADAMTS-12 shares a structural multidomain complex architecture with the rest of members of the family. This domain organization includes a signal peptide, a pro-domain involved in maintaining enzyme latency and a catalytic domain that contains the consensus sequence HEXXHGXXHD involved in the coordination of the zinc atom necessary for catalytic activity of the enzyme. This sequence ends in an Asp residue which distinguishes ADAMTSs from other metalloproteases such as MMPs. This catalytic region is followed by a disintegrin-like domain, a central thrombospondin-1 (TSP-1) motif, a cysteine-rich domain, a spacer region and a variable number of TSP-1 repeats, three in the case of ADAMTS-12 (Figure 1). A structural hallmark of ADAMTS-12 is the presence of a second spacer region ending in four additional TSP-1 repeats [3].

Preparation

Recombinant ADAMTS-12 with a FLAG epitope at the carboxy-terminal region can be obtained form 293-EBNA cells stably transfected with a pCEP plasmid containing the full-length cDNA of the metalloprotease [5]. These cells can be scraped from culture plate, suspended in a buffer containing 50 mM Tris-HCl pH 7.4, and 500 mM NaCl and rotated for 2 hours at 4 ºC. Then, the cell-layer-associated fraction diluted 1:1 (v/v) in the same buffer but lacking NaCl, can be separated from the rest of the cell components by centrifugation. Then, supernatant is loaded onto an anti-FLAG affinity gel column and the recombinant ADAMTS-12 eluted using and a FLAG peptide. Purification process may be assessed by western blot using an anti-FLAG-M2 antibody.

Biological aspects

Functional relevance of ADAMTS-12 can be inferred from those studies aimed at evaluating the implication of this metalloprotease in tumor progression or in arthritic diseases. Most remarkable aspects are the following:

ADAMTS12 and cancer.

Different studies have revealed that *ADAMTS12* acts as a tumor-suppressor gene. Thus, it has been shown how the exogenous expression of *ADAMTS12* in Madin-Darby canine kidney (MDCK) cells hinders the tumorigenic effects of the hepatocyte growth factor through the blocking of the Ras-MAPK signalling pathway [5]. Moreover, the presence of this metalloprotease prevents the ability of bovine aortic endothelial cells to form tubules upon stimulation with VEGF, which could be associated with an angio-inhibitory effect of ADAMTS-12. Additionally, tumors induced in immunodeficient SCID mice using A549 lung adenocarcinoma cells displayed a remarkable growth deficiency when overexpressing ADAMTS-12 in comparison with animals injected with A549 parental cells, supporting the role of this enzyme as an anti-tumor protease. These data have also pointed out the relevance of the TSP-1 repeats in these processes as exosites within the metalloprotease structure. Thus, when MDCK cells expressed a truncated form of ADAMTS-12 lacking the TSP-1 repeats, they did not show the anti-tumorigenic effects described for MDCK cells expressing the entire protein. Consequently, the metalloprotease and disintegrin domains of ADAMTS-12 are insufficient to elicit the tumor-suppressor properties of this metalloprotease [5].

Recent studies have reinforced the role of ADAMTS-12 as a tumor-suppresor enzyme. Thus, it has been found how *ADAMTS12* is epigenetically silenced in tumor cells from different sources [9]. In particular, methylation levels of *ADAMTS12* promoter were very high in a colon cancer sample panel that included both cancer cell lines and tumor samples, whereas it was found not or barely methylated in normal cells and tissues. Moreover, colon cancer cell lines did not show appreciable levels of *ADAMTS12* expression, unless cells were treated with demethylating agents. However and similar to what has been found in gastrointestinal and pancreatic carcinomas, *ADAMTS12* expression was higher in colon tumor samples cells than in normal tissues. The explanation for this situation resides in that ADAMTS-12 is produced by the stromal cells surrounding neoplastic cells and not by the tumor cells themselves, which was confirmed using different approaches. For instance, immunofluorescence techniques allowed the localization of this protease in the proximity to alpha smooth muscle actin positive cells, which suggests that cancer-associated fibroblasts could be

responsible for *ADAMTS12* expression. By contrast, ADAMTS-12 staining resulted negative in the case of tumor cells. *ADAMTS12* expression in fibroblasts was verified through the use of co-cultures of colon fibroblasts with colon cancer cells. In fact, these fibroblasts showed a 2-fold to 5-fold increase in *ADAMTS12* expression when co-cultured with colon cancer cells that when cultured alone. Furthermore, this expression could be associated with a functional effect as colon cancer cells showed minor growth rates and an increase in apoptosis when co-cultured with colon fibroblasts in comparison to the colon tumor cell line cultured alone. Consequently, *ADAMTS12* expression in colon fibroblasts could be part of a protective response aimed to compensate for the epigenetic silencing of this gene in tumor cells [9]. Taken collectively, these results emphasize the relevance of ADAMTS-12 as a new member of the growing category of proteases with anti-tumor properties [10].

Phenotypic analysis of the *Adamts12*-deficient mouse has confirmed the role of this metalloprotease as a tumor-protective enzyme [11]. This mouse develops normally and does not show any obvious phenotype. However, different models to analyze the angiogenesis process *in vivo*, including malignant keratinocyte transplantation, aortic ring assay and Matrigel plug, supported that this protease exhibits anti-angiogenic properties. Additionally, both intact ADAMTS-12 and a catalytically inactive form of ADAMTS-12 showed a similar ability to inhibit the spreading of endothelial cells. These data were in line with the previous results indicating that antitumor functions of ADAMTS-12 do not depend on its metalloprotease domain [5].

ADAMTS12 and arthritis.

Aggrecan degradation constitutes a hallmark in the pathology of arthritis [12]. In this regard, two ADAMTSs, ADAMTS-4 and ADAMTS-5, are the two major aggrecanases and their activities play an important role in the destruction of articular cartilage, thus contributing to the severity of disease [13]. Nevertheless, aggrecan degradation is not the only key event that can be associated with arthritis. For instance, degradation of COMP has been linked to the pathogenesis of arthritis as its cleaving products have been identified in synovial fluid and serum of patients suffering from rheumatoid arthritis and osteoarthritis [14]. Consequently, the identification of those enzymes responsible for COMP degradation constitutes a challenge to shed light on the

mechanisms underlying the progress of the disease and to develop new therapeutic strategies. In this regard, two different studies have pointed out how *ADAMTS12* expression is upregulated in osteoarthritis [15, 16]. It has been also shown that ADAMTS-12 can degrade COMP [6, 7]. Furthermore, α_2-macrobulin can inhibit COMP degradation by ADAMTS-12 and the mechanism underlying this inhibitory effect resembles that described to block both ADAMTS-4 and ADAMTS-5 aggrecan-degrading activities [17], which consists in the cleavage of α_2-macroglobulin bait region resulting in enzyme entrapment. These data clearly support the role of ADAMTS-12 as a metalloprotease involved in the development of arthritic diseases.

In summary, different studies performed to characterize *ADAMTS12* indicate that its loss-of-function may favour tumor growth or progression, whereas its gain-of-function can be involved in the development of arthritic diseases. The recent generation of the mouse deficient in this gene [11], constitutes a valuable tool to better understand the role of this specific metalloprotease in cancer and pathogenesis of arthritis.

Distinguishing Features

Two aspects can be considered characteristic of the ADAMTS-12 regarding to its structure and biology respectively. As has been previously indicated, domain organization of this metalloprotease shows a second spacer region followed by four more TSP-1 repeats, which has been only described for an ADAMTS-7 isoform [18], but not for the rest of the members of the family. And, concerning expression patterns, *ADAMTS12* expression is higher in tumor samples than in normal tissues. However this elevated expression can not be associated to tumor progression. In fact, the stromal cells surrounding the malignant cells are responsible for this expression, which could form part of a mechanism that prevents tumor progression.

Related Peptidases

Attending to different structural features, MMPs, ADAMs and the rest of ADAMTSs are metalloproteases related with ADAMTS-12. Particularly, all of them are

extracelullar proteases that show a consensus sequence at their catalytic region with three histidine residues necessary to bind zinc, which is necessary for their activity. But, among these enzymes, ADAMTS-7 is the closest partner of ADAMTS-12 not only due to its second spacer domain and the second set of TSP repeats identified in these metalloproteases but also because of functional aspects. Thus, it has been described that COMP can mediate in the binding of these two particular ADAMTSs to the chondrocytes surface. It is noteworthy that the four C-terminal thrombospondin domains of ADAMTS-7 and ADAMTS-12 are required for the interaction with COMP, highlighting the role of the thrombospondin repeats as mediators in the recognition of possible ADAMTS substrates or cofactors [6, 19]. Interestingly, treatment of cartilage explants with the inflammatory cytokines TNF-α and IL-1β increased COMP degradation fragments [20]. However, these fragments are not detected in the presence of anti-ADAMTS-7 and anti-ADAMTS-12 antibodies, suggesting that these two ADAMTSs may be active elements in the link between the inflammatory response mediated by these cytokines and the degradation of ECM components.

Further reading

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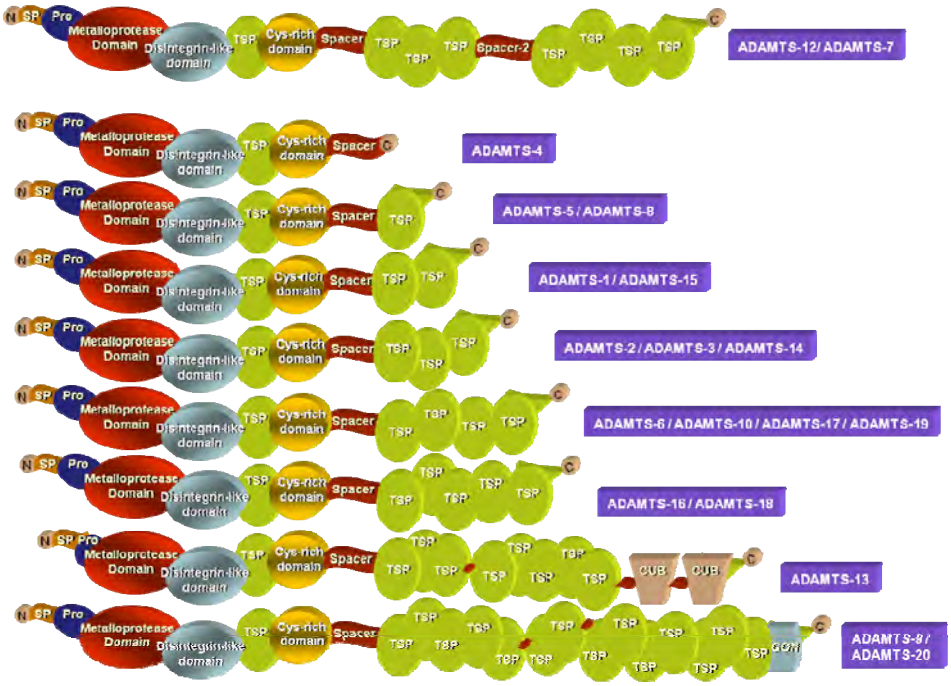

Domain organization of ADAMTS-12. Structures of the main domains from the remaining members of the ADAMTS family are also shown. N and C indicate the amino-terminus and carboxy-terminus of the protein, respectively; SP, signal peptide; Pro, prodomain (shorter in the case of ADAMTS-13); TSP, thrombospondin type 1; Cys-rich, cysteine-rich; CUB, for complement C1r/C1s, Uegf, Bmp1; GON, similar to the GON1 protein in *Caenorhabditis elegans.*

II. Estudio del papel de la metaloproteasa ADAMTS-12 en cáncer mediante el empleo de modelos celulares

Desde la identificación de los primeros miembros de la familia ADAMTS este grupo de metaloproteasas ha cobrado una notable relevancia debido a su intervención en numerosos procesos fisiológicos y patológicos incluyendo el cáncer. En concreto, se ha descrito que los dominios trombospondina funcionan en muchos de ellos como supresores tumorales inhibiendo la angiogénesis. Con estos antecedentes, nos planteamos el intentar dilucidar las propiedades de ADAMTS-12, la primera de las 9 ADAMTSs identificadas por nuestro laboratorio y de función desconocida. Como primer paso para avanzar en este propósito, empleamos la línea celular MDCK como modelo *in vitro* para evaluar sus propiedades en el contexto tumoral. Paralelamente, analizamos su efecto sobre células endoteliales y desarrollamos una primera aproximación *in vivo* a través del trasplante de la línea tumoral A549 en ratones SCID. Como resultado, pudimos describir el comportamiento anti-tumoral de esta proteasa.

Artículo 2: María Llamazares, Álvaro J. Obaya, **Ángela Moncada-Pazos**, Ritva Heljasvaara, Jesús Espada, Carlos López-Otín y Santiago Cal. "The ADAMTS12 metalloproteinase exhibits antitumorigenic properties through modulation of the Ras-dependent ERK signalling pathway".

Journal of Cell Science 120: 3544-3552 (2007).

Aportación personal al trabajo

Me incorporé a este proyecto a mi llegada al laboratorio, sirviéndome para iniciarme en las técnicas básicas de cultivos celulares, microscopía y de identificación de proteínas. En concreto, contribuí en este articulo realizando tareas de establecimiento y mantenimiento de las líneas celulares, participé en los ensayos *in vivo* sobre ratones inmunodeficientes y colaboré en la redacción del manuscrito del trabajo.

3544 Research Article

The ADAMTS12 metalloproteinase exhibits anti-tumorigenic properties through modulation of the Ras-dependent ERK signalling pathway

María Llamazares[1,*], Alvaro J. Obaya[2], Angela Moncada-Pazos[1], Ritva Heljasvaara[1,‡], Jesús Espada[1], Carlos López-Otín[1] and Santiago Cal[1,§]

[1]Departamento de Bioquímica y Biología Molecular and [2]Departamento de Biología Funcional (Fisiología), Instituto Universitario de Oncologia, Universidad de Oviedo, 33006-Oviedo, Asturias, Spain

*Present address: Institute for Molecular and Tumor Research, Philipps-Universität, Marburg, Germany
‡Present address: Biocenter Oulu and Department of Medical Biochemistry and Molecular Biology, University of Oulu, Finland
§Author for correspondence (e-mail: santical@uniovi.es)

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Summary

Members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of proteolytic enzymes are implicated in a variety of physiological processes, such as collagen maturation, organogenesis, angiogenesis, reproduction and inflammation. Moreover, deficiency or overexpression of certain ADAMTS proteins is directly involved in serious human diseases, including cancer. However, the functional roles of other family members, such as ADAMTS12, remain unknown. Here, by using different in vitro and in vivo approaches, we have evaluated the possible role of ADAMTS12 in the development and progression of cancer. First, we show that expression of ADAMTS12 in Madin-Darby canine kidney (MDCK) cells prevents the tumorigenic effects of hepatocyte growth factor (HGF) by blocking the activation of the Ras-MAPK signalling pathway and that this regulation involves the thrombospondin domains of the metalloproteinase. We also show that addition of recombinant human ADAMTS12 to bovine aortic endothelial cells (BAE-1 cells) abolishes their ability to form tubules upon stimulation with vascular endothelial growth factor (VEGF). Additionally, tumours induced in immunodeficient SCID mice injected with A549 cells overexpressing ADAMTS12 show a remarkable growth deficiency in comparison with tumours formed in animals injected with parental A549 cells. Overall, our data suggest that ADAMTS12 confers tumour-protective functions upon cells that produce this proteolytic enzyme.

Key words: Cell-cell adhesion, Hepatocyte growth factor, Cell migration, E-cadherin

Introduction

The human ADAMTS family comprises 19 structurally complex metalloproteinases containing a variable number of type-1 thrombospondin (TSP-1) domains in the region of their carboxy-termini (Cal et al., 2002; Llamazares et al., 2003; Porter et al., 2005). Functional studies have demonstrated the participation of these enzymes in a variety of processes, such as collagen maturation, organogenesis, proteoglycan degradation, inhibition of angiogenesis, reproduction and inflammation. Furthermore, deficiency or overexpression of specific ADAMTS proteins is directly involved in serious human diseases. Human ADAMTS proteins can be classified into seven subfamilies in accord with their structural and biochemical features. Notably, ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8 and ADAMTS9 are able to cleave cartilage aggrecan and can contribute to cartilage degradation in osteoarthritic diseases (Porter et al., 2005). In particular, ADAMTS5 is now considered to be the major functional aggrecanase because mice deficient in this enzyme show a marked reduction in the severity of cartilage destruction in comparison with wild-type mice (Glasson et al., 2005; Stanton et al., 2005). ADAMTS2, ADAMTS3 and ADAMTS14 are aminoprocollagen peptidases (Colige et al., 2005; Colige et al., 2002; Wang et al., 2003), and ADAMTS2 deficiency causes Ehlers-Danlos syndrome VIIc in humans and dermatosparaxis in animals, a disease characterized by a decrease in the tensile strength and integrity of the skin, joints and other connective tissues (Colige et al., 1999). ADAMTS13 is the proteinase responsible for cleaving von Willebrand factor, and mutations in the *ADAMTS13* gene cause a severe blood disease named thrombotic thrombocytopenic purpura (Levy et al., 2001). ADAMTS9 (Clark et al., 2000; Somerville et al., 2003) and ADAMTS20 (Llamazares et al., 2003; Somerville et al., 2003) are named the GON-ADAMTS proteins as their gene sequences show a high degree of sequence similarity with the *Caenorhabditis elegans gon-1* gene, which is essential for formation of gonads (Blelloch et al., 1999). Mutations in *ADAMTS10* cause an autosomal recessive disorder of connective tissue called Weill-Marchesani syndrome (Dagoneau et al., 2004). ADAMTS1 and ADAMTS8 are potent angio-inhibitory enzymes (Vazquez et al., 1999), and *Adamts1*-deficient mice display altered morphology and function of organs and reduced fertility (Shindo et al., 2000). It is remarkable that certain members of this metalloproteinase family are dysregulated in tumours of varying origins. These include ADAMTS6 and ADAMTS18 in breast cancer (Porter et al., 2004), ADAMTS19 in osteosarcoma (Cal et al., 2002), ADAMTS20 in breast and colon carcinomas (Llamazares et

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al., 2003) and ADAMTS4 and ADAMTS5 in glioblastomas (Held-Feindt et al., 2006).

Despite significant advances in our understanding of the biological functions of some of these enzymes, the roles of other family members, such as ADAMTS12, remain unknown. The ADAMTS12 metalloproteinase was originally identified and cloned in our laboratory by using human foetal lung cDNA samples (Cal et al., 2001) and has been proposed to participate in the initiation and progression of arthritis through its ability to degrade cartilage oligomeric matrix protein (Kevorkian et al., 2004; Liu et al., 2006). However, at present, no data are available concerning a putative role for ADAMTS12 in the development and progression of cancer. To this end, we have analyzed the effects of the exogenous expression of ADAMTS12 in MDCK cells. This cell line constitutes a good model system to study changes in cellular phenotype because it undergoes striking morphological changes, from cobblestone to long-spindle shapes, following HGF treatment, an effect known as 'scattering' (Weidner et al., 1990). Moreover, MDCK cells grown in 3D collagen gels form cyst structures, and HGF stimulation induces tubulogenesis (O'Brien et al., 2002), a phenotype relevant to renal development and carcinogenesis. Early in tubule development, cells form long invasive extensions that represent a partial epithelial-mesenchymal transition (EMT) (O'Brien et al., 2004). The morphological changes that accompany the EMT occur through activation of Met, the HGF receptor, switching on a complex signalling pathway that finally leads to downregulation of E-cadherin, upregulation of vimentin and secretion of several extracellular proteinases that are essential for matrix remodelling (Birchmeier et al., 2003).

Here, we report that expression of ADAMTS12 in MDCK cells prevents the tumorigenic effect caused by HGF stimulation and show that this effect derives from the inhibition of components of the Ras-MAPK signalling pathway. We also show that addition of recombinant human ADAMTS12 to bovine aortic endothelial cells (BAE-1 cells) abolishes their ability to form tubules upon stimulation with VEGF. Finally, we report that tumours induced in immunodeficient mice injected with A549 cells overexpressing ADAMTS12 exhibit a marked growth deficiency in comparison with tumours formed in mice injected with control A549 cells. Overall, our data indicate that ADAMTS12 could act as an angio-inhibitory proteinase with the ability to confer anti-tumorigenic properties to epithelial or endothelial cells.

Results

Generation of stable MDCK clones expressing ADAMTS12

MDCK cells were transfected with the full-length cDNA for ADAMTS12 (Fig. 1A), and the expression of the recombinant protein in the stable clones was examined by both RT-PCR and western blot analysis (Fig. 1B). These analyses allowed us to detect the expression of ADAMTS12 in MDCK clones TS12-4 and TS12-22. A specific band of molecular mass ~175 kDa was visualized by western blot using antibodies against ADAMTS12 and the FLAG epitope (Fig. 1B, and data not shown). Additionally, immunocytochemical analysis of these clones with the antibody against FLAG allowed the ADAMTS12 protein to be localized on the cell surface (Fig. 1C). Likewise, and consistent with previous studies (Cal et al., 2001), the entire protein was not detected in the cell medium. The MDCK clone stably transfected with the empty pCEP vector (cMDCK) did not show immunoreactivity for the antibodies against both ADAMTS12 and FLAG in these assays, and it was subsequently used as a negative control. Clones TS12-4 and TS12-22 were chosen for further evaluation of the putative phenotypic changes accompanying ADAMTS12 expression in MDCK cells.

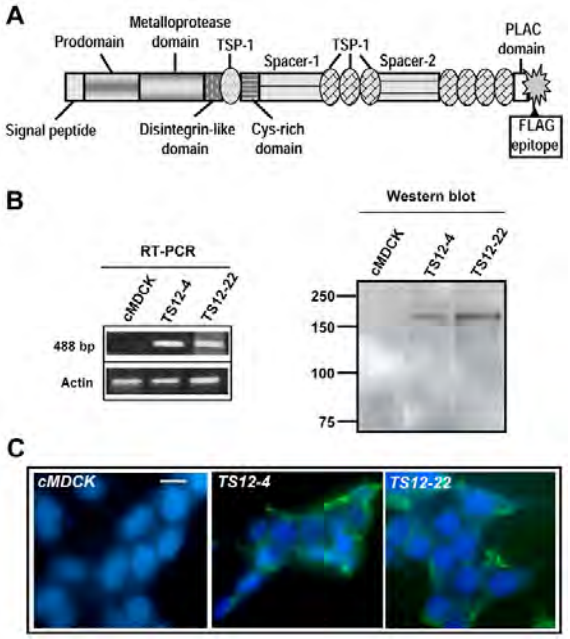

Fig. 1. Characterization of MDCK ADAMTS12 stable transfectants. (A) Domain organization of ADAMTS12 showing the position of the FLAG epitope. (B) RT-PCR and western blot analysis of the ADAMTS12-expressing clones TS12-4 and TS12-22, using the H-142 antibody against ADAMTS12. The cMDCK clone was used as a negative control in these assays. In RT-PCR experiments, amplification of mRNA encoding β-actin was used to ascertain RNA integrity and ensure equal loading. (C) Immunostaining of non-permeabilized cMDCK, TS12-4 and TS12-22 cells using an anti-FLAG antibody. Bar, 10 μm.

MDCK ADAMTS12 clones are refractory to the scattering effect of HGF treatment

Following HGF stimulation, the scattering effect in MDCK cells occurs concurrently with the disassembly of junctional components, which leads to a rapid loss of cell-cell adhesions (Singh et al., 2004). To evaluate whether these effects were modified in the selected ADAMTS12-expressing clones, TS12-4 and TS12-22 clones were cultured in the presence or absence of HGF (50 ng/ml), and morphological changes were visualized 16 hours later. The levels of production of ADAMTS12 and its localization do not seem to be affected by stimulation with the growth factor (data not shown). As can be seen in Fig. 2, cMDCK, TS12-4 and TS12-22 clones formed colonies of similar morphology when cultured in the absence

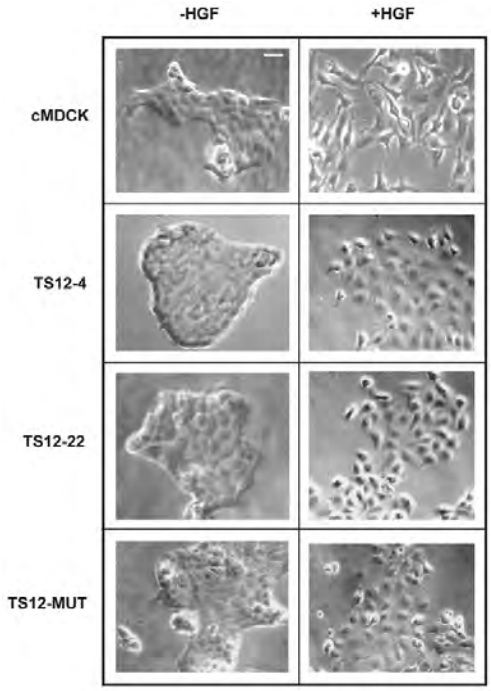

Fig. 2. MDCK ADAMTS12-expressing clones are refractory to the scattering effect of HGF stimulation. The control cMDCK clone shows reduced cell-cell adhesion and has a spindle-shape morphology upon treatment with HGF. By contrast, TS12-4 and TS12-22 still form epithelial-like colonies after 16 hours of incubation with HGF. A MDCK clone that expresses a catalytically inactive mutant version of ADAMTS12 (TS12-MUT) is also refractory to the scattering effect. Bar, 50 μm.

of HGF. However, following HGF treatment, the cMDCK clone showed the long spindle-shape morphology and reduced cell-cell adhesion characteristic of epithelial cells treated with this growth factor. By contrast, TS12-4 and TS12-22 clones were clearly refractory to HGF stimulation as the cells remained in contact with each other and formed epithelial-like colonies, and the scattering could only be occasionally observed in some cells surrounding the colonies. Likewise, we also assayed a MDCK clone that expresses an ADAMTS12 form containing changes in two key amino acids in the catalytic domain, resulting in an enzymatically inactive enzyme (Cal et al., 2001). Detection of this mutant ADAMTS12 was performed as described for TS12-4 and TS12-22 clones (data not shown). Fig. 2 illustrates that this clone, TS12-MUT, was also refractory to the morphological changes that are expected upon HGF stimulation. This observation, together with the lack of effect of the broad-range metalloproteinase inhibitor ilomastat on the HGF-stimulated TS12-4 and TS12-22 clones (data not shown), suggests that the described effects are not caused by the catalytic domain of the enzyme. Moreover, no changes were observed in the proliferation rates of the selected clones, indicating that ADAMTS12 expression in MDCK cells counteracts the HGF-mediated effects on cell-cell contacts and motility.

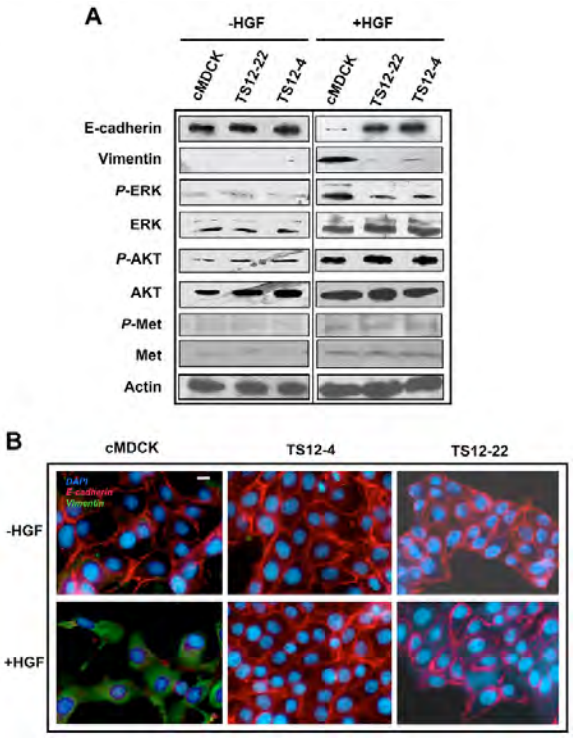

Fig. 3. ADAMTS12 negatively regulates the HGF signalling pathway. (A) Levels of different components of the HGF pathway (E-cadherin, vimentin, ERK, *P*-ERK, AKT, *P*-AKT and *P*-Met) were evaluated by western blot in the selected cMDCK, TS12-4 and TS12-22 clones, in both non-stimulated (– HGF) and stimulated (+ HGF) conditions. Ectodomain shedding of Met was also analyzed in the conditioned medium from these clones (Met). (B) Immunostaining of E-cadherin and vimentin in the selected clones without (– HGF) and with (+ HGF) stimulation by hepatocyte growth factor. Bar, 10 μm.

ADAMTS12 negatively regulates the HGF signalling pathway

HGF induces cell proliferation and migration through its tyrosine kinase receptor Met (Zhang and Vande Woude, 2003). We asked whether the inhibition of the scattering effect observed in MDCK cells transfected with ADAMTS12 could occur through this metalloproteinase exerting a negative regulatory signal upon the HGF pathway. To address this question, we prepared whole-cell extracts from both growth-factor-treated MDCK clones and untreated control clones, and levels of different components of the HGF pathway were evaluated by western blot. The results shown in Fig. 3A revealed that, in the absence of HGF stimulation, there were no differences in the levels of E-cadherin, vimentin, ERK, phosphorylated ERK (*P*-ERK), AKT, phosphorylated (*P*-AKT) and phosphorylated Met (*P*-Met) among the three clones analyzed, cMDCK, TS12-4 and TS12-22. Likewise, we have found that the expression of the metalloproteinase did not increase the ectodomain shedding of Met. By contrast, clear differences in the levels of some of the analyzed proteins were apparent when cells were stimulated with the growth factor (+

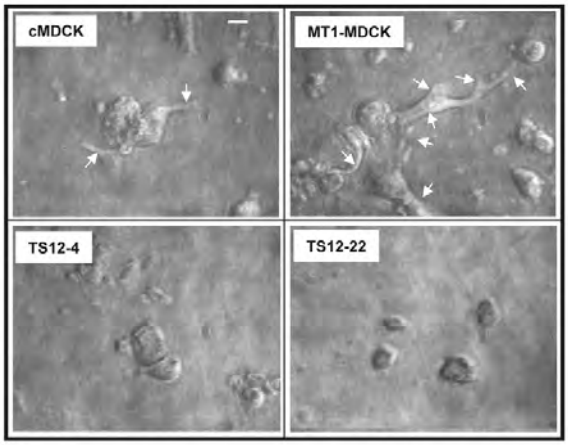

Fig. 4. MDCK cells expressing ADAMTS12 fail to form tubular structures in a 3D collagen matrix. Selected clones expressing ADAMTS12, TS12-4 and TS12-22 were grown within a collagen gel for seven days. Morphological changes such as long extensions (arrows) from the cysts are clearly observed in cMDCK and MT1-MDCK clones. Bar, 50 μm.

HGF, Fig. 3A). Thus, E-cadherin expression was clearly diminished in the cMDCK control clone upon HGF stimulation, whereas, in the ADAMTS12 clones, its expression was similar in both stimulated and non-stimulated cells. In addition, the cMDCK clone produced high levels of vimentin upon HGF stimulation, a change that was not observed in the TS12-4 and TS12-22 clones. Similarly, the levels of the active phosphorylated form of ERK (*P*-ERK) were significantly lower in the clones that expressed ADAMTS12 than in the corresponding controls following HGF treatment. Finally, we also observed that the levels of both AKT and phosphorylated AKT were similar among the analyzed clones in both stimulated and non-stimulated samples.

To further evaluate the observed differences between ADAMTS12-producing clones and control cells, we next performed an immunocytochemical analysis of E-cadherin and vimentin expression in these cells, both in the presence and absence of HGF. As can be seen in Fig. 3B, and in agreement with the results obtained by western blot analysis, cell-surface staining for E-cadherin was clearly detected in TS12-4 and TS12-22 clones, in both stimulated and non-stimulated samples, but negligible or no staining was observed for vimentin. By contrast, the response of cMDCK control cells to HGF stimulation was characteristic of an EMT as vimentin was clearly visible in HGF-treated cells and as E-cadherin levels were virtually undetectable following treatment with the growth factor (Fig. 3B). These results lead us to conclude that expression of ADAMTS12 can negatively regulate the HGF signal-transduction pathway. Moreover, the lowered levels of *P*-ERK detected in the ADAMTS12-expressing MDCK cells when compared with control MDCK cells suggest that the effects mediated by this metalloproteinase arise from an inhibition of the Ras-MAPK cascade and are independent of Met activation.

MDCK clones expressing ADAMTS12 fail to form tubular structures in a 3D collagen lattice

HGF mediates tubular morphogenesis in MDCK cells when grown within 3D collagen networks (Ridley et al., 1995). To evaluate the possible influence of ADAMTS12 expression on MDCK tubulogenic properties, TS12-4, TS12-22 and cMDCK clones were cultured in 3D collagen gels and stimulated with HGF, as described above. As a positive control for this experiment, we used a MDCK clone (MT1-MDCK) that expresses MT1-MMP, a membrane metalloproteinase that induces morphological changes to generate large cavities in the collagen gel (Hotary et al., 2000; Kadono et al., 1998). As can be seen in Fig. 4, MDCK cells that express ADAMTS12 form cysts when cultured in the presence of HGF in 3D collagen gels. However, these clones develop neither any tubular structures nor even any spiking or scattering of the cells that could indicate an early EMT (O'Brien et al., 2004). By contrast, morphological changes were observed in both cMDCK and MT1-MDCK clones. Thus, around 30% of the cysts formed by cMDCK cells, and 41% of those formed by MT1-MDCK cells, showed long extensions from the edge, which were clearly detectable after 7 days of incubation. By contrast, less than 10% of the cysts formed by TS12-4 and TS12-22 clones showed extensions, and such extensions were considerably shorter than those formed by the cMDCK and MT1-MDCK clones. These proportions were maintained when the tubule formation was followed for a longer period (data not shown). These findings indicate that ADAMTS12 expression might affect the ability of MDCK cells to invade a collagen matrix and constitute additional evidence concerning the inhibitory effects that this metalloproteinase can cause on the HGF signal-transduction cascade.

ADAMTS12 lacking thrombospondin domains does not inhibit EMT in HGF-stimulated MDCK cells

To test whether the thrombospondin domains of ADAMTS12 could be involved in the inhibitory effects observed in the TS12-4 and TS12-22 clones, we prepared a new clone, MDCK-ΔTS12, that stably expresses a truncated form of the protein lacking the TSP-1 domains (Fig. 5A,B). Scattering analysis, culture in 3D collagen gels and study of vimentin and E-cadherin expression revealed that this clone does not show any of the inhibitory effects described for those clones that express the full-length protein (Fig. 5C). Similar results were obtained when this assay was performed in the presence of the metalloproteinase inhibitor ilomastat (data not shown), which suggests that these effects are not due to the enzymatic activity of the protein. The two bands detected by western blot analysis of cell extracts could correspond to the entire protein (top band) and the mature ΔTS12 (bottom band), once the prodomain is removed in the trans-Golgi by a furin-like protease. According to these findings, the metalloproteinase and disintegrin domains are not sufficient to trigger the effects observed in the TS12-4 and TS12-22 clones. Hence, the presence of the angio-inhibitory TSP-1 domains as exosites within the metalloproteinase structure seems to be required to induce the negative regulatory effect following HGF stimulation.

Production, purification and enzymatic analysis of recombinant ADAMTS12

To further examine the relevance of ADAMTS12 in EMT

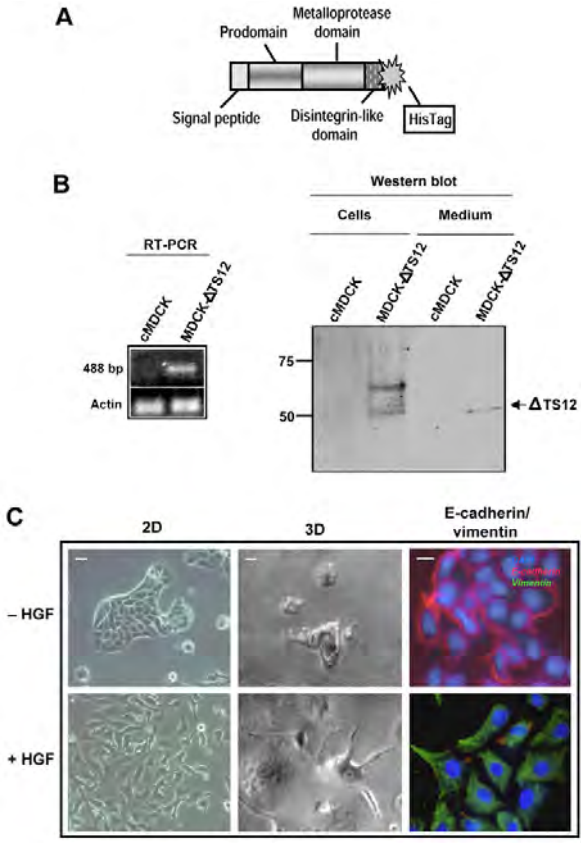

Fig. 5. Cells expressing a truncated ADAMTS12 lacking thrombospondin domains (ADAMTS-ΔTS12) undergo an EMT following HGF stimulation. (A) Domain organization of ADAMTS-ΔTS12. The position of the HisTag epitope is indicated. (B) Expression analysis of ADAMTS-ΔTS12 by RT-PCR and western blot using an antibody against the HisTag. In both cases, the cMDCK clone was used as a control. Truncated ADAMTS12 is located in the conditioned medium of cells that stably express this form of the enzyme. In RT-PCR, amplification of the mRNA encoding β-actin was used to ascertain RNA integrity and ensure equal loading. (C) Scattering assay (2D), tubulogenesis assay (3D) and immunostaining of E-cadherin and vimentin of the ADAMTS-ΔTS12-expressing cells in the absence (– HGF) or presence (+ HGF) of hepatocyte growth factor. Bars, 30 μm (left, centre); 10 μm (right).

processes, we next generated 293-EBNA cells stably expressing this enzyme (EBNA-TS12), following a strategy similar to that described above for MDCK cells. It was possible to recover the metalloproteinase from the membrane-associated fraction by using a buffer containing 0.5 M NaCl, suggesting that, after secretion, this proteinase is immobilized around the pericellular space (Fig. 6A). After purification of the recombinant ADAMTS12 using an anti-FLAG affinity gel column, we performed a western blot analysis with an antibody against FLAG that allowed the detection of the purified ADAMTS12 (Fig. 6A). The purified enzyme was employed to test its activity by using aggrecan as a potential substrate. An

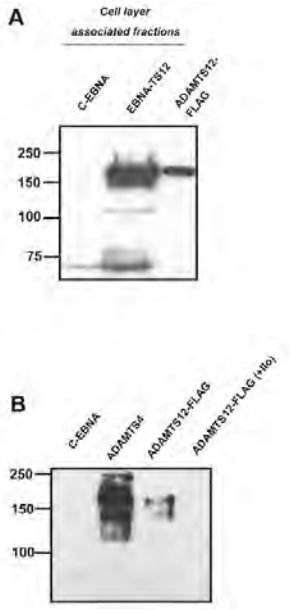

Fig. 6. Purification process and enzymatic activity of ADAMTS12. (A) Western blot analysis of ADAMTS12 during the purification process. c-EBNA and EBNA-TS12 indicate cell-layer-associated fractions from 293-EBNA cells transfected with an empty vector, and the equivalent fraction from 293-EBNA cells expressing ADAMTS12, respectively. Recombinant purified ADAMTS12 is also indicated. (B) Following incubation of aggrecan with recombinant ADAMTS4 (lane 2), purified ADAMTS12 (lane 3) or an equivalent fraction purified from c-EBNA cells (lane 1), samples were deglycosylated and the aggrecan degradation products were detected by using the BC-3 antibody. In lane 4, purified ADAMTS12 was previously incubated with the metalloproteinase inhibitor ilomastat (+Ilo). Molecular mass markers (kDa) in both panels are indicated on the left.

equivalent fraction derived from a 293-EBNA clone stably transfected with an empty vector was used as a negative control (c-EBNA), whereas ADAMTS4, a potent aggrecanase, was used as positive control in this assay. As shown in Fig. 6B, the isolated recombinant ADAMTS12 displayed a clear aggrecanase activity, as assessed by western blot analysis using the BC-3 antibody that detects aggrecan degradation products. Moreover, this activity is abolished when the enzyme is pre-incubated with the metalloproteinase inhibitor ilomastat. These results confirm that recombinant ADAMTS12 produced in 293-EBNA cells is enzymatically active and thereby useful for the analysis reported below.

Effects of ADAMTS12 on VEGF-induced tubulogenesis in BAE-1 cells

To analyze whether the exogenous administration of ADAMTS12 could also affect tubulogenesis in a different system, we chose the endothelial cell line BAE-1, which forms capillary networks in collagen lattices when cultured in the presence of VEGF (Maeshima et al., 2004). In these conditions, purified ADAMTS12 or the corresponding cell-layer-associated

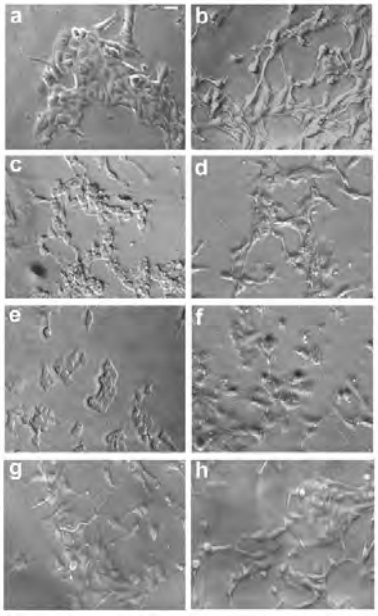

Fig. 7. Effect of ADAMTS12 on tubulogenesis of BAE-1 cells. BAE-1 cells were cultured within a collagen gel for 2 days in the absence (a) or presence (b–f) of 100 ng/ml VEGF. 10 μl of the cell-layer-associated fraction from 293-EBNA cells that express ADAMTS12 (EBNA-TS12) (c), or the same amount of the equivalent fraction from c-EBNA cells (d), were added to the culture. BAE-1 cells were grown in the presence of 10 μl of purified ADAMTS12 (e), or the same amount of the equivalent fraction purified from c-EBNA cells (f). Addition of 50 μl of conditioned medium from cells that express truncated ADAMTS12 (g) or from control cells transfected with an empty vector (h) does not affect the ability of BAE-1 cells to form a capillary network in the presence of VEGF. Bar, 30 μm.

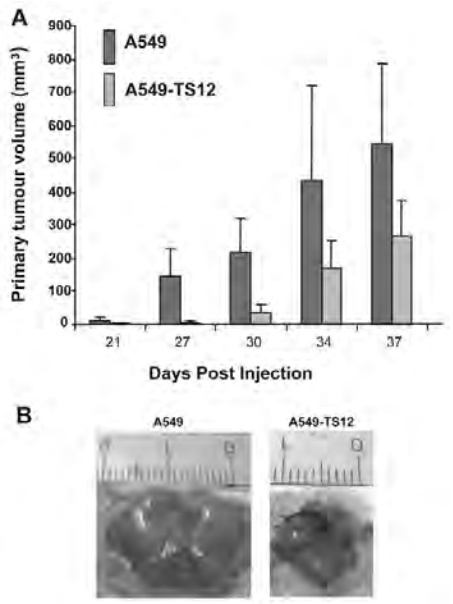

Fig. 8. Inhibition of subcutaneous tumour growth by ADAMTS12. (A) SCID mice were injected with parental A549 cells or with cells from an A549 clone (A549-TS12) expressing ADAMTS12. Tumour growth was followed until day 37, and tumour volumes were calculated after measurements were taken with a calliper. (B) Representative subcutaneous tumours at day 37 following injection are shown for mice that received A549 or A549-TS12 cells

fraction from 293-EBNA cells expressing ADAMTS12 were added to the cell cultures, and tubulogenesis was evaluated 48 hours later. As a negative control, BAE-1 cells under the same culture conditions were incubated with equivalent fractions of 293-EBNA cells stably transfected with the empty vector. As can be seen in Fig. 7, BAE-1 cells cultured in the presence of VEGF formed tubules and ~64% of cell clusters contained capillary structures. By contrast, formation of these capillary networks was drastically reduced in the presence of ADAMTS12, and less than 10% of cell clusters showed any tubular structures. Control cells, grown either without the recombinant ADAMTS12, or in the presence of the cell-layer-associated fraction from control 293-EBNA cells, clearly showed tubular morphogenesis in >40% of the cell clusters. Similar values were obtained when cells were grown in the presence of the protein lacking the thrombospondin domains (ΔTS12), which was carried out by adding to the BAE-1 cells conditioned medium from the MDCK cells that expressed this truncated form of the enzyme. From these assays, we conclude that, in this system, ADAMTS12 inhibits the formation of VEGF-induced tubular structures in BAE-1 cells. It cannot be ruled out that the metalloproteinases can interact with the growth factor. This situation would resemble that found for ADAMTS1, in which this metalloproteinase binds to VEGF through the thrombospondin domain of its C-terminal region (Luque et al., 2003).

ADAMTS12 inhibits tumour growth in vivo

A549 is a human lung adenocarcinoma cell line that forms primary tumours when injected into immunodeficient mice (Rao et al., 2005; Wedge et al., 2002). To evaluate the effect of ADAMTS12 expression in the in vivo tumour formation assay by A549 cells, we first selected a stable clone, A549-TS12, that expresses ADAMTS12. The presence of the metalloproteinase was determined by RT-PCR, western blot and immunocytochemistry (data not shown). A549-TS12 cells were subcutaneously injected in the flank of four SCID mice. Control parental A549 cells were injected in the same conditions in a further four animals, and the evolution of tumour growth was followed for five weeks. As can be seen in Fig. 8A,B, A549-TS12 cells overexpressing the metalloproteinase display a significantly reduced tumour growth rate in comparison with A549-derived tumours. Thus, after 37 days, the average tumour volume in mice that received the A549-TS12 clone was only ~50% of the tumour volume of mice that received the A549 parental cells. Moreover, in the case of the A549-TS12 clone, the tumours were macroscopically visible four weeks after cells injection, whereas the tumours produced by the parental A549 cells could already be detected by three weeks after injection. These results indicate that ADAMTS12 might also confer anti-tumour properties in vivo.

Discussion

ADAMTS12 is a complex metalloproteinase that contains multiple TSP-1 repeats in its C-terminal region and whose expression has been detected mainly in some human foetal tissues as well as in a variety of carcinomas and cancer cell lines (Cal et al., 2001). As a first step to study the putative functional relevance of ADAMTS12 in tumour-associated processes, we have analyzed in this work whether the expression of this proteinase could affect the morphological changes that MDCK cells undergo following HGF treatment. This cellular model has been successfully used to evaluate how different members of the matrix metalloproteinase (MMP) family promote the generation of an invasive phenotype in MDCK cells engineered to overproduce these enzymes (Hotary et al., 2000; Kadono et al., 1998; Kang et al., 2000). Following a similar strategy, and after selection of two MDCK clones that expressed ADAMTS12, we first observed that these clones were very refractory towards undergoing the scattering effect characteristic of MDCK cells upon HGF stimulation. Likewise, within collagen gels, the ADAMTS12-producing cells could form cysts, but neither tubules nor other morphological alterations such as spindle-shaped extensions were observed on the surface of the cysts. Western blot and immunocytochemical analysis showed that HGF stimulation did not downregulate E-cadherin or upregulate vimentin expression in the ADAMTS12-expressing clones, as would otherwise be expected for MDCK cells treated with this growth factor (Bolos et al., 2003).

Analysis of the putative signalling pathways underlying the observed effects induced by ADAMTS12 expression implicated the Ras-MAPK pathway in these events, as deduced from the fact that levels of active phosphorylated ERK were diminished in the ADAMTS12-expressing clones compared with control cells. This signalling cascade is involved in the regulation of epithelial tubule development in MDCK cells (O'Brien et al., 2004) and is necessary and sufficient to initiate tubulogenesis (Hellman et al., 2005). Moreover, sustained activation of ERK seems to be crucial for cell scattering (Liang and Chen, 2001). Our data are consistent with the possibility that ADAMTS12 inhibits the cellular processes that regulate the EMT in HGF-stimulated MDCK cells.

How could ADAMTS12 mediate these effects? The above results were obtained by using MDCK clones that expressed the entire metalloproteinase. However, after performing the same assays with cells producing a mutant ADAMTS12 protein lacking the thrombospondin domains, we observed that this truncated enzyme was unable to inhibit the effects induced by HGF, thereby suggesting that the C-terminal TSP-1 domains of ADAMTS12 might regulate this process. Moreover, MDCK cells that expressed a catalytically inactive ADAMTS12 form were also refractory to the scattering effect. Consistent with this proposal, inhibition of endothelial cell migration by TSP-1 occurs through the interaction with its receptor CD36, and cellular levels of TSP-1 are regulated by tumour-suppressor genes and oncogenes (Ren et al., 2006). It has also been reported that HGF turns off TSP-1 expression in MDCK cells to avoid negative regulatory signals on cell proliferation and migration and that this downregulation depends on the MAPK pathway and operates independently of the phosphoinositide 3-kinase and Stat3 pathways (Zhang and Vande Woude, 2003). Furthermore, and in relation to the possibility that other ADAMTS proteins could play similar roles, it has been reported recently that ADAMTS1 from *Xenopus* also negatively modulates the Ras-MAPK pathway through inhibition of ERK phosphorylation and that the C-terminal region of the protein containing the TSP-1 domains is necessary and sufficient for this function (Suga et al., 2006). Interestingly, both ADAMTS1 and ADAMTS12 are enzymes that undergo several proteolytic processing events to generate several fragments that might play different roles (Cal et al., 2001; Rodriguez-Manzaneque et al., 2000). The balance between the various generated fragments could influence the pro- or anti-metastatic activities of ADAMTS12, as has been shown for ADAMTS1 (Liu et al., 2006; Masui et al., 2001).

To evaluate the potential anti-tumorigenic effects of ADAMTS12 in vivo, we analyzed the growth of the poorly differentiated non-small-cell lung cancer cell line A549, engineered to produce ADAMTS12, in immunodeficient SCID mice. The A549 parental cell line has been used previously to determine that downregulation of the MMP-9 metalloproteinase has a significant inhibitory effect on tumour growth and progression (Rao et al., 2005). Conversely, we observed that A549 cells expressing ADAMTS12 form subcutaneous tumours in SCID mice, but the tumour volumes were considerably smaller than those produced by parental A549 cells. This observation suggests that ADAMTS12 expression might delay tumour growth, which agrees with our in vitro findings on the potential anti-tumour effects of this metalloproteinase. These results, based on using an animal model, open the possibility for future work aimed at evaluating the regulatory roles of ADAMTS12 in other in vivo tumour-associated processes such as angiogenesis or metastasis.

In conclusion, the results presented in this work suggest that ADAMTS12 could confer tumour-protective functions. In agreement with this idea, it has been recently established that Ki-*Ras*-transformed mouse colonocytes expressing high levels of Myc promote the formation of highly vascularized tumours. This effect is due, at least in part, to the downregulation of a series of angio-inhibitory proteins, including ADAMTS12 (Dews et al., 2006). The finding of anti-tumorigenic effects for a metalloproteinase is not unprecedented as it has also been described for other enzymes of this catalytic class, including several MMPs (Acuff et al., 2006; Balbin et al., 2003; Folgueras et al., 2004; Houghton et al., 2006). These findings point to the occurrence of alternative proteinase functions in cancer and also reinforce the need for analyzing the tumour 'degradome' – the entire set of proteinases produced by a specific tumour in a certain stage of development (Lopez-Otin and Overall, 2002). These proteinase-profiling approaches might also contribute to the identification of therapeutic targets for the future design of specific inhibitors that would block only the unwanted activity of these enzymes during tumour progression (Overall and Lopez-Otin, 2002). Generation of mice lacking ADAMTS12, now in progress, will provide an essential tool to understand better the relevance of this enzyme in tumour-associated processes.

Materials and Methods

Cell culture, transfection and cDNA constructs

MDCK, 293-EBNA, A549 and BAE-1 cells were routinely maintained in DMEM medium containing 10% heat-inactivated foetal bovine serum and 100 U/ml penicillin and 50 μg/ml streptomycin. Full-length cDNA encoding ADAMTS12 was amplified by PCR from the vector pcDNA3-ADAMTS12-HA (Cal et al., 2001),

and it was cloned between the *Hin*dIII and *Not*I sites of a modified pCEP4 expression vector (Life Technologies). Additionally, two oligonucleotides (5′-GGCCGACTACAAGGACGACGATGACAAG-3′ and 5′-GGCCCTTGTCATCGTCGTCCTTGTAGTC-3′) were used to introduce a FLAG epitope at the *Not*I site. The generated plasmid, pCEP-TS12, was transfected into MDCK and 293-EBNA cells using the lipofectamine reagent (Life Technologies), as recommended by the manufacturer. MDCK and 293-EBNA clones stably expressing ADAMTS12 or transfected with the empty vector to be used as a negative control were selected in the presence of 2 μg/ml puromycin (Sigma-Aldrich). When indicated, a MDCK clone expressing a mutant ADAMTS12 (TS-12 MUT) was employed. This form of ADAMTS12 contains two mutations in the metalloproteinase domain to produce a catalytically inactive enzyme (Cal et al., 2001). MT1-MMP cDNA was a generous gift from M. Seiki (Institute for Medical Science, University of Tokyo, Japan) and was employed to generate MDCK cells that stably expressed this proteinase. MDCK cells were also transfected with a cDNA encoding a truncated form of ADAMTS12 with a premature stop codon between the Cys-rich and the first TSP-1 domains (MDCK-ΔTS12). A His-tag tail was added to the C-terminus of this protein to facilitate its detection by western blot. To detect this truncated form of ADAMTS12, 300 μl of the conditioned medium was precipitated with an equal volume of chilled acetone.

Western blotting

For western blot analysis, the proteins were resolved by 8 or 12% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and subsequently probed with the indicated antibodies, as recommended by the manufacturers. The primary antibodies used for detection of specific proteins were anti-FLAG-M2 (Sigma-Aldrich), H-142 (anti-ADAMTS12; Santa Cruz Biotech), anti-p44/42 mitogen-activated protein kinase (MAPK), anti-phospho-Akt (Ser473) and anti-Akt (Cell Signalling Technology); anti-ERK, BC-3, anti-VEGF and anti-vimentin (Abcam); anti-E-cadherin (kindly supplied by A. Cano, IIB, Madrid, Spain); anti-MT1-MMP (kindly supplied by A. G. Arroyo, CNIC, Madrid, Spain); and anti-HisTag (GE Healthcare). Anti-*P*-Met antibody was from Biosource International (Camarillo, CA) and anti- Met antibody (clon DL-21) was from Upstate (Charlottesville, VA). Immunoreactive proteins were visualized using HRP-peroxidase-labelled anti-rabbit or anti-mouse secondary antibody and the ECL detection system (Pierce).

Reverse transcription-PCR amplification (RT-PCR)

When indicated, presence of ADAMTS12 in the selected clones was assayed by RT-PCR. Total RNA from these clones was isolated by guanidium thiocyanate-phenol-chloroform extraction, and the cDNA synthesis was carried out using the RNA PCR kit from Perkin-Elmer Life Sciences, as specified by the manufacturer. After reverse transcription using 1 μg of total RNA and random hexamers as primers, PCR was performed with two specific ADAMTS12 oligonucleotides, (5′-TCACGACGTGGCTGTCCTTCT-3′ and 5′-ACCACAG TGTCTGGCAGACGT-3′), corresponding to the metalloproteinase domain. cDNA quality was verified by control RT-PCR reactions using primers derived from the sequence of β-actin.

Immunocytochemical analysis

MDCK cells stably expressing ADAMTS12, or control cells carrying an empty vector, were fixed with 4% paraformaldehyde in phosphate-buffered saline. The non-permeabilized cells were blocked with 15% foetal bovine serum in the same buffer. To detect recombinant ADAMTS12, blocked slides were incubated for 2 hours with the primary antibody against FLAG, followed by 2 hours of incubation with a secondary fluorescein-conjugated sheep anti-mouse antibody (GE Healthcare). Detection of E-cadherin and vimentin was carried out basically as described above except that cells were permeabilized with 0.5% Triton X-100 for 5 minutes before adding the primary antibodies. Secondary antibodies were conjugated with Cy3 and Cy2 (Jackson ImmunoResearch Laboratories), respectively. In all samples, 4′,6′-diamino-2-phenylindole hydrochloride (DAPI) was added at 100 ng/ml to visualize DNA in the cell nucleus. Images were obtained using fluorescence microscopy and a digital camera.

Production and purification of recombinant ADAMTS12

Production and purification of recombinant ADAMTS12 was performed as described by Colige et al. (Colige et al., 2002) for ADAMTS14, with some modifications. In brief, 293-EBNA cells expressing ADAMTS12 (EBNA-TS12) were scraped from the culture plate, suspended in a buffer containing 50 mM Tris-HCl, pH 7.4, and 500 mM NaCl and rotated for 2 hours at 4°C. After centrifugation, the supernatant (cell-layer-associated fraction) was diluted 1:1 (v/v) in the same buffer lacking NaCl, and loaded onto an anti-FLAG affinity gel column (Sigma-Aldrich). Elution was carried out by using a FLAG peptide (Sigma-Aldrich), as recommended by the manufacturer. The purification process was followed by western blot using the anti-FLAG-M2 antibody. The same process was applied to an EBNA clone stably transfected with an empty vector.

Enzyme activity assays

The proteolytic activity of recombinant ADAMTS12 was assessed using aggrecan as a potential substrate. To do this, recombinant purified protein was incubated with 500 nM aggrecan from bovine articular cartilage (Sigma-Aldrich) in a buffer containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl and 10 mM $CaCl_2$ at 37°C for 16 hours. Next, fragments were enzymatically de-glycosylated using aggrecan chondroitinase ABC (0.1 U per 10 μg of aggrecan; Sigma-Aldrich) at 37°C for 1 hour, followed by 2 hours incubation at 37°C with 0.002 U per 10 μg aggrecan of endo-β-galactosidase (Sigma-Aldrich). Reaction products were analyzed by western blot by using the BC-3 antibody. When indicated, the broad-range inhibitor of metalloproteinases ilomastat (Calbiochem) was employed (5 μM final concentration). As a positive control, aggrecan digestion was carried out by ADAMTS4 (Chemicon International) under the same experimental conditions.

Scattering assay

Selected MDCK cell clones were seeded in quadruplicate in a 24-well tissue-culture plate (1×10^4 cells) and were allowed to attach for 6 hours. Next, 50 ng/ml HGF (R&D Systems) was added to two samples of each quadruplicate and pictures were taken after 16 hours of incubation, using a video camera attached to a Zeiss Axiovert 200 M motorized inverted microscope.

Tubulogenesis in 3-D collagen gels

Tubulogenesis assays were carried out using a 3D collagen cell culture system (Chemicon International), following the manufacturer's instructions. Briefly, selected MDCK clones (1.5×10^3 cells) were mixed with 200 μl of chilled collagen and allowed to gel at 37°C in 24-well plates. Next, DMEM medium containing 3% bovine foetal serum and 30 ng/ml HGF was added to the wells. This medium was renewed every 2-3 days and photographs were taken after 7 days, and the percentage of cysts with extensions was quantified using a 40× objective on an inverted microscope (Axiovert 200, Carl Zeiss). In the case of BAE-1 cells, tubule formation was induced by adding 100 ng/ml VEGF to 5×10^3 cells per well growing in the collagen gel, and photographs were taken after 2 days, as described by Maeshima et al. (Maeshima et al., 2004). To semi-quantify tubulogenic activity, the percentage of cell clusters containing tubular structures was determined. When indicated, different amounts of recombinant ADAMTS12 directly extracted from the 293-EBNA cell layer or after purification were added to the wells.

Animals and subcutaneous tumours

Male adult severe combined immunodeficient mice (SCID; C.B-17/IcrCrl-scid-BR) were obtained from Charles River Laboratories (Sulzfeld, Germany), and animal care and experimentation was carried out following institutional guidelines approved by the local animal review board. Mice were kept in sterile cages bedded with sterilized soft wood granulate and fed irradiated rat chow ad libitum with autoclaved water in a 12-hour light-dark cycle. All manipulations were performed in a laminar-flow hood and the mice were intraperitoneally anaesthetized with a mixture of 50 mg/kg ketamine and 10 mg/kg xylazine. For euthanasia, animals were given a lethal dose of ketamine and xylazine. To induce subcutaneous tumours, suspensions of A549 cells or A549 that expressed recombinant ADAMTS12 (A549-TS12) were subcutaneously injected (6×10^6 cells in 0.5 ml PBS) into the left side of four SCID mice. Selection of the clone A549-TS12 was carried out following a procedure similar to that described above for the generation of stable ADAMTS12-expressing MDCK and 293-EBNA cells. The health status of mice and appearance of tumours was monitored routinely and the animals remained healthy throughout the entire experiment. Tumour size was measured with a calliper and tumour volume was determined using the formula: $V=0.4\times A\times B^2$, where A is the largest dimension of the tumour and B is the smallest dimension.

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III. Análisis epigenético del gen ADAMTS12 en tumores de diverso origen

Los resultados previos describen las propiedades antitumorales de ADAMTS-12. En nuestro afán por caracterizar el papel de esta proteína en el cáncer, nos propusimos dar un nuevo paso intentando esclarecer la posible pérdida de su función mediante mecanismos epigenéticos durante la progresión tumoral. Para ello, investigamos en primer lugar el estado de metilación de su promotor, donde previamente habíamos identificado una isla CpG. Mediante PCRs específicas de metilación y secuenciación de bisulfito, pusimos de manifiesto la frecuente hipermetilación en tejido tumoral de colon frente a tejido sano. No obstante, descubrimos que paradójicamente los niveles de expresión eran mayores en el tejido cancerígeno. Mediante localización inmunofluorescente pudimos señalar a las células del estroma como las responsables de la producción de la proteína. Finalmente, empleamos chips de metilación que nos permitieron hacer extensible esta situación de hipermetilación a tumores distintos de los carcinomas colorrectales.

Artículo 3: **Ángela Moncada-Pazos**, Álvaro J. Obaya, Mario F. Fraga, Cristina G. Viloria, Gabriel Capellá, Mireia Gausachs, Manel Esteller, Carlos López-Otín y Santiago Cal. “The *ADAMTS12* metalloprotease gene is epigenetically silenced in tumor cells and transcriptionally activated in the stroma during progression of colon cancer”.

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Aportación personal al trabajo

En este trabajo corrió a mi cargo la mayor parte de la labor experimental, incluyendo los análisis epigenéticos, para los cuales fui instruida en el laboratorio del Dr. Manel Esteller bajo la supervisión del Dr. Mario Fraga. Realicé también los ensayos con líneas celulares así como las inmunofluorescencias sobre tejidos. Finalmente, colaboré en la elaboración del manuscrito y las figuras.

2906 Research Article

The *ADAMTS12* metalloprotease gene is epigenetically silenced in tumor cells and transcriptionally activated in the stroma during progression of colon cancer

Angela Moncada-Pazos[1], Alvaro J. Obaya[2], Mario F. Fraga[3], Cristina G. Viloria[1], Gabriel Capellá[4], Mireia Gausachs[4], Manel Esteller[3], Carlos López-Otín[1] and Santiago Cal[1,*]

[1]Departamento de Bioquímica y Biología Molecular and [2]Biología Funcional, Area de Fisiología, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, 33006 Oviedo, Spain
[3]Cancer Epigenetic Laboratory, Spanish National Cancer Centre (CNIO), 28029 Madrid, Spain
[4]Laboratori de Recerca Translacional, IDIBELL, Institut Català d'Oncologia, L'Hospitalet de Llobregat, 08907 Barcelona, Spain
*Author for correspondence (santical@uniovi.es)

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Summary

Proteases have long been associated with tumor progression, given their ability to degrade extracellular matrix components and facilitate invasion and metastasis. However, recent findings indicate that different proteases can also act as tumor-suppressor enzymes. We have recently reported that lung carcinoma cells expressing the ADAMTS-12 metalloprotease show a remarkable impairment of growth in immunodeficient mice as compared with parental cells. Here, we show that *ADAMTS12* promoter is hypermethylated in cancer cell lines and tumor tissues. Interestingly, *ADAMTS12* expression in the stromal cells surrounding epithelial malignant cells is higher than in the paired normal tissues. Moreover, the expression of this metalloprotease in colon fibroblasts co-cultured with colon cancer cell lines is higher than in those cultured alone. Furthermore, the expression of *ADAMTS-12* by these fibroblasts is linked with an anti-proliferative effect on tumor cells. Based on these findings, we hypothesize that ADAMTS-12 is a novel anti-tumor protease that can reduce the proliferative properties of tumor cells. This function is lost by epigenetic silencing in tumor cells, but concurrently induced in stromal cells, probably as part of a response of the normal tissue aimed at controlling the progression of cancer.

Key words: Metalloprotease, Thrombospondin, Methylation, Degradome

Introduction

Proteases perform fundamental processing events in multiple biological processes in all living organisms (Lopez-Otin and Bond, 2008). To date, more than 560 proteolytic enzymes and homologs have been cataloged in the human genome (Puente et al., 2003), which reflects their relevance to physiological and pathological conditions, including cancer. In fact, proteolytic activity facilitates spreading of tumor cells and formation of distant metastasis through degradation of protein components of the extracellular matrix (Overall and Lopez-Otin, 2002). However, the relationship between proteolytic enzymes and cancer is much more complex because these enzymes target a variety of substrates distinct from extracellular matrix components and influence all stages of tumor development (Egeblad and Werb, 2002; Freije et al., 2003). Recent functional studies have also revealed that some proteases exhibit potent anti-tumor functions (Lopez-Otin and Matrisian, 2007). An illustrative example is that of MMP-8, a matrix metalloprotease showing a protective role in cancer through its ability to regulate the inflammatory response induced by carcinogens (Balbin et al., 2003). Furthermore, MMP-8 expression by breast tumors correlates with a lower incidence of lymph node metastasis and confers good prognosis to breast cancer patients (Gutierrez-Fernandez et al., 2008). These findings have contributed to extend the known functions of cancer-associated proteases and emphasize the importance of targeting specific proteases for cancer treatment (Lopez-Otin and Overall, 2002; Martin and Matrisian, 2007).

The ADAMTS (a disintegrin and metalloprotease with thrombospondin domains) family comprises 19 extracellular metalloproteases closely related to MMPs and ADAMs (Cal et al., 2002; Apte, 2004; Porter et al., 2005). Gain or loss of function of different ADAMTSs are involved in serious human diseases such as osteoarthritis (Bondeson et al., 2008) and thrombotic thrombocytopenic purpura (Sadler, 2008). In relation to tumorigenesis, some ADAMTSs are overexpressed in tumors of different sources (Porter et al., 2005; Rocks et al., 2008), whereas other family members have been cataloged as tumor-protective enzymes (Lopez-Otin and Matrisian, 2007). Thus, different reports point out the angio-inhibitory properties of ADAMTS-1 (Iruela-Arispe et al., 2003; Kuno et al., 2004; Lee et al., 2006). Moreover, *ADAMTS1* expression has been found epigenetically silenced in colorectal tumors (Lind et al., 2006). Furthermore, *ADAMTS8, ADAMTS9* and *ADAMTS18* gene promoters are also hypermethylated in several carcinomas (Dunn et al., 2004; Jin et al., 2007; Lo et al., 2007). ADAMTS-12 is another member of this family and was originally identified and cloned in our laboratory (Cal et al., 2001). We have recently described that this enzyme can modulate the Ras-dependent ERK (extracellular signal-regulated kinase) signalling pathway in MDCK (Madin-Darby canine kidney)

cells, and that subcutaneous tumors induced by A549 lung carcinoma cells expressing *ADAMTS12* display a considerable growth deficiency as compared with those induced by parental cells (Llamazares et al., 2007). To further examine the relevance of ADAMTS-12 as a putative tumor-suppressor enzyme, we have analyzed the mechanisms controlling its expression in cancer cells and tissues with the finding that *ADAMTS12* is epigenetically silenced in tumor cell lines from multiple sources, whereas it is transcriptionally activated in stroma cells. Our data strongly suggest that ADAMTS-12 is part of a protective host response against tumor progression derived from the intimate crosstalk between tumor cells and the surrounding stroma.

Results

ADAMTS12 gene promoter is hypermethylated in colorectal carcinomas and colon cancer cell lines

Analysis of 2.0 kb upstream and 0.5 kb downstream of the first ATG codon allowed the identification of a CpG island in this region, suggesting the potential influence of CpG methylation in the transcription of the *ADAMTS12* gene. Methylation-specific PCR (MSP) amplification of a 138 bp promoter within the CpG island (Fig. 1A) was performed on a set of paired normal and tumor colon samples. As can be seen in Fig. 1B, the methylation level of *ADAMTS12* promoter was higher in 12 out of 19 (63.2%) biopsies of primary colorectal tumors than in the corresponding normal tissue. Similarly, MSP amplification using genomic DNA from the microsatellite-stable (MSS) SW620, SW480, COLO 205 and HT29 colon carcinoma cell lines, and the microsatellite-unstable (MSI) DLD-1, HCT 15, HCT-116, LoVo and RKO cell lines showed that *ADAMTS12* promoter is hypermethylated in all of them, with the exception of LoVo cells (Fig. 1B; Table 1).

We next performed high-resolution bisulfite genome sequencing of 26 CpG sites within the CpG island identified (Fig. 1A). Eleven colon carcinoma samples were selected for this assay, attending to the different intensities of PCR products in previous MSP amplifications. Seven of these samples were more methylated in the tumor than in their paired normal tissues (Fig. 1C; Table 1). Among them, tumor samples 426, 467 and 557 were highly methylated in most of the 26 CpG sites examined (70-90%). By contrast, their paired normal tissues were much less methylated (20-40%). Four additional tumor samples, 340, 401, 478 and 566, showed percentages of methylation ranging between 20 and 60%, but their paired normal tissues were scarcely methylated (below 10%). Samples 404 and 496 showed similar levels of methylation in both tumor and normal tissues, whereas samples 397 and 555 were more methylated in normal tissues. Parallel analysis carried out in colon carcinoma cell lines revealed the high frequency of methylation at these CpG sites, independently of the microsatellite instability status. Thus, percentages of 80-90% were determined for SW620, SW480 and COLO 205 (MSS cell lines), and for

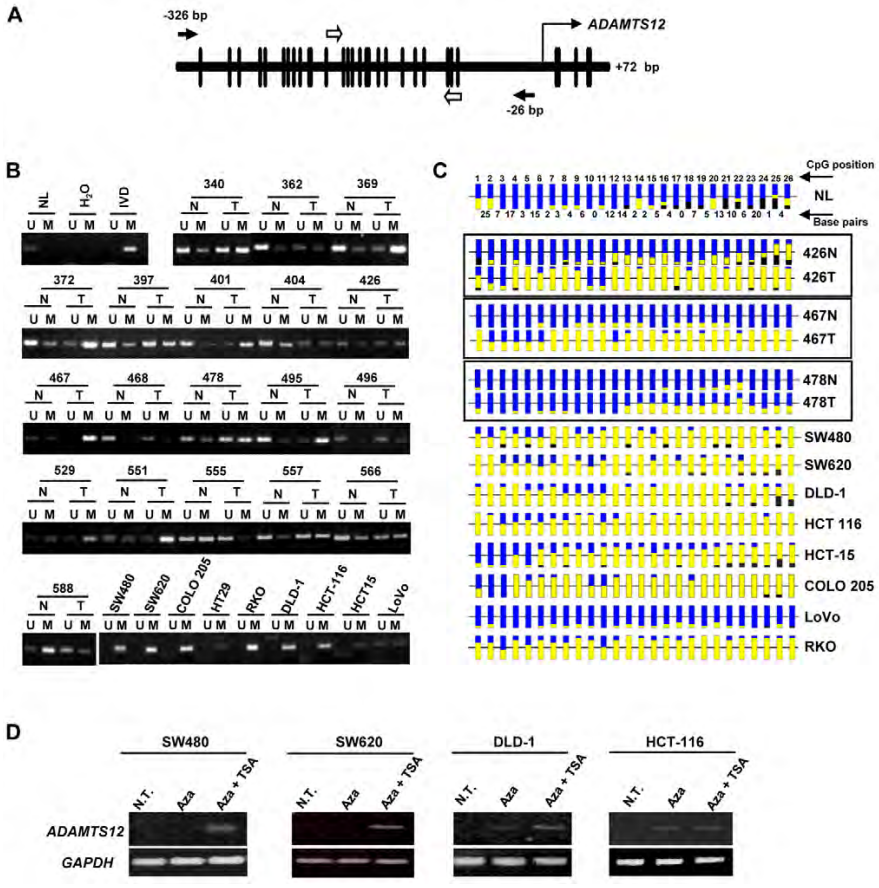

Fig. 1. Epigenetic silencing of *ADAMTS12* in colon cancer. (A) Schematic representation of the *ADAMTS12* CpG island. An interval of 2.0 kb upstream and 0.5 kb downstream from the first ATG codon of *ADAMTS12* was analyzed for CpG islands. Thin black arrow indicates the transcription start site; white arrows indicate MSP primers and thick black arrow BSP primers. (B) Methylation status of *ADAMTS12* in primary colon carcinomas and colon cancer cell lines. PCR-methylation analysis was carried out in paired normal (N) and tumor tissues (T), using the specific primers for either methylated (M) or modified unmethylated DNA (U). Genomic DNA from human lymphocytes was used as a control for unmethylated DNA (NL), and the CpGenome Universal Methylated DNA as control for methylated DNA (IVD). (C) Methylation density of *ADAMTS12* in colon cancer. Primary tumor samples and colon cancer cells were subjected to bisulfite treatment, and PCR amplification was performed using BSP primers. Relative positions of each CpG analyzed and the distance in bp are indicated in the control for unmethylated DNA (NL). Methylation percentages for the rest of the tumor samples examined are indicated in Table 1. Colors shows the methylation density of each CpG: yellow, methylated; blue, not methylated; grey, not present. (D) Activation of *ADAMTS12* expression in colon cancer cell lines. Indicated cell lines were treated with 5′-aza-2′-deoxycitidine (Aza), or Aza + TSA. *ADAMTS12* expression was assayed by RT-PCR. N.T., no treatment. *GAPDH* mRNA was amplified as an internal control.

Table 1. Hypermethylation of the *ADAMTS12* promoter

Cell lines	Patient sample number	Microsatellite instability	Mean CpG site methylation (%)		Methylation status
			Normal	Tumor	
	340	MSS	9.80	25.80	M
	362	ND	ND	ND	M
	369	MSS	ND	ND	M
	372	MSI	ND	ND	M
	397	ND	49.33	13.75	U
	401	MSI	0.64	20.33	M
	404	MSS	17.39	22.05	U/M
	426	MSS	21.42	82.25	M
	467	MSS	9.23	84.07	M
	468	MSS	ND	ND	U/M
	478	MSS	4.33	25.96	M
	495	MSS	ND	ND	M
	496	MSS	11.11	19.61	U/M
	529	ND	ND	ND	U/M
	551	MSS	ND	ND	M
	555	MSS	35.34	20.07	U
	557	MSS	31.61	70.76	M
	566	MSS	7.40	59.24	M
	588	ND	ND	ND	U
DLD-1		MSI	85.74		M
COLO 205		MSS	82.21		M
HCT-116		MSI	86.51		M
HCT 15		MSI	66.79		M
HT29		MSS	ND		M
LoVo		MSI	9.58		U
RKO		MSI	80.76		M
SW480		MSS	82.14		M
SW620		MSS	84.10		M

MSP amplification using genomic DNA from the microsatellite-stable COLO 205, HT29, SW620 and SW480 colon carcinoma cell lines, and the microsatellite-unstable DLD-1, HCT 15, HCT-116, LoVo and RKO cell lines was used to measure methylation of the *ADAMTS12* promoter. Samples were also analyzed from paired normal and primary colorectal tumor tissue from 19 patients.

U, unmethylated; M, methylated; MSS, microsatellite-stable; MSI, microsatellite-unstable; ND, not determined.

DLD-1, HCT-116 and RKO (MSI cell lines) (Fig. 1C; Table 1). The percentage of methylation for HCT 15 cells was 66.8% and, consistent with the above MSP analysis, *ADAMTS12* gene promoter was found barely methylated in LoVo cells (Table 1).

The above results strongly suggested that promoter methylation could silence *ADAMTS12* gene expression. To examine this possibility, we selected the MSS cell lines SW480 and SW620, and the MSI cell lines DLD-1 and HCT-116 to perform RT-PCR amplification. As illustrated in Fig. 1D, none of these cell lines expressed *ADAMTS12*. It is also remarkable that none of the additional cells employed in the methylation analysis showed appreciable levels of *ADAMTS12* expression (not shown). Then, we investigated whether *ADAMTS12* expression could be restored by treatment with the demethylating agent 5′-aza-2′-deoxycitydine (Aza). This treatment was carried out with or without the histone deacetylase inhibitor trichostatin A (TSA), which has also been used to examine the methylation status of *ADAMTS18* (Jin et al., 2007). As shown in Fig. 1D, *ADAMTS12* expression could be reinstated following drug treatment. Moreover, this activation was observed in DLD-1 and HCT-116 cells even in the absence of TSA. Taken together, these results provide evidence for the epigenetic silencing of *ADAMTS12* expression in colon carcinomas.

Expression of *ADAMTS12* in colon carcinomas

Next, we used RT-PCR to study expression levels of *ADAMTS12* in the set of colon cancer samples previously used to examine their methylation status (Table 1). Interestingly, and in contrast to what could be expected from the previous results, *ADAMTS12* expression levels were higher in tumor samples than in normal tissues (Fig. 2). These tumor biopsies contained at least 50% tumor cell content but no attempt at tumor-cell enrichment was made. A similar result was obtained when PCR amplification was performed using a set of five commercially available paired normal and tumor cDNA samples from colon carcinomas (Fig. 2). As a positive control for this analysis, we performed PCR amplification of *SFRP1*, a gene known to be silenced by methylation in colon cancer (Aguilera et al., 2006).

Induction of *ADAMTS12* expression in stromal cells

A possible explanation for above unexpected result is that *ADAMTS12* is produced by stromal cells in the immediate vicinity of neoplastic epithelial cells and not by the tumor cells themselves. To evaluate this possibility, we performed an immunofluorescence study using 20 colon carcinoma tissue sections. As can be seen in Fig. 3A, double immunofluorescent staining for ADAMTS-12 and alpha smooth muscle actin (α-SMA) showed a close approximation of ADAMTS-12 immunoreactivity to α-SMA-positive cells. By contrast, there was no staining in epithelial cells. Moreover, detailed examination of 55 tissue areas revealed that 60% of areas of well-differentiated and moderately differentiated carcinomas showed a strong immunoreactivity for ADAMTS-12 (Table 2), and only two areas of the moderately differentiated carcinomas lacked staining for ADAMTS-12. However, only 36% of areas of advanced poorly differentiated carcinomas showed strong immunostaining for the enzyme, whereas 48% of these areas lacked staining, which is consistent with the relative low proportion of myofibroblasts in these

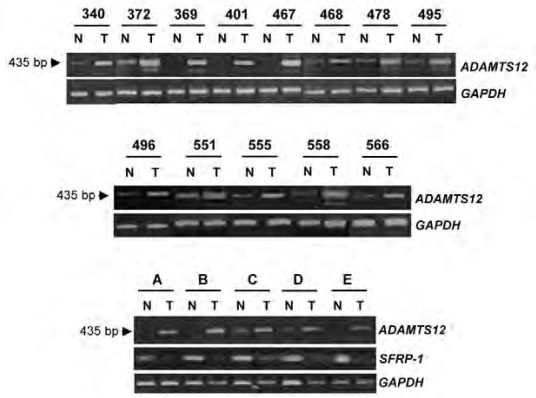

Fig. 2. *ADAMTS12* expression in human colon carcinomas. Top and middle panels: total RNA from the indicated colon carcinoma samples was isolated and *ADAMTS12* expression was assayed by RT-PCR (N, normal; T, tumor). The remaining colon carcinoma samples employed in this work were discarded for this analysis because of RNA quality. Bottom panel: amplification of *ADAMTS12* from a set of five (A-E) commercially available paired normal and tumor first-strand cDNA samples from colon carcinomas. Amplification of *SFRP1* was used as positive control. In all experiments, RT-PCR of *GAPDH* mRNA was used as an internal control.

undifferentiated tumours. Expression in normal mucosa or adenomatous polyps was not or hardly detectable. These data indicate that the expression of *ADAMTS12* is due to fibroblastic-like cells present in the stroma surrounding malignant cells. Moreover, these results point to a correlation between the expression of this metalloprotease and the histopathologic grade of tumors.

To examine whether colon fibroblasts could express the metalloprotease gene, we carried out co-cultures of CCD-18Co colon fibroblasts with colon cancer cells, and used quantitative RT-PCR to compare the differences in *ADAMTS12* expression levels with those detected in fibroblasts cultured alone. To discard any possible presence of tumor cells mixed with the fibroblasts following co-culture, absence of RT-PCR amplification of the carcinoma marker *EpCAM* (Trzpis et al., 2007) was also assayed (not shown). As illustrated in Fig. 3B, fibroblasts showed about twofold higher *ADAMTS12* expression when co-cultured with SW620 and DLD-1 cells, and fivefold higher levels in the case of co-culture with SW480 colon cancer cells. Additionally, we found that TGF-α (4.5-fold), TGF-β1 (3.3-fold), and IL-6 (twofold) also raised *ADAMTS12* expression in these fibroblasts. By contrast, treatment with IL-10 did not modify the expression levels of this metalloproteinase. Next, we examined whether the enhanced expression of *ADAMTS12* observed in fibroblasts co-cultured with tumor cells could be promoted by any of the analyzed cytokines. To this end, we employed different blocking antibodies and found that a considerable reduction of the metalloprotease expression was observed when blocking antibodies against TGF-β1 were added to the co-cultures, but not in the presence of blocking antibodies against TGF-α (Fig. 3C). *ADAMTS12* expression was not detected in tumor cells in the course of these experiments (not shown).

Then, we asked whether this induced expression of *ADAMTS12* in colon fibroblasts could be associated with any functional effect. To evaluate this possibility, we selected SW620 (MSS) and DLD-1 (MSI) cells because of the similar enhancement of *ADAMTS12* expression caused in CCD-18Co fibroblasts (Fig. 3B). Growth rates for the colon cancer cells were calculated during the exponential growth phases in co-culture with colon fibroblasts. Cancer cells cultured alone on the inserts were used as control. Results indicated that both cell lines had growth defects. The doubling time for DLD-1 cells in co-culture with fibroblasts was 16.9 hours, whereas it was 13.9 hours when the cell line was cultured alone. This difference was even more significant in the case of SW620 cells: the doubling times were 31.5 hours in co-culture and 25.7 hours in the absence of fibroblasts. We also determined the percentage of apoptotic cells in the assayed conditions after 96 hours of incubation (Fig. 3D). Analysis of DLD-1 revealed that the apoptotic cell population was 34% in co-culture with colon fibroblasts, and 25.5% when the tumor cell line was cultured alone. Potency of apoptosis induction by the fibroblasts was higher in case of SW620 cells: 36.5% of tumor cells were apoptotic in co-culture, but only 12.5% in the absence of fibroblasts. This study indicates the occurrence of a decrease in tumor cell proliferation concomitant with enhanced *ADAMTS12* expression.

Taken together, these findings indicate that ADAMTS-12 is mainly secreted by the stromal cells surrounding cancer cells in colon carcinomas. This stromal expression of *ADAMTS12* might suggest the occurrence of a protective response, which tries to compensate for the epigenetic silencing of this gene in malignant cells.

ADAMTS12 expression is epigenetically silenced in tumors of different origin

The above results obtained in colon cancer tissues and cell lines prompted us to extend our study on *ADAMTS12* expression to tumors of different origins through methylation analysis of additional tissues and cell lines. We employed the Illumina GoldenGate methylation platform to study the DNA methylation status of two CpG positions within the *ADAMTS12* CpG island in a panel of normal tissues and cancer cell lines. As shown in Fig. 4, *ADAMTS12* was found to be highly hypermethylated in most of the analyzed tumor cell lines (21 out of 29 for probe P250, and 26 out of 29 for probe E52). By contrast, methylation was scarcely detected or not detected in the normal tissues examined. Overall, these results provide additional evidence to indicate that *ADAMTS12* is an epigenetically silenced gene in tumor cells of diverse origin and to support its potential role as a tumor suppressor gene. Interestingly, this study also showed that the promoter region of *ADAMTS12* is not methylated in LoVo cells (probe P250), which perfectly agrees with the results obtained from MSP and bisulfite-sequencing PCR (BSP) analysis (Fig. 1). However, this gene is highly methylated in these cell lines in a region contained within its first exon (E52). Thus, it could not be ruled out that methylation in positions other than those previously examined by BSP in this work could control the silencing of *ADAMTS12* expression.

Discussion

In this work, we have found that the *ADAMTS12* gene is subjected to a generalized methylation process in colorectal tumors and in a variety of tumor cells from different origins, a mechanism of growing relevance in the inactivation of tumor-suppressor genes (Esteller, 2007). We have also proven that *ADAMTS12* expression can be induced in CCD-18Co fibroblasts and is associated with a decrease in proliferation and an increase in apoptosis of the adjacent tumor epithelial cells. Finally, histopathologic analysis has revealed that ADAMTS-12 is detected in stromal cells from well-differentiated and moderately differentiated tumors rather than in

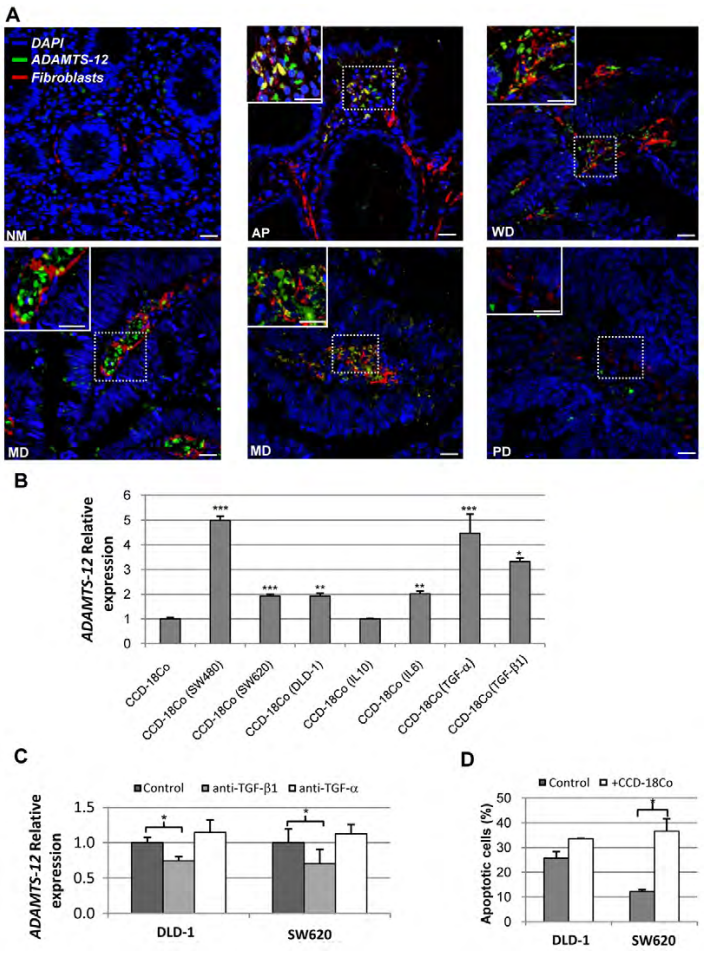

Fig. 3. Induction of *ADAMTS12* expression in stromal cells. (A) Immunofluorescence detection of ADAMTS-12 in colon carcinoma tissue sections. Detection of activated fibroblasts was performed using an anti-SM α-actin monoclonal antibody. ADAMTS-12 was detected using the H-142 antibody from Santa Cruz Biotechnology. Representative tissue sections corresponding to normal mucosa (NM), adenomatous polyp (AP), and well-differentiated (WD), moderately differentiated (MD) and poorly differentiated (PD) carcinomas are shown. Insets show higher magnifications of sections marked with a square. Scale bars: 50 μM. (B) Induction of *ADAMTS12* expression was assayed by co-culture of CCD-18Co fibroblasts with the indicated colon cancer cells, or through treatment with the indicated cytokines. After 36 hours, *ADAMTS12* expression was determined by quantitative PCR. Actin expression was simultaneously analyzed as internal control. (C) Anti-TGF-β1 blocking antibodies reduce *ADAMTS12* expression by fibroblasts co-cultured with tumor cells. A remarkable reduction of *ADAMTS12* expression was detected when anti-TGF-β1 blocking antibodies were added to the co-cultures, whereas the presence of these antibodies did not show any direct effect on the metalloprotease expression. (D). Induction of apoptosis in colon cancer cells. DLD-1 and SW620 cells were co-cultured with CCD-18Co fibroblasts or cultured alone, and the percentage of apoptotic tumor cells was determined after 96 hours. (B,C,D) Data are means ± s.e.m. Statistical significance was determined using a Student's *t*-test (****P*<0.001, ***P*<0.01, **P*<0.05).

those from advanced poorly differentiated carcinomas. Taken together, these results reinforce the proposal that ADAMTS-12 is a novel protease that can be included in the growing category of proteolytic enzymes with tumor-defying properties (Lopez-Otin and Matrisian, 2007).

ADAMTS-12 belongs to a family of metalloproteases that has been commonly associated with cancer (Rocks et al., 2008). Nevertheless, the functional relevance of these enzymes in tumor progression is still unclear and in some cases contradictory. On the one hand, ADAMTS-4 and ADAMTS-5 contribute to invasiveness of glioblastoma cells through their ability to cleave brevican (Held-Feindt et al., 2006), therefore acting as pro-tumor proteases. On the other hand, and similar to our findings on ADAMTS-12, ADAMTS-8, ADAMTS-9 and ADAMTS-18 have been proposed to act as anti-tumor enzymes because the genes encoding these proteases have been found hypermethylated in tumors of different origins (Dunn et al., 2004; Jin et al., 2007; Lo et al., 2007). Interestingly, ADAMTS-1 plays a dual role in cancer because it exhibits both pro- and anti-tumor properties (Vazquez et al., 1999; Masui et al., 2001; Iruela-Arispe et al., 2003; Kuno

Table 2. *ADAMTS12* expression and histopathologic grade in colorectal carcinoma

Sample type		Number of areas examined	Degree of staining		
			–	+/–	+
Adenomatous polyp		5	3 (60%)	2 (40%)	0 (0%)
Adenocarcinoma	Well-differentiated	5	0 (0%)	2 (40%)	3 (60%)
	Moderately differentiated	20	2 (10%)	6 (30%)	12 (60%)
	Poorly differentiated	25	12 (48%)	4 (16%)	9 (36%)
All types		55	17 (31%)	14 (25.4%)	24 (43.6%)

Twenty samples of adenomatous polyps and different grades of carcinomas were immunostained for ADAMTS-12, and 55 tissue areas examined in detail. The number and percentage of areas stained were calculated for each different group. – indicates no staining, +/– moderate staining, + intense staining. Staining resulted negative in the case of normal mucosa.

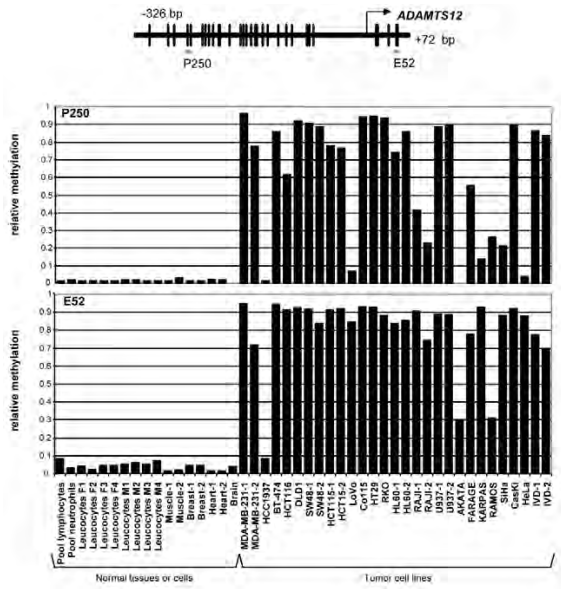

Fig. 4. DNA methylation analysis of the *ADAMTS12* promoter in a panel of normal tissues and cancer cell lines using bead arrays. Methylation was assessed at two CpG sites within the *ADAMTS12* CpG island using Illumina Goldengate Methylation Arrays. The first CpG site was located 250 bp upstream of the transcriptional start site (P250) and the second CpG site was located 52 bp downstream the transcriptional start point (E52). The amount of bisulfite-modified target DNA that hybridizes to each spot of the Illumina chip was quantified and standardized over a scale from 0.0 to 1.0 (effectively 0% and 100% likelihood of gene promoter hypermethylation, respectively).

et al., 2004; Lee et al., 2006; Lind et al., 2006). An explanation for these controversial effects might derive from the fact that this metalloprotease undergoes proteolytic processing events and the generated forms display tumor-protective or tumor-promoting properties depending on the cleavage site (Liu et al., 2006). It has been reported that the ADAMTS-1 thrombospondin domains are responsible for this protective role through their ability to sequester growth factors like vascular endothelial growth factor (Luque et al., 2003; Kuno et al., 2004). The capacity of thrombospondin domains to inhibit the function of growth factors, together with their pro-apoptotic actions (Mirochnik et al., 2008), could also help to explain the effects herein described in colon cancer cells in the presence of ADAMTS-12.

Histopathologic analysis has revealed that ADAMTS-12 is detected in the stroma of well-differentiated tumors rather than in advanced carcinomas. It is noteworthy that immunofluorescent double-staining analysis for ADAMTS-12 and α-SMA resembles that shown for periostin, which is expressed by stromal cells in colon carcinoma (Kikuchi et al., 2008). Periostin is a secreted extracellular matrix protein found in close proximity to the cells responsible for its expression, the α-SMA-positive cancer-associated fibroblasts. The roles of periostin in cancer appear to be quite diverse, but studies with periostin-deficient mice have shown that this protein negatively regulates tumor growth by promoting capsule formation. This suggests that, similar to our proposal for ADAMTS-12, periostin can also function as an anti-tumor protein (Shimazaki and Kudo, 2008).

In this work, we also found that *ADAMTS12* expression is enhanced in colon fibroblasts co-cultured with colon cancer, which is linked to an anti-tumor effect on cancer cells. On this basis, we speculate that the expression of this metalloprotease could form part of a stromal response promoted by cancer-associated fibroblasts to control the pro-angiogenic properties induced by different soluble growth factors. These fibroblasts, also termed myofibroblasts, are the predominant cell type in the stroma of most carcinomas (Ronnov-Jessen et al., 1996) and constitute a heterogeneous population of cells that cannot always be identified as α-SMA-positive (Sugimoto et al., 2006). These cells are phenotypically different from fibroblasts present in normal tissue, but similar to those found in wounds during the healing process (Gabbiani, 2003). Myofibroblasts are intricately interwoven with the epithelial tumor cells (Micke and Ostman, 2004) and, therefore, different pro-tumor or anti-tumor responses can be elicited by the stroma as a consequence of its interaction with the malignant cells (Mueller and Fusenig, 2004).

Among the cytokines assayed in this study, TGF-α and TGF-β1 induced the highest levels of *ADAMTS12* expression when the human colon fibroblasts were cultured alone. However, the use of blocking antibodies in co-cultured assays suggests that TGF-β1 could be one of the factors responsible for expression of the metalloprotease as a consequence of the crosstalk between tumor and stromal cells. This finding perfectly agrees with previous studies showing that TGF-β1 is one of the main inducers of *ADAMTS* gene expression, including that of *ADAMTS12*, in different conditions (Cal et al., 2001; Wang et al., 2003). Moreover, Porter et al. have also reported that *ADAMTS12* mRNA levels are higher in stromal fibroblasts than in epithelial tumor cells from breast cancer (Porter et al., 2004), a type of tumor that commonly expresses high levels of TGF-β1 (Chang et al., 2007). Altogether, these findings indicate that, following its induction in stromal fibroblasts through the action of factors such as TGF-β1, ADAMTS-12 might act as a tumor-protective protease in colon cancer and also in tumors of different origin such as breast cancer. Finally, the fact that other members of this metalloprotease family have also been found in stromal cells but not in the corresponding cancer cells (Cross et al., 2005), leads us to propose that stromal expression of different *ADAMTSs* could be a common feature in tumor development or progression.

In summary, we provide new data indicating a dual regulation of *ADAMTS-12* expression in colon carcinoma: epigenetic inactivation in epithelial malignant cells and induction in myofibroblasts, suggesting that this metalloprotease could form part of a protective stromal response aimed at limiting tumor progression. Our data also suggest that ADAMTS-12 might have potential clinical applications because this enzyme could be a prognosis marker not only in colon cancer but also in other tumors in which ADAMTS-12 is detected (Cal et al., 2002). However, further studies will be necessary to determine the precise role of *ADAMTS12* expression during progression of colon cancer. Likewise, analysis of susceptibility to colon cancer in *Adamts12*-null mice, currently ongoing in our laboratory, will help to clarify the precise role of this enzyme in the context of the growing number of tumor-defying proteases that are known to be produced by human cells.

Materials and Methods

DNA samples

Genomic DNAs from paired normal and tumor colon samples were obtained from 19 patients undergoing surgery at the Catalan Institute of Oncology (Barcelona, Spain). Genomic DNA from human lymphocytes was used as a control for unmethylated DNA, and the CpGenome Universal Methylated DNA (Chemicon), for methylated

DNA. A set of five paired normal and tumor colon cDNA samples to evaluate *ADAMTS12* expression was purchased from Clontech.

Cell culture

Colon cancer cell lines and CCD-18Co fibroblasts were from the American Type Culture Collection. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 50 μg/ml streptomycin.

CpG island identification and DNA methylation analysis

An interval of 2.0 kb upstream and 0.5 kb downstream from the first *ADAMTS12* ATG codon was analyzed for CpG island identification using the Methyl Primer Express Software v1.0, (https://produtcs.appliedbiosystems.com). This program was also used to design MSP primers. Primers for the unmethylated reaction were 5′-GAGTTTGGGAGGAAGATGTATT-3′ and 5′-CTAACAATATCCACTTTCA-ACAAAA-3′, and for the methylated reaction 5′-GAGTTCGGGAGGAAGAT-GTATC-3′ and 5′-ACAATATCCGCTTTCGACG-3′. To analyze methylation density of genomic DNA, two BSP primers, 5′-TGGTTTGGGGGTTTTTTATT-3′ and 5′-AACTAAACACCTTTTTCCCCTC-3′, were designed using the indicated program. Bisulfite-modified genomic DNA from paired normal and tumor tissues and cell lines was used as template.

RT-PCR amplification

Total RNA from tissue samples and cell lines was isolated by guanidium thiocyanate-phenol-chloroform extraction and reverse-transcription reactions were carried out with 300 ng of RNA, using the Thermoscript RT-PCR system with Platinum Taq polymerase (Invitrogen). The *ADAMTS12*-specific primers used were 5′-CAG-AAAGGACATCTTGCTGG-3′ and 5′-TCCTGGCAGAAGGTGCATTC-3′. PCR amplifications were performed under the following conditions: one cycle at 95°C for 1 minute, and 35 cycles at 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds. *GAPDH* was amplified as an internal control.

Treatment of colon cancer cell lines with demethylating agents

Colon cancer cells were seeded in 10 mm dishes and incubated in culture media with 10 μM Aza for 3 days. For the combined treatment, cells were treated for 3 days with Aza and subsequently with 10 ng/ml TSA for 24 hours. *ADAMTS12* expression before and after treatment was determined by RT-PCR.

Co-culture assays and cytokine treatment

Induction of *ADAMTS12* expression in colon fibroblasts was examined through their co-culture with colon cancer cell lines or by cytokine treatment. For co-cultures, subconfluent CCD-18Co fibroblasts in six-well dishes were cultured with cell-culture inserts (8.0 μm pore size; BD Biosciences) overlaid with the indicated colon cancer cell lines and incubated for 36 hours in DMEM medium containing 0.25% FBS. Cytokines were added to CCD-18Co fibroblasts cultured in six-well dishes at 2 ng/ml final concentration (except TGF-α, which was used at 25 ng/ml). Then, 9 μl of a 1:5 dilution of cDNA was employed in quantitative PCR using TaqMan probe HS00917112_m1, TaqMan Master Mix and AbiPrism 7900HT (Applied Biosystems). When indicated, anti-TGF-α or anti-TGF-β1 blocking antibodies (Calbiochem) were added to the co-cultures at 1.5 μg/μl. Actin expression was analyzed as an internal control. Statistical significance was determined using a Student's *t*-test. For determination of cell growth, overlaid colon cancer cell lines (SW620 and DLD-1) were trypsinized and counted directly in a hemocytometer. After plotting total cell number against time, data were adjusted to exponential curves from where doubling-time and regression values were calculated. For apoptosis analysis, 5×10^5 cells were evaluated using the Anexin V-FITC/IP kit from Sigma-Aldrich. Each cell population was identified in a Cytomics FC500 (Beckman-Coulter).

Tissue immunofluorescence analysis

Paraffin-embedded colon tissues from 20 different tumors were cut into 5-micron sections and mounted onto glass slides. The sections were deparaffinized in xylene followed by ethanol hydration. Samples were permeabilized for 30 minutes with 0.1% Triton X-100 and incubated for 15 minutes in PBS with 0.1 M glycine. Nonspecific labeling was blocked with 5% BSA in PBS for 30 minutes. Sections were incubated overnight at 4°C with an anti alpha smooth muscle primary antibody (α-SMA, dilution 1:50; DakoCytomation) to detect activated fibroblasts. After incubation, sections were washed with PBS and PBS-Tween 20 (0.5% v/v), and then incubated with Alexa Fluor 546-conjugated goat anti-mouse antibody (dilution 1:500; Invitrogen) for 45 minutes at room temperature. After washing with PBS and PBS-Tween, sections were then incubated with a second primary antibody against ADAMTS-12 (H-142, dilution 1:25; Santa Cruz Biotechnology) overnight at 4°C and washed. H-142 is a polyclonal antibody raised against amino acids 1441-1582 of the human ADAMTS-12. Alexa Fluor 488-conjugated goat anti-rabbit antibody (dilution 1:200; Invitrogen) was added and incubated for 45 minutes at room temperature. The corresponding negative controls were set by omitting the anti-ADAMTS-12 antibody. We then washed the slides and stained nuclei with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at 1 μg/ml in Vectashield mounting medium (Vector Laboratories). Images were obtained using a confocal microscope, Leica TCS-SP2-AOBS.

Analysis of DNA methylation status using bead arrays

Methylation analysis of *ADAMTS12* promoter in a panel of normal tissues and cancer cell lines was assessed using Illumina Goldengate Methylation Arrays (Bibikova et al., 2006). The two CpG sites analyzed were located 250 bp upstream and 52 bp downstream of the *ADAMTS12* transcriptional start point (E52). Thge amount of bisulfite-modified target DNA that hybridizes to each spot of the Illumina chip was quantified and standardized over a scale from 0.0 (0% likehood of gene promoter methylation) to 1.0 (100% likelihood of gene promoter hypermethylation).

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IV. Análisis mutacional del gen ADAMTS15 en cáncer colorrectal humano

ADAMTS-15 es otra proteína identificada en nuestro laboratorio y a la que trabajos previos en la bibliografía habían apuntado como un posible supresor tumoral mutado en cáncer de colon. Dados estos precedentes, juzgamos necesario investigar en profundidad los mecanismos de inactivación de *ADAMTS15*, y a tal fin llevamos a cabo un análisis mutacional de todos los exones de su gen así como un estudio del estado de metilación en cáncer colorrectal. A diferencia de *ADAMTS12*, *ADAMTS15* no está silenciado epigenéticamente, aunque pudimos detectar diversas mutaciones en su secuencia codificante, potencialmente deletéreas para la función de la proteína. Adicionalmente, realizamos ensayos funcionales que contribuyeran a explicar el significado de su pérdida de función, encontrando que la presencia de ADAMTS-15 disminuía el crecimiento y la invasión tumoral y estaba correlacionada inversamente con el grado de diferenciación histológico.

Artículo 4: Cristina G. Viloria, Álvaro J. Obaya, **Ángela Moncada-Pazos**, María Llamazares, Aurora Astudillo, Gabriel Capellá, Santiago Cal y Carlos López-Otín. “Genetic inactivation of ADAMTS15 metalloprotease in human colorectal cancer”.

Cancer Research 69: 4926-4934 (2009).

Aportación personal al trabajo

Contribuí en este proyecto llevando a cabo los análisis epigenéticos y parte de los estudios funcionales, concretamente algunos de los ensayos de invasión con líneas celulares. Además participé en la elaboración del manuscrito así como de las figuras que lo acompañan.

Research Article

Genetic Inactivation of ADAMTS15 Metalloprotease in Human Colorectal Cancer

Cristina G. Viloria,[1] Alvaro J. Obaya,[2] Angela Moncada-Pazos,[1] María Llamazares,[1] Aurora Astudillo,[3] Gabriel Capellá,[4] Santiago Cal,[1] and Carlos López-Otín[1]

[1]Departamento de Bioquímica y Biología Molecular and [2]Biología Funcional, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, [3]Servicio de Anatomía Patológica, Hospital Central de Asturias, Oviedo, Spain and [4]Laboratori de Recerca Translacional, IDIBELL, Institut Català d'Oncologia, L'Hospitalet de Llobregat, Barcelona, Spain

Abstract

Matrix metalloproteinases have been traditionally linked to cancer dissemination through their ability to degrade most extracellular matrix components, thus facilitating invasion and metastasis of tumor cells. However, recent functional studies have revealed that some metalloproteases, including several members of the ADAMTS family, also exhibit tumor suppressor properties. In particular, *ADAMTS1, ADAMTS9,* and *ADAMTS18* have been found to be epigenetically silenced in malignant tumors of different sources, suggesting that they may function as tumor suppressor genes. Herein, we show that *ADAMTS15* is genetically inactivated in colon cancer. We have performed a mutational analysis of the *ADAMTS15* gene in human colorectal carcinomas, with the finding of four mutations in 50 primary tumors and 6 colorectal cancer cell lines. Moreover, functional *in vitro* and *in vivo* studies using HCT-116 and SW-620 colorectal cancer cells and severe combined immunodeficient mice have revealed that ADAMTS15 restrains tumor growth and invasion. Furthermore, the presence of ADAMTS15 in human colorectal cancer samples showed a negative correlation with the histopathologic differentiation grade of the corresponding tumors. Collectively, these results provide evidence that extracellular proteases, including ADAMTS15, may be targets of inactivating mutations in human cancer and further validate the concept that secreted metalloproteases may show tumor suppressor properties. [Cancer Res 2009;69(11):4926–34]

Introduction

The establishment of causal relationships between proteolytic activities and tumor progression has prompted to consider proteases as suitable targets for anticancer therapy (1–5). However, an increasing body of evidence indicates that some proteases can confer antitumorigenic properties (6, 7). Among them, there are several members of the a disintegrin and metalloproteinase with thrombospondin domains (ADAMTS) family including ADAMTS1, ADAMTS8, ADAMTS9, ADAMTS12, ADAMTS15, and ADAMTS18 (7). All of them share a complex structural design characterized by the presence of a metalloproteinase domain linked to a variety of specialized ancillary domains including a series of thrombospondin-1 repeats (8–11). ADAMTS are secreted enzymes, which, on secretion, remain located at the pericellular space through interactions of their thrombospondin-1 motifs and spacer regions with extracellular matrix components (11, 12). Several reports have shown that the tumor suppressor activities of ADAMTS1 and ADAMTS8 derive from the angio-inhibitory capacities of their thrombospondin-1 domains (13, 14), whereas ADAMTS12 inhibits tumor growth through its ability to modulate the Ras-dependent extracellular signal-regulated kinase (ERK) signaling pathway (15). In addition to these biochemical studies, genetic and epigenetic analyses have supported the proposal that ADAMTS family members may act as antitumor proteases. Thus, *ADAMTS1, ADAMTS9*, and *ADAMTS18* have been identified as epigenetically silenced genes in several carcinomas (16–18). The putative role of ADAMTS15 as a potential tumor-protective protease has recently emerged after the finding that its overexpression is a sign of favorable outcome in breast cancer patients (19). Furthermore, mutational analysis of most protein coding genes in a small set of human breast and colorectal carcinomas has revealed the occurrence of some mutations in *ADAMTS15* (20, 21). However, no functional studies have been done to evaluate the potential consequences of these mutations in tumor development and progression.

In this work, we have investigated the potential role of ADAMTS15 as a candidate tumor suppressor protease through an exhaustive analysis of both *ADAMTS15* genetic and epigenetic alterations in a large collection of human colorectal carcinomas. We have also performed a series of functional analyses to assess the protective nature of ADAMTS15, with the finding that depletion of this protease markedly promotes *in vitro* and *in vivo* tumor growth and invasion, whereas overexpression of the enzyme reverses the phenotype. Finally, data obtained from human tissue microarrays show a negative correlation between *ADAMTS15* expression and differentiation grade of colon carcinomas. Overall, our data indicate that ADAMTS15 is a metalloprotease with tumor suppressor properties.

Materials and Methods

Mutational analysis. Genomic DNA from a set of 50 normal/tumor paired colorectal tissue samples were obtained from patients subjected to surgery at Institut Català d'Oncologia. PCR amplification of *ADAMTS15* coding exons was done as described by Sjoblom et al. (20). Sequence data were analyzed using Mutation Surveyor software (20). Both tumor and normal paired tissue samples were resequenced to validate potential mutations and discard germ-line variants. Potential effect of an amino acid change in the ADAMTS15 protein function was predicted *in silico* using the Sorting Intolerant From Tolerant algorithm (22). This computational Web-based approach calculates a score, ranging from 0 to 1, which corresponds to the probability that a particular change in an amino acid is tolerated.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

C.G. Viloria and A.J. Obaya contributed equally to this work.

Requests for reprints: Carlos López-Otín, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, 33006 Oviedo, Spain. Phone: 34-985-104201; Fax: 34-985-103564; E-mail: clo@uniovi.es.

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This software uses alignments among the members of one family to predict if an amino acid is essential for the function of the protein.

cDNA constructs. *ADAMTS15* full-length human cDNA (23) was subcloned in pcDNA3.1 expression vector. The same cDNA was employed to delete a guanine at position 2547 (pcDNA-*ADAMTS15*ΔG2547 vector; ADAMTS15_G849fs protein). To this end, cassette mutagenesis was carried out by overlap extension (24) using the following primers: TS15bamFOR 5′-CCAAATGGGATCCCTATGGCC-3′, TS15REV 5′-CGCGGAGCACGGCCC-CAGCTGCCAGCCAC-3′, TS15FOR 5′-GCAGCTGGGGCCGTGCTCCGC-GAGCTGCGG-3′, and TS15hindREV 5′-AAGCTTGCACGGCCTCAGGAC-3′. An additional construct was generated using a modified pCEP4 expression vector and a FLAG epitope at the COOH-terminal region of the protein.

Cell culture and transfection. Colorectal tumor cell lines HCT-116, SW-480, SW-620, LoVo, DLD-1, and Caco-2 were purchased from the American Type Culture Collection. Cells were routinely maintained in DMEM, with the exception of SW-620 cells that were grown in L-15 medium. Cells were transfected with the Lipofectamine reagent, and stable clones were selected in the presence of 2 μg/mL puromycin.

DNA methylation analysis and reverse transcription-PCR analysis. Methyl Primer Express version 1.0 software (Applied Biosystems) was used to identify CpG islands in an interval of 2.0 kb upstream and 1.5 kb downstream from the first ATG codon. The same program was used to design primers to amplify methylated or unmethylated DNA in both promoter and first exon regions. To evaluate *ADAMTS15* expression, cDNA was synthesized using 1 μg purified RNA and the ThermoScript Reverse Transcription-PCR System (Invitrogen). To quantitatively assess *ADAMTS15* expression levels in colorectal cancer cells, quantitative reverse transcription-PCR was done using the Taqman probe Hs01054400_g1 from Applied Biosystems. All reactions were done in quadruplicates.

RNA interference. A panel of four *ADAMTS15* specific short hairpin RNA (shRNA; 5′-TCTTCAGACCTGCGACGCTGCTTCTATTC-3′, 5′-AGA-GAAGCTGCTCTGTCATTGAGGACGAT-3′, 5′-GCTACAACCACAGCAC-CAACCGGCTCACT-3′, and 5′-TCTGTGTCCAAGGCAAGTGCATCAAGGCT-3′) introduced into the pRetroSuper (pRS) vector was purchased from OriGene. SW-620 or 293-EBNA cells were transfected with these vectors or with pRS control vector and selected with 1 μg/mL puromycin.

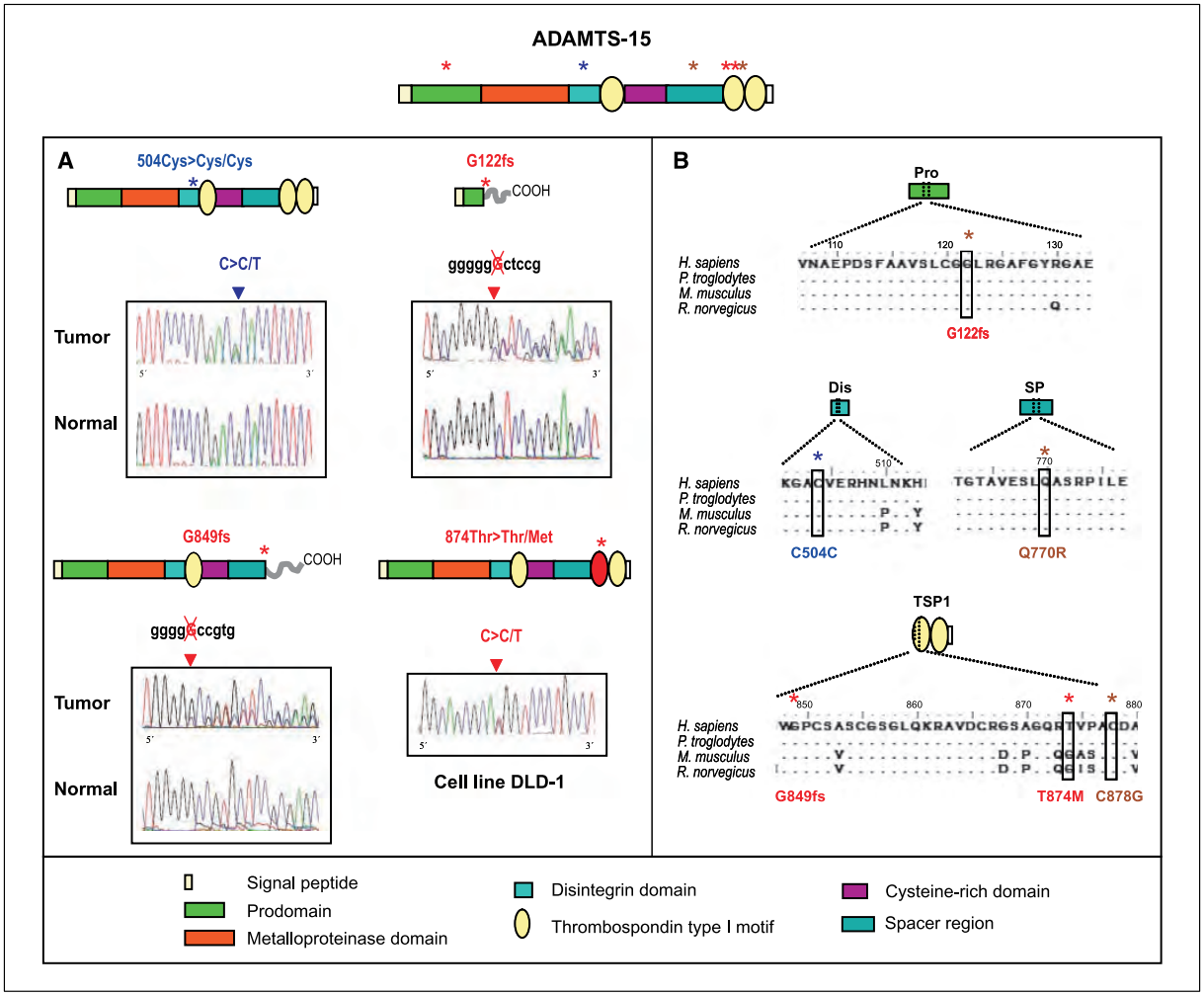

Figure 1. Somatic mutations in *ADAMTS15* gene. *A*, domain organization of ADAMTS15 and sequencing electropherograms obtained from paired normal/tumor colorectal samples and cell lines. *Arrowheads,* positions of somatic mutations; *asterisks,* nonsynonymous mutations (*red*), synonymous mutation (*blue*), and previously reported mutations in colorectal cancer (*brown*). *B*, alignment of the identified mutations with ADAMTS15 orthologues from the indicated species. Sequences are fully conserved with the exception of the indicated amino acids. Altered amino acids by somatic mutations are boxed.

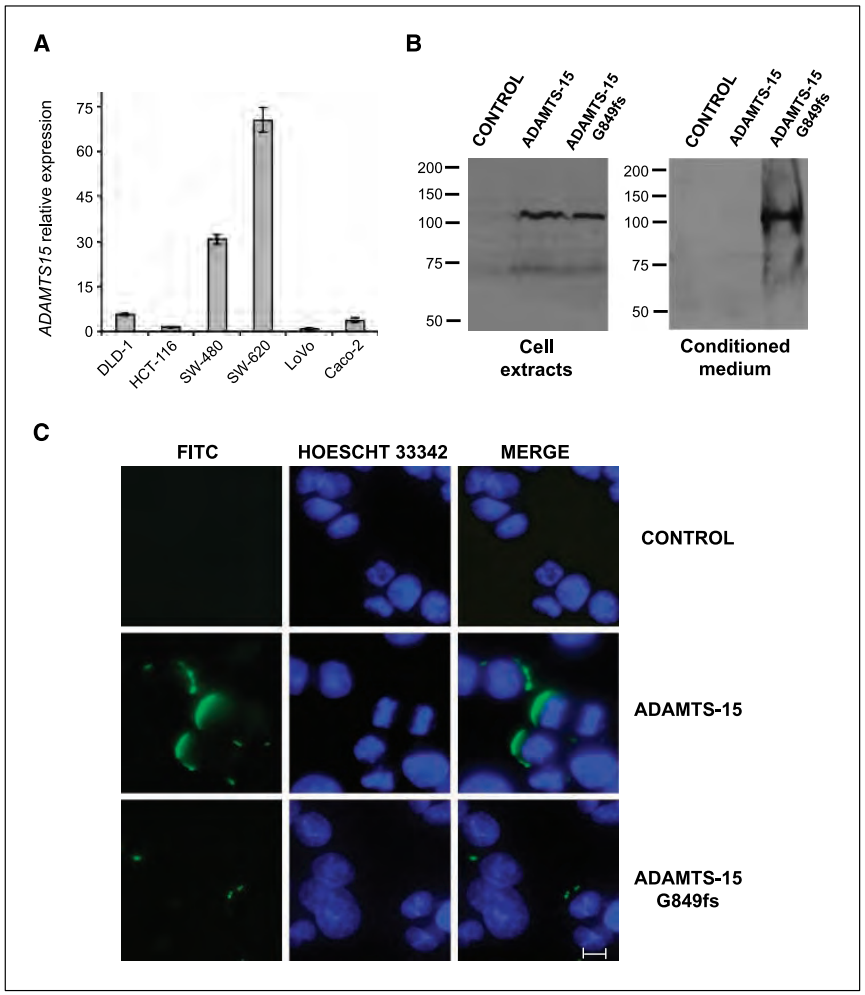

Figure 2. ADAMTS15 expression and localization in HCT-116 cells. *A,* relative expression of *ADAMTS15* determined by quantitative reverse transcription-PCR in a panel of six human colorectal cancer cell lines. *B,* detection of recombinant forms of wild-type ADAMTS15 and ADAMTS15_G848fs mutant was determined in HCT-116 lysates and in conditioned medium using the H-135 antibody. *C,* immunofluorescence analysis. The same antibody was employed to detect both wild-type and mutant ADAMTS15 in HCT-116. A secondary FITC-conjugated goat anti-rabbit antibody was used. Cell nuclei were stained with Hoechst 33342. *Bar*, 10 μm.

Colony formation assays. HCT-116 cells plated in 60 mm dishes were transfected with the wild-type or mutant *ADAMTS15*ΔG2547 expression vectors along with the corresponding control vector. Then, cells were trypsinized, plated in 6-well dishes, and cultured in medium containing 0.4 mg/mL geneticin for 15 days. SW-620 cells were also transfected with the interfering constructs along with the control vector as described above. Colonies were stained with 0.5% crystal violet in 20% methanol and counted in eight randomly selected microscopic fields.

Invasion assays. In vitro invasion potential was evaluated using 24-well Matrigel-coated invasion chambers with an 8 μm pore size (BD Biosciences). For HCT-116, 5×10^4 cells were allowed to migrate for 48 h using 5% fetal bovine serum as a chemoattractant. In the case of SW-620 cells, invasion of 1×10^5 cells was evaluated after 96 h using 2% fetal bovine serum. Cells that reached the lower surface were stained with crystal violet and counted in 10 randomly selected microscopic fields.

Western blot. Western blot analysis was done using the following primary antibodies: H-135 anti-ADAMTS15 (Santa Cruz Biotechnology), anti-p44/42 mitogen-activated protein kinase, anti-phospho-p44/42 mitogen-activated protein kinase (Cell Signaling Technology), anti-FLAG-M2 (Sigma-Aldrich), and anti-actin (Abcam). Immunoreactive bands were visualized using horseradish peroxidase-labeled secondary antibodies and chemiluminescent horseradish peroxidase substrate (Millipore). To detect recombinant proteins, the indicated cells were transfected with pCEP-*ADAMTS15*-FLAG (15), pcDNA-*ADAMTS15*ΔG2547, or the corresponding empty vector. Cell extracts along with the conditioned medium were used for Western blot analysis.

Immunostaining. Two human tissue arrays with normal and tumor colorectal samples were obtained from the Hospital Universitario Central de Asturias and used to evaluate *ADAMTS15* expression. After dewaxing and rehydrating, samples were incubated overnight with H-135 antibody at 1:200 dilution. Sections were incubated with EnVision System-labeled polymer horseradish peroxidase anti-rabbit (DAKO) followed by incubation with diaminobenzidine colorimetric reagent (DAKO) and counterstaining with hematoxylin. To detect ADAMTS15 in HCT-116 cells, immunofluorescence was carried out as described (15).

***In vivo* tumorigenicity.** Experimentation with severe combined immunodeficient mice (C.B-17/IcrCrl-scid-BR; Charles River Laboratories) followed institutional guidelines approved by the local animal review board. To induce subcutaneous tumors, two groups of six severe combined immunodeficient mice were injected at one flank with 7×10^6 cells of two different SW-620-ADAMTS15–interfered clones (sh clone2 and sh clone12). As control, the opposite flank in each animal was injected with a stable clone transfected with the pRS empty vector. Tumor growth was monitored as described (15). Histopathologic characteristics of the tumors were evaluated in H&E-stained sections.

Results

Genetic and epigenetic analysis of *ADAMTS15* in human colorectal cancer. Genomic DNA from a collection of 50 human colorectal cancer cases and 6 colon cancer cell lines was screened for mutations through the eight coding exons and intron-exon junctions of the *ADAMTS15* gene. Combining colorectal cancer samples and cell line data, four heterozygous mutations were identified, three mutations in patients and one mutation in cell lines (Fig. 1). All these changes were shown to be somatic by analysis of DNA from normal tissues of the same individuals. No correlation between the appearance of mutations and histopathologic features was observed (Supplementary Table S1). In tumors, one of these changes was a single-base substitution and the other two were single-base deletions (Fig. 1*A*). The observed nucleotide substitution consisted of a cytosine-to-thymine transition at nucleotide 13777 of the gene (exon 4), resulting in a silent mutation at codon 504. Hence, the cysteine residue encoded by this codon remains unaltered in heterozygosis (504Cys>Cys/Cys; Fig. 1*A* and *B*). The remaining changes were nonsynonymous mutations and consequently may directly affect the ADAMTS15 protein function. Thus, the coding changes corresponding to deletion 366ΔG in exon 1 (G122fs; Fig. 1*A*) would cause a frameshift generating a truncated protein of 244 amino acids (compared with 950 in the wild-type protein), only containing the signal peptide and part of the prodomain. In the case of deletion 24544ΔG (G849fs; Fig. 1*A*), it would also produce a frameshift finally leading to the production of an altered protein of 947 amino acids and lacking the last two thrombospondin-1 motifs. These deletions were heterozygously detected in the same tumor sample and may cause the loss of ADAMTS15 normal function.

Mutational analysis of colorectal cancer cell lines revealed the presence of one point mutation in DLD-1 cells, consisting of a single-base substitution 24616C>C/T (874Thr>Thr/Met) in exon 8 (Fig. 1*A* and *B*). This change would affect the first of the two contiguous thrombospondin-1 domains present at the COOH-terminal region. Interestingly, we also detected a heterozygous polymorphism in exon 4 (13589T>T/C; 442Tyr>Tyr/His) that had not been described previously. This single nucleotide polymorphism was also detected in two tumor/normal colorectal samples and in LoVo cells and affects a tyrosine residue that is conserved in the disintegrin domain of nine different analyzed species (not shown). We next evaluated the potential effect of the $Thr^{874}Met$ mutation in metalloprotease function using the Sorting Intolerant From Tolerant algorithm (22). The normalized probability for a $Thr^{874}Met$ substitution at the second thrombospondin-1 domain of ADAMTS15 was <0.01, thus predicting a deleterious effect. Similarly, the germ-line variant $Tyr^{442}His$ was also predicted to be deleterious. Overall, these results indicate that, with the exception of the silent mutation C13777T ($Cys^{504}Cys$), the functional relevance of which is uncertain, all remaining mutations found in this mutational analysis of *ADAMTS15* in colon cancer could affect the normal function of the protein.

Additionally, we examined whether *ADAMTS15* could be epigenetically silenced through methylation. To do this, we first determined the occurrence of CpG islands in its 5′ region and performed a methylation-specific PCR amplification analysis in a variety of randomly selected colorectal tissue samples. Moreover, the *ADAMTS15* expression pattern in the same samples was analyzed. No relation between expression levels, normal or tumor status of the tissues, and promoter methylation was observed (Supplementary Table S1; Supplementary Fig. S1). Consistent with these results, *ADAMTS15* did not show the expression pattern characteristic of epigenetically silenced genes (Supplementary Fig. S1).

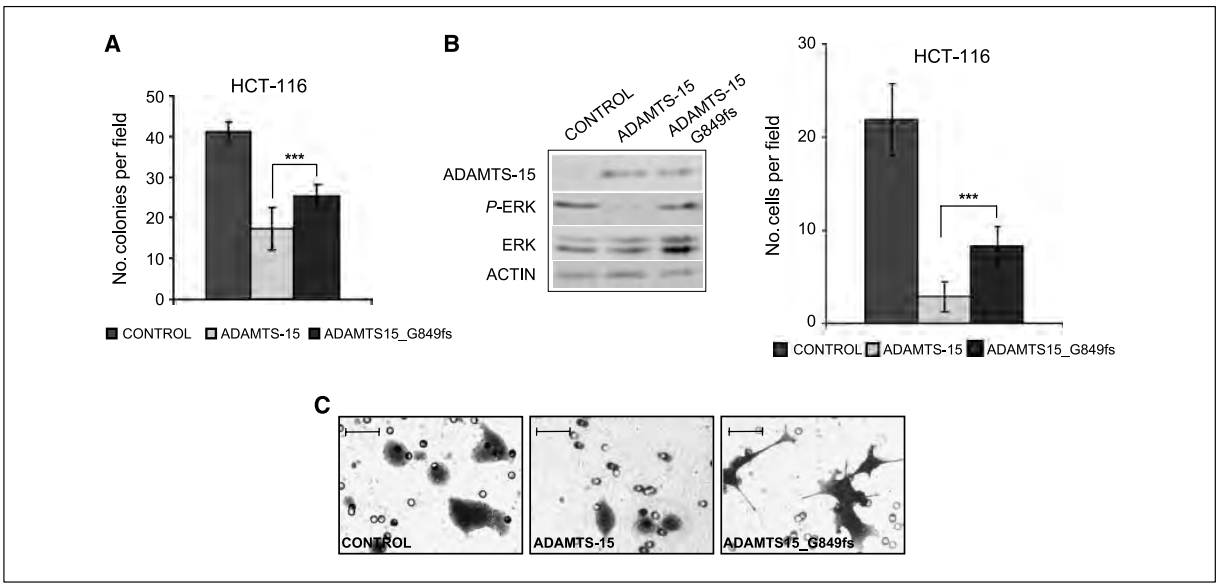

Figure 3. *ADAMTS15* expression modifies colony-forming ability and invasion properties of HCT-116 colorectal cancer cells. *A,* average colony formation after 15 days of selected HCT-116 cells expressing *ADAMTS15* and *ADAMTS15_G849fs. Bars,* SD. ***, $P < 0.005$, Student's *t* test. *B, left,* expression of *ADAMTS15* and *ADAMTS15_G849fs* in HCT-116 clones was determined by Western blot analysis; *right,* HCT-116 cells expressing *ADAMTS15* and *ADAMTS15_G849fs* were plated in Matrigel invasion chambers and the average number of invasive cells was evaluated. *Bars,* SD. ***, $P < 0.005$. Levels of ERK and phospho-ERK (*P-ERK*; *left*) were evaluated by Western blot of HCT-116 cells expressing *ADAMTS15* or *ADAMTS15_G849fs. C,* morphologic changes induced in HCT-116 *ADAMTS15* and *ADAMTS15_G849fs* cells. *Bar,* 40 μm.

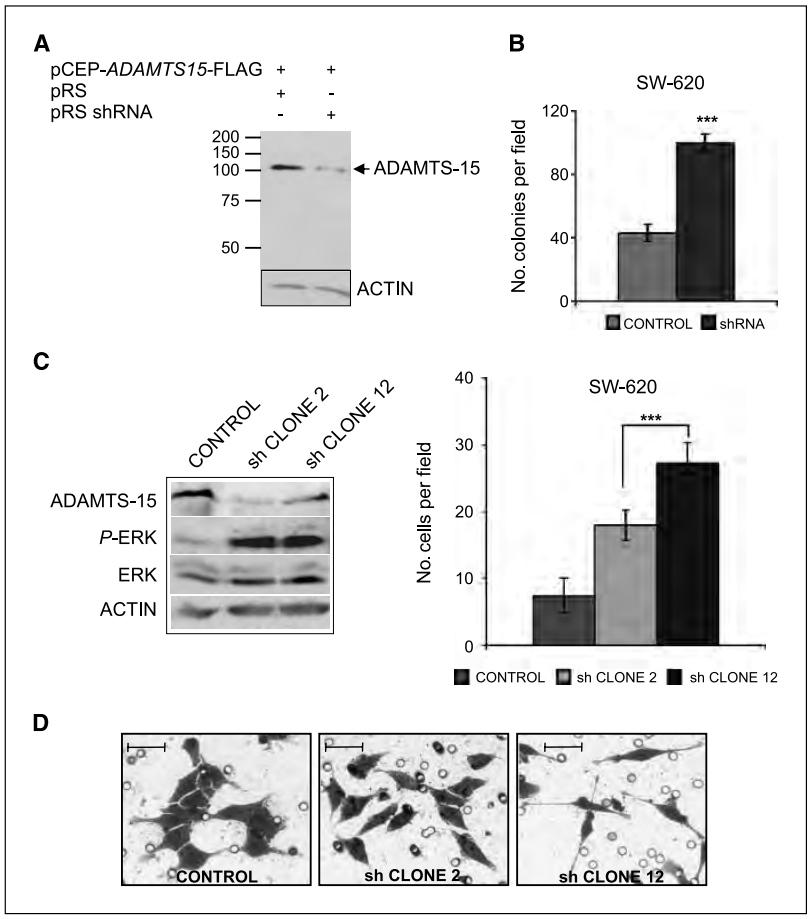

Figure 4. *ADAMTS15* down-regulation modifies colony-forming ability and invasion properties of SW-620 colorectal cancer cells. *A,* 293-EBNA cells were cotransfected with the *ADAMTS15* epitope-tagged cDNA construct (pCEP-*ADAMTS15*-FLAG) and a set of four shRNA interfering plasmids (pRS-shRNA) or with the control vector (pRS). *ADAMTS15* expression levels were evaluated by Western blot analysis. *B,* average colony formation selected ADAMTS15-interfered SW-620 cells. *Bars,* SD. ***, $P < 0.005$, Student's *t* test. *C, left, ADAMTS15* expression in two interfered SW-620 clones was determined by Western blot; *right,* ADAMTS15-interfered SW-620 cells along with control cells were plated in Matrigel invasion chambers, and the average number of invasive cells was evaluated. *Bars,* SD. ***, $P < 0.005$. Levels of ERK and phospho-ERK (*left*) were also evaluated. *D,* morphologic changes induced in ADAMTS15-interfered SW-620 cells versus control. *Bar,* 40 μm.

G849fs mutation alters the localization of ADAMTS15. To examine the functional relevance of the G849fs alteration, we constructed a vector containing the *ADAMTS15* cDNA harboring this mutation. HCT-116 cells were used for this analysis, as they do not express *ADAMTS15* (Fig. 2*A*). Western blot analysis using the H-135 antibody on whole-cell lysates identified specific bands on extracts from both ADAMTS15 and ADAMTS15_G849fs HCT-116 transfected cells (Fig. 2*B*). These bands were absent in cells transfected with an empty vector, indicating that they likely correspond to the wild-type and mutant forms of ADAMTS15, as their molecular masses agree well with those predicted for these proteins, which are very similar in size but distinct at their COOH-terminal ends (ADAMTS15, ~103 kDa; ADAMTS15_G849fs, ~102 kDa). However, only the mutant form can be detected in conditioned medium (Fig. 2*B*). Moreover, immunostaining of HCT-116 cells expressing intact ADAMTS15 indicated that the protease remained attached to the extracellular matrix following secretion (Fig. 2*C*). These results suggest that the thrombospondin-1 units are important to retain the full-length ADAMTS15 protein attached to the cell surface, because the absence of thrombospondin-1 domains as occurs in ADAMTS15_G849fs drives the protein to the cell medium. Similar results were obtained in 293-EBNA cells transfected with vectors expressing wild-type and mutant forms of ADAMTS15 tagged with a FLAG epitope (data not shown), thus ruling out the possibility that these changes in protease localization are cell line specific. These data indicate that different forms of ADAMTS15 may show different cellular localizations, which could result in the abrogation of the normal function of the protease or in the acquisition of novel functional properties.

***ADAMTS15* expression in colorectal cancer cell lines reduces colony formation and cell invasion.** HCT-116 cells expressing exogenous ADAMTS15 were selected to examine the ability of the enzyme to induce antitumor properties. As shown in Fig. 3*A*, ectopic expression of wild-type *ADAMTS15* strongly inhibited colony formation (58.1% colony reduction) compared with control cells, whereas expression of the ADAMTS15_G849fs form exhibited a substantially diminished inhibitory effect (38.4% colony reduction). Similar results were obtained in SW-480 colorectal cells (data not shown). Following transfection in HCT-116 cells, individual clones were isolated and screened for *ADAMTS15* expression by quantitative reverse transcription-PCR and Western blot (Fig. 3*B, left*; data not shown). Clones expressing >10-fold *ADAMTS15* and *ADAMTS15_G489fs* mRNA levels compared with control cells were selected for further analysis. As shown in Fig. 3*B* (*right*), ectopic expression of *ADAMTS15* considerably inhibited invasion of HCT-116 cells (86.8% reduction). Likewise, the mutant form of the protein conferred a diminished invasive phenotype to cells (61.6% reduction; Fig. 3*B, right*). Altogether, these findings indicate that

overexpression of ADAMTS15 protein in HCT-116 colon cancer cells results in reduction of both plating efficiency and *in vitro* invasive properties, thus reinforcing the proposed role for ADAMTS15 as a tumor-protective metalloprotease.

Then, and considering that both *Xenopus* ADAMTS1 (25) and human ADAMTS12 (15) modulate the Ras-dependent ERK pathway, we examined the levels of the phosphorylated form of ERK in HCT-116 cells expressing ectopic ADAMTS15 and ADAMTS15_G849fs. As shown in Fig. 3*B* (*left*), phospho-ERK levels were very low in cells expressing wild-type ADAMTS15. By contrast, cells expressing the mutant protein retained the ability to phosphorylate this kinase, indicating that the COOH-terminal thrombospondin-1 domains of ADAMTS15 are required for an effective inhibition of ERK activity. Moreover, a catalytically inactive ADAMTS15 also confers antitumor properties to HCT-116 (Supplementary Fig. S2), suggesting that these domains are responsible for its antitumor role. Furthermore, ADAMTS15_G849fs overexpression induced clear morphologic changes in the invasive HCT-116 cells as illustrated by the fact that ADAMTS15_G849fs-expressing cells were larger, flatter, and spindle-shaped in contrast to the smaller and round-shaped morphology of both control and *ADAMTS15*-transfected cells (Fig. 3*C*). Additionally, we observed that neither wild-type ADAMTS15 nor the mutant-expressing cells showed altered motility in wound-healing assays (not shown).

Down-regulation of *ADAMTS15* by shRNA increases clonogenicity and invasive properties of colon cancer cells. According to their significant *ADAMTS15* expression levels (Fig. 2*A*), SW-620 cells were selected to perform RNA interference experiments. To do that, four shRNA constructs were employed to target different regions of *ADAMTS15*. First, and to evaluate the effectiveness of the shRNAs, 293-EBNA cells were transfected either with the pCEP-*ADAMTS15*-FLAG and the set of four shRNA plasmids or with pCEP-*ADAMTS15*-FLAG and the pRS empty vector. Western blot analysis revealed that the immunoreactive signal corresponding to ADAMTS15 was greatly reduced in the interfered cells when compared with control cells (Fig. 4*A*). Then, we examined the functional consequences of knocking down this protease. To this end, polyclonal populations of SW-620 cells transfected with the shRNA plasmids were plated along with the control cells, and their colony-forming ability was tested 15 days later. Figure 4*B* shows that *ADAMTS15* down-regulation confers an increased clonogenicity to SW-620 colon cancer cells.

Following puromycin selection, individual clones were expanded and *ADAMTS15* expression levels analyzed by quantitative reverse transcription-PCR and Western blot (Fig. 4*C, left*; data not shown). Clones 2 and 12 showed a similar reduction in *ADAMTS15* mRNA levels (51.9% and 53.5%, respectively) and were selected to evaluate their invasive potential using Matrigel-coated invasion chambers. As shown in Fig. 4*C* (*right*), these clones exhibited an enhancement of their invasive properties compared with cells transfected with the empty vector (1.4- and 2.6-fold, respectively). Moreover, and consistent with the above findings in the HCT-116 clones expressing *ADAMTS15*, levels of phospho-ERK were significantly augmented in both clones (Fig. 4*C, left*). Furthermore, *ADAMTS15* down-regulation also induced morphologic changes, which are reminiscent of the spindle-shaped phenotype observed previously in HCT-116 cells expressing mutant ADAMTS15_G849fs (Fig. 4*D*). Collectively, these data are also consistent with the proposal that ADAMTS15 is an antitumor protease.

We next evaluated whether the observed *in vitro* functional alterations elicited by ADAMTS15 expression could also be extended to *in vivo* models. To this purpose, we examined the tumorigenic potential of ADAMTS15-depleted cells in subcutaneously injected severe combined immunodeficient mice. Consistent with the above *in vitro* data, the volume of tumors generated by ADAMTS15-interfered clones 2 and 12 versus control cells was significantly augmented over time (Fig. 5*A*). Histopathologic analysis revealed a higher percentage of necrotic regions in tumors derived from both interfered clones when compared with control tumors (Fig. 5*B*). Other histologic markers such as angiogenesis or

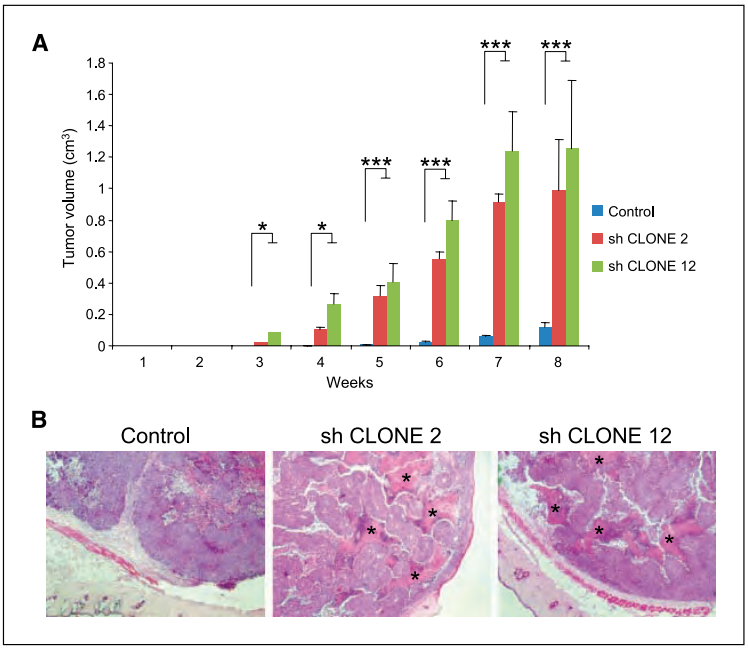

Figure 5. *In vivo* subcutaneous tumor growth of SW-620 ADAMTS15-interfered clones. *A,* severe combined immunodeficient mice were injected with control SW-620 cells or with the ADAMTS15-interfered clones (2 or 12). Tumor growth was followed during 8 wk postinjection. *Bars,* SD. *, $P < 0.05$; ***, $P < 0.005$, Student's *t* test. *B,* representative H&E staining of formalin/paraffin-embedded tumors obtained after 8 wk of subcutaneous growth (×20). *Asterisk,* necrotic tumor region.

local invasion showed no remarkable differences among the different tumors. In all cases, tumors were classified as poorly differentiated adenocarcinomas (Fig. 5*B*; data not shown). Collectively, these results support the conclusion that ADAMTS15 exhibits an *in vivo* antitumorigenic effect.

***ADAMTS15* expression negatively correlates with the histopathologic differentiation grade in colon adenocarcinoma.** To further evaluate the putative clinical relevance of *ADAMTS15* expression in colon cancer, we performed an immunohistochemical analysis of two tissue arrays containing normal and tumor colorectal specimens (Fig. 6). ADAMTS15 was detected in normal samples, preferentially in the apical side of the colon mucosa. However, in most analyzed samples, ADAMTS15 expression was reduced in the tumor tissue in comparison with the corresponding normal epithelia. Moreover, ADAMTS15 immunostaining inversely correlated with the histopathologic grade of colorectal tumors (Fig. 6). Thus, we evaluated *ADAMTS15* expression levels in 77 areas ranging from adenomatous polyps to different grades of colon adenocarcinoma. Immunoreactivity was scored as shown in Supplementary Table S2. The intensity of immunostaining in adenomatous polyps varied among samples: 25% of areas with an intense staining, 37.5% with a slight staining, and the remaining 37.5% areas with negative staining. A direct correlation between ADAMTS15 down-regulation and tumor development was assessed by the observation that positive ADAMTS15 staining was found in 58.3% of well-differentiated tumors, 21% of moderately differentiated tumors, and 0% of poorly differentiated colorectal carcinomas. These data suggest that ADAMTS15 could represent an effective differentiation marker for the histopathologic grading of colonic adenocarcinoma.

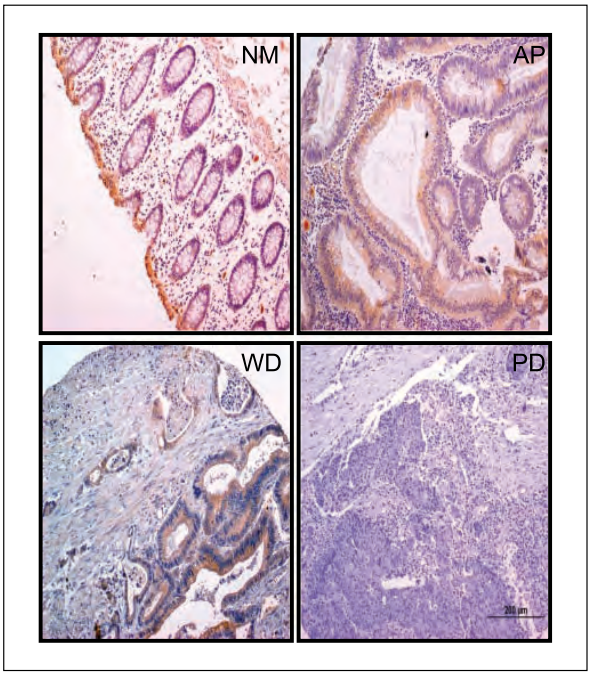

Figure 6. *ADAMTS15* expression inversely correlates with histopathologic differentiation grade in human colorectal carcinomas. Human colorectal tissue arrays were immunostained for ADAMTS15 and four representative tissue sections are shown. ADAMTS15 shows positive immunostaining in luminal epithelia of normal mucosa (*NM*). Adenomatous polyp (*AP*) also shows strong immunoreactivity to ADAMTS15. Well-differentiated adenocarcinoma (*WD*) still presents significant expression. Poorly differentiated high-grade adenocarcinoma (*PD*) does not show staining for the metalloprotease.

Discussion

In this work, we provide functional evidence that ADAMTS15 is a new enzyme in the growing category of tumor-defying proteases (7). The gene encoding this metalloprotease was originally cloned in our laboratory as part of our long-term studies aimed at identification of proteases associated with tumor progression (23). However, its precise physiologic and pathologic relevance is currently unknown. The first indication regarding a potential protective role for ADAMTS15 derived from the observation that low *ADAMTS15* expression levels coupled to high *ADAMTS8* levels conferred poor prognosis to breast cancer patients (19). Moreover, *ADAMTS15* was identified as one of the so-called CAN genes found to be mutated in a small set of colorectal cancers (20). To provide functional support to the putative relevance of ADAMTS15 as a tumor suppressor protease, we extended the mutational analysis of this metalloprotease gene to a panel of 50 colorectal cancers and 6 colon cancer cell lines. This work allowed us to identify four new mutations, validating the proposal that *ADAMTS15* is mutated in human colon carcinomas. Next, we performed different functional approaches aimed at evaluating the antitumorigenic properties of ADAMTS15 using cellular models. As a first step to this purpose, we proceeded to exogenously express *ADAMTS15* in HCT-116 cells, which led to a considerable reduction in both colony formation and invasive capacities of this cell line. Moreover, functional analysis of selected *ADAMTS15*-knockdown SW-620 clones showed an enhancement of their invasive capacities and growing ability when compared with control clones. These data provide the first functional evidence on the tumor suppressor properties of ADAMTS15.

Furthermore, we could observe in these colon cancer cell lines that defective or tumor-derived mutant ADAMTS15 invasive clones exhibited morphologic alterations characteristic of cells that have lost their epithelial features and acquired a mesenchymal phenotype. Epithelial-to-mesenchymal transition is a characteristic feature of cells undergoing invasion, which is an essential step for transformed cells to progress and metastasize in distant organs (26). In agreement with these data, we also found that the Ras/mitogen-activated protein kinase oncogenic pathway, known to induce epithelial-to-mesenchymal transition events, is modulated by ADAMTS15. Thus, we observed a marked inhibition of ERK phosphorylation in *ADAMTS15*-expressing HCT-116 clones and an activation in the *ADAMTS15* knockdown SW-620 clones. Previous studies have shown that sustained activation of Ras/mitogen-activated protein kinase pathway is involved in promoting cellular processes associated with tumor progression (27). The presence of thrombospondin-1 domains in ADAMTS15 could be responsible for switching off this pathway. In fact, ectopic expression of thrombospondin-1 markedly inhibits tumor formation (28). Moreover, expression of thrombospondin-1 is up-regulated by the tumor suppressor gene p53 and down-regulated by oncogenes such as Myc and Ras (29). Furthermore, it has been reported that both *Xenopus* ADAMTS1 and human ADAMTS12 can also inhibit this pathway (15, 25). These two ADAMTS undergo proteolytic processing events to generate fragments with prometastatic or antimetastatic activities depending on the presence of thrombospondin-1 domains in them (15, 30, 31). In fact, it has been proven

that these domains confer angio-inhibitory properties to different ADAMTS (13) and may be involved in the modulation of the ERK signaling pathway (15, 25). Interestingly, the 24544ΔG mutation identified in our *ADAMTS15* mutational analysis would result in an enzyme lacking the last two thrombospondin-1 domains. We have engineered an ADAMTS15 isoform mimicking this situation, which shows reduced invasive properties compared with the full-length protein. Moreover, cells expressing this form displayed higher levels of phosphorylated ERK than those detected in cells expressing intact ADAMTS15. These data would indicate that the thrombospondin motifs are essential for ADAMTS15 to act as a tumor suppressor protease. Furthermore, we have also found that the presence of these domains is important to immobilize the protease in the pericellular space following its secretion, which would facilitate a putative interaction of the thrombospondin-1 domains with their receptors as a mechanism to inhibit ERK activation (25). In contrast, the form lacking these domains is secreted to the cell medium, thus hindering this putative ERK-inhibitory function.

Consistent with the above data, *in vivo* experiments based on subcutaneous injection of the interfered clones in severe combined immunodeficient mice revealed that ADAMTS15 knockdown markedly promotes *in vivo* tumor growth. Additionally, we carried out an immunohistochemical analysis of ADAMTS15 in colorectal tissue samples with the finding that this metalloprotease is preferentially detected at the apical side of the colonic normal mucosa, whereas its presence shows a clear decay within the tumor tissue. Interestingly, the negative correlation between *ADAMTS15* expression and the differentiation status of the colorectal tumors agrees with the suggested protective properties of ADAMTS15 in tumors of colorectal origin.

We have also examined whether *ADAMTS15* can be an epigenetically silenced gene in colon cancer, a quite common mechanism to inactivate several tumor suppressor genes in colorectal carcinomas (32). Moreover, *ADAMTS1* and *ADAMTS18* have been found to be hypermethylated in these malignancies (16, 18). However, our results revealed that this is not the case for *ADAMTS15* because an analysis of paired normal/tumor samples and cDNAs from colon carcinomas did not show the expected expression pattern for epigenetically silenced genes. Thus, genetic mutations rather than epigenetic silencing seem to be the mechanism that contributes to *ADAMTS15* inactivation in some cases of human colorectal carcinomas. The total number of identified mutations in the *ADAMTS15* gene is relatively low but similar to that found in other genes reported as novel targets of genetic inactivation in cancer (20, 21, 33). Nevertheless, it is very interesting that mutation events may result in the inactivation of this protease gene in other human malignant tumors as assessed by the recent findings showing that *ADAMTS15* is mutated in pancreatic (33) and lung cancers,[5] although no functional analysis of these mutations has been reported yet. Additionally, it is also noteworthy that *ADAMTS15* gene is located in chromosome 11q24.3 between markers *D11S912* and *D11S969*, a region of frequent loss of heterozygosity in different tumors including colorectal cancer (34). This fact would provide a new clue on the potential relationship between loss-of-function of *ADAMTS15* and development of colorectal cancer.

In summary, the results presented in this work provide strong support to the proposal that ADAMTS15 is a metalloprotease with tumor-protective functions. This finding, together with previous observations on the antitumorigenic properties of different proteases (7), emphasizes the need to more precisely characterize the cancer degradome (35). These approaches will contribute to identify those proteolytic enzymes that constitute appropriate therapeutic targets and to distinguish them from those that confer tumor protection. Ultimately, the structural and functional discrimination between protease targets and antitargets in cancer will be very helpful to design new inhibitors aimed to specifically block the unwanted proteolytic activities associated with tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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V. Análisis mutacional de la familia ADAM en melanoma

Las nuevas estrategias de secuenciación y de estudio bioinformático han posibilitado los análisis mutacionales a gran escala en múltiples tipos de cáncer. Dado este escenario nos propusimos tomar parte en un proyecto liderado por la Dra. Yardena Samuels dirigido a la búsqueda de mutaciones en genes de la familia ADAM en melanoma. El análisis de los 19 genes humanos de ADAMs, reveló que un 34% de los tumores presentaba mutaciones que afectaban a 8 genes de este grupo proteolítico. A continuación, los dos miembros con una mayor frecuencia de alteraciones génicas, *ADAM7* y *ADAM29,* se seleccionaron para evaluar funcionalmente el efecto biológico de sus mutaciones. Considerando que ambas proteínas carecen de capacidad catalítica, nos centramos en intentar estimar los posibles cambios en adhesión y migración. Como resultado, describimos que las mutaciones identificadas modulan la capacidad de las células de melanoma para unirse a componentes de la matriz extracelular y de migrar a través de ella.

Artículo 5: Xiaomu Wei, **Ángela Moncada-Pazos**, Santiago Cal, Clara Soria-Valles, Jared Gartner, Udo Rudloff, Jimmy C. Lin, NISC Comparative Sequencing Program, Steven A. Rosenberg, Carlos López-Otín y Yardena Samuels. “Analysis of the disintegrin-metalloproteinases family reveals *ADAM29* and *ADAM7* are often mutated in melanoma”.

Human Mutation 32: E2148-2175 (2011).

Aportación personal al trabajo

En este trabajo participé llevando a cabo la mayor parte de los ensayos funcionales. Concretamente, realicé ensayos de adhesión y de migración con las células que expresaban las proteínas mutantes y la proteína natural sobre diferentes sustratos habituales en la matriz extracelular. Asimismo, colaboré en la escritura del artículo y en la elaboración de sus figuras.

HUMAN MUTATION Mutation in Brief 32: E2148-E2175 (2011) Online

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Analysis of the Disintegrin-metalloproteinases Family Reveals ***ADAM29*** and ***ADAM7*** Are Often Mutated in Melanoma

Xiaomu Wei[1], Angela Moncada-Pazos[2], Santiago Cal[2], Clara Soria-Valles[2], Jared Gartner[1], Udo Rudloff[3], Jimmy C. Lin[4], NISC Comparative Sequencing Program[5], Steven A. Rosenberg[3], Carlos López-Otín[2], and Yardena Samuels[1]*

[1] Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health (NIH), Bethesda, Maryland, 20892, USA..[2] Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología (IUOPA), Universidad de Oviedo, 33006-Oviedo, Spain [3] Surgery Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892, USA..[4] Ludwig Center for Cancer Genetics and Therapeutics, and Howard Hughes Medical Institute at the Johns Hopkins Kimmel Cancer Center, Baltimore, Maryland 21231, USA. [5]NIH Intramural Sequencing Center, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA.

*Correspondence to Yardena Samuels; E-mail:samuelsy@mail.nih.gov.

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ABSTRACT: We performed a mutational analysis of the 19 disintegrin-metalloproteinases (ADAMs) genes in human cutaneous metastatic melanoma and identified eight to be somatically mutated in 79 samples, affecting 34% of the melanoma tumors analyzed. Functional analysis of the two frequently mutated ADAM genes, *ADAM29* and *ADAM7* demonstrated that the mutations affect adhesion of melanoma cells to specific extracellular matrix proteins and in some cases increase their migration ability. This suggests that mutated ADAM genes could play a role in melanoma progression. ©2011 Wiley-Liss, Inc.

KEY WORDS: Somatic mutation, Melanoma, ADAM7, ADAM29

INTRODUCTION

The matrix metalloproteinase (MMP) proteolytic enzymes have recently been shown to be frequently mutated in melanoma (Palavalli, et al., 2009). The MMPs are only one family within a superfamily of zinc-based proteinases, the metzincins (Stocker and Bode, 1995). Another family of metzincins is the ADAMs (a disintegrin and metalloproteinase) which are membrane anchored glycoproteins with several biological functions encompassing cell adhesion, cell fusion and signaling. About half of the ADAMs have a consensus metalloproteinase catalytic sequence, giving them proteolytic activity, whereas the rest have cell adhesion roles. Well characterized ADAM family members include ADAM17 (TACE) which causes ectodomain shedding of various membrane-associated molecules including TNF-α and EGF-related ligands (Peschon, et al., 1998), as well as ADAM10 which has been shown to act via the Notch pathway (Qi, et al., 1999) and through shedding of ephrins (Hattori, et al., 2000). Although the function of some of the ADAMs has been characterized, the function of most family members and their involvement in cancer is as yet unknown (Blobel, 2005; Schlondorff and Blobel, 1999).

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Several studies suggest a direct role of ADAM family in human cancer development and progression. For example, ADAM17 is an upstream regulator of the EGF signaling pathway, one of the most commonly altered signal transduction pathways in cancer. Additionally, the presence of distinct somatic mutations in several members of the ADAM family further suggests a direct involvement of these genes in cancer genesis (Dalgliesh, et al., 2010; Parsons, et al., 2008; Pleasance, et al., 2010; Sjoblom, et al., 2006; TCGA, 2008). However, previous reports investigating the extent of ADAM mutations in cancer are incomplete as they either did not investigate the entire ADAM gene family or were limited to particular cancer types (Sjoblom, et al., 2006; Wood, et al., 2007).

In this study, we systematically analyzed the entire ADAM gene family in a large panel of human melanomas. Melanoma is the most common fatal skin cancer and despite years of research, metastatic disease has a dismal prognosis. The median patient survival is six months following diagnosis of late-stage disease, with fewer than 5% surviving five years (Jemal, et al., 2009). Identification of genes affected by somatic mutations in metastatic melanomas should provide new opportunities for clinical intervention. Our comprehensive genetic study identified two frequently mutated ADAM genes, *ADAM29* (MIM# 604778) and *ADAM7* (MIM# 607310).

Functional analysis of a subset of mutations identified in ADAM29 showed them to increase their adhesion to collagen I, II and IV. In contrast, analysis of ADAM7 mutations showed reduced cell adhesion to collagen IV and laminin and increased melanoma cell migration. Our study is the first comprehensive mutational analysis of the ADAM family in human cancer and provides evidence for the association of ADAM in melanoma tumorigenesis.

MATERIALS AND METHODS

Tumor Tissues

A panel of pathology-confirmed metastatic melanoma tumor resections, paired with apheresis-collected peripheral blood mononuclear cells, was collected from 79 patients enrolled in IRB-approved clinical trials at the Surgery Branch of the National Cancer Institute. Pathology-confirmed melanoma cell lines were derived from mechanically or enzymatically dispersed tumor cells, which were subsequently cultured in RPMI 1640 + 10% FBS at 37^{o}C in 5% CO_2 for 5-15 passages. Genomic DNA was isolated using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA).

PCR, sequencing and mutational analysis of melanoma samples

PCR and sequencing was done as previously described (Palavalli, et al., 2009; Prickett, et al., 2009; Viloria, et al., 2009). The primary phase mutation screen was analyzed using Consed (Gordon, et al., 1998). Variants were called using Polyphred 6.11 (Bhangale, et al., 2006) and DIPDetector (Hansen N., unpublished), an indel detector for improved sensitivity in finding insertions and deletions. Sequence traces of the secondary screen were analyzed using the Mutation Surveyor software package (SoftGenetics, State College, PA).

In this study all coding exons of the ADAM gene superfamily in 31 melanoma patients were included into the screen and a total of 401 exons from the ADAM were extracted from genomic databases (Supp. Table S1). These exons as well as at least 15 intronic bases at both the 5' and 3' ends including the splicing donor and acceptor sites, were amplified by using polymerase chain reaction (PCR) from cancer genomic DNA samples using the primers listed in Supp. Table S2. Out of the 367 exons amplified, 96% were successfully sequenced using dye terminator chemistry.

Validation of somatically mutated genes was performed in an additional 48 tumor samples. A total of 20,098 PCR products, spanning 7Mb of tumor genomic DNA, were generated and sequenced. Sequence data for each amplicon were evaluated for quality within the target region. To avoid PCR or sequencing artifacts we re-amplified and re-sequenced amplicons that had alterations.

The DNA mutation numbering system used in this study was based on cDNA sequence. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Construction of wild-type and mutant *ADAM29* and *ADAM7* expression vector

Human *ADAM7* (NM _003817.2) and *ADAM29* (NM_014269.4) were purchased from Open Biosystems and PCR cloned into the mammalian expression vector pCDF-MCS2-EF1-Puro™ (Systems Biosciences, Inc.,

Mountain View, CA) or pCDNA3.1 (-) (Invitrogen, Molecular Probes) via the XbaI and NotI restriction sites. Flag tag was introduced into 3' end of the gene. Cloning primers for *ADAM7* are: 5' - GCTGTCTAGAGCCACCATGAAGATGTTACTCCTGCTGCATTGCCTTGGG - 3' and 5' - ATCAGCGGCCGCCTACTTATCGTCGTCATCCTTGTAATCCTTGGCACTTTG - 3'. Cloning primers for *ADAM29* are 5'- GCTGTCTAGAGCCACCATGAAGATGTTACTCCTGCTGCATTGCCTTGGGG - 3' and 5'- ATCAGCGGCCGCCTACTTATCGTCGTCATCCTTGTAATCGGAGGGCGTCA - 3'. The *ADAM7* and *ADAM29* mutants were generated using Phusion PCR for site-directed mutagenesis.

Generation of Mel-STR and A375 stable pooled clones

Mel-STR cells were maintained in RPMI-1640 supplemented with 10% FBS. Cells were seeded in T175 flasks at 70% confluent the day before transfection. pcDNA3.1(-) empty vector or *ADAM7* (WT, p.H243Y, p.M359I, p.E639K, p.S703N) were transfected into cells using Fugene6 (Roche, Indianapolis, IN) following manufacturers protocol. 48 hrs after transfection, cells were selected using normal growth medium supplemented with 300μg/ml G418.

To make lentivirus for *ADAM29* A375 stable pooled clones, pCDF-*ADAM29* (WT, p.E111K, p. S112F, p.S115F, p.E176K, p.I257F, p.G434D, p.E503K) constructs were co-transfected into HEK 293T cells seeded at $1.5X10^6$ per T75 flask with pVSV-G and pFIV-34N (kind gifts from Todd Waldman, Georgetown University) helper plasmids using Fugene6 (Roche, Indianapolis, IN) . Virus was harvested 48 hrs after transfection. A375 cells were seeded at 1.5×10^6 cells per T75 flask 24 hr prior to infection. 24 hrs after infection, cells were selected using normal growth medium supplemented with 3ng/ml puromycin.

Proliferation assay

Mel-STR or A375 cells were seeded in 96 well plates at 250 cells per well in normal growth medium and incubated for 13-17 days. Proliferation of cells were monitored every 48 hrs. Cells were incubated in 50 μl 0.2% SDS/well at 37°C for 2 hrs. 150 μl/well SYBR Green I solution (1:750 SYBR Green I (Invitrogen-Molecular Probes) diluted in dH_20) were added to cells before analyzing using a BMG Labtech FLOUstar Optima.

Reverse Transcription PCR

Total RNA was extracted from melanocytes, melanoma cells and pooled clones following the manufacturer's protocol for RNeasy Mini Kit (Qiagen #74101). Total RNA was eluted in 30 μl DEPC-treated dH20. A total of 1μg of total RNA was used for single strand cDNA synthesis using a SuperScript III First Strand kit (Invitrogen #18080-051). cDNA was amplified using the oligo dT20 primer supplied in the kit. PCR primers used for *ADAM7* message are 5′-ACACGGAAGGATTTTGATCATGTTG - 3′ and 5′-GGATTGGCTCAGTCCTTATCTGCTG - 3′. , *ADAM29* PCR primers are 5'- GATCTGGACCAATAAAAACCTCATTGTAGTAGATGATGTAAGGAA - 3' and 5'- CATTTTTGATACCACAGTGACCAACACGGTCACCTAAGG - 3'. *GAPDH* primers (forward primer: 5′- TGGAAGGACTCATGACCACA - 3′, reverse primer: 5′- TGCTGTAGCCAAATTCGTTG -3′) were used as a control. The product was then analyzed on a 1% agarose gel.

Adhesion Assay

Adhesion capacity of different cell pools was analyzed with the ECM Cell Adhesion Array kit (Colorimetric) (EMD Biosciences, ECM540 96 wells) following manufacturer's instructions.

Migration assays

Mel-STR *ADAM7* stable clones were seeded into migration wells (8.0 μm – BD Biocoat, BD Biosciences) at 10,000 cells per well in serum-free medium in top chambers and incubated for 24 hrs. The bottom chamber contain normal growth medium. Migrated cells were fixed and stained using Hema 3 Stat Pack.

RESULTS

The human ADAM family consists of 19 genes (Supp. Table S1). While previous studies listed in Supp. Table S3 investigated the ADAM gene family mutational status, results are not comprehensive. As a few of the ADAM genes were found to be altered in melanoma previously, we decided to systematically evaluate the mutation status of all the gene family members in a large panel of cutaneous metastatic melanoma samples. The coding exons of

the ADAMs gene superfamily were genetically evaluated in 31 melanoma patients. To determine whether a mutation was somatic (i.e., tumor specific) we examined the sequence of the gene in genomic DNA from normal tissue (derived from blood) of the relevant patient. This allowed the identification of eight genes containing somatic mutations. Genes found to have one non-synonymous mutation or more were then further analyzed for mutations in an additional 48 melanomas. Through this approach, we identified 41 mutations in eight genes, thus affecting 34% of the melanoma tumors analyzed (Table 1).

Table 1. Mutations Identified in ADAMs

Gene[a]	Ref Seq accession*	CCDS accession*	No. of mutations (% tumors affected)[#]	Tumor	Exon	Nucleotide[†]	Amino Acid[†]	Functional domain
ADAM7	NM_003817.2	CCDS6045.1	12 (12.7%)	1T	1	c.40C>T	p.P14S	Propeptide
				12T	2	c.91C>T	p.R31C	Propeptide
				32T	2	c.106C>T	p.P36S	Propeptide
				98T	5	c.316C>T	p.H106Y	Propeptide
				80T	6	c.539T>C	p.V180A	None
				72T	9	c.727C>T	p.H243Y	Reprolysin
				21T	10	c.905G>A	p.G302E	Reprolysin
				39T	10	c.905G>A	p.G302E	Reprolysin
				23T	11	c.1077G>A	p.M359I	Reprolysin
				103T	15	c.1598G>A	p.G533E/LOH	Cysteine-rich
				21T	17	c.1915G>A	p.E639K	None
				39T	20	c.2108G>A	p.S703N	None
ADAM10	NM_001110.2	CCDS10167.1	1 (1.3%)	37T	5	c.526C>T	p.H176Y	None
ADAM12	NM_003474.4	CCDS7653.1	2 (2.5%)	50T	19	c.2135G>A	p.G712E/LOH	None
				34T	23	c.2677C>T	p.P893S	None
ADAM18	NM_014237.2	CCDS6113.1	5 (6.3%)	81T	6	c.508C>T	p.P170S	None
				37T	10	c.851T>G	p.V284G	Reprolysin
				23T	11	c.1032G>A	p.M344I	Reprolysin
				39T	12	c.1085T>A	p.M362K	Reprolysin
				106T	15	c.1607C>T	p.S536L	Cysteine-rich
ADAM19	NM_033274.3	CCDS4338.1	1 (1.3%)	6T	14	c.1498C>T	p.Q500X/LOH	Disintegrin
ADAM28	NM_014265.4	CCDS34865.1	5 (6.3%)	13T	3	c.194G>A	p.G65E	Propeptide
				55T	6	c.401G>A	p.G134E	Propeptide
				26T	13	c.1349G>A	p.G450E	Disintegrin
				24T	14	c.1445C>T	p.S482F/LOH	Disintegrin
				64T	14	c.1505G>A	p.G502D	Cysteine-rich
ADAM29	NM_014269.4	CCDS3823.1	14 (15.2%)	7T	1	c.267C>G	p.I89M	Propeptide
				55T	1	c.331G>A	p.E111K	Propeptide
				41T	1	c.335C>T	p.S112F	Propeptide
				104T	1	c.344C>T	p.S115F	Propeptide
				39T	1	c.391G>A	p.D131N/LOH	Propeptide
				7T	1	c.526G>A	p.E176K	None
				83T	1	c.701C>T	p.S234F	Reprolysin
				1T	1	c.769A>T	p.I257F	Reprolysin
				55T	1	c.914G>A	p.G305E	Reprolysin
				32T	1	c.1033G>A	p.D345N	Reprolysin
				91T	1	c.1208G>A	p.G403D	None
				23T	1	c.1301G>A	p.G434D	Disintegrin
				106T	1	c.1507G>A	p.E503K	Cysteine-rich
				17T	1	c.1597C>T	p.H533Y	Cysteine-rich
ADAM33	NM_025220.2	CCDS13058.1	1 (1.3%)	37T	10	c.914C>T	p.A305V	Reprolysin

*Accession numbers for mutated ADAMs in Santa Cruz and GenBank. #Number of non-synonymous mutations observed and percent of tumors affected for each of the 8 genes in the panel of 79 melanoma cancers. DNA mutation numbering system was based on cDNA sequence.
†Nucleotide and amino acid change resulting from mutation. "X" refers to stop codon.
"LOH" refers to cases wherein the wild-type allele was lost and only the mutant allele remained.
"None" refers no functional known domain.
Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.
[a]GenBank accession numbers for adams with mutations are: *ADAM7 (NM_003817.2), ADAM10 (NM_001110.2), ADAM12 (NM_003474.4), ADAM18 (NM_014237.1), ADAM19 (NM_033274.2), ADAM28 (NM_014265.4), ADAM29 (NM_014269.4) and ADAM33 (NM_025220.2).*

The observed somatic mutations could either be "driver" mutations, on which tumor growth is dependent, or "passenger" events that confer no selective advantage to tumor growth. In the eight genes found to be mutated, 41 non-synonymous and 10 synonymous somatic mutations were identified, yielding a N:S (non-synonymous: synonymous) ratio of 4.1:1, a value significantly higher than the N:S ratio of 2:1 predicted for nonselected passenger mutations (p <0.03) (Sjoblom, et al., 2006), suggesting that many of these are likely to be "driver" mutations. Most somatic mutations base substitutions in our screen were C>T/G>A transitions and were significantly greater than other nucleotide substitutions (p <0.002) (Supp. Figure S1), which is reminiscent of the mutation pattern reported previously caused by ultraviolet light exposure (Greenman, et al., 2007).

One of the most frequently mutated genes, *ADAM7*, harbored two alterations in the same residue (p.G302E), forming a mini-hotspot. Evaluation of the mutation status of *ADAM7* in commercially available melanoma cell lines revealed two additional mini-hotspots. The mutation p.H243Y found in the SK-Mel5 melanoma line was also

present in the 72T sample, and the melanoma cell line SK-Mel28 was found to harbor the p.M359I mutation which was also detected in the 23T melanoma sample. The three mini-hotspots occur within the same functional reprolysin domain (Supp. Figures S2 and S3A).

Eight of the genes found to be mutated in our melanoma panel were previously found to be altered in several cancer types (highlighted in Supp. Table S3). Only four of these were found to be mutated in melanoma. Notably, seven of the previously observed alterations lie close to the mutations detected in this study. ADAM18 which was formerly found to be mutated on residue p.T583I in glioma was mutated on residue p.S536L in our melanoma panel. ADAM28 which was previously reported to harbor a mutation in residue p,D470N in melanoma was identified to harbor two neighboring somatic mutations (p.G450E and p.S482F) in our melanoma panel. ADAM19 which was reported to contain the p.L425V alteration in the disintegrin domain had a p.Q500X alteration in the same domain in our studies. Finally, the most frequently mutated gene, ADAM29, was previously found to be mutated in melanoma in the recently published whole genome report by the Sanger Center (Pleasance, et al., 2010). Importantly, all previously identified mutations (p.V205I, p.C534X and p.G589E) lie in the same domains as our newly discovered mutations (Table 2 and Supp. Figure S3).

Table 2. Locations of Novel and Previously Identified ADAMs Mutations

	COSMIC				79 Melanoma samples (Samuels Lab)			
Gene Name[a]	Nucleotide	Amino Acid	Primary Tissue	Functional Domain	Nucleotide	Amino Acid	Tumor	Functional Domain
ADAM18	c.1748C>T	p.T583I	glioma	cysteine-rich	c.1607C>T	p.S536L	106T	Cysteine-rich
ADAM19	c.1273C>G	p.L425V	upper_aerodigestive_tract carcinoma	Disintegrin	c.1498C>T	p.Q500X	6T	Disintegrin
ADAM28	c.1408G>A	p.D470N	malignant_melanoma	Disintegrin	c.1349G>A	p.G450E	26T	Disintegrin
					c.1445C>T	p.S482F	24T	Disintegrin
ADAM29	c.92C>T	p.P31L	large_intestine carcinoma	Propeptide	c.267C>G	p.I89M	7T	Propeptide
ADAM29	c.613G>A	p.V205I	large_intestine carcinoma	Reprolysin	c.701C>T	p.S234F	83T	Reprolysin
ADAM29	c.1602T>A	p.C534X	pancreas carcinoma	cysteine-rich	c.1597C>T	p.H533Y	17T	Cysteine-rich
ADAM29	c.1766G>A	p.G589E	malignant_melanoma	cysteine-rich				

Seven previously identified ADAMs mutations lie close to novel mutations found in 79 melanoma samples. Detailed information of nearby mutations is shown in the table.
Alterations on the left side are previously published mutations, ones on the right side are identified in 79 melanoma samples. Neighbouring mutations are highlighted as the same color.
The position of nucleotide mutations corresponds to that in the coding sequence of each gene, where position 1 is the A of ATG initiation codon.
[a]GenBank accession numbers for adams with mutations are: *ADAM18 (NM_014237.1), ADAM19 (NM_033274.2), ADAM28 (NM_014265.4) and ADAM29 (NM_014269.4)*

The clinical information associated with the melanoma tumors containing somatic ADAM mutations is provided in Supp. Tables S4 and S5. There was no association of the detected mutation pattern with any of the analyzed clinical or pathological characteristics of the melanoma patients.

ADAM7 was one of the most frequently mutated ADAM genes, as it was found to be somatically mutated in over 12% of melanoma cases (Table 1). In addition, taking into account a background mutation rate in melanoma of 11 mut/Mb (paper under review), the observed mutations in *ADAM7* are significantly different to that expected by chance (p= 1.32E-06). The alterations identified in ADAM7 occurred in residues that are highly conserved evolutionarily, retaining identity in rat and mouse (Supp. Figure S4). Furthermore, the identified somatic mutations clustered onto various important functional domains of the ADAM family (Supp. Figure S3A). While mutations p.P14S, p.R31C, p.P36S and p.H106Y all occur in the propeptide domain. Mutations p.H243Y, p.G302E and p.M359I are within the reprolysin domain. To provide a computational estimation of the effects of the different missense mutations, we used the SIFT (sorting intolerant from tolerant) algorithm (Ng and Henikoff, 2003). This data is presented in Supp. Table S6 and shows that at least six of the eleven alterations would affect protein function. Interestingly, in contrast to melanoma cells, no *ADAM7* was found to be expressed in human melanocytes (Supp. Figure S5). The combination of this data suggests that mutant *ADAM7* is likely to function as a driver in melanoma.

Based on the potential functional relevance of the altered residues in ADAM7, and the fact that ADAM7 does not have protease activity we chose to clone the two cytoplasmic mutations p.E639K and p.S703N as well as the p.H243Y and p.M359I mutations for further studies. To test the effects of these mutations on ADAM7 function we created stable pooled clones in human melanoma Mel-STR cells (Gupta, et al., 2005) which harbor wild-type *ADAM7* (Supp. Table S7). As seen in Supp. Figure S6A the cell clones expressed similar levels of wild-type or mutant ADAM7. These clones were then used for further studies. We first observed that expression of wild-type or mutant ADAM7 did not affect the growth rate of Mel-STR cells in tissue culture (Supp. Figure S6B). Then, and

because ADAM family members are known to modulate tumor cell adhesion by interactions with proteins in the basal lamina (Edwards, et al., 2008), we evaluated whether the mutations in ADAM7 affected its adhesion to different extracellular matrix components. For this purpose, we used the above described Mel-STR pooled clones expressing wild-type or mutant ADAM7 in an adhesion assay. This assay revealed that mutant ADAM7 conferred significantly reduced binding on collagen IV (p.M359I, p.E639K and p.S703N) and laminin-1 (p.H243Y, p.E639K and p.S703N) compared with the wild-type expressing cells ($P < 0.005$, *t*-test) (Figure 1).

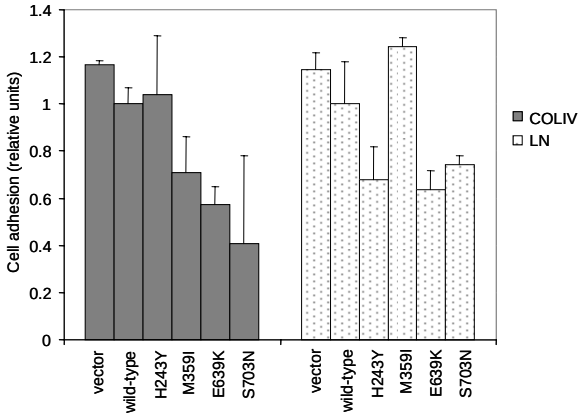

Figure 1. Mutant ADAM7 modulates cell adhesion. Adhesion properties of Mel-STR cells overexpressing either the wild-type or mutant ADAM7 forms were assessed using the ECM Cell Adhesion Array kit. Collagen IV (COLIV), laminin (LN), (n=2).

As previous studies reported that reduced adhesion facilitates cell migration (Touab, et al., 2002; Yamagata, et al., 1989), our finding that cells expressing mutant ADAM7 have reduced collagen IV and laminin-1 adhesion prompted us to investigate whether these cells also have increased migration ability. As can be seen in Figure 2A, Mel-STR control cells migrated through the porous membrane. Interestingly, cells expressing wild-type ADAM7 resulted in > 60% reduction in migrating cells (171 +/- 6.6 versus 70+/- 17.7). When the ADAM7 mutant expressing cells were tested in the same assay, two mutations (p.E639K and p.S703N) increased the cell migration capabilities compared to wild-type ADAM7 (122+/- 18.5 and 210 +/- 33.6 $P < 0.001$, *t*-test). Based on these results we can conclude that wild-type ADAM7 inhibits cell migration and that some ADAM7 mutations alleviate this, which might suggest that these mutations might be causing a loss of function effect.

To further our functional analysis of ADAM gene somatic alterations, we next focused on *ADAM29* which was found to be somatically mutated in over 15% of melanoma cases (Table 1) making it the most highly mutated ADAM in this screen. Several criteria suggest *ADAM29* to also be a driver in melanoma (1) *ADAM29* has previously been shown to harbor somatic mutations in other cancer types (Table 2), (2) the observed mutations in *ADAM29* are significantly higher to that expected by chance (p= 7.73E-08) (3) 14 non-synonymous (N) and 0 synonymous (S) somatic mutations were identified in *ADAM29*, yielding a N:S ratio of 14:0, which is significantly higher than the N:S ratio of 2:1 predicted for non-selected passenger mutations (Sjoblom, et al., 2006) ($P < 0.003$) and (4) SIFT analysis shows that seven of the 14 alterations in ADAM29 would affect its protein function (Supp. Table S8).

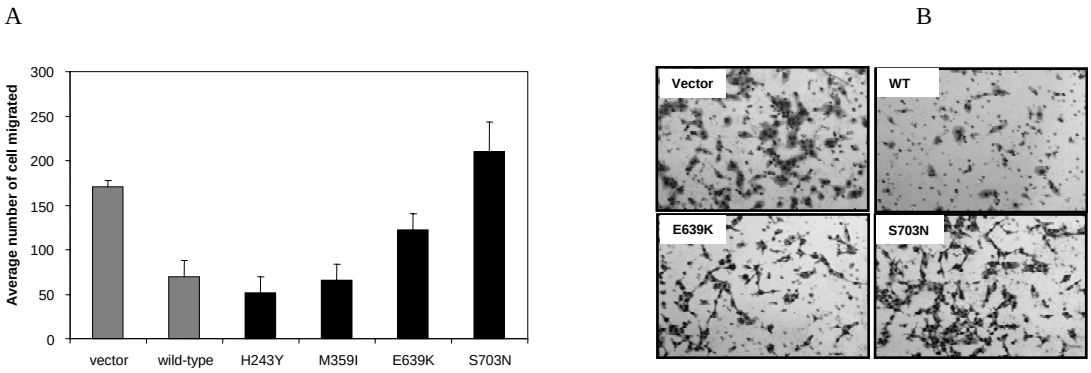

Figure 2. Effects of ADAM7 mutation on cell migration. (A) Mel-STR cells overexpressing wild-type or mutant *ADAM7* were seeded in Boyden chambers and assessed for their migration abilities. Graph indicates the number of cells that migrated 24 hrs after seeding (n=3). (B) Representative pictures of migrated cells.

On this basis, we chose to functionally evaluate some of the mutations identified in *ADAM29* as well. Somatic mutations in ADAM29 occurred mainly in the propeptide and reprolysin domains (Supp. Figure S3B). As the reprolysin domain encodes the catalytic portion of the protein and ADAM29 does not have protease activity, we decided to mainly focus on the mutations found in the propeptide domain especially as an interesting cluster of mutations was observed in this domain involving the p.E111K; p.S112F and p.S115F alterations (Supp. Figure S3B).

To determine whether mutations in ADAM29 affected cell growth, we created stable pooled clones expressing wild-type ADAM29 and seven tumor derived mutants (p.E111K, p.S112F, p.S115F, p.E176K, p.I257F, p.G434D and p.E503K) in A375 cells, which were shown to harbor wild-type *ADAM29* (Supp. Table S9). A similar expression level of the ADAM29 constructs was observed (Supp. Figure S7A). Expression of wild-type or mutant ADAM29 did not affect the growth rate of A375 cells in tissue culture (Supp. Figure S7B). In contrast, expression of the various ADAM29 mutants substantially increased cell adhesion to collagen I and IV compared to cells expressing wild-type ADAM29 ($P < 0.005$, *t*-test (Figure 3). Furthermore, five of the ADAM29 mutants (p.S112E, p.S115F, p.I257F, p.G434D and p.E503K) showed significantly increased adhesion compared to wild type ADAM29 to collagen II ($P < 0.005$, *t*-test (Figure 3). Binding to other extracellular matrix proteins such as fibronectin, laminin, tenascin and vitronectin showed some differential binding between wild type and some of the ADAM29 mutants, but this was not as striking as the binding to collagen I, II and IV (Supp. Figure S8).

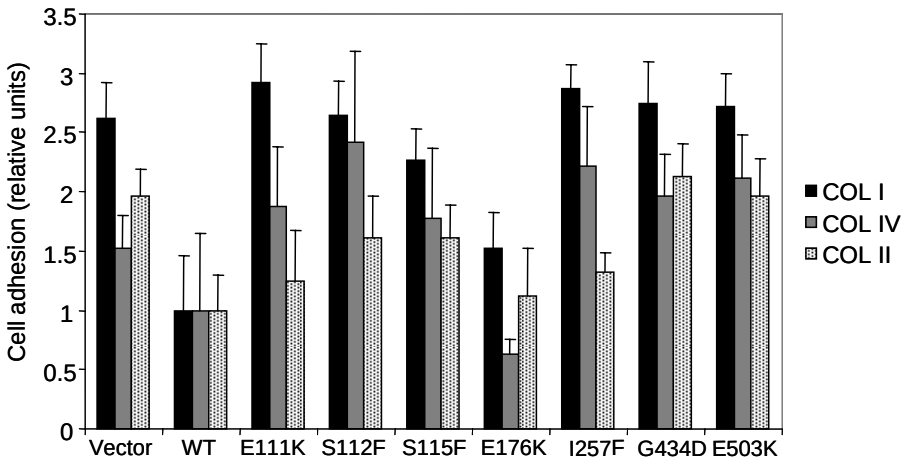

Figure 3. Mutant ADAM29 modulates cell adhesion on collagen I, II and IV. Adhesion properties of A375 cells overexpressing either the wild-type or mutant ADAM29 forms were assessed with the ECM Cell Adhesion Array kit. collagen (COL), wild type (WT) (n=2).

DISCUSSION

We have performed the first systematic mutational analysis of the ADAMs gene family in any human cancer type leading to the discovery of two frequently mutated ADAM genes, *ADAM7* and *ADAM29*. The somatic mutations identified in this study were confirmed by multiple PCR and sequencing reactions. The mutations are novel and multiple mini-hotspot alterations were identified in *ADAM7*. The changes in ADAM7 and ADAM29 affect highly conserved residues. Our in silico analysis predicted that some of the mutated residues in ADAM7 and ADAM29 would affect tumorigenic phenotypes of melanoma cells. This was experimentally tested by cloning the relevant nucleotide alterations into ADAM7 as well as ADAM29 cDNA. Functional assays confirmed that four of the mutations in ADAM7 (p.H243Y, p.M359I, p.E639K and p.S703N) have altering effects on the wild-type protein function. Specifically, although cells expressing wild-type ADAM7 adhere to collagen IV and laminin, the four mutated proteins reduce this ability significantly. Intriguingly, in contrast to ADAM7, seven of the ADAM29 alterations (p.E111K, p.S112F, p.S115F, p.E176K, p.I257F, p.G434D and p.E503K) increase the adhesion of melanoma cells to collagen I and collagen IV, compared to wild-type ADAM29. These results emphasize the need to test the role of each ADAM and its related mutations in an individual manner to precisely define its functional role in cancer.

ADAM enzymes are zinc endopeptidases and contain a conserved zinc binding motif within the reprolysin family zinc metallopeptidase domain (PF01421). Of all the presumed functional human ADAM genes, 13 encode proteins that possess the characteristic reprolysin-type active site (HEXGHXXGXXHD) in the metalloproteinase domain followed downstream by the ‘‘Met turn” a key signature of metzincin enzymes (Bode, et al., 1993), indicating functional proteolytic capability (Edwards, et al., 2008). Several ADAM family members (including ADAM2, ADAM11, ADAM18, ADAM23 and ADAM32) lack all three conserved histidines found within the zinc-binding motif which prevents zinc binding and proteolytic activity. ADAM7 contains the “Met turn” as well as the three conserved histidines, but is missing a glutamic acid in the second position of the motif, which is one of the critical features in the Zn-binding active site (Supp. Figure S9). This suggests that the metalloproteinase domain in ADAM7 may play roles in protein folding and protein–protein interactions rather than cleaving components of extracellular matrix (Lopez-Otin and Bond, 2008). Interestingly, one of the ADAM7 mini-hotspot alterations discovered in our study occurs at the Met-Turn (p.M359I), which as mentioned above is highly

conserved throughout the ADAM gene family, suggesting that this mutation would have substantial functional effects on the protein.

A role in signal transduction of this ADAM gene is further supported by the fact that the cytoplasmic domain of ADAM7 includes a phosphorylation site and a Src homology region 3 (SH3) binding domain. In particular, the motif LKQVQSP is recognized by those SH3 domains with non-canonical class II recognition specificity and the motif QVQSPPT is predicted as a proline-directed kinase phosphorylation site in higher eukaryotes. Interestingly, the p.S703N mutation is found in this location and might therefore be involved in signalling.

Indeed, many ADAM cytoplasmic domains contain serine and proline residues, with ADAM7 containing the consensus class I (R*XX*P*XX*P) and class II (P*XX*P*X*R) ligands for interaction with SH3 domains of various intracellular proteins (Lopez-Otin and Hunter, 2010; Seals and Courtneidge, 2003). These motifs also exist in other ADAM family members such as ADAM1, ADAM15 and ADAM17 but their positions as well as the neighboring sequences are different among the diverse ADAMs. It is therefore possible that each ADAM could form a unique complex of cytoplasmic proteins through specific protein-protein interactions.

Bioinformatics analysis of the ADAM7 and ADAM29 mutations was performed based on sequence homology to other ADAM proteins in the family or in other species (Supp. Figure S10) (Ruan, et al., 2008). Both the p.E639K and p.S703N mutations in ADAM7 were outside of known existing protein family domains that were present in ADAM7 and were not significantly evolutionarily conserved (Ng and Henikoff, 2003). It must be noted that domains, such as the Reprolysin, ADAM_CR, Pep_M12B_propep have more conserved residues among the different protein family members (Finn, et al., 2008). The most proximal annotated domain to the two mutations is the transmembrane domain, which demarks how the protein is situated in the plasma membrane of the cell. The locations of the p.E639K and p.S703N mutations fall in the cytoplasmic region of the protein, which is highly variable among the different species and family members and would probably affect protein interactions within the cell, potentially altering intracellular signaling.

Several cancer genome wide studies (Jones, et al., 2008; Sjoblom, et al., 2006; Wood, et al., 2007) clearly show that the cancer genetic landscape is made up of few genes that are frequently mutated (cancer ‘mountain’ genes) and a much larger number of genes that are infrequently mutated (cancer ‘hill’ genes) (Jones, et al., 2008). One main challenge is to decipher which of the discovered mutations have a functional role in cancer progression and are thus ‘drivers’ and which of the mutations are ‘passengers’. While no direct inhibitors of ADAM genes have reached clinical development yet, targeting altered ADAM-mediated effector pathways has very recently entered clinical development: for example, targeting ADAM17-mediated ligand cleavage to inhibit Erb receptor signaling through inhibition of its secretase activity, or ADAM10 regulated Notch signaling are prime examples for such efforts. The novel gamma secretase inhibitor RO4929097 (Tolcher, et al., 2010) or the Notch inhibitor MK0752 (Deangelo, et al., 2010) have recently completed phase I clinical trials. As we find that several of the novel alterations have a functional effect on the protein suggesting that these are indeed drivers in melanoma, additional research on the role of the gene family in human cancers might discover novel therapeutic avenues.

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VI. Generación de ratones deficientes en ADAMTS-12, una proteína implicada en angiogénesis y progresión tumoral

Los estudios previos nos permitieron describir, mediante diversas aproximaciones *in vitro*, el comportamiento de la metaloproteasa ADAMTS-12 como un supresor tumoral. Siguiendo esta línea de investigación y en nuestro afán por comprender mejor el papel de esta proteína, procedimos a la generación de un modelo murino deficiente en este enzima. Observamos que la ausencia de Adamts-12 no comprometía la viabilidad ni el desarrollo de los ratones. No obstante, al someter a los animales a protocolos para evaluar la angiogénesis, detectamos que los ratones mutantes mostraban un incremento de los fenómenos angiogénicos. Asimismo, el empleo de técnicas complementarias *ex vivo* mostró que la presencia de ADAMTS-12 causa directamente una inhibición de la angiogénesis. Estos resultados refuerzan la idea de que ADAMTS-12 desarrollaría un papel supresor tumoral durante la progresión del cáncer, y que este rol descansaría, al menos parcialmente, en su capacidad para bloquear la angiogénesis.

Artículo 6: Mehdi El Hour, **Ángela Moncada-Pazos**, Silvia Blacher, Anne Masset, Santiago Cal, Sarah Berndt, Julien Detilleux, Lorin Host, Álvaro J. Obaya, Catherine Maillard, Jean Michel Foidart, Fabien Ectors, Agnès Nöel y Carlos López-Otín. “Higher sensitivity of *Adamts12*-deficient mice to tumor growth and angiogenesis”.

Oncogene 29: 3025-3032 (2010).

Aportación personal al trabajo

Mi participación en este trabajo incluyó diversas etapas de la generación de los ratones deficientes en *Adamts-12*, y posteriormente del establecimiento y mantenimiento de la colonia a partir de los primeros ratones quiméricos. Por otro lado, contribuí al diseño experimental de este proyecto y colaboré en la realización de los ensayos funcionales. Finalmente, participé en la redacción del manuscrito y en la elaboración de las figuras.

Oncogene (2010) 29, 3025–3032

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SHORT COMMUNICATION

Higher sensitivity of *Adamts12*-deficient mice to tumor growth and angiogenesis

M El Hour[1], A Moncada-Pazos[2], S Blacher[1], A Masset[1], S Cal[2], S Berndt[1], J Detilleux[1], L Host[1], AJ Obaya[2], C Maillard[1], JM Foidart[1], F Ectors[3], A Noel[1] and C Lopez-Otin[2]

[1]*Laboratory of Tumor and Developmental Biology, Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), University of Liège, Liège, Belgium;* [2]*Departamento de Bioquimica y Biologia Molecular, Universidad de Oviedo, Instituto Universitario de Oncologia, Asturias, Spain and* [3]*GIGA-Transgenesis, University of Liège, Liège, Belgium*

ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) constitute a family of endopeptidases related to matrix metalloproteinases. These proteases have been largely implicated in tissue remodeling and angiogenesis associated with physiological and pathological processes. To elucidate the *in vivo* functions of ADAMTS-12, we have generated a knockout mouse strain (*Adamts12*$^{-/-}$) in which *Adamts12* gene was deleted. The mutant mice had normal gestations and no apparent defects in growth, life span and fertility. By applying three different *in vivo* models of angiogenesis (malignant keratinocyte transplantation, Matrigel plug and aortic ring assays) to *Adamts12*$^{-/-}$ mice, we provide evidence for a protective effect of this host enzyme toward angiogenesis and cancer progression. In the absence of Adamts-12, both the angiogenic response and tumor invasion into host tissue were increased. Complementing results were obtained by using medium conditioned by cells overexpressing human ADAMTS-12, which inhibited vessel outgrowth in the aortic ring assay. This angio-inhibitory effect of ADAMTS-12 was independent of its enzymatic activity as a mutated inactive form of the enzyme was similarly efficient in inhibiting endothelial cell sprouting in the aortic ring assay than the wild-type form. Altogether, our results show that ADAMTS-12 displays antiangiogenic properties and protect the host toward tumor progression.
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Keywords: ADAMTS-12; angiogenesis; tumor suppression

Introduction

Cancer progression depends not only on the acquisition of new properties by neoplastic cells, but also on a complex cross talk occurring between tumor cells and their microenvironment implicating different types of cells, soluble mediators and cell membrane-associated molecules (Nyberg *et al*., 2008). The contribution of proteolytic enzymes to cancer progression has long been associated with their ability to degrade extracellular matrix components and has been recently extended to their capacity to control the activity and bioavailability of these mediators (Egeblad and Werb, 2002; Cauwe *et al*., 2007; Overall and Blobel, 2007). Recently, the generation of animal models involving gain or loss of function of matrix metalloproteinases (MMPs) has led to the surprising discovery of tumor-suppressive function for some proteases (Lopez-Otin and Matrisian, 2007). These host-protective proteases are not produced by tumor cells, but mainly by tumor infiltrating cells including inflammatory cells (MMP-8) (Balbin *et al*., 2003) and fibroblastic cells (MMP-19) (Jost *et al*., 2006). These recent findings have broken the dogma of proteases as simple positive regulators of cancer progression and emphasize the urgent need in identifying individual proteases as host-protective partners or tumor-promoting agents.

Among proteases with putative tumor-suppressive functions are the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), MMP-related enzymes characterized by the presence of at least one thrombospondin type I domain (TSP-1) (Cal *et al*., 2001; Porter *et al*., 2004, 2005; Bai *et al*., 2009). Their multi-domain structure endows these secreted proteins with various functions including the control of cell proliferation, apoptosis, adhesion and migration (Noel *et al*., 2008; Rocks *et al*., 2008; Bai *et al*., 2009). It is worth noting that ADAMTS-1 and ADAMTS-8 display antiangiogenic properties (Vazquez *et al*., 1999; Liu *et al*., 2006). Our recent studies have highlighted the antitumor properties exhibited by ADAMTS-12 (Cal *et al*., 2001; Llamazares *et al*., 2007). In accordance with this concept of ADAMTS-12 being a host-protective enzyme, *ADAMTS12* is epigenetically silenced in human tumor samples and tumor cell lines (Moncada-Pazos *et al*., 2009). However, the exact contribution of ADAMTS-12 during the different steps of cancer progression including angiogenesis remains to be elucidated. To address this important issue, we have generated mutant mice lacking the *Adamts12* gene. *Adamts12* deficiency did not cause obvious abnormalities

Correspondence: Dr A Noel, Laboratory of Tumor and Developmental Biology, Groupe Interdisciplinaire de Génoprotéomique Appliquée-Cancer (GIGA-Cancer), University of Liège, Tour de Pathologie (B23), CHU Sart-Tilman, Liège B-4000, Belgium.
E-mail: agnes.noel@ulg.ac.be
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during embryonic development or in adult mice. Therefore, mutant mice provide a novel and useful tool to investigate Adamts-12 functions in pathological angiogenesis. Through different complementary approaches, we provide evidence that Adamts-12 protects the host against tumor angiogenesis, growth and invasion.

Results

Adamts12-*deficient mice are viable without any obvious phenotype*

The biological functions of ADAMTS-12, a metalloprotease-related enzyme overexpressed in human cancers (Porter *et al*., 2004), are poorly understood. To establish a mutant mouse strain deficient for *Adamts12* gene (KO, *Adamts12*$^{-/-}$ mice), the targeting vector was designed to replace the exons 6 and 7 (corresponding to the N-terminal part of the catalytic domain) by a neomycin-phosphoglycerate kinase (NEO-PGK) cassette (Figure 1a) and to introduce a frame shift. Embryonic stem clones generated by homologous recombination (Figure 1b) were injected into C57Bl/6J blastocysts to generate chimeric males. Heterozygous mice from the F1 generation were intercrossed to generate *Adamts12*-deficient mice that were obtained in the Mendelian ratio. We checked mice genotypes by PCR (Figure 1c).

The expression of *Adamts12* was determined by reverse transcriptase (RT) PCR in different organs. In WT mice, *Adamts12* was expressed in some organs, including the ovary, mammary gland, uterus, lung, ear cartilage and lymph node (Figure 1d). *Adamts12* was not expressed in the heart, kidney, bone, liver, brain, intestine, testis, muscle, skin, eyes and spleen (data not shown). As expected, tissues from *Adamts12*$^{-/-}$ mice did not produce any Adamts-12 in all organs tested, as assessed by RT–PCR amplification using primers targeting the catalytic domain (Figure 1d) and the prodomain (data not shown). These RT–PCR analysis show that no residual mRNA was produced in mutant mice because of the frame-shift introduced during exon replacement.

Despite *Adamts12* deficiency, mutant mice developed normally were fertile and had long-term survival rates indistinguishable from those of their wild-type (WT) counterpart. No obvious phenotype was detected. These findings clearly indicate that *Adamts12* is dispensable for embryonic and adult mouse development and growth.

Adamts12 *deficiency affects tumoral angiogenesis*

In the course of the phenotyping of *Adamts12*$^{-/-}$ mice, we evaluated its expression in pathological conditions. *Adamts12* expression was first investigated during laser-induced choroidal neovascularization by RT–PCR analyses performed at various time points after laser burn (Lambert *et al*., 2003). *Adamts12* expression was never detected at any stage of choroidal neovascularization (data not shown), excluding the potential implication of Adamts-12 in choroidal angiogenesis.

The overexpression of *ADAMTS12* in various human cancers (Cal *et al*., 2001) prompted us to explore its putative functions in tissue remodeling associated with cancer development. We applied three models of angiogenesis, which have been previously successfully used to evaluate the contribution of different metalloproteinases during angiogenic processes (Masson *et al*., 2002; Berndt *et al*., 2006, 2008). The transplantation system is a highly sensitive tool to inspect the kinetic of early steps of host stromal response to tumor signals (Mueller and Fusenig, 2004; Jost *et al*., 2007, 2008). Malignant PDVA cells issued from carcinogen treatment of murine keratinocytes (Fusenig *et al*., 1983) precultured on a type I collagen gel were transplanted onto WT ($n=14$) and *Adamts12*-deficient mice ($n=14$). An early endothelial cell migration toward the tumor layer can be visualized through double immunostaining carried out to distinguish tumor cells (keratin positive) and vessels (CD31 positive cells or type IV collagen-positive basement membrane) (Blacher *et al*., 2008). Two different patterns of invasion were observed 21 days after transplantation (Figure 2). A low vascularized profile scored + was characterized by blood vessel infiltration of the collagen gel without reaching the tumor layer. Then, once blood vessels have reached tumor cell layers, malignant keratinocytes formed tumor sprouts that invaded downward the remodeled host tissue and were intermingled with new vessels (Figure 2). Such highly vascularized pattern was scored + + +. Tumor vascularization and invasion was improved in *Adamts12*$^{-/-}$ mice. Indeed, 86% of transplants were highly vascularized (scored + + +), whereas 14% of samples were scored +. In WT mice, the percentage of transplants scored + + + fell down to 57% and that of low vascularized transplants reached 43% ($P=0.027$, χ^2-test) (Figure 2a).

To further confirm this distinct invasive profile, an original method of computerized image analysis (Blacher *et al*., 2008) allowing a concomitant quantification of blood vessel recruitment and tumor cell invasion was applied to all samples. The distribution of tumor cell and endothelial cell densities was determined as a function of distance to the top of the tumor (Figures 2b and c). With this aim, a grid formed from the dilatation of tumor boundaries was superposed onto tumor and vascularization images as previously described (Blacher *et al*., 2008). The degree of superimposition of curves corresponding to vessel and tumor densities determines tumor vascularization. Yellow spots in Figure 2 delineate areas of tumor in which tumor cells and blood vessels were intermingled, reflecting tumor vascularization. In *Adamts12*$^{-/-}$ mice, invasion and migration process took place to a much larger extent (Figures 2b and c; $P<0.05$). A stronger invasion characterized by a deeper extension of tumor cells was observed in *Adamts12*$^{-/-}$ mice (migration up to $L_{max}=3.55\pm0.4$ mm in KO mice versus $L_{max}=2.5\pm0.4$ mm in WT mice, $P<0.05$, Mann–Whitney test). In addition, blood vessels migrated roughly toward tumor cells leading to increased overlapping area of tumor cells and blood vessels (Figures 2b and c, yellow spot). Indeed, the area of

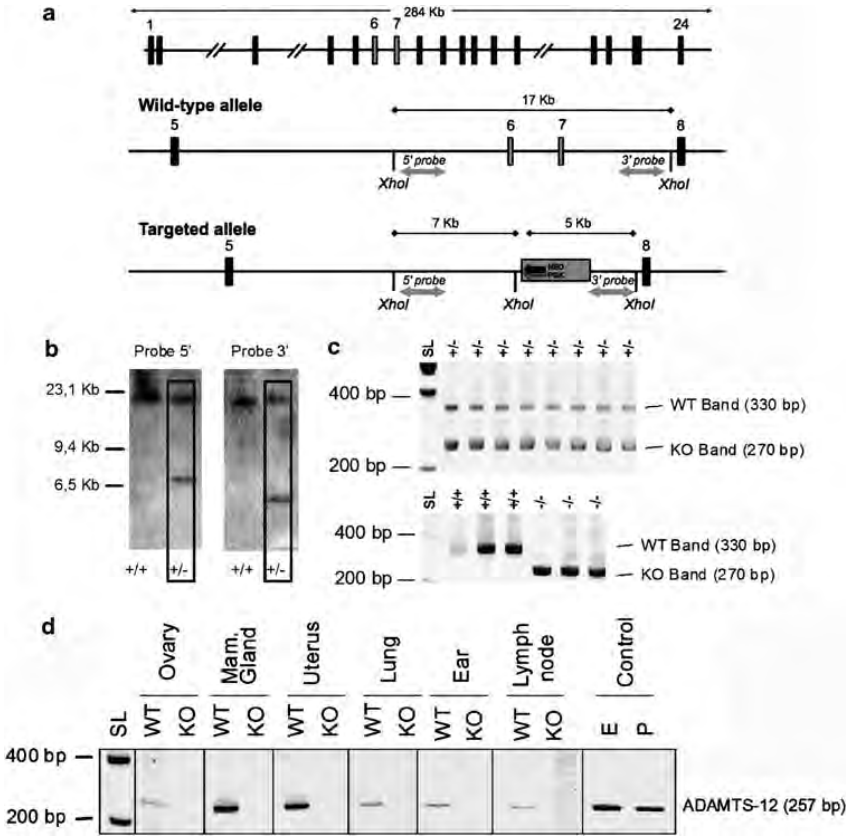

Figure 1 Targeted disruption of mouse *Adamts12* gene. (**a**) Schematic illustration of the targeting strategy (top, WT allele; bottom: targeted allele). A genomic clone encoding *Adamts12* was obtained from a mice 129/SvJ strain genomic DNA library. The exons 6 and 7 were replaced by a neomycin-phosphoglycerate kinase (NEO-PGK) cassette. Predicted sizes of Xho I fragments are indicated (rectangle, exon; double-headed arrows: probes for detecting the homologous recombination event). (**b**) Xho I Southern blot analysis of $+/+$ and $+/-$ ES129 cells. The targeted embryonic stem (ES) clones obtained by homologous recombination were then injected into blastocysts (10–15 cells per blastocyst of day 4). (**c**) Example of PCR genotyping from tail biopsies of *Adamts12* WT ($+/+$), *Adamts12* heterozygous ($+/-$) and homozygous *Adamts12* ($-/-$) mice generated in C57BL/6 genetic background. (**d**) RT–PCR analysis of *Adamts12* expression in WT and KO mouse tissues. Positive controls are embryo (E) and placenta (P). Representative gels are displayed with the expected size (bp) of RT–PCR products on the right.

vessels intermingled with tumor cells was twice higher in mutant mice (0.27 versus 0.13 mm^2 in WT mice).

These data indicate that *Adamts12* deficiency in host tissue is associated with an acceleration of the angiogenic response and increased tumor invasion into the host tissue. It is worth noting that malignant keratinocytes used in this model did not express *Adamts12 in vitro*, whereas human ADAMTS-12 was detected in tumor transplants. The RT–PCR analysis of tumor cell layer and the host compartment separated through laser microdissection revealed that *Adamts12* was expressed by host cells rather than by tumor cells (Figure 2d). These results show an induction of host *Adamts12* expression following tumor cell transplantation *in vivo*. These data are in line with our recent study showing that *ADAMTS12* expression by fibroblasts is enhanced in co-culture with cancer cells (Moncada-Pazos *et al*., 2009). They are also in full agreement with a previous report showing that *ADAMTS12* as other family members (*ADAMTS2*, *7*, *8* and *10*) are predominantly expressed in stromal fibroblasts from human mammary tissues, but not in breast cancer cells (Porter *et al*., 2004). Altogether, these observations suggest that fibroblastic cells are an important source of ADAMTS-12 and respond to the presence of tumor cells by producing this enzyme.

Adamts-12 affects in vivo *and* ex vivo *angiogenesis*

The angiogenic response to basic fibroblast growth factor (bFGF) was then investigated in WT and mutant mice. Matrigel (500 µl) containing heparin (10 U/ml) and bFGF (250 ng/ml) were injected subcutaneously into mice ($n = 16$). To quantify functional vessel recruitment, hemoglobin concentrations were measured in the plugs harvested 7 days after injection (Berndt *et al*., 2006). The angiogenic response induced by bFGF was again higher

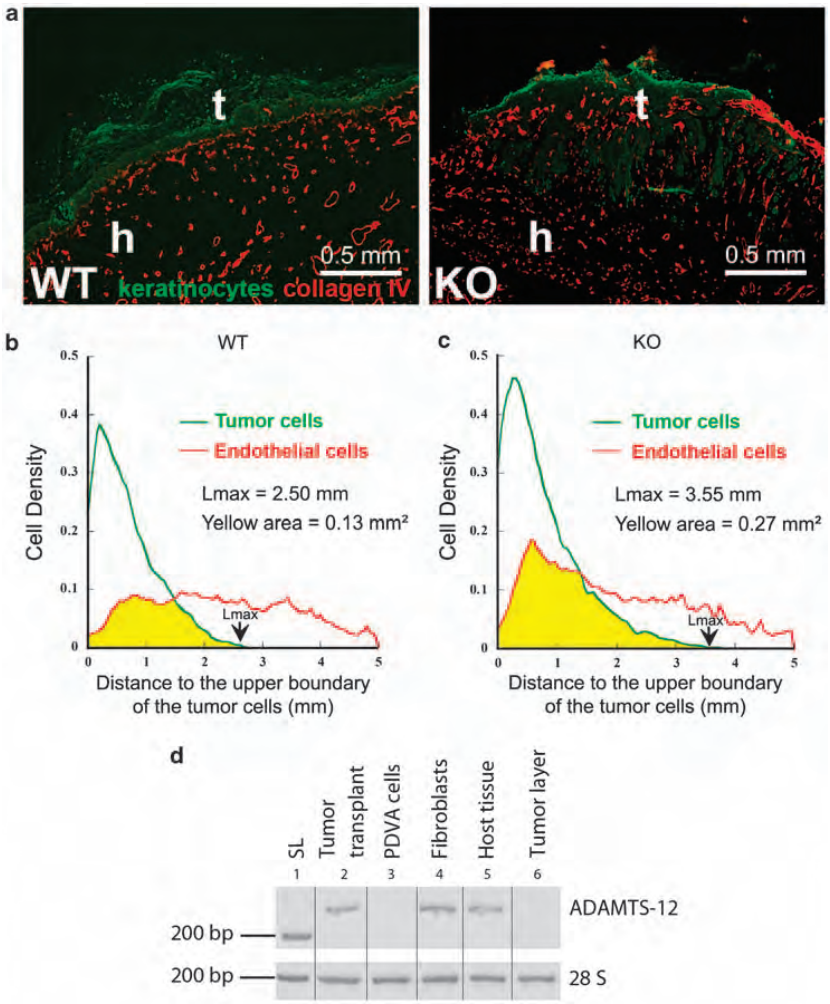

Figure 2 Histological analysis of tumor transplanted into mice. Malignant murine PDVA keratinocytes precultured on a collagen gel were covered by a silicone chamber and transplanted for 3 weeks on the back of 6- to 8-week-old mice *Adamts12*$^{+/+}$ (WT) or *Adamts12*$^{-/-}$ (KO) mice ($n=14$ per experimental groups). (**a**) Double immunostaining of tumor transplant resected from WT (left panel) and KO mice (right panel) were carried out on cryostat section to identify tumor cells (anti-keratin antibody, green, rabbit polyclonal antibody, Dako, Glostrup, Denmark) and vessels (anti-type IV collagen or anti-CD31, red guinea pig polyclonal antibody, Sigma-Aldrich, St Louis, MO, USA). t, tumor; h, host tissue. Magnification: ×40. Bar, 0.5 mm. (**b**, **c**) Tumor and endothelial cell density distributions determined by computerized image analysis with the Aphelion 3.2 software from Adsis (Meythet, France). After image binarization/segmentation, the upper boundary of the tumor was automatically detected and a grid was constructed with the successive dilations ($n=1, 2, 3\ldots$) of this upper boundary. Tumor and vessel densities were determined on each interval of the grid. Results were drawn in function of the distance to the upper tumor limit. Intermingling of tumor cells and vessels is shown by superimposition of the respective curves (yellow areas). The maximal distance of tumor cell invasion (L_{max}) is indicated by an arrowhead. (**d**) RT–PCR analysis of Adamts-12 expression in the whole tumor transplant (2, tumor transplant), in PDVA cells cultured *in vitro* (3, PDVA cells), in *in vitro* cultures of fibroblasts (4, fibroblasts), in the host tissue compartment of tumor transplant (5, host tissue) and in the tumor layer of tumor transplant (6, tumor layer). The mRNA of the tumor layer and the host compartment were extracted from samples isolated here with laser microdissection performed on tumor transplant cryostat sections. SL (1): molecular weight (smart ladder).

in *Adamts12*$^{-/-}$ than in WT mice. In WT mice, the mean hemoglobin content was two-fold reduced (0.65 ± 0.1 Hb (mg/ml) per mg of plug in WT mice versus 1.65 ± 0.5 Hb (mg/ml) per mg of plug in *Adamts12*$^{-/-}$ mice), and the percentage of poorly vascularized plugs (hemoglobin concentration lower than 0.5 Hb (mg/ml) per mg of plug) reached 56% (9 of 16) in WT mice, whereas it was <12% (2 of 16) in mutant mice ($P<0.05$).

To further study the contribution of Adamts-12 in neovessel formation, aortic explants issued from mutant or WT mice were embedded into a three-dimensional type I collagen gel in the presence of serum

corresponding to their genotype (Figure 3a). An increased angiogenic response was observed in *Adamts12*$^{-/-}$ mice as evidenced by increased sprout density (Figure 3b, left graph) and vessel length (Figure 3b, right graph). As the formation of capillary-like structures is associated with the spreading out of mural cells, we performed immunohistochemical labeling on whole mount of aortic rings. Interestingly, increased mural cell recruitment detected by NG2 labeling was observed in IB4-positive capillaries issued from *Adamts12*$^{-/-}$ mice (Figure 3c). Computerized quantification revealed that the mean area of colocalization between IB4-positive endothelial cells and NG2-positive mural cells was twice higher in sprouts issued from *Adamts12*$^{-/-}$ mice as compared with those from WT explants (Figure 3d). These data reflect an increased maturation of blood vessels in the absence of Adamts-12. Altogether, these findings indicate that *ADAMTS12* is a negative regulator of angiogenesis.

The finding that *Adamts12* deficiency resulted in an acceleration of the angiogenic response led us to postulate that cells overexpressing ADAMTS-12 would have opposite effect and would inhibit it. To address this question, we next evaluated the impact of medium conditioned by a population of MCF7 cells overexpressing human FLAG-tagged ADAMTS-12 (MCF7p ADAMTS-12 FLAG) on neovessel formation in another experimental model using aortic rings issued from a rat. The presence of ADAMTS-12 in the conditioned media was assessed by western blot (Figure 4d) using an anti-FLAG antibody allowing the detection of a specific band of molecular mass $\approx$175 kDa. In accordance with our hypothesis, the medium of cells overexpressing ADAMTS-12 inhibited the angiogenic response as revealed by a reduction vessel length (Figure 4b) and vessel maturation through pericyte coverage (Figure 4c).

We next determined whether the antitumorigenic effect of ADAMTS-12 could be ascribed to its catalytic function. With this aim, new MCF7 transfectants were generated to produce inactive mutants of ADAMTS-12 or intact ADAMTS-12. Point mutations of two key amino acids of the catalytic site (H465Q/E466A) were performed as previously described (Cal *et al*., 2001). The mutated ADAMTS-12 form was produced at similar levels than the WT form as assessed by western

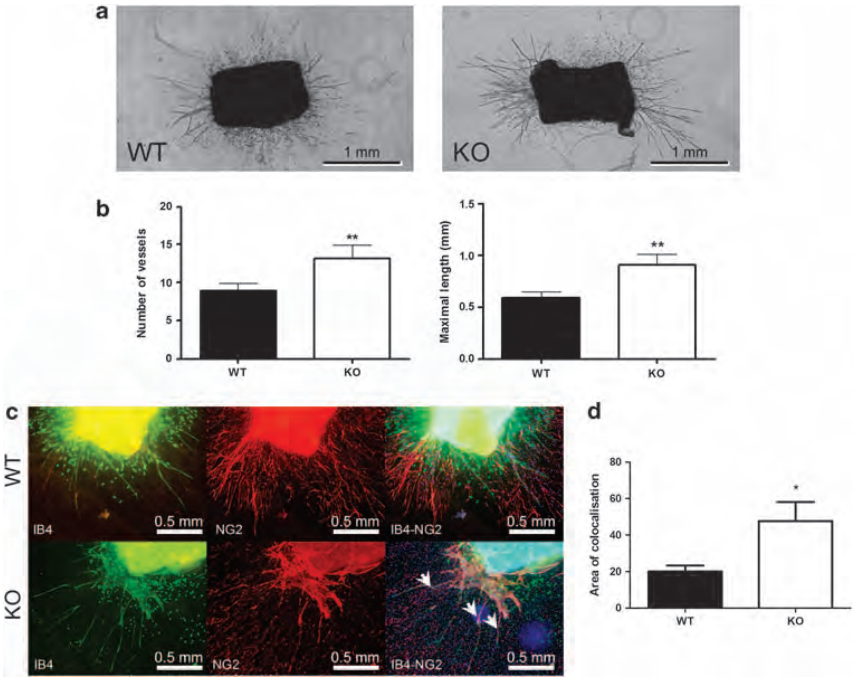

Figure 3 Capillary outgrowth from mouse aortic rings cultured in a collagen gel. (**a**) Morphological observation of explants of mouse thoracic aorta issued from *Adamts12*$^{+/+}$ (WT) and *Adamts12*$^{-/-}$ mice (KO). Magnification: $\times$25. Bar, 1 mm. (**b**) Computerized quantification. Two parameters are shown: number (left graph) and maximal length of vessels (right graph). $^{**}P<0.01$ (Mann–Whitney test). (**c**) Characterization of cells spreading out of aortic rings. Immunostainings were performed on whole-mount rings to identify endothelial cells (Griffonia Simplifolia isolectine B4/Alexa Fluor 488, green, Molecular Probes Inc., Eugene, OR, USA), pericytes (rabbit anti-NG2 chondroitin sulfate proteoglycan antibody, red, Sigma-Aldrich) and pericytes covering endothelial cells (IB4 + NG2, merged in yellow) (white arrowheads). Magnification: $\times$40. Bar, 0.5 mm. (**d**) The area of colocalization (mm^2) between pericytes and endothelial cells was determined by using a computer-assisted method quantification by implementing an algorithm using MATLAB7.1 software. The assay was conducted by using at least triplicate culture per condition and results are those of a representative assay out of three. $^{*}P<0.05$ (Mann–Whitney test).

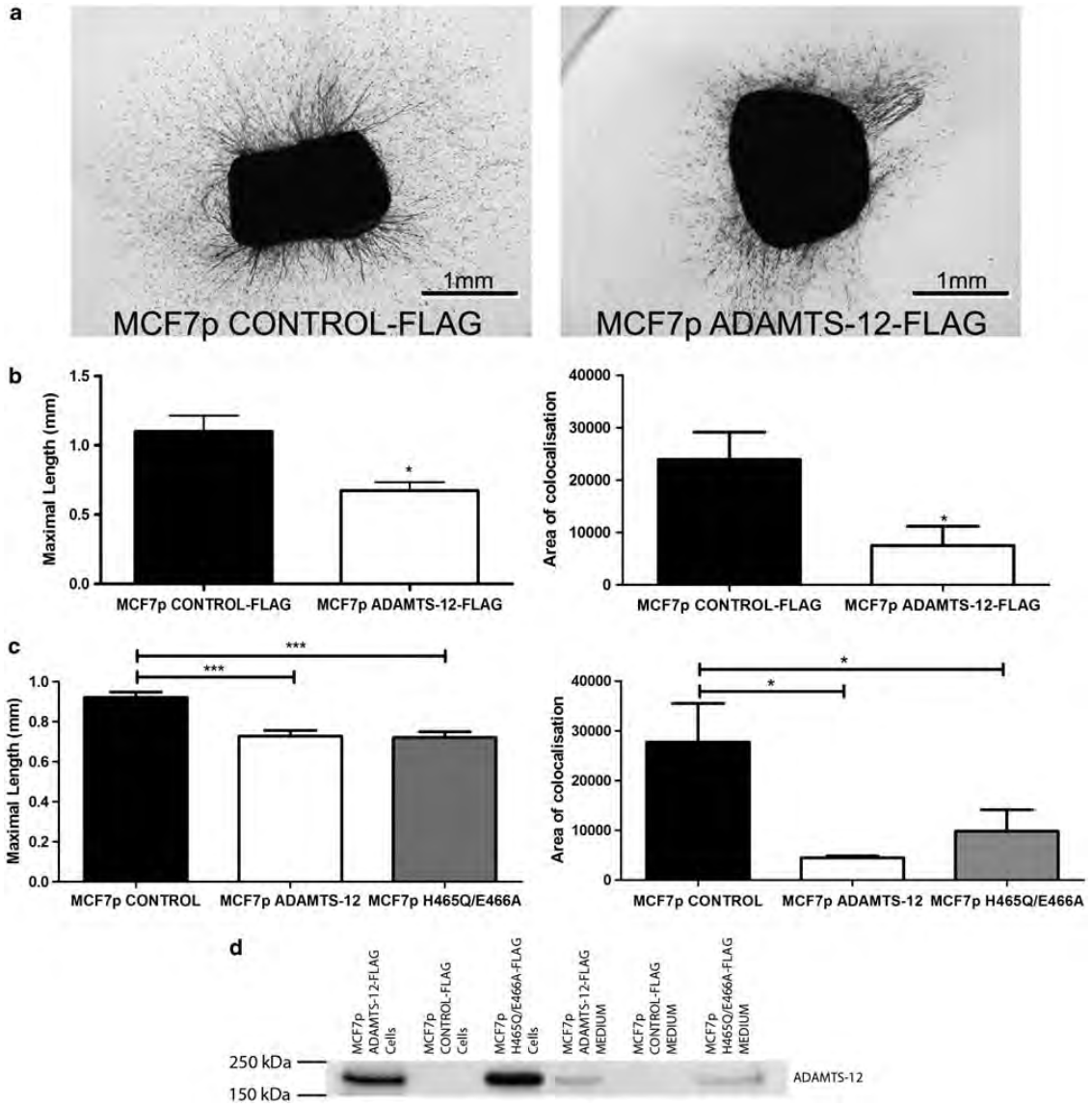

Figure 4 Capillary outgrowth from rat aortic rings cultured in the presence of ADAMTS-12. Wistar rat aortic explants were cultured in MCDB-131 medium (2.5 ml) supplemented with medium (2.5 ml) conditioned by a population of breast adenocarcinoma MCF7 cells expressing (MCF7pADAMTS12-FLAG) or not (MCF7pCONTROL-FLAG) tagged ADAMTS-12. (**a**) Morphological observation of aortic explants in the absence of ADAMTS-12 (MCF7pCONTROL-FLAG) or in the presence of ADAMTS-12 (MCF7pADAMTS-12-FLAG). Magnification: × 25. Bar, 1 mm. (**b**) Maximal length of vessels was quantified by computerized image analysis (left panel). The area of colocalization (mm^2) of pericytes and endothelial cells was determined by using a computer-assisted method applied to immunostainings performed on whole mounts (see legend of Figure 3) (right panel). (**c**) Impact of point mutation of ADAMTS-12 catalytic site. Populations of MCF7 cells expressing WT ADAMTS-12 (MCF7pADAMTS-12), inactive mutant ADAMTS-12 (MCF7pH465Q/E466A) or not expressing ADAMTS-12 (MCF7pCONTROL) were generated by stable transfection, and their conditioned media were tested in the rat aortic ring assay. The double point mutation in the catalytic site did not affect the inhibitory effect of ADAMTS-12 on vessel sprouting (left panel) and on vessel coverage by pericytes (right panel). Similar results were obtained with two clones of each transfectants. The assay was conducted by using at least triplicate culture per condition and results are those of a representative assay out of three. $*P<0.05$; $***P<0.001$ (Mann–Whitney test). (**d**) Western blot analysis of the ADAMTS-12-expressing MCF7 cells (MCF7p ADAMTS-12-FLAG), control cells (MCF7p CONTROL-FLAG) and the mutated ADAMTS-12 (MCF7p H465Q/E466A-FLAG) in cell extracts and conditioned media concentrated 10-fold using Amicon Ultra (Millipore, Billerica, MA, USA). ADAMTS-12 was visualized by using an antibody raised against the Flag epitope with anti-FLAG-M2 antibodies (Sigma-Aldrich).

blotting (Figure 4d). These mutations in the catalytic domain did not impair the antiangiogenic effect induced by medium conditioned by transfectants (Figures 4d and e). Indeed, the medium conditioned by cells overexpressing intact ADAMTS-12 or mutated ADAMTS-12 inhibited the spreading out of endothelial cells as assessed by a reduction of vessel length (Figure 4d) and of vessel maturation through pericyte coverage (Figure 4e). These results suggest that the catalytic activity of ADAMTS-12 is dispensable for its angioinhibitory function.

Our data are in line with our previous report showing that ADAMTS-12 inhibits the formation of vascular endothelial growth factor-induced tubular structures in BAE-1 cells (Llamazares *et al*., 2007). The finding of antiangiogenic effects for a member of the ADAMTS family is not unprecedented as it has also been described for ADAMTS-1 and ADAMTS-8 (Vazquez *et al*., 1999; Dunn *et al*., 2006). However, the originality of our findings relies on the identification of a key host ADAMTS-12 contribution in the host protection toward the angiogenic response induced by tumor cells. This report based on the generation of a novel loss-of-function animal model provides the first demonstration of the antiangiogenic properties of a single host ADAMTS. Moreover, in contrast to ADAMTS-1 whose proteolytic activity is apparently required for its antiangiogenesis property (Iruela-Arispe *et al*., 2003), the angioinhibitory property of ADAMTS-12 does not depend on its catalytic activity. We cannot ruled out the possibility that ADAMTS-12 can sequester vascular endothelial growth factor through its C-terminal thrombospondin domain(s) and the spacer region as previously described for ADAMTS-1 (Luque *et al*., 2003). Addressing this issue would require a careful dissection of the complex C-terminal part of ADAMTS-12 containing seven thrombospondin repeats, two spacers and a PLAC domain, which is out of the scope of this study.

In conclusion, we propose that the ADAMTS-12 production induced in response to the presence of cancer cells could have a beneficial protective effect toward tumor growth and invasion. The new generation of *Adamts12*-deficient mice provides a suitable tool to give new insights into the *in vivo* functions of this enzyme, which are presently unknown. In addition, this new transgenic tool paves the way for further investigations on the biological functions of ADAMTS-12 in various pathological conditions in which the enzyme is putatively involved such as asthma (Kurz *et al*., 2006) and arthritis (Bai *et al*., 2009).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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VII. Estudio de la respuesta inflamatoria en ratones deficientes en Adamts12

La disponibilidad de un modelo murino sin Adamts-12 nos animó a intentar comprender mejor sus implicaciones fisiológicas evaluando su respuesta en procesos inflamatorios y durante la cicatrización de heridas. De esta forma, pudimos detectar que la falta del enzima exacerbaba los síntomas en enfermedades inflamatorias como colitis, pancreatitis o choque séptico. Los síntomas clínicos eran coherentes, además, con la existencia de múltiples indicadores inflamatorios que resultaron estar incrementados a nivel molecular. Los tejidos afectados presentaban a menudo un aumento de la presencia de neutrófilos. Mediante el aislamiento y cultivo de neutrófilos, comprobamos que ADAMTS-12 era capaz de promover la apoptosis de este tipo celular. Estos datos indican que esta metaloproteasa participaría en la respuesta inflamatoria, presumiblemente modulando la apoptosis fisiológica de neutrófilos. Finalmente, describimos que en ausencia de Adamts-12 la cicatrización de heridas en piel se ve ralentizada.

Artículo 7: **Ángela Moncada-Pazos**, Álvaro J. Obaya, María Llamazares, Ritva Heljasvaara, María F. Suárez, Agnès Nöel, Santiago Cal y Carlos López-Otín. “ADAMTS-12 metalloprotease is necessary for normal inflammatory response”.

(Manuscrito).

Aportación personal al trabajo

En este trabajo me encargué del mantenimiento de la colonia de ratones, así como de los protocolos *in vivo* para analizar las distintas condiciones inflamatorias y la cicatrización de heridas y del análisis de los resultados obtenidos en todos ellos. También participé con la obtención de muestras y análisis de expresión y contribuí a los estudios histológicos. Asimismo, realicé los aislamientos, cultivos y mediciones de apoptosis de neutrófilos. Finalmente, participé en la elaboración del manuscrito y figuras bajo la supervisión de los Drs. Santiago Cal y Carlos López-Otín.

ADAMTS-12 metalloprotease is necessary for normal inflammatory response

*Angela Moncada-Pazos, †Alvaro J. Obaya, *María Llamazares, *‡Ritva Heljasvaara,

*María F. Suárez, §Agnès Noël, *Santiago Cal and *Carlos López-Otín

*Departamento de *Bioquímica y Biología Molecular and †Biología Funcional, Instituto Universitario de Oncología, Universidad de Oviedo, Oviedo, Spain. ‡ Oulu Center for Cell-Matrix Research, Biocenter Oulu and Department of Medical Biochemistry and Molecular Biology, University of Oulu, Finland. §Laboratory of Tumor and Developmental Biology, University of Liège, Tour de Pathologie, Liège, Belgium*

M.L. present address: *Philipps-Universität Marburg, Klinik für Dermatologie und Allergologie, Dermatologische Forschung, Marburg, Germany.*

Running title: Loss of *Adamts12* impairs inflammation

Correspondence: Carlos López-Otín. Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain. Telephone: +34 985104201. E-mail: clo@uniovi.es

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ABSTRACT

ADAMTSs (a disintegrin and metalloprotease with thrombospondin domains) are a family of enzymes with both adhesion and proteolytic functions, which have been implicated in distinct pathologies. In this work, we have investigated the putative role of ADAMTS-12 during inflammation by using a mouse model deficient in this metalloprotease. Control and mutant mice were subjected to different experimental conditions in order to induce colitis, pancreatitis, endotoxic sepsis and wound healing. We have observed that *Adamts12*-deficient mice exhibit more severe inflammation and a delayed recovery from these challenges when compared with their wild-type littermates. These changes are accompanied by an increase in inflammatory markers including several cytokines, as assessed by microarray expression analysis and proteomic-based approaches. Interestingly, the clinical symptoms observed in *Adamts12*-deficient mice are also concomitant with an elevation in the number of neutrophils in the affected tissues. Isolation and *in vitro* culture of neutrophils have demonstrated that the presence of ADAMTS-12 is responsible for neutrophil apoptosis induction, and that this effect can be inhibited, at least partially, by blocking CD36. On the basis of these results, we propose that ADAMTS-12 is implicated in the inflammatory response by modulating normal neutrophil apoptosis.

INTRODUCTION

Metalloproteases regulate many biological processes in all living organisms through their ability to specifically cleave peptide bonds in a variety of substrates. Thus, these enzymes are essential for cell proliferation and differentiation, fertilization, morphogenesis, shedding of cell surface proteins, cell signaling, angiogenesis, hemostasis or apoptosis (1). Experiments involving gain- or loss-of-function of metalloprotease genes have placed these enzymes as main actors in numerous physiological and pathological processes, including inflammation. Most experiments in this regard have been performed with different members of the matrix metalloproteinase (MMP) family (2). Thus, MMP-8 is a collagenase mainly expressed in neutrophils and with the ability to cleave several chemokines (3). Our studies with a mouse model lacking *Mmp8* have shown that this protease is necessary for normal inflammatory responses, exerting a protective role during tumor development, induced sepsis, wound healing, lung fibrosis or asthma (4-8), while facilitating development of hepatitis (9). Other MMPs also play roles as inflammatory modulators such as MMP-2 (gelatinase A) by inactivating monocyte chemoattractant protein-3 (10), or MMP-7 (matrilysin) by contributing to create local chemokine gradients by facilitating shedding of the chemokine-binding protein syndecan-1 (11). Additionally, different members of the ADAM (a disintegrin and metalloproteinase) family of membrane-anchored metalloproteases have also been linked to inflammatory processes (12). For example, ADAM17 cleaves and activates TNF-α, a potent pro-inflammatory cytokine and important mediator of distinct pathologies like Crohn's disease (13). However, to date, limited information is available regarding the implication of the ADAMTS (ADAMs with thrombospondin domains) family of metalloproteases in inflammatory processes.

The ADAMTSs are a family of 19 secreted metalloproteases showing structural similarities to MMPs or ADAMs such as the presence of a zinc-chelating motif essential for the enzymatic activity. However, the presence of different thrombospondin domains within their

architecture constitutes a distinctive structural hallmark of ADAMTSs (14). These thrombospondin domains, together with other ancillary domains, constitute an adhesion region which likely act by modulating substrate binding, determining cell localization or sequestering factors in the extracellular matrix. Some members of the ADAMTS family, such as ADAMTS-4 and ADAMTS-5, have been related to inflammatory processes including arthritic diseases (15). Therefore, it is tempting to speculate that the altered function of any of these ADAMTSs putatively involved in inflammation could contribute to the development of a variety of different human diseases. In this regard, it is well established that abnormal and persistent immune responses are responsible for pathologies such as inflammatory bowel diseases, rheumatoid arthritis, atherosclerosis or allergies. All these conditions have increased their prevalence during the past decades and now constitute a major concern for public health with limited therapeutic opportunities (16). There is growing evidence that these pathologies are caused by a complex interaction between multiple susceptibility loci and environmental factors. Accordingly, the identification of genes involved in inflammatory diseases and the further characterization of their mechanisms of action represent suitable strategies for future clinical improvement of these conditions.

ADAMTS-12 was first identified and cloned in our laboratory more than 10 years ago (17), but few data are available concerning its biological functions. In contrast, there is growing information about the putative pathological relevance of this enzyme. Thus, ADAMTS-12 has been associated with osteoarthritis due to its ability to degrade the cartilage components aggrecan and cartilage oligomeric matrix protein (18, 19). Moreover, ADAMTS-12 has been linked to asthma after the finding that the gene encoding this metalloprotease is a locus of increased susceptibility to asthma (20). In neoplastic diseases, ADAMTS-12 has been described to act as an anti-tumor protease. In this regard, we have previously reported that this metalloprotease inhibits pro-tumor activities of MDCK and BAEC cells as well as *in vivo* tumor

growth of A549 cells in SCID mice (18). Furthermore, *ADAMTS12* is hypermethylated in tumors from different origins and, at least in colorectal cancer, the epigenetic silencing occurring in tumor cells is compensated by its overexpression in myofibroblasts of the surrounding stroma (21, 22). To try to elucidate the biological roles of this enzyme, we have recently established a mouse strain lacking *Adamts12*, which shows no obvious phenotype but exhibits significant differences in cancer susceptibility by comparison with control mice (23). Thus, *Adamts12*-deficient mice are more susceptible to cancer development, exhibiting increased angiogenesis and tumor invasion.

In the present work, we have studied the putative role of ADAMTS-12 in inflammation. For that purpose, we have examined the mouse model lacking *Adamts12* under distinct inflammatory conditions including ulcerative colitis, sepsis, pancreatitis and wound healing, with the finding that the absence of this protease causes increased inflammation. Moreover, we have analyzed the inflammatory response of *Adamts12*-deficient tissues at the molecular level, and observed that several inflammatory markers are also increased at RNA and protein levels, and correlate with the observed clinical features. Finally, we have found that *Adamts12* null mice undergo an excessive accumulation of neutrophils due to their reduced apoptosis. On the basis of these results, we propose that ADAMTS-12 exerts a protective role in inflammatory pathologies, avoiding a detrimental concentration of neutrophils by inducing their appropriate apoptosis.

MATERIALS AND METHODS

Animals

Adamts12$^{-/-}$ mice were generated as previously described (23), and then backcrossed to a C57BL6 strain. Genotyping was performed by PCR with DNA extracted from mice tails. A minimum number of 9 mice aged 8 to 10 weeks were used in all experiments. Mice were housed under specific pathogen-free conditions and following the guidelines of the Committee on Animal Experimentation of the Universidad de Oviedo, Oviedo, Spain.

Induction of colitis

Animals were treated with 2% (w/v) dextran sulfate sodium salt (DSS, MP Biomedicals, Santa Ana, CA, USA) in drinking water for 7 days, and followed by 1 day of regular water. For induction of chronic colitis, mice were treated with 5% DSS during 10 days, and then allowed to recover for 15 additional days. Control mice were given the same drinking water without DSS. Body weight and clinical symptoms of colitis were monitored daily following the scoring system previously described (24): 0 for normal stool, 1 for soft stool, 2 for very soft stool with blood traces and 3 for diarrhea and rectal bleeding. At day 8 in acute colitis model (and at day 25 in chronic colitis model), mice were sacrificed and colons were removed, measured and washed. Fractions of cecum and of proximal and distal colon were processed for histological analysis or frozen for protein extraction.

LPS-induced sepsis model

Adamts12$^{-/-}$ and *Adamts12*$^{+/+}$ mice were injected intraperitoneally (i.p.) with 8.5 µg/kg body weight of ultrapure lipopolysaccharide (LPS, Sigma, St. Louis, MO, USA) in saline solution. Controls were injected with saline solution. Body weight was monitored daily. At day 2 or 8, mice were anesthetized and blood samples were taken by intracardiac puncture after which animals were sacrificed and liver and spleen removed for histological and proteomic studies. Cell population abundances in blood were determined by flow cytometry by using a combination of specific antibodies against murine Ly-6G (BD Biosciences 551461), CD45 (550994), CD45R (552094), CD3e (553061), Ly-6G and Ly-6C (553129) and F4/80 (552958).

Cerulein-induced pancreatitis

For induction of acute pancreatitis, animals were injected i.p. hourly with 50 µg cerulein/kg body weight for 10 h. Untreated controls were injected with saline solution solely. One hour after the last injection, animals were anesthetized, blood was removed by intracardiac puncture and pancreas isolated for further analyses. Serum levels of amylase and lipase were determined enzymatically in a Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Histological analyses and MPO-immunohistochemistry

Immediately after extraction, tissue samples were fixed with 4% formaldehyde for 24 h, washed with 70% ethanol and embedded in paraffin. Sections were subjected to hematoxylin and eosin staining, and analyzed by an expert pathologist. In the colitis model, the grade of affected surface, number of ulcers, presence of inflammatory infiltrate and reparative changes were evaluated. Pancreatitis was assessed based on the significance of edema, cellular infiltrate and

necrosis. For interacinar septa quantification, we performed 6 measurements per animal slide (n=9 *Adamts12*$^{-/-}$ and 10 *Adamts12*$^{+/+}$) with ImageJ software. The number of polymorphonuclear (PMNs) cells was determined by myeloperoxidase (MPO) immunohistochemical detection. The reaction was carried out with an anti-MPO antibody (Thermo Fisher Scientific, Waltham, MA, USA) in the Discovery XT System (Ventana, Oro Valley, AZ, USA). First, unmasking was performed by heating at pH 8.4 with CC1 buffer (Ventana). Then, slides were blocked with the casein based Antibody block (Ventana) and incubated with the antibody at 37 ^{o}C, for 1 h and at 1:50 dilution in Ab diluent (Ventana). The secondary antibody used was OmniMap anti-Rb HRP (Ventana) and detection was carried out with ChromoMap DAB (Ventana). Contrast staining was performed with hematoxylin and Bluing reagent (Ventana). After staining, the number of MPO-positive cells in equivalent areas of ulceration was determined in slides from *Adamts12* proficient and deficient mice (6 fields per slide, n=4 per group).

RNA expression arrays

Whole RNA from distal colon of *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$mice was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and additionally purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Concentration and quality of samples was determined using an Agilent 2100 Bioanalyzer, and those with best quality were selected for hybridization with GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix, Santa Clara CA, USA), following manufacturer's instructions. Quality control of microarray data was performed using Affymetrix Expression Console. Data are expressed as base 2 exponentials. For comparison between the two genotypes, we selected those genes that were no significantly different in the absence of treatment, but that were differentially expressed after colitis induction. Bioinformatic analysis was performed using the Babelomics tools platform (http://babelomics.bioinfo.cipf.es).

Proteomic studies and MALDI-ToF analysis

Colons from *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$mice were homogenized in TUCT (7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris-HCl pH 8.5). 50 µg of each sample were labeled with 400 pmol of a specific fluorophore (GE Healthcare, Waukesha, WI, USA): CyDye 3 (WT sample), CyDye 5 (KO sample) and CyDye 2 (pool of WT and KO sample 1:1). Labeled samples were combined and UCDA (8 M urea, 4% CHAPS, 130 mM DTT and 2% IEF buffer) was added in a 1:1 ratio. Then, UCda (8 M urea, 4% CHAPS, 13 mM DTT and 1% IEF buffer) was added up to 450 µl final volume. Samples were loaded in a strip holder and a 24 cm IPG strip pH gradient 3–11 non linear (GE Healthcare) was placed over it. Isoelectrofocusing (IEF) was performed for 26 h at a gradient voltage on an IPGphor Unit (GE Healthcare). Then, the strip was successively equilibrated in SES-DTT (6 M urea, 30% glycerol, 2% SDS, 75 mM Tris-HCl pH 6.8, 0.5% DTT and bromophenol blue), and in SES-IA (6 M urea, 30% glycerol, 2% SDS, 75 mM Tris-HCl pH 6.8, 4.5% iodoacetamide and bromophenol blue), and mounted on top of a 13% SDS–PAGE in a Hoefer S600 device (Ettan DALT Six, GE Healthcare). After SDS–PAGE, CyDye-labelled proteins were visualized directly by scanning using a Typhoon^{TM} 9400 imager (GE Healthcare). The excitation laser wavelengths were 488 nm for Cy2, 532 for Cy3 and 633 for Cy5. The emission filters employed were of 520 nm band pass (BP) 40, 580 nm BP 30 and 670 nm BP 30 respectively. The gel was scanned, analyzed with Progenesis SameSpots software (NonLinear Dynamics) and stained with SYPRO Ruby (Molecular Probes, Invitrogen). Differential spots were excised, washed with 25 mM ammonium bicarbonate/acetonitrile (70:30), dried and incubated with 12 ng/µl trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, for 1 h at 60 ^{o}C. Peptides were purified with ZipTip C18 (Millipore, Billerica, MA, USA), eluted with 1 µl of CHCA (α-cyano-4-hydroxycinnamic acid, Waters) and analyzed by mass spectrometry on a time-of-flight mass spectrometer (Voyager-DE STR,

Applied Biosystems, Foster City, CA, USA). Data from 200 laser shots were collected to produce a mass spectrum which was analyzed with Data explorer version 4.0.0.0 (Applied Biosystems).

Western blotting

Proteins were separated by electrophoresis and transferred to PVDF membranes (Millipore). Blots were blocked with 5% non-fat dry milk in TBS-T buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.05% Tween-20) for 1 h at room temperature and incubated overnight at 4^{o} C with 3% BSA in TBS-T with either 0.2 μg /ml anti-S100A8 or anti-S100A9 (R&D Systems Minneapolis, MN, USA), and in 3% non-fat dry milk in TBS-T buffer with 0.2 μg/ml anti-hemopexin or 0.2 μg/ml anti-calgranulin A/B (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were then incubated with 10 ng/ml rabbit anti goat horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA), donkey anti rabbit horseradish peroxidase (GE Healthcare), and goat anti mouse horseradish peroxidase (Jackson ImmunoResearch), respectively. Finally, blots were washed with TBS-T and developed with Immobilon Western chemiluminescent HRP substrate (Millipore). Chemiluminescent images were taken in a Fujifilm LAS3000 mini.

Quantitative RT-PCR analysis

Total RNA from tissue samples and cell lines was isolated by guanidium thiocyanatephenol-chloroform extraction and reverse transcription reactions were carried out with 300 ng of RNA, using the Thermoscript RT-PCR system (Invitrogen). Then, 9 μl of a 1:5 dilution of cDNA was employed in quantitative PCR using the TaqMan probe HS00917112_m1

and TaqMan Master Mix in an AbiPrism 7900HT (Applied Biosystems), and following manufacturers' instructions.

Neutrophil isolation and culture

Neutrophils from *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice were isolated from peritoneal lavage as previously described (25). Briefly, mice were injected i.p. with 1 ml of casein (bovine sodium salt, Sigma) 9% w/v in PBS, left overnight and injected with a second casein dose the next morning. Mice were sacrificed 3 h after the second injection and neutrophils were obtained by peritoneal lavage with 8 ml of PBS. Cells were then washed and levels of apoptotic neutrophils determined by flow cytometry in a Cytomics FC500 (Beckman-Coulter, Brea, CA, USA), as annexin V and Ly6G positive cells using the anti-Ly6G antibody and the Annexin V-FITC/IP kit (Sigma, APOAF-50TST 047K4115). For co-culture with ADAMTS-12 overexpressing cells, human neutrophils were isolated from peripheral blood of healthy donors. Briefly, 4 ml of blood were mixed with 4 ml of a sterile solution of NaCl 0.9%. Diluted blood was then added to 4 ml of Lymphoprep™ and centrifuged for 20 min at 800 rcf. After separation, supernatant was removed and the pellet containing granulocytes and erythrocytes was suspended in 12 ml of erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). After 10 min, this suspension was centrifuged for 5 min at 400 rcf; supernatant was removed and the remaining pellet was washed with 800 µl of erythrocyte lysis buffer. The final pellet containing purified granulocytes (mainly neutrophils) was resuspended in RPMI medium and counted. $2x10^5$ cells were co-cultured in 6 well plates with control EBNA cells or EBNA cells overexpressing ADAMTS-12 (EBNA-TS12) (18). When indicated, blocking antibodies anti-CD36 (Abcam ab17044, Abcam, Cambridge, MA, USA) or anti-CD47 (Abcam ab3283) were employed at 2.5

µg/ml. After 15 h, neutrophils were collected, washed and analyzed through flow cytometry to determine apoptosis levels using the Annexin V-FITC/IP kit.

Wound healing assay

Skin wounds were performed as described previously (5). Briefly, animals were anesthetized by isoflurane inhalation and the dorsal area was shaved and disinfected. Two full-thickness excisions were made with a 8-mm Acu-Punch (Acuderm, Fort Lauderdale, FL, USA) in both flanks. Healing was monitored by taking pictures 1, 3, 5, 7 and 9 days after injury. Wound area was calculated for each time point with the ImageJ software. At the different time points, animals were sacrificed by CO_2 inhalation and wounds and surrounding skin were harvested and fixed for histological analysis.

Statistical analysis

All statistical analyses were carried out with the GraphPad Prism 5.0 software. Data are presented as means ± SEM. The occurrence of significant differences between groups was determined with the Student's t-test (for Gaussian distributions) or the Mann-Whitney test (non-parametric). P-values under 0.05 were considered significant.

RESULTS

***Adamts12* deficiency results in increased inflammation and higher susceptibility to DSS-induced colitis**

Adamts12$^{-/-}$ mice have no apparent defects in growth or life span. However, under certain experimental conditions, they show increased angiogenesis and tumor growth (23). To better elucidate the biological roles of this protease, we analyzed its putative functional relevance during inflammation. Thus, and given that ADAMTS-12 is produced by stromal cells in human colorectal carcinomas (21, 22), we first examined the effect of this enzyme in DSS-induced colitis. To this purpose, 8 to 10-week-old *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice were challenged with 2% DSS in drinking water for 7 days, followed by 1 day of water without DSS. In this model of acute colon injury, weight loss, ulceration and inflammation occur within 4-7 days after treatment (24). No significant differences were observed in lethality rate or weight loss between mutant and control mice. In contrast, differences in clinical features (diarrhea and rectal bleeding) were apparent during the last days of treatment and the recovery phase (Figure 1A). Likewise, significantly reduced colon length, a symptom of inflammation, was also observed in *Adamts12*-null mice (mean=5.61±0,14 SEM cm) compared with wild-type mice (mean=6.30±0,19 SEM cm) (Figure 1B). Histological examination of colon revealed that both *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ tissues are normal when untreated. However, after DSS treatment, large areas of ulceration, cell infiltration and thickening of the mucosa were evident, especially in the tissues collected from mutant mice, where the extent of surface affectation was higher (mean 65% in mutant mice *vs.* 28% in control mice) (Figure 1C and 1D). After this initial treatment, we challenged a new group of mice with 5% DSS during 10 days and allowed them to recover for 15 days, in order to study in detail the reparative changes occurring after an important tissue damage. Under these conditions, we found that clinical recovery is delayed in *Adamts12*$^{-/-}$ mice. Thus, whereas the increment in body weight subsequent to the initial loss

started immediately after removal of DSS in *Adamts12*$^{+/+}$ mice (days 10-11), it did not begin in *Adamts12*$^{-/-}$ animals before day 15 (Figure 1E). Moreover, histological analysis of colon samples extracted from the surviving animals (5 *Adamts12*$^{-/-}$ and 4 *Adamts12*$^{+/+}$), revealed that ulceration, inflammation and reparative changes occurred more intensively in mutant mice than in the littermate controls. Thus, we applied a histological score ranging from 0 to 32 (0-14 for inflammation component; 0-12 for reparative symptoms and 0-6 for post-reparative changes), and determined a final summative score of 8, 7, 10, 11 and 13 for the five mutant animals, and 4, 8, 9 and 2 for the corresponding wild-type littermates (Supplementary Table 1). Altogether, these data suggest a protective role for Adamts-12 in the development of ulcerative colitis.

Increased inflammation in *Adamts12*$^{-/-}$ mice colon is associated with neutrophil accumulation

Recruitment of inflammatory cells at ulceration sites is one of the necessary steps for healing and restoration of normal mucosa (26). However, after this acute response, which is mainly mediated by neutrophils, resolution of inflammation is required: neutrophils undergo apoptosis and are eliminated by macrophages. We investigated whether increased inflammation observed in *Adamts12*$^{-/-}$ mice could be due to a sustained presence of neutrophils in the affected tissue. For this purpose, we examined by immunohistochemistry with an anti-MPO antibody the number of infiltrated neutrophils in areas of ulceration. As shown in Figure 1D, the number of infiltrated neutrophils was clearly increased in *Adamts12*-deficient tissue. The number of MPO-positive cells per field in ulcers was also determined, being significantly higher in the damaged tissue of *Adamts12*$^{-/-}$ mice than that of the wild-type controls (p-value=0.0002, *Adamts12*$^{-/-}$ mean=24.46 positive cells per field *vs.* 11.75 for *Adamts12*$^{+/+}$; Figure 1D). The sustained presence of neutrophils in colon would generate a pro-inflammatory environment that could

contribute to explain the prolonged clinical symptoms and delayed recovery observed in DSS-treated *Adamts12*$^{-/-}$ mice and even lead to a chronic disease in these mutant animals.

Several inflammation markers are elevated at the transcriptional and proteomic level in the absence of Adamts-12

To further characterize the differences between *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice, we performed an mRNA expression profile and a DIGE-based proteomic study of untreated and DSS-treated colon tissues prepared from mutant and control mice. At the transcriptional level, mRNA profiles from affected colons of *Adamts12*$^{-/-}$ mice revealed augmented expression of several inflammation markers. We defined as differentially expressed genes those which expression was increased or decreased at least $2^{1.5}$-fold (≈2.83-fold) compared with controls. Under this definition, 219 genes were found upregulated in *Adamts12*$^{-/-}$ mice compared with their wild-type littermates, and due to the colitis induction. Out of 45,100 targets analyzed, we selected 26 that were normal in controls but highly overexpressed (more than $2^{2.5}$ times relative expression, ≈5.66-fold) in mutant animals under DSS treatment (Figure 2). Many of these genes can be considered as inflammation markers, since they are directly involved in processes relevant to immune response. In fact, a bioinformatic analysis to identify functionally-related enriched genes revealed an enrichment of a series of well-defined functional pathways in KEGG (http://www.genome.jp/kegg/ pathway.html). Thus, *Adamts12*$^{-/-}$ mice showed a significant over-expression of genes involved in the cytokine and cytokine-receptors interaction (*p*-value = 4.2×10^{-6}) and of genes expressed during hematopoiesis, specifically in the neutrophil lineage (*p*-value = 6.63×10^{-7}). This group of genes includes those encoding several interleukins, the calgranulins S100A8 and S100A9, and the chemokines Ccl11 or Cxcl10. Additionally, matrix metalloproteinase genes such as *Mmp3* and *Mmp13* as well as *Hdc* and *Aldhla3* genes necessary

for histamine synthesis and metabolism have also been indirectly related to inflammation (27, 28). Interestingly, one of the most overexpressed genes, *Il6*, encodes a chemokine involved in neutrophil clearance, which is very consistent with the observed increased number of neutrophils in colon from mutant mice (29). Finally, it is remarkable that few genes were found to be downregulated (55 genes with a decrease of $2^{-1.5}$ or higher) in *Adamts12*-deficient mice, and they were ascribed to distinct biological functions.

We then explored putative differences at the protein level between mice from both genotypes. For this purpose, we performed a 2-D DiGE combined with MALDI-ToF analysis of colon samples from *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ animals treated with DSS. Similar to the situation observed at the transcriptional level, most differences were due to proteins accumulated in mutant tissue compared to their littermate controls (Figure 3A). Among these, we identified several proteins, including galectin-1, hemopexin, transgelin, S100A6, S100A8 and S100A9, which could be of interest in the context of inflammatory responses. The differences observed in these candidates were validated through 2D-western blotting (Figure 3B and data not shown). S100A8 and S100A9 were also found increased at the mRNA level, thus ruling out a protein accumulation caused by the absence of proteolytic processing by Adamts-12, but reinforcing the above results and representing an additional validation. None of the other proteins mentioned were found transcriptionally over-expressed. We then performed a mono-dimensional western blot analysis of all the candidate proteins identified by proteomics in samples from several *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ animals. In the case of galectin-1, S100A6 and transgelin, we were unable to validate the previously obtained results in every sample analyzed, and thus they could not be considered as uniform differential markers (data not shown). Nevertheless, hemopexin, scarcely detectable in untreated samples, was confirmed as a differentially incremented protein in all *Adamts12*$^{-/-}$ tissues compared with controls (Figure 4). Moreover, and as shown in the 2-D western blot analysis (Figure 3B), the hemopexin isoforms that accumulated in *Adamts12*$^{-/-}$

colons are specific isoforms that were barely or not detected in samples from *Adamts12*$^{+/+}$ animals. This finding, together with the fact that no expression differences were found for hemopexin at the transcriptional level, suggests that Adamts-12 could be involved in the post-translational processing of hemopexin. Finally, western blot analysis confirmed that S100A8, S100A9, and calprotectin, a heterodimeric complex formed by combination of S100A8 and S100A9, were also increased in all *Adamts12*$^{-/-}$ tissues compared to wild-type controls (Figure 4).

Adamts12$^{-/-}$ mice show delayed recovery after LPS induced sepsis

Given the above described relevance of Adamts-12 in colitis, we decided to analyze its putative role in a model of global acute inflammation, by comparing the host response to LPS-induced sepsis in *Adamts12*-proficient and -deficient mice. To this purpose, 8 to 10-week-old mice were injected i.p. with 8.5 µg LPS per g of body weight. At this concentration, no significant differences regarding mortality rate were detected between both genotypes (data not shown). However, we found that after a loss of approximately 10% in body weight by day 2 after challenge with the endotoxin, wild-type mice initiated the recovery phase, whereas mutant mice extended weight loss until day 3-4 (Figure 5A). Not even at the time of sacrifice (day 8) *Adamts12*$^{-/-}$ mice had finished their convalescence. These results indicate that Adamts-12 also plays a role in the inflammatory response during sepsis. One of the major features achieved during sepsis is the increase of leukocytes in blood. To evaluate this question, we examined blood samples from *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice 2 and 8 days after LPS-injection by flow cytometry and detected distinct patterns of response between the two genotypes. Thus, at day 2 after LPS-injection, the number of monocytes and lymphocytes was reduced in *Adamts12*$^{-/-}$ mice when compared with controls, while no differences were found in levels of granulocytes (Figure 5B). By day 8 after sepsis induction, neutrophils decreased to normal levels in wild-type animals,

whereas in *Adamts12*$^{-/-}$ animals their amount remained elevated (Figure 5B). These findings are in good agreement with the clinical symptoms described above and are indicative of an altered immune response in the absence of Adamts-12.

We next investigated possible differences affecting the altered molecular targets identified in colitis. To this end, we performed western blot analysis for hemopexin, S100A8 and S100A9 in protein extracts from spleen and liver of LPS-treated mice at day 8. As shown in Figure 5C, S100A8 and S100A9 resulted to be differential markers for *Adamts12*$^{-/-}$ also during sepsis. S100A9 was increased in both genotypes as a consequence of treatment, but this increase was markedly higher in all examined *Adamts12*$^{-/-}$ tissues. The levels of S100A8 were also augmented due to sepsis induction in spleen extracts, and at lower extent in liver extracts from the mutant mice. However, in liver, the amount of S100A8 was constantly higher in *Adamts12*$^{-/-}$ samples. In spleens, the accumulation of S100A8 in mutant mice was not so evident, but when the antibody detected calprotectin (25 KDa), the difference was marked. The anti-S100A9 antibody was also able to detect calprotectin in both tissues, showing an accumulation of the heterodimer in *Adamts12*$^{-/-}$ samples (Figure 5C). Altogether, these data reinforce the relevance of Adamts-12 in the inflammatory response.

Higher affectation of *Adamts12*$^{-/-}$ mice pancreas after cerulein treatment

Pancreatitis is another acute inflammatory disease with serious consequences in humans, including multiple organ failure and even death. In mice, intraperitoneal injection of cerulein induces acute pancreatitis which is very pronounced after 10 h of treatment. Therefore, *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice were challenged with cerulein and sacrificed 10 h after protocol initiation to evaluate putative differences in their inflammatory response. The extent of disease was examined by measurement of serum levels of pancreatic enzymes (lipase and

amylase) and through histological analysis of pancreatic tissue. Levels of pancreatic enzymes were more increased in mutant mice compared with wild-type animals, indicating a higher affectation. Thus, levels of serum amylase were 26,450 ± 2,176 and 29,224 ± 2,177 U/l in *Adamts12*$^{+/+}$ and *Adamts12*$^{-/-}$ mice, respectively, while those of lipase were 789.0 ± 83.19 and 941.1 ± 84.52 U/l. Additionally, we found increased edema in specimens from *Adamts12*$^{-/-}$ mice (Figure 6A). The edema, which is one of the most significant features of pancreatitis, is visible as an augmented size of septa between lobes, acini and cells. The interlobular, interacinar and intercellular distances were measured in hematoxylin-eosin slides from both genotypes, proving the existence of significant difference in interacinar edema between the genotypes. Thus, the mean distance between pancreatic acini in treated mice, was 5.03 ± 0.23 µm in mutant animals and 4.06 ± 0.21 µm in wild-type mice (Figure 6A). Finally, to evaluate putative molecular parallelisms with the previously described inflammation models, we examined levels of S100A8, S100A9 and hemopexin in pancreas of control and pancreatitis-induced mice. We found an accumulation of hemopexin in *Adamts12*$^{-/-}$ tissue equivalent to that defined for colitis, although not significant differences were detected for the inflammatory markers S100A8 and S100A9 (Figure 6B and not shown). Collectively, these results indicate that Adamts-12 may also play a role during the development of acute pancreatitis.

Adamts-12 induces neutrophil apoptosis

All the above results suggest a relevant role for Adamts-12 in the inflammatory response. To better understand the mechanism by which this contribution could be carried out, we explored the putative link between the observed neutrophil accumulation and the presence or absence of the protease. It is well known in this regard that, once neutrophil activities during acute response have been completed, these cells die by apoptosis and undergo macrophage clearance. Based on

these considerations, we asked whether Adamts-12 could be necessary for appropriate neutrophil apoptosis. To evaluate this, we isolated peritoneal neutrophils from *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice after injection of casein, which acts as a sterile agent that induces neutrophil elicitation. Then, cells isolated from the neutrophil-enriched peritoneal exudates were analyzed by flow cytometry and levels of apoptotic cells were determined. This analysis revealed a marked reduction in neutrophil apoptosis in the absence of Adamts-12: 10.24% of neutrophils from wild-type animals were apoptotic *vs.* 5.25% from mutant mice (Figure 7A).

We then asked whether this effect on apoptosis could also be detected *in vitro*, facilitating the development of a system suitable for further studies. Thus, we isolated non-activated neutrophils from blood of healthy human donors and co-cultured them for 16 h with control EBNA cells or EBNA cells overexpressing ADAMTS-12. Afterwards, we analyzed apoptosis by flow cytometry. We found that in the presence of ADAMTS-12, the percentage of apoptotic cells (pre-apoptotic and death apoptotic cells) raised from 56.7% to 68.4% (Figure 7B). A possible explanation for the involvement of ADAMTS-12 in neutrophil apoptosis could be its interaction with some cell surface proteins, such as CD36 or CD47, which have been previously described to modulate neutrophil transmigration and clearance (30, 31). For this purpose, we performed co-cultures of human neutrophils with EBNA-TS12 or EBNA-control cells in the presence or absence of blocking antibodies against CD36 or CD47. Whereas no significant effects on apoptosis were observed after blocking CD47 (data not shown), we detected a reduction of apoptosis when CD36 was inhibited. Thus, the percentage of apoptotic neutrophils in the presence of ADAMTS-12 decreased from 72.72% to 63.72% when blocking CD36, and the abundance of living cells raised from 27.12% to 36.07%. In contrast, it produced no effect in the absence of ADAMTS-12 (Figure 7C).

From previous works (21, 22), we know that *ADAMTS12* is expressed by human myofibroblasts, which are one of the predominant cells in stroma in proinflammatory

environments such as wound healing and cancer. In the models we have studied in this work, ADAMTS-12 could be controlling neutrophil apoptosis through cell components of stroma, like activated fibroblasts. Nevertheless, we also asked whether neutrophils themselves could be expressing this metalloprotease. For this purpose, we performed qRT-PCR to analyze *ADAMTS12* expression in neutrophils of murine and human origin, compared to control human lymphocytes. Whereas *ADAMTS12* expression in lymphocytes was minimal, we detected significant expression in neutrophils from both species. Indeed, relative expression of *ADAMTS12* was 7,430-fold and 189-fold higher in human and murine neutrophils compared to control human lymphocytes.

Delayed wound healing in *Adamts12*$^{-/-}$ mice

To further enrich the phenotypic description of *Adamts12*$^{-/-}$ mice, we studied the cutaneous wound repair in *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ animals. During this process, inflammation, secretion of granulative and definitive extracellular matrix, and re-epithelialization phenomena are involved and allow the healing and formation of regenerated skin. Given the participation of ADAMTS-12 in neutrophil apoptosis and the fact that metalloproteases are essential for degradation of extracellular matrix compounds, we hypothesized that this enzyme could be relevant for wound healing. We performed full-thick 8-mm excisions in the skin of *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice and monitored their closure over time. We found that healing was delayed in *Adamts12*$^{-/-}$ skin, where 50% closure was achieved 55 h after injury, whereas it took only 33 h in control mice (Figure 8A). Approximately 100 h after performing the incision, 15.5% of the initial wound remained opened in mutant mice *vs.* 10.2% in wild-type controls (*p*-value < 0.0001). Under histological examination, wounds from *Adamts12*-deficient mice showed a slightly slower re-epithelialization: at day 7, a total of 7 out of 8 wild-type wounds were

completely re-epithelializated, compared to 5 out of 8 in mutant animals. Moreover, wild-type animals regenerated the wounded skin by developing complete skin adnexals whereas in many mutant animals non- or very rudimentary structures were visible under stratum basale. Thus at day 7 after injury, 1 out of 5 *Adamts12*$^{-/-}$ animals and 3 out of 7 *Adamts12*$^{+/+}$ mice had developed adnexals, whereas at day 9, only 3 out of 8 among mutant mice in contrast to 7 out of 8 in the wild-type group had developed these structures (Figure 8B). These observations indicate that Adamts-12 would also be necessary for normal wound repair, likely acting during the inflammation phase.

DISCUSSION

The current view of proteases as modulators of precise proteolytic processing events is far away from the traditional one in which they were conceived as mere degradative enzymes in charge of protein catabolism (32, 33). Nowadays, more than 560 human proteases have been identified (34). These proteases establish intimate interactions with very specific substrates and are strictly regulated over time and space through control of their expression, activity and localization. As a result, most proteases perform very specific proteolytic cleavages on a number of different substrates and influence multiple biological processes (1). Hence, predicting the phenotype of animal models deficient in a certain protease is very difficult and often offers unexpected findings. To date, several mouse models lacking ADAMTSs have been generated with a heterogeneous spectrum of phenotypes including those found in *Adamts4*- and *Adamts5*-deficient mice, that are protected from osteoarthritis (35), and in *Adamts13*-deficient mice, that show increased inflammation due to enhanced extravasation of neutrophils (36).

In the present work, we have found that loss-of-function of Adamts-12 enhances mice susceptibility to inflammatory processes. This phenotype wass not limited to a certain tissue as it was a common phenomenon affecting multiple organs. Thus, mice lacking *Adamts12* exhibited an increased damage subsequent to inflammation in colon, pancreas and during endotoxic shock. Hence, *Adamts12*$^{-/-}$ mice subjected to induction of experimental colitis showed a more pronounced loss of weight, significant bowel shortening, and other clinical symptoms as diarrhea and fecal bleeding, which are typical signs of increased inflammation. Similar results were observed during pancreatitis, as histopathological analysis of pancreas revealed a more pronounced edema in tissues from mutant mice. Additionally, induction of systemic sepsis highlighted the higher inflammatory response taking place in *Adamts12*-deficient mice. Further gene expression and proteomic studies confirmed and extended these differences, as most differential RNAs and proteins identified in these mutant mice are indicators of increased

inflammation. These were the cases of the pro-inflammatory cytokines IL-6, IL-11 or CCL-11, and the calcium-binding proteins S100A8 and S100A9, important amplifiers and markers of acute and chronic inflammation (37), which were increased at both RNA and protein level in *Adamts12*-deficient mice. The latter two proteins form the heterodimer calprotectin, currently used as a diagnostic indicator for inflammatory bowel diseases when present in feces, and that was also found accumulated in *Adamts12*$^{-/-}$ mice. Taken collectively, all these observations indicate that Adamts-12 is necessary for normal inflammatory response in mice.

We also found that the more severe inflammatory symptoms observed in *Adamts12*-deficient mice were accompanied by an increase in neutrophilia in the affected tissues, which provided us a first clue to initiate the search for the mechanism underlying the phenotypic and molecular differences present in these mutant mice. In normal conditions, and immediately after a harmful stimulus, neutrophils migrate towards the affected tissue and contribute to the development of an immune response. Thus, these cells produce pro-inflammatory cytokines that attract other inflammatory cells and amplify the response as well as release toxic substances aimed at eliminating pathogens. Nevertheless, when this delicate balance is altered and excessive neutrophils are present, they can promote chronic inflammation and directly cause damage to the host tissue (38). Normally, after neutrophils exert their function in the acute phase response, they suffer a massive apoptosis and are phagocytosed by macrophages. On this basis, we hypothesized that a defect in apoptosis in the absence of Adamts-12 could be responsible for the increased number of neutrophils. To test this possibility, we analyzed changes in apoptotic death in neutrophils from human and murine origin, with the finding that the presence of this metalloprotease triggered an increment in their apoptosis. In this regard, numerous data are available describing the interaction between thrombospondins or ADAMTSs and the surface proteins CD36 and CD47, which are implicated in neutrophil death and clearance by macrophages and monocytes (30, 39). Thus, ADAMTS-13 binds CD36 through its

thrombospondin type 1 repeats (40), whereas CD47 interacts with thrombospondin-1 and thrombospondin-2 (TSP-1 and TSP-2) (31). TSP-1 and TSP-2 have in their structure three tandem thrombospondin type 1 repeats, which are structural motifs present in all ADAMTSs, including ADAMTS-12. Hence, this enzyme may also have the possibility to interact with CD36 or CD47. Moreover, and because mice deficient in *Adamts12* lack 8 thrombospondin type-1 repeats, the potential loss of interaction with CD36 or CD47 could explain the observed defects in neutrophil apoptosis. To evaluate this hypothesis, we measured changes in programmed cell death of human cells due to ADAMTS-12 in the presence or absence of blocking antibodies against CD36 or CD47. These experiments revealed an implication of CD36 in the involvement of human ADAMTS-12 for neutrophil apoptosis. Consistent with these results, we propose that ADAMTS-12 would be necessary *in vivo* for the restraint and resolution of inflammatory response, likely by interacting with CD36 or other proteins through its thrombospondin-1 repeats, and allowing normal neutrophil elimination. This model exclusively implies the adhesion function of ADAMTS-12. Nevertheless, blocking of CD36 only partially reverted apoptosis to the levels observed in the absence of exogenous ADAMTS-12. Besides the possibility of an incomplete inhibition by the antibody, we do not rule out the potential effect of alternative mechanisms modulating apoptosis in neutrophils. Also in this regard, we found that some hemopexin isoforms were accumulated in *Adamts12*-deficient mice. A possible explanation for this finding is that this metalloprotease would be necessary for hemopexin processing. If this was the case, the absence of ADAMTS-12 could lead to an altered function of hemopexin, which is mainly involved in sequestering the extracellular free-heme and in its recovery in liver (41). Free-heme is released during several types of cell damage, causing oxidative effects and promoting inflammation. Indeed, it has been proven that heme is able to delay spontaneous apoptosis of human neutrophils (42). Finally, ADAMTS-12 might be involved in other processes apart from neutrophil apoptosis, such as neo-vascularization or

matrix remodeling and therefore impairing normal healing of ulcers in colitis or wounds in skin, as well as appendages regeneration.

In summary, in this work we provide evidence on how *Adamts12*-deficiency is responsible for increased inflammation in mice and reduced apoptosis of neutrophils as well as impaired wound healing. Furthermore, we know from previous studies that Adamts-12 is also implicated in angiogenesis, apoptosis of tumor cells and modification of the extracellular matrix (18, 21, 23). Therefore, albeit Adamts-12 is not essential for life and its absence is not dramatic under normal conditions in mouse, probably due to functional compensation by other ADAMTSs, the contribution of this enzyme to a variety of inflammatory processes highlights its relevant role in the context of the large and growing complexity of proteolytic systems operating in life and disease.

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FIGURE LEGENDS

Figure 1. Increased susceptibility of *Adamts12*-deficient mice to colitis. A) Clinical score in *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice during treatment. B) Colon length at sacrifice after 2% DSS treatment. C) Microscopic images of the normal colonic mucosa and after DSS treatment (left panel) and percentage of affected colon surface in colitis-induced mice from both genotypes. D) Myeloperoxidase (MPO) staining in DSS-treated colons and quantification of MPO positive brown cells per field in affected mucosa. Red arrows indicate some of the MPO-positive cells. E) Body weight change of *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ mice during and after treatment with 5% DSS. All experiments were carried out with at least 12 animals per genotype.

Figure 2. Microarray analysis in colon from *Adamts12*$^{+/+}$ and *Adamts12*$^{-/-}$ mice. Heat map representing the relative expression levels of selected genes.

Figure 3. Identification of differential proteins in colon from DSS-treated *Adamts12*$^{+/+}$ and *Adamts12*$^{-/-}$ mice. A) Representative DiGE image with tissue extracts from *Adamts12*$^{+/+}$ (CyDye 3, green) and *Adamts12*$^{-/-}$ (CyDye 5, red) mice. Some of the differential proteins are indicated. B) Differentially produced proteins identified by MALDI-ToF were confirmed by 2-D western blot. In each blot, uni-dimensional experiments of both *Adamts12*$^{-/-}$ and *Adamts12*$^{+/+}$ tissues are identifiable on the right. β-actin was used as a loading control.

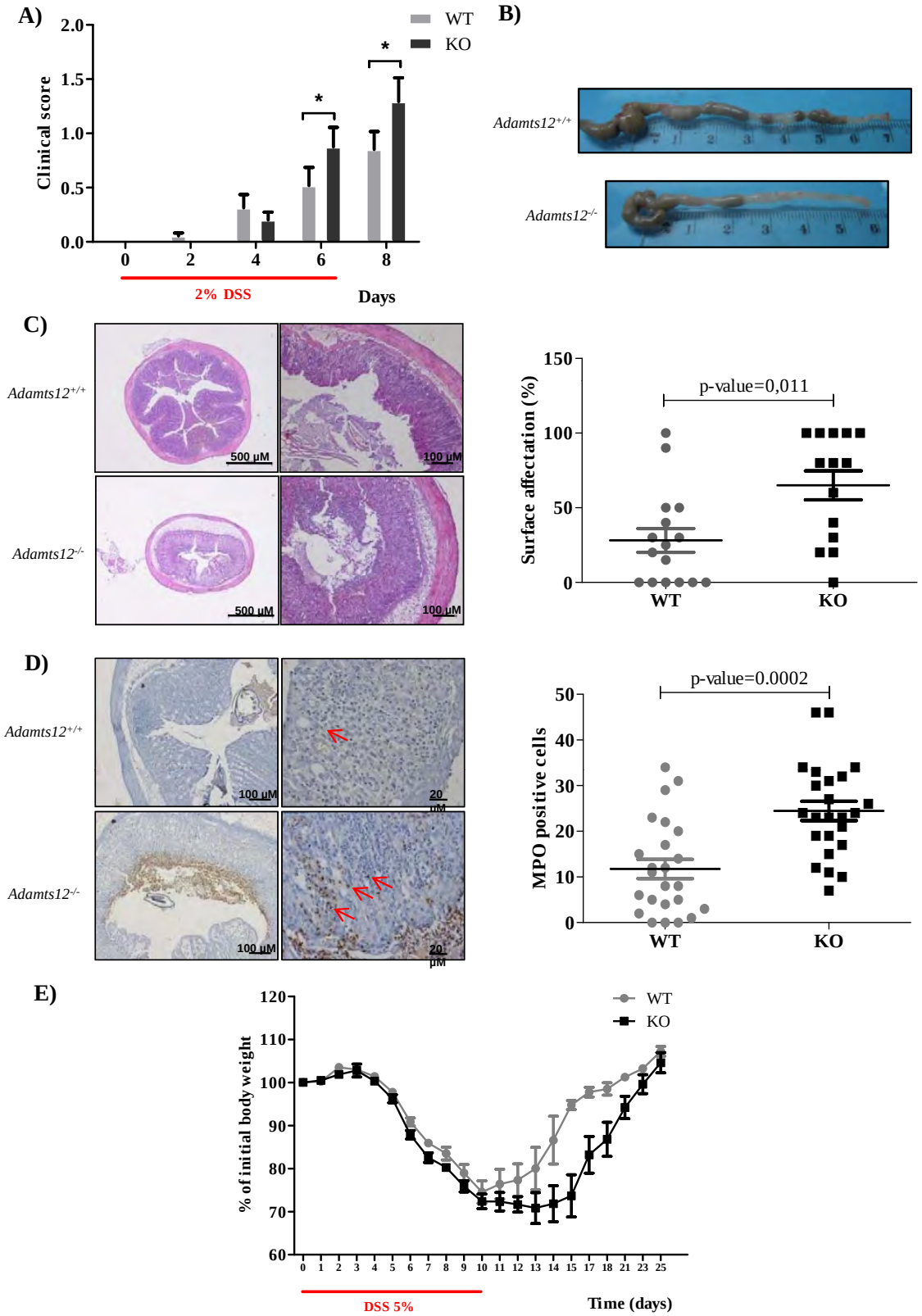
Figure 4. Validation of proteins accumulated in colon of *Adamts12*$^{-/-}$ mice by western blot. S100A8, S100A9, calprotectin and hemopexin were found to be increased in the absence of Adamts-12 in a set of 3 *Adamts12*$^{-/-}$ mice compared to 3 *Adamts12*$^{+/+}$ animals. β-actin was used as a loading control.

Figure 5. Increased susceptibility to LPS-induced sepsis in *Adamts12*$^{-/-}$ mice. A) Variation in body weight expressed as percentage of initial measures. Mice lacking *Adamts12* show a delayed recovery in terms of gain of body weight, when compared with wild-type animals. B) Levels of leukocytes in peripheral blood 2 and 8 days after LPS injection. At day 2 after treatment, *Adamts12*$^{-/-}$ mice show reduced number of lymphocytes whereas at day 8 the percentage of neutrophils is still elevated. C) S100A8 and S100A9 are elevated in tissues of *Adamts12*$^{-/-}$ mice after induction of sepsis. Experiments were carried out with at least 12 animals per group.

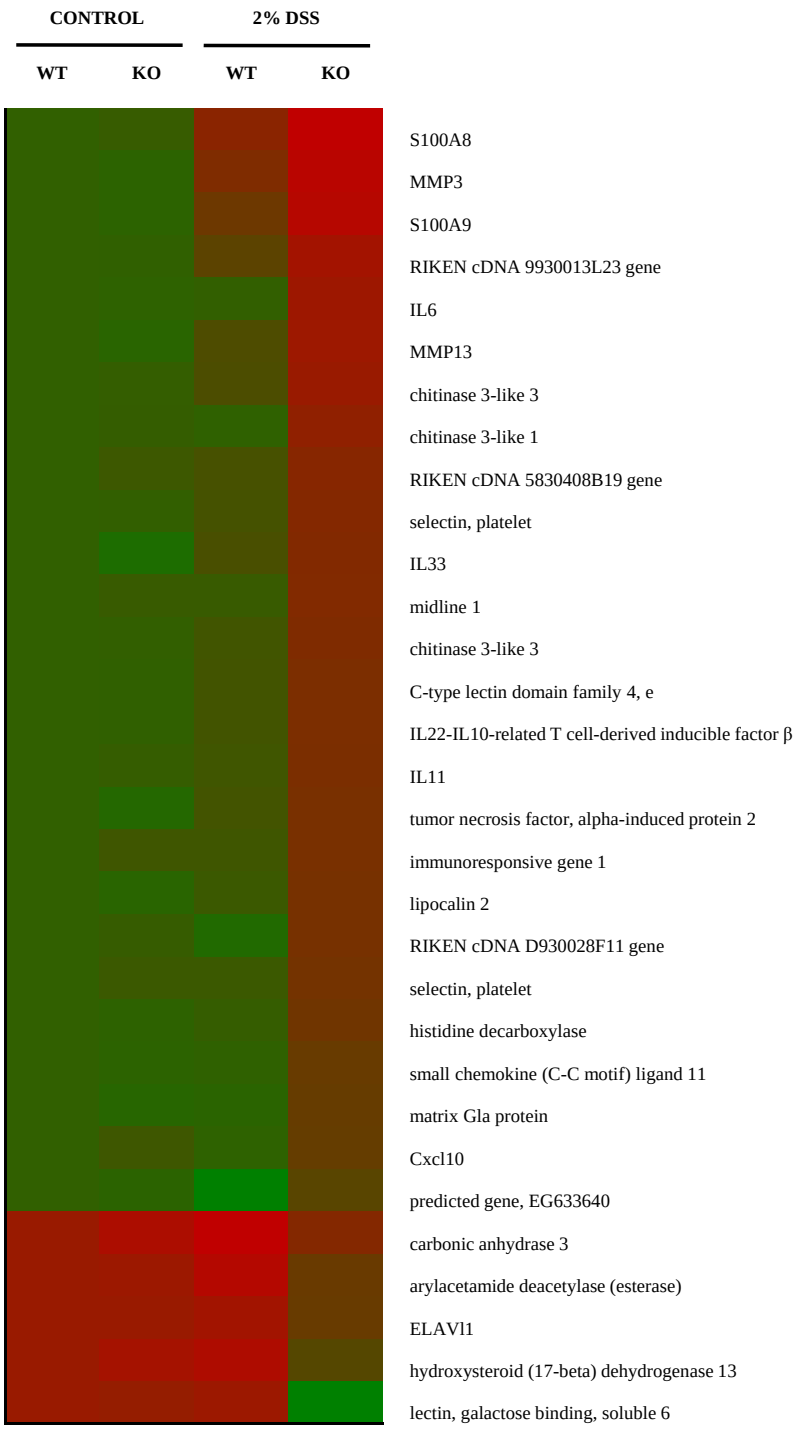
Figure 6. Increased affection of *Adamts12*$^{-/-}$ mice after cerulein-induced pancreatitis. A) Edema, one of the main indicators of pancreatitis was increased in *Adamts12*$^{-/-}$ mice after treatment with cerulein. B) Increased leves of hemopexin in pancreatitis of mice deficient in *Adamts12*. 10 WT and 9 *Adamts12*$^{-/-}$ animals were used in this experiment.

Figure 7. Role of ADAMTS-12 in neutrophil apoptosis. A) Apoptosis of peritoneal neutrophils from *Adamts12*$^{-/-}$ (n=7) and *Adamts12*$^{+/+}$ (n=6) mice after casein induction. B) Percentage of live and apoptotic human neutrophils after co-culture with EBNA-TS12 or EBNA-control. C) Apoptosis in human neutrophils in co-culture with EBNA-TS12 or EBNA-control and in the absence or presence of blocking antibody against CD36.

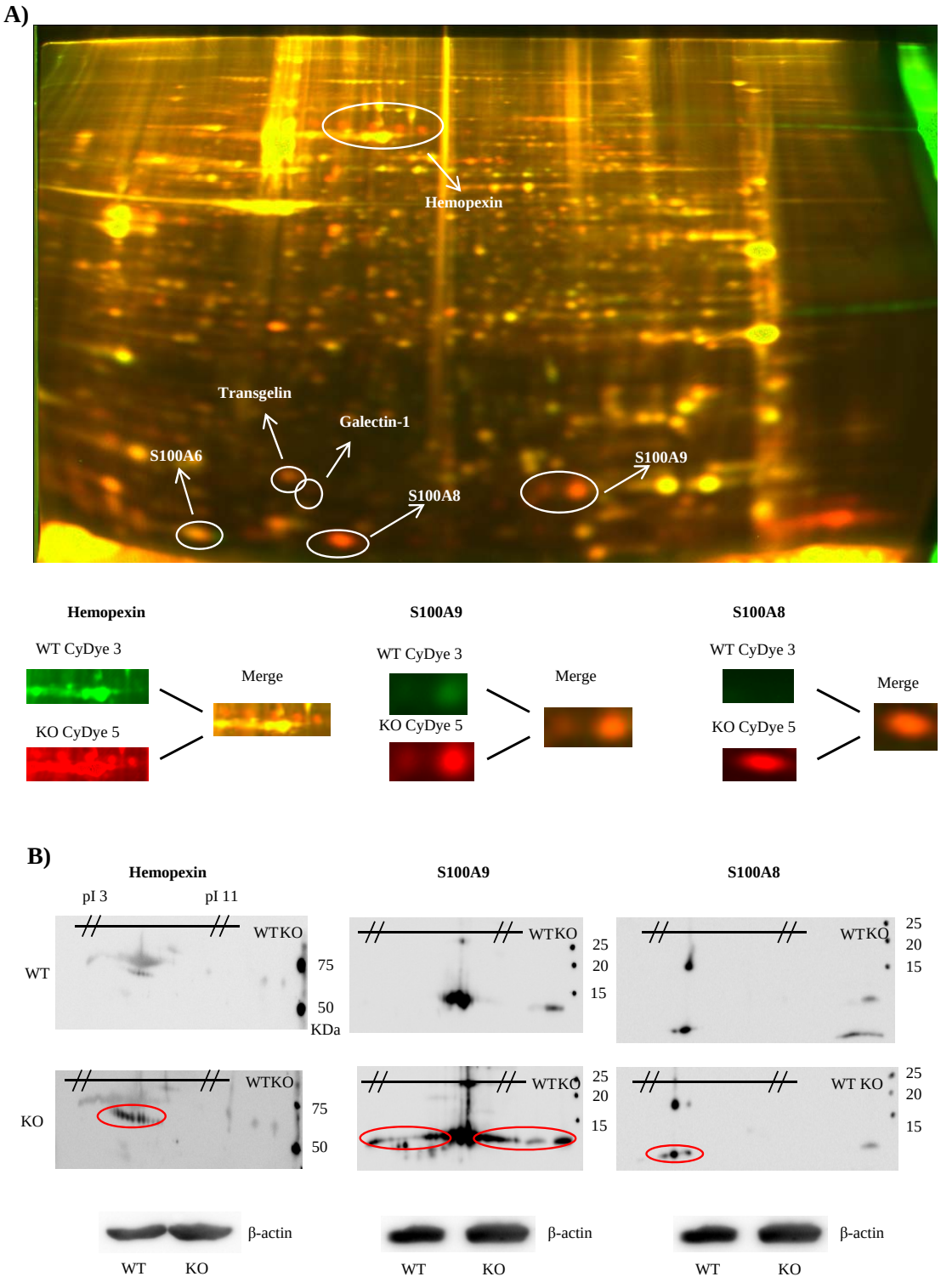
Figure 8. Wound healing is impaired in *Adamts12*$^{-/-}$ mice. A) Representative images during wound healing in *Adamts12*$^{+/+}$ and *Adamts12*$^{-/-}$ mice. B) Histological view of skins from *Adamts12*$^{+/+}$ and *Adamts12*$^{-/-}$ mice at days 1 and 7 after injury. The presence of skin appendages in wild-type tissues is indicated with arrows in the box. All experiments were carried out with at least 12 animals per genotype.



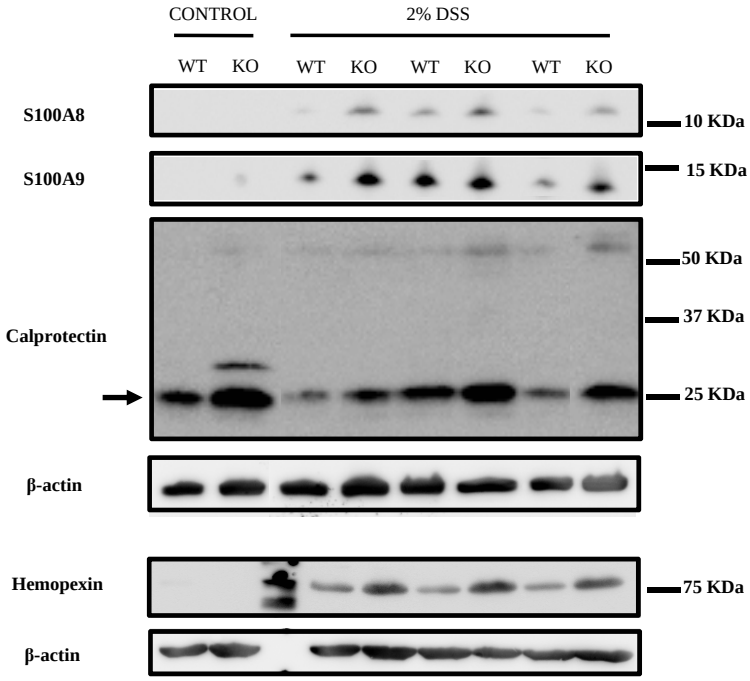
Moncada-Pazos *et al*. Figure 1



Moncada-Pazos *et al*. Figure 2



Moncada-Pazos *et al*. Figure 3



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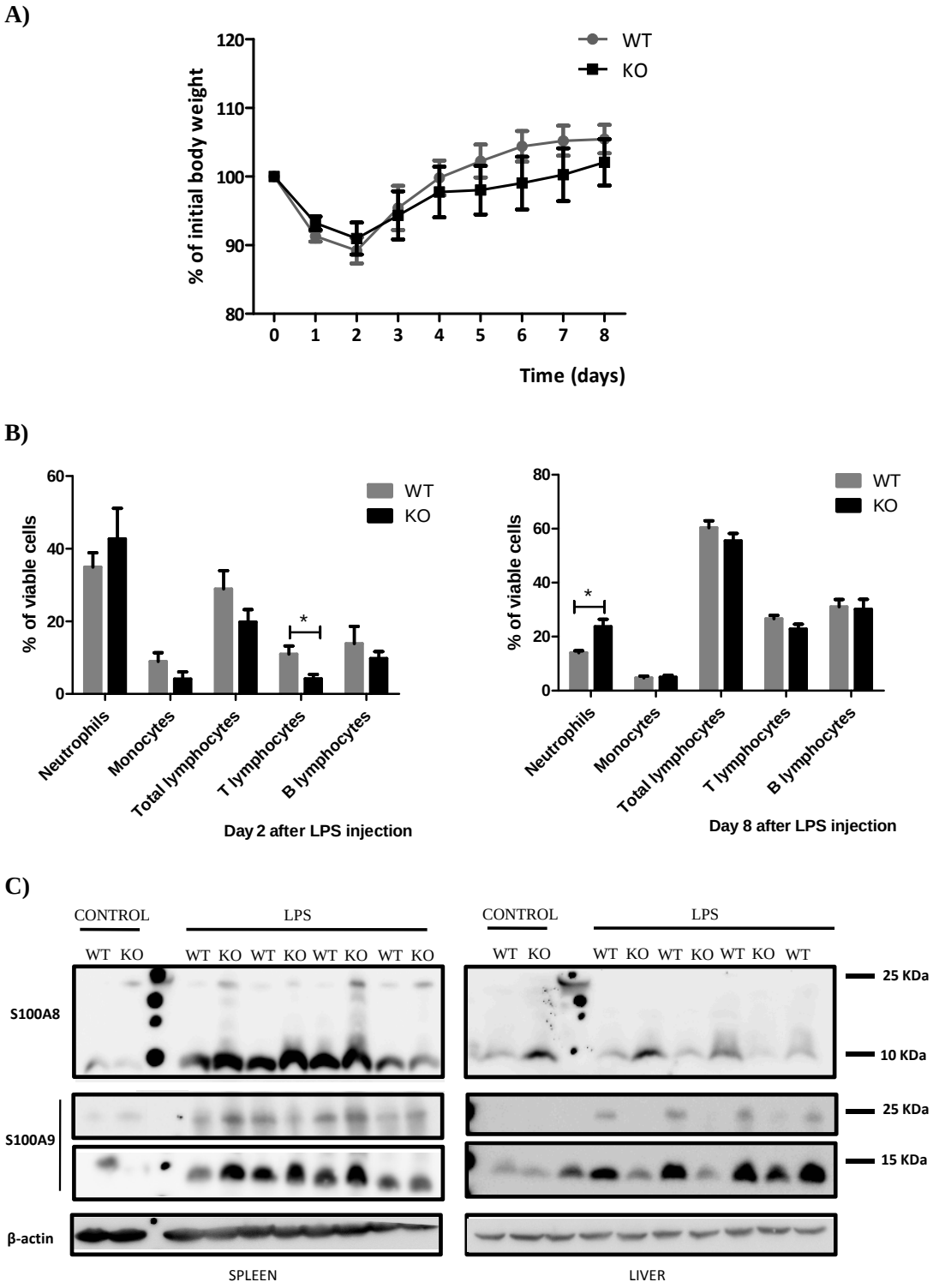
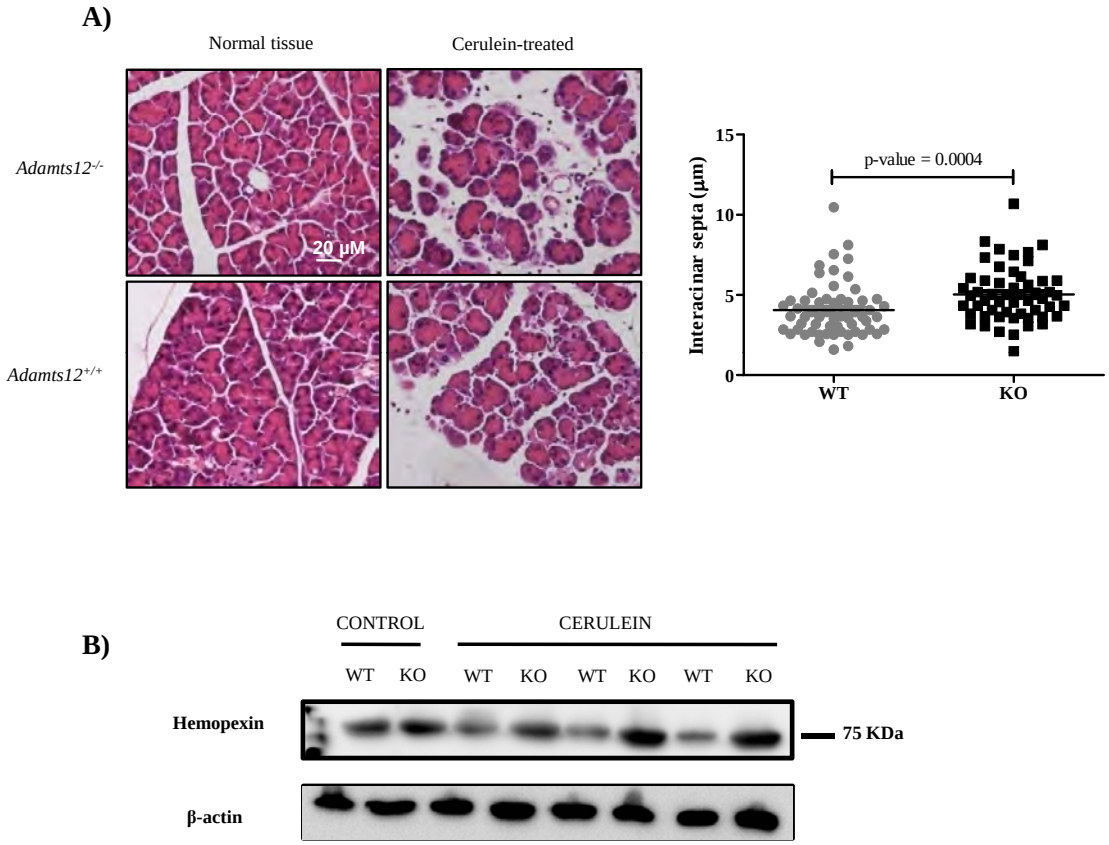
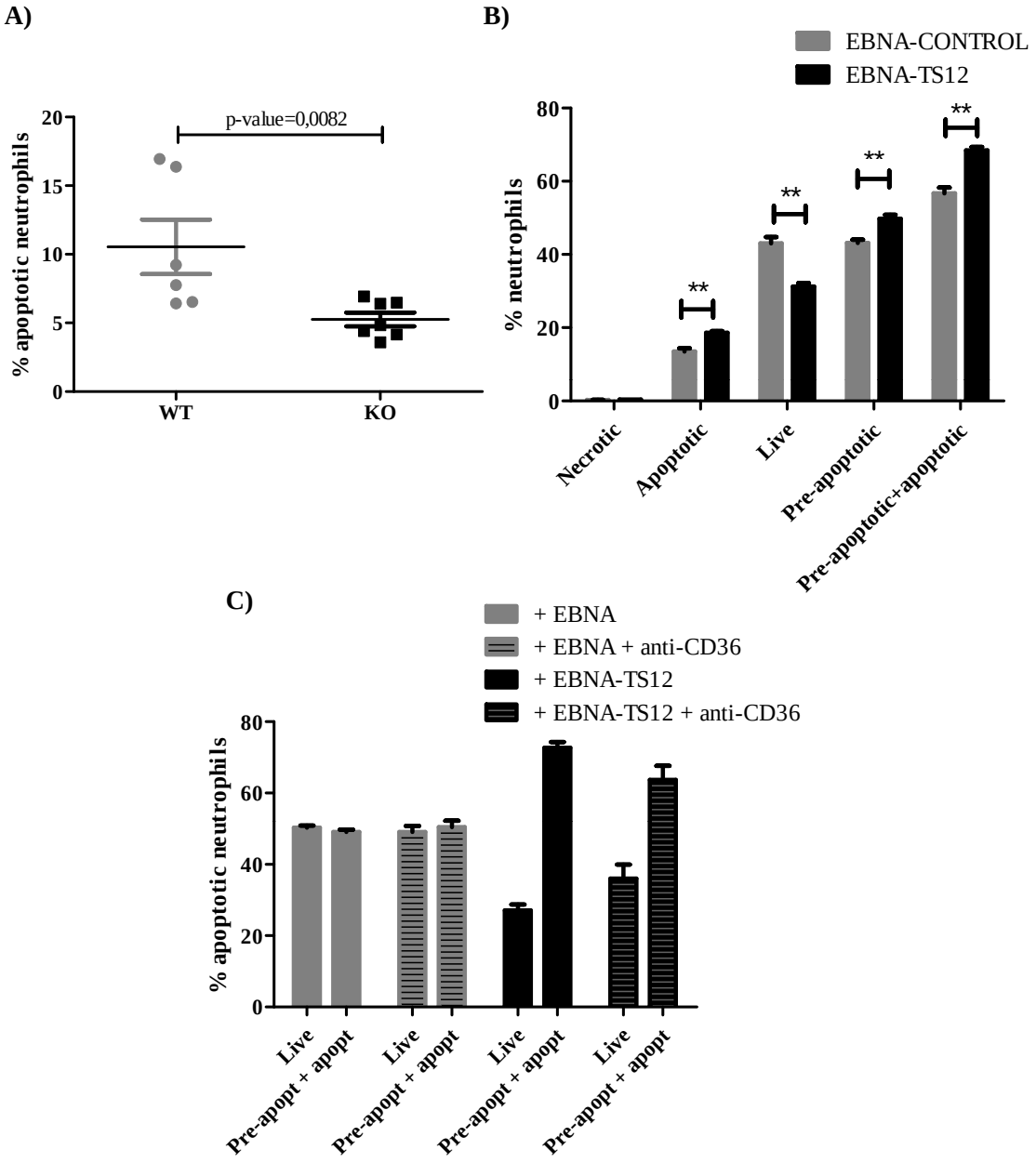


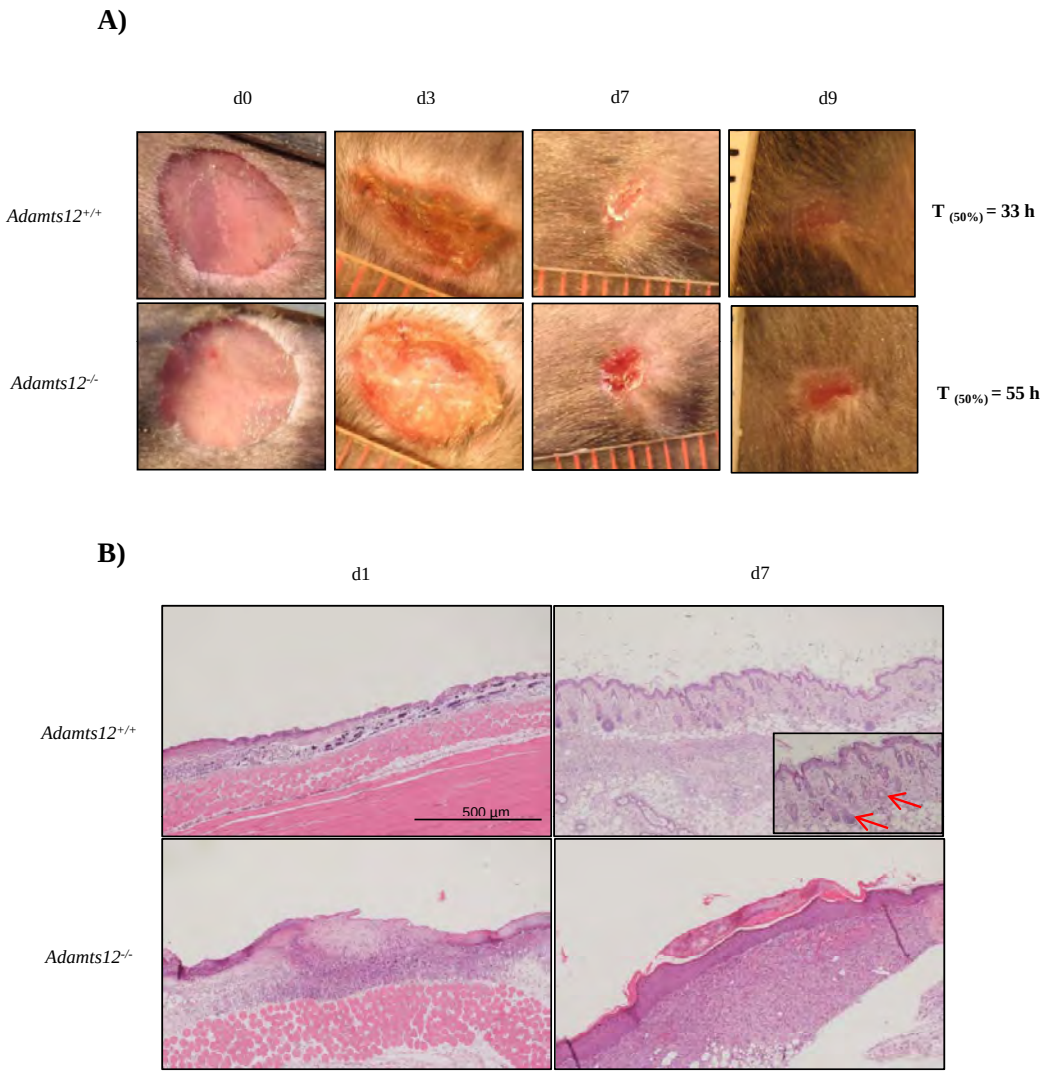
Moncada-Pazos *et al*. Figure 5



Moncada-Pazos *et al*. Figure 6



Moncada-Pazos *et al*. Figure 7



Moncada-Pazos *et al*. Figure 8

			INFLAMMATION					REPARATIVE CHANGES				POST-REPARATIVE CHANGES							
	SECT	DEPTH	Lymph	Neutr	MALT	Crypts	Ulcer	Gran	Epith	Arch	Reg	Hyp	Dys	Atro	Fibr	A	B	C	SCORE
WT	7/9	1	1	1	0	0	0	0	0	0	1	0	0	1	0	*2*	*1*	*1*	***4***
WT	5/9	3	1	2	1	0	1	1	0	0	1	1	0	0	0	*5*	*2*	*1*	***8***
WT	8/11	3	1	2	1	1	1	1	0	0	1	0	0	1	0	*6*	*2*	*1*	***9***
WT	7/11	1	1	0	0	0	0	0	0	0	0	0	0	1	0	*1*	*0*	*1*	***2***
KO	8/10	4	1	1	1	0	1	1	1	0	1	1	0	0	0	*4*	*3*	*1*	***8***
KO	7/9	2	1	1	0	0	1	1	0	0	1	1	0	1	0	*3*	*2*	*2*	***7***
KO	7/9	3	1	2	0	0	1	1	1	0	1	1	1	1	0	*4*	*3*	*3*	***10***
KO	6/8	4	1	1	1	1	0	1	3	0	1	0	1	1	0	*4*	*5*	*2*	***11***
KO	7/10	4	1	1	1	0	2	1	3	0	1	1	1	1	0	*5*	*5*	*3*	***13***

SECT *= number of affected sections / total number of sections*

DEPTH *= maximum depth of inflammation*
- *1: mucosae*
- *2: submucosae*
- *3: muscular*
- *4: subserosa*
- *5: serosa*

INFLAMMATION (0-14)

Lymph *= presence of lymphocytes*
Neutr *= neutrophil infiltrate*
MALT *= mucosae-associated lymphoid tissue*
- *0: negative*
- *1: light*
- *2: moderate*
- *3: severe*

Crypts *= presence of neutrophils in crypts*
Ulcer *= mucosae ulceration*
- *0: negative*
- *1: focal*
- *2: extensive*

REPARATIVE CHANGES (0-12)

Gran *= granulation tissue*
- *0: negative 1: positive*

Epith *= re-epithelialization*
- *0: negative*
- *1: focal (<25% of section)*
- *2: extensive (>25% of section)*
- *3: presence of scamous metaplasia*

Arch *= architecture alteration*
- *0: negative*
- *1: light*
- *2: moderate*
- *3: severe*
- *4: presence of pseudopolyps*

Reg *= regenerative changes*
- *0: negative*
- *1: focal*
- *2: extensive*

POST-REPARATIVE CHANGES (0-6)

Hyp *= hyperplasia*
Dys *= dysplasia*
- *0: negative*
- *1: light*
- *2: moderate-severe*

Atro *= atrophy*
Fibr *= fibrosis*
- *0: negative*
- *1: focal*
- *2: extensive*

SCORE = INFLAMMATION + REPARATIVE CHANGES + POST-REPARATIVE CHANGES (0-32)

Supplementary Table 1

VIII. Análisis de diversos compuestos naturales como inhibidores de agrecanasas

La relevancia de las ADAMTSs en procesos patológicos se ha establecido con abundantes datos experimentales. Entre estos, cabe destacar el papel que desarrollan las agrecanasas-1 y -2 (ADAMTS-4 y ADAMTS-5) en las patologías articulares. Convertir estos conocimientos en beneficios clínicos pasa necesariamente por la búsqueda de tratamientos que se dirijan selectivamente a las moléculas de interés. Con esta idea como referente, nos planteamos aproximarnos a una investigación traslacional en este campo evaluando la capacidad de ciertos componentes naturales para bloquear específicamente a las agrecanasas. Para ello, seleccionamos una serie de flavonoides presentes en vegetales y llevamos a cabo una exploración preliminar utilizando un sustrato sintético. De esta forma, identificamos a la luteolina como un compuesto capaz de inhibir ADAMTSs y no MMPs. A continuación, determinamos las características catalíticas de la luteolina sobre el agrecano, así como sobre la degradación de componentes del cartílago mediante cultivo de tejidos de ratón *ex vivo*. Los resultados obtenidos sitúan a la luteolina como un potencial agente prototípico que, a través de mejoras farmacológicas, tendría aplicación en el tratamiento de las enfermedades degenerativas articulares.

Artículo 8: **Ángela Moncada-Pazos**, Álvaro J. Obaya, Cristina G. Viloria, Carlos López-Otín y Santiago Cal. “The nutraceutical flavonoid luteolin inhibits ADAMTS-4 and ADAMTS-5 aggrecanase activities”.

Journal of Molecular Medicine 89: 611-619 (2011).

Aportación personal al trabajo

Mi contribución a este trabajo incluyó la evaluación inicial de una batería de compuestos naturales y el estudio del efecto de la luteolina sobre el agrecano. También participé en los análisis de expresión mediante PCR cuantitativa y en la medición de la degradación en explantes de cartílago cultivados. Por último, contribuí a la elaboración del texto y de las figuras que componen el artículo.

J Mol Med
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ORIGINAL ARTICLE

The nutraceutical flavonoid luteolin inhibits ADAMTS-4 and ADAMTS-5 aggrecanase activities

Angela Moncada-Pazos · Alvaro J. Obaya · Cristina G. Viloria · Carlos López-Otín · Santiago Cal

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Abstract A disintegrin and metalloprotease with thrombospondin domains (ADAMTS)-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) are metalloproteases involved in articular cartilage degradation and represent potential therapeutic targets in arthritis treatment. We explore herein the ability of different natural compounds to specifically block the destructive action of these enzymes. Following a preliminary screening using carboxymethylated transferrin as substrate, we focused our interest on luteolin due to its inhibitory effect on ADAMTS-4 and ADAMTS-5 activities using aggrecan and fluorogenic peptides as substrates. However, matrix metalloproteinases (MMPs) activities on these substrates result less affected by this flavonoid. Moreover, incubation of mouse chondrogenic ATDC5 cells in the presence of luteolin clearly decreases the release of aggrecan fragments mediated by aggrecanases under the same conditions in which aggrecanolysis mediated by MMPs is detected. Additionally, glycosaminoglycan levels in culture medium of murine cartilage explants stimulated with interleukin-1-alpha plus retinoic acid are reduced by the presence of the flavonoid. This inhibition takes place through blockade of ADAMTS-mediated aggrecanolysis, while MMPs activity is not or poorly affected. These results suggest that luteolin could be employed as a prototypic modifying disease-agent to create new chondroprotective compounds aimed to specifically block the unwanted aggrecanase activities in arthritic diseases.

Keywords Arthritis · Biochemistry · Inflammation · Extracellular matrix

A. Moncada-Pazos · C. G. Viloria · C. López-Otín · S. Cal
Departamento de Bioquímica y Biología Molecular,
Instituto Universitario de Oncología ,Universidad de Oviedo,
33006 Oviedo, Spain

A. J. Obaya
Departamento de Biología Funcional,
Instituto Universitario de Oncología, Universidad de Oviedo,
33006 Oviedo, Spain

S. Cal (✉)
Departamento de Bioquímica y Biología Molecular,
Universidad de Oviedo. c/ Fernando Bongera s/n,
33006 Oviedo, Spain
e-mail: santical@uniovi.es

Introduction

Proteolytic degradation of articular cartilage is a key pathological feature of arthropathies such as osteoarthritis (OA) and rheumatoid arthritis (RA) [1]. Particularly, degradation of aggrecan and type II collagen represents a hallmark of arthritis pathology [2]. Aggrecan is a proteoglycan with a core protein of high molecular mass (~250 kDa), consisting of two globular domains at the NH_2 terminus and a single globular domain at the COOH terminus, separated by a large domain heavily modified with glycosaminoglycans (GAG) [3]. The region linking the two NH_2 terminus globular domains is the interglobular (IGD) domain, which is highly sensitive to proteolysis. Thus, nine different cleavage sites within this domain have been characterized following aggrecan incubation with purified proteases. Nevertheless, only two major cleavages appear to occur in human tissues [3]. One site is between VDIPEN^{341} and ^{342}FFGVG and this cleavage is attributed to the action of different members of the matrix metalloproteinase (MMP) family. The other site is between NITEGE^{373} and ^{374}ARGSVI and is cleaved by ADAMTSs (a disintegrin and metalloprotease with thrombospondin domains) [1]. The functional consequences of aggrecan

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cleavage are distinct depending on the site undergoing proteolytic breakdown. Thus, while the involvement of MMPs in arthritis through type II collagen cleavage is well established [4], their role in aggrecanolysis remains controversial. Some studies have suggested that MMPs, through the N^{341}–^{342}F cleavage of aggrecan [4], are involved in the normal cartilage turnover whereas ADAMTS cleavage at E^{373}–^{374}A plays a major role in pathological loss of aggrecan from cartilage.

Functional studies support the main role of two members of the ADAMTS family, ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2), in pathological aggrecanolysis [5]. Indeed, mice lacking MT1-MMP [6] or MMP-9 [7] show increased cartilage degradation, whereas *Adamts5*-null mice are protected from cartilage degradation [8]. These and other studies have highlighted that therapeutic blockade of aggrecanases could be an option for arthritis treatment [5]. In this regard, versatile health benefits of nutraceutical flavonoids indicate that these compounds might be employed as anti-inflammatory compounds [9]. Moreover, several flavonoids have emerged as potential anti-arthritis drugs due to their metalloprotease inhibitory activities. For instance, a series of green tea catechin gallate ester has been found effective against different members of the MMP and ADAMTS families [10]. Also, nobiletin, a flavonoid isolated from citrus peels, effectively interferes with *Adamts4* and *Adamts5* gene expression and hence, aggrecan degradation mediated by these enzymes is hampered by treatment with this flavonoid [11].

The present study has examined the inhibition of aggrecanases by luteolin, a flavonoid widely distributed in the plant kingdom, especially in celery and green pepper [12]. Healthy benefits of luteolin have been evaluated indicating that this flavonoid might protect against cancer, microbial infections, and some chronic diseases [12]. Different mechanisms have been associated with the anti-inflammatory properties of luteolin, including inhibition of lipopolysaccharide-induced interleukin (IL)-6 production [13] or NF-kappa B activation [14]. In this work, we report that luteolin can also inhibit aggrecan degradation mediated by ADAMTS-4 and ADAMTS-5. Moreover, we have employed different biochemical approaches, cell-based assays, and murine cartilage explants to determine that luteolin is a better inhibitor of aggrecanases than of MMPs.

Materials and methods

Materials

Dulbecco's Eagle's medium (DMEM/F12), fetal bovine serum (FBS), antibiotics, and glutamine were from Life Technologies, Inc., (Paisley, UK). Human apo-transferrin and sodium selenite, chondroitinase ABC from *Proteus vulgaris*, keratanase from *Pseudomonas* sp., aggrecan from bovine articular cartilage, retinoic acid, dimethylmethylene blue, and human transferrin were from Sigma-Aldrich (St. Louis, MO, USA). Transferrin was carboxymethylated using nonradioactive iodoacetic acid [15]. BC-3 antibody was from Abcam (Cambridge, United Kingdom), and BC-14 was from MD Biosciences (Zürich, Switzerland). Curcumin, resveratrol, z-guggulsterone, piceatannol, luteolin, genistein, GM6001 (Ilomastat), dissolved in dimethylsulfoxide (DMSO) following manufacturer's indications; and recombinant MMP-7 were from Calbiochem (Darmstadt, Germany). Recombinant MMP-2, MMP-13, ADAMTS-4, ADAMTS-5, IL-1β, IL-1α, and the fluorogenic peptide Mca-K-P-L-G-L-Dpa-A-R-NH_2 (substrate IX) were from R&D Systems (Minneapolis, MN, USA). SensoLyte® 520 Aggrecanase-1 Assay Kit was from AnaSpec (Fremont, CA, USA). Fluorogenic peptide Dabcyl-K-E-L-A-E-L-R-E-S-T-S-Glu (Edans) (TS5-peptide, sequence taken from peptide FasL1, patent US7,312,045B2, www.freepatentsonline.com) was from JPT Peptide Technologies (Berlin, Germany). ATDC5 cells were a generous gift from Dr. Oreste Gualillo (Complejo Hospitalario Universitario de Santiago de Compostela, Spain).

Enzymatic assays

Proteolytic assay on carboxymethylated transferrin (Cm-Tf) (60 μM) was carried out for 16 h at 37°C in 15 μL aggrecanase reaction buffer (ARB; 50 mM Tris–HCl at pH 8.5 containing 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, and 0.05% Brij 35). Metalloproteases were employed at 50 nM, with the exception of MMP-7, which was employed at 40 nM. Reactions were stopped by addition of reducing SDS-PAGE sample buffer containing 20 mM EDTA. Digestion products were analyzed by SDS-PAGE gels and stained using Coomassie Brilliant Blue R-250. For aggrecanase assays, recombinant aggrecan (20 μg) was incubated with the indicated enzymes in 100 μL of ARB. Reactions were stopped by adding an equal volume of double strength glycosaminoglycan buffer (200 mM sodium acetate, 50 mM Tris–HCl pH 6.8, and 100 mM EDTA). Samples were deglycosylated with 0.01 units of chondroitinase/10 μg of aggrecan, and 0.01 units of keratanase/10 μg aggrecan for 16 h at 37°C, and then precipitated with acetone and centrifuged at 3,000×*g* for 15 min. Pellets were dried and resuspended in 20 μL of reducing SDS-PAGE sample buffer. Samples were monitored by Western-blot analysis in polyvinylidene fluoride (PVDF) membranes with BC-3 or BC-14 antibodies [16]. Blots were scanned and the band intensity quantified using ImageJ program. Kinetic analysis using fluorogenic peptide were made in a LS55 PerkinElmer Life

Science spectrofluorometer (λ_{ex}=320 nm and λ_{em}=405 nm for the fluorogenic peptide IX, and λ_{ex}=340 nm and λ_{em}= 500 nm for TS5-peptide). Routine assays were performed using a substrate concentration of 10 μM for peptide IX and 50 μM for TS5-peptide. Metalloproteases were assayed at 10 nM and the enzymatic activity detected as increase in fluorescence at 37°C for different times. ADAMTS-4 activity was assayed using the SensoLyte® 520 Aggrecanase-1 Assay Kit according to manufacturer's instructions. DMSO concentration in the enzymes assays was <1%. When indicated, preincubation with the specified concentrations of the different inhibitors was performed for 30 min at 37°C. IC_{50} values were calculated from plots of activity versus inhibitor concentration in a semilogarithmic representation.

Chondrocyte cultures

ATDC5 cells were routinely maintained in DMEM/F12 medium supplemented with 5% FBS, antibiotics, glutamine, apo-transferrin, and sodium selenite [17]. To evaluate aggrecanolysis, ATDC5 cells were cultured for 48 h in serum-free DMEM/F12 containing 100 μg/mL aggrecan. When indicated, IL-1β (10 ng/mL) and/or luteolin (10 μM) were added to the cultures. Media quenched with EDTA prior to incubation with the substrate served as assay blanks [18]. To evaluate aggrecan degradation products in chondrocyte cultures, 1 mL of conditioned medium was precipitated with five volumes of acetone and deglycosylation was carried out as described above. For comparative studies, all PVDF membranes were processed simultaneously and Western analysis was performed under identical conditions. Vehicle only (DMSO) was added to control cells not treated with luteolin, to ensure that the observed effects are ascribable to luteolin.

Analysis of glycosaminoglycans release using cartilage explants

Murine femoral head cartilages from 4-week-old mice were harvested and placed into a 48-well culture plate. Explants were maintained in a humidified incubator as previously described [19]. After 2 days, explants were placed in serum-free medium and stimulated or not with IL-1α (10 ng/mL) and a retinoic acid (10 μM), in the presence or absence of 100 μM luteolin. Glycosaminoglycan content in the media was measured using the dimethylmethylene blue assay [20]. Results are expressed as the percentage of proteoglycan release relative to the total proteoglycan in the media and digested cartilage. Mouse care and handling were conducted according to the Institutional Animal Care and Use guidelines for animal research.

RNA extraction and RT-qPCR

Total RNA was isolated by guanidium thiocyanate–phenol–chloroform extraction and quantified in a NanoDrop. Reverse transcription reactions were carried out with 2.5 μg of RNA, using the Thermoscript RT-PCR system with random hexamers and following manufacturer's instructions. Then 4.5 μL of a 1:3 dilution of cDNA were employed for qPCR using Taqman hydrolysis probes: Mm00556068_m1 (*Adamts4*, NM_172845.2), Mm00478620_m1 (*Adamts5*, NM_011782.2), Mm00439506_m1 (*Mmp2*, NM_008610.2), Mm01168419 (*Mmp7*, NM_010810.4), Mm00439491_m1 (*Mmp13*, NM_008607.1), and 4352339E (GAPDH) as a reference gene to normalize. TaqMan Master Mix and the 7300 Real-Time PCR System (Applied Biosystems) were employed. PCR conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression levels were calculated with the $\Delta\Delta C_q$ method, using the BioRad Gene Expression macro for Excel (Microsoft Corporation). Values were calculated referring all expression to the lowest one that was given the value "1." Value "0" was given when expression was not detected.

Statistical analysis

All data are presented as mean±SEM. The analysis of released GAGs was done using Student's *T* test (Prism 5 GraphPad software). *P* value <0.05 was considered significant.

Results

Inhibitory activity of natural agents against aggrecanases

Curcumin, resveratrol, z-guggulsterone, piceatannol, luteolin, and genistein were selected to examine their relevance as possible aggrecanase inhibitors [21]. These products were initially tested at 5 μM on ADAMTS-4 proteolytic activity using Cm-Tf as substrate. Ilomastat was included as a positive control for inhibition. Among all these agents, luteolin showed the highest capacity to block ADAMTS-4 activity as a clear reduction of Cm-Tf degradation can be observed with respect to the reaction without the flavonoid (Fig. 1a). Similar results were obtained when using ADAMTS-5 (not shown). Then, we analyzed the capacity of luteolin to inhibit aggrecanases in comparison to MMPs. As can be observed in Fig. 1b, the presence of this flavonoid hampers the proteolytic activity of both ADAMTS-4 and ADAMTS-5 on Cm-Tf in the micromolar range. Pixel density analysis allowed calculating an IC_{50}

of ~10 μM, and degradation of this substrate was totally abolished at 50 μM luteolin (Fig. 1b). However, MMP-2, MMP-7, and MMP-13 remain active at concentrations up to 100 μM luteolin (Fig. 1b). These results indicate that luteolin greatly influences aggrecanase activities, but MMPs activities result less affected in the micromolar concentration range employed. In consequence, luteolin could be a potential inhibitor of the ADAMTS-mediated proteolysis.

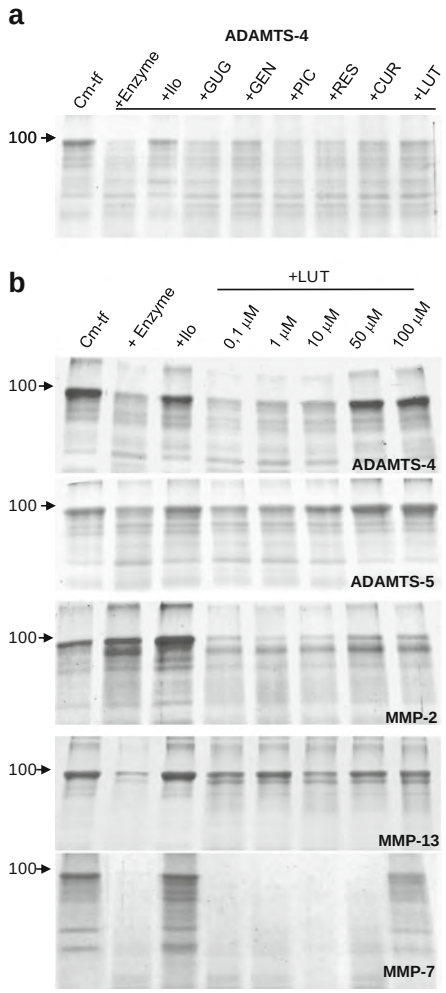

Fig. 1 Luteolin inhibits Cm-Tf cleavage by aggrecanases. **a** SDS-PAGE showing the potential ability of different natural products to block ADAMTS-4 enzymatic activity on Cm-Tf. *Lane 1* Cm-Tf alone, *Enzyme* indicates Cm-Tf degradation by ADAMTS-4 without preincubation with any agent. +*Ilo* indicates that the enzyme was preincubated with Ilomastat. The remaining lanes show the results following preincubation with z-guggulsterone (+*GUG*), genistein (+*GEN*), piceatannol (+*PIC*), resveratrol (+*RES*), curcumin (+*CUR*), and luteolin (+*LUT*). **b** Effect of luteolin on aggrecanase and MMP activities. ADAMTS-4, ADAMTS-5, MMP-2, MMP-13, and MMP-7 were preincubated with luteolin (+*LUT*) at the indicated concentrations. Please note that the metalloproteases were employed at 50 nM, with the exception of MMP-7, which was employed at 40 nM

Luteolin inhibits ADAMTS-mediated aggrecan cleavage

Then, we wanted to examine whether luteolin could also inhibit ADAMTS-4 and ADAMTS-5 activity on aggrecan. To this end, we employed bovine aggrecan as substrate and aggrecanolytic activities were detected using BC-3 antibody. This antibody recognizes the newly generated amino-terminal ^{374}ARGSVI sequence following digestion of the IGD of aggrecan by different ADAMTSs (Fig. 2a), but does not recognize this sequence when present within a peptide spanning the cleavage site [3]. Aggrecan catabolites following degradation by ADAMTS-4 or ADAMTS-5 were detected as immune reactive bands at ~250 kDa (Fig. 2b). To examine whether luteolin could restrain these degradative activities, aggrecanases were preincubated with the flavonoid at 1, 10, and 50 μM, respectively. Result in Fig. 2b shows that aggrecan degradation products are scarcely detected when luteolin is present at 10 μM and essentially absent at 50 μM, indicating an inhibitory effect of the flavonoid on ADAMTS activities.

To evaluate whether luteolin could affect aggrecan digestion by MMPs, aggrecan was incubated with MMP-13 and MMP-7 in the presence or absence of the flavonoid, and degradation products were detected using BC-14. This antibody recognizes the ^{342}FFGVG amino-terminal sequence generated by MMPs degradation of IGD domain of aggrecan (Fig. 2a). In the absence of the flavonoid, BC-14 immunoreactive fragments following MMP-13 digestion were mainly detected at ~150 kDa. In the case of MMP-7, major degradation products were detected at ~150 and ~70 kDa (Fig. 2b). Preincubation of both MMPs with luteolin at the same concentrations assayed for ADAMTSs caused a slight but not significant decrease of MMP activities as BC-14 immunoreactive bands can be detectable at 50 μM luteolin.

Peptide substrate assays

Fluorogenic peptide Mca-K-P-L-G-L-Dpa-A-R-NH_2 was employed to assess the inhibitory effect of luteolin on MMP-2, MMP-7, and MMP-13. Data were fit to sigmoid curve to calculate IC_{50} values. Results indicate that luteolin is a better inhibitor for MMP-13 (IC_{50}=18.3 μM) than for MMP-2 and MMP-7 (IC_{50}=40.9 and 68.2 μM, respectively; Fig. 3a). The above peptide is not a suitable substrate for ADAMTSs, and hence the SensoLyte® 520 Aggrecanase-1 Assay Kit was employed to calculate an IC_{50} value of 9.8 μM for luteolin, highlighting the inhibitory effect of this flavonoid on ADAMTS-4. To evaluate ADAMTS-5 activity in the presence of luteolin, the fluorogenic TS5-peptide was

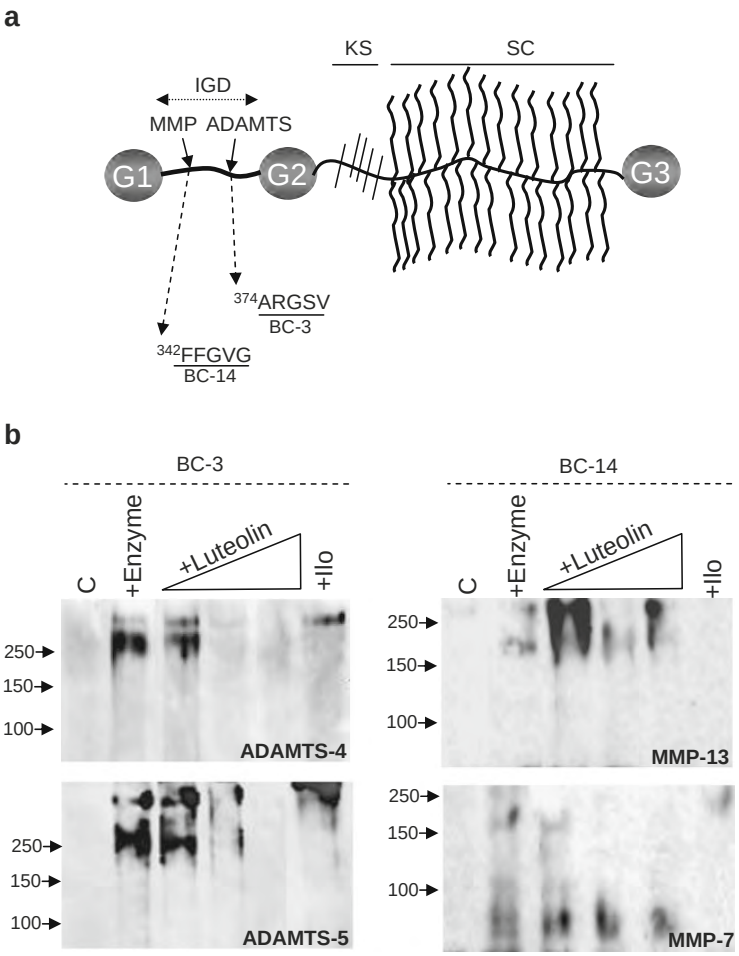

Fig. 2 Effect of luteolin on aggrecan digestion by aggrecanases and MMPs. **a** Aggrecanases (ADAMTS) and MMPs cleavage sites in the aggrecan IGD domain. *G1*, *G2*, and *G3* indicate aggrecan globular domains 1, 2, and 3, respectively; *KS*, keratan sulphate; *CS*, chondroitin sulphate. The newly generated amino-terminal sequences following aggrecanases or MMPs digestion are indicated. **b** Western-blot analysis of aggrecan degradation by ADAMTS-4, ADAMTS-5, MMP-13, and MMP-7 in absence (+*Enzyme*) or presence of luteolin (+*Luteolin*) at the concentrations indicated in "Results." The ^{374}ARGSVI and ^{342}FFGVG immunoreactive fragments were detected by the BC-3 and BC-14 antibodies respectively. *C* indicates aggrecan incubated in the absence of enzyme and luteolin. +*Ilo* indicates that the corresponding metalloprotease was preincubated with ilomastat

employed and MMP-7 was used for comparative analysis as this MMP hydrolyzes this substrate better than MMP-2 and MMP-13 (not shown). Again, ADAMTS-5 was strongly inhibited by luteolin (IC_{50}=12.2 μM, Fig. 3b), whereas the flavonoid did not show a significant inhibitory effect on MMP-7 (IC_{50}>250 μM). These substrates were also used to determine that luteolin acts as a noncompetitive inhibitor of ADAMTS-mediated proteolysis (Fig. 3c).

Effects of luteolin on aggrecanolytic activities of ATDC5 cells

ATDC5 cells were stimulated with IL-1β or left untreated, and then treated or untreated with luteolin. mRNA expression analysis for *Adamts4*, *Adamts5*, *Mmp2*, and *Mmp13* was assessed by qRT-PCR after 48 h of incubation (Fig. 4a). Luteolin was assayed at 10 μM, as this concentration is within a range that we had previously examined to discard any effect on ATDC5 cell viability (not shown). This experiment allowed determining expression of *Adamts4*, *Adamts5*, and, at lower level, of *Mmp13* in the absence of both IL-1β and luteolin. *Mmp7* expression was not detected. The presence of luteolin at 10 μM caused a decrease of both *Adamts4* and *Adamts5* expression levels whereas *Mmp2* expression remained stable, suggesting that this *MMP* is constitutively expressed. Detectable but low *Mmp13* expression was also observed when the flavonoid was present. Similar expression analysis was performed following IL-1β treatment of ATDC5 cells, showing an enhanced expression of *Adamts4*, *Adamts5*, and *Mmp2*, but *Mmp13* expression was again undetectable in the absence of luteolin (Fig. 4a). However, combination in the culture of both the flavonoid and the cytokine moderately decreased *Adamts4* and *Mmp2* expression, whereas *Adamts5* expression was markedly reduced respecting the cells grown in the presence of IL-1β alone. Similarly to what happened in the absence of IL-1β, *Mmp13* expression is detected at a low expression level in the presence of luteolin.

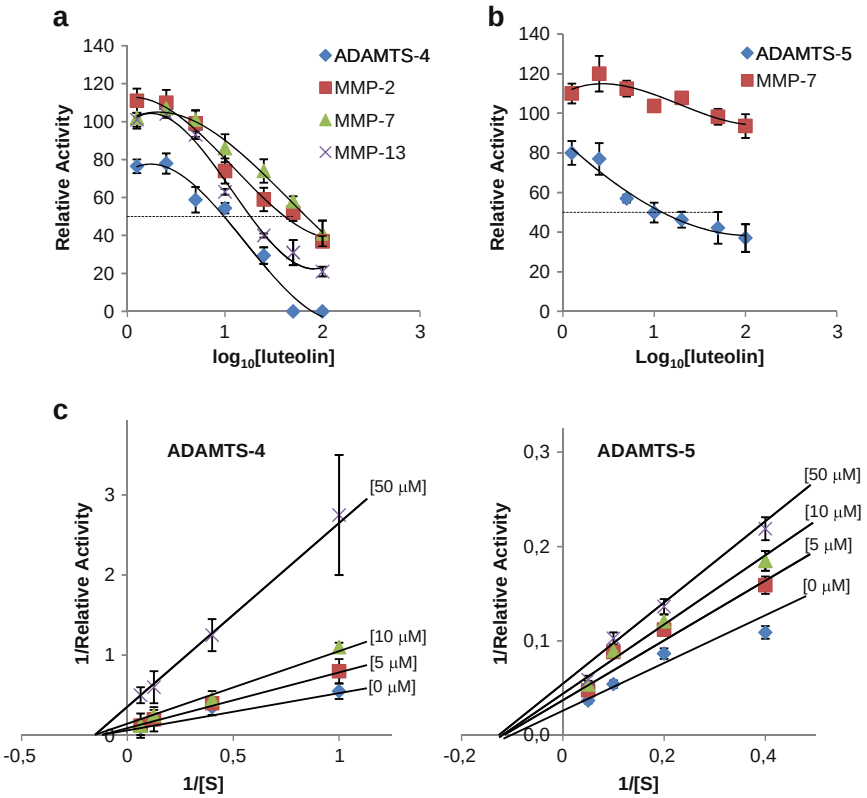

Fig. 3 Effect of luteolin on the digestion of fluorogenic peptide substrates by aggrecanases and MMPs. **a** Fluorogenic peptide substrate IX for MMP-2, MMP-7, and MMP-13, and the Senso-Lyte® 520 Aggrecanase-1 Assay Kit for ADAMTS-4 were used for monitoring metalloprotease activities in the presence of various concentrations of luteolin as described in "Materials and Methods." **b** Similar assay using the fluorogenic TS5-peptide, a common substrate for MMP-7, and ADAMTS-5. *Dashed lines* indicate 50% ctivity. **c** Lineweaver–Burk plots for ADAMTS-4 and ADAMTS-5 determined in the presence of luteolin at the concentrations indicated in *brackets*

Regarding aggrecanolytic activities, conditioned media were analyzed to detect aggrecan degradation products. Treatment with IL-1β considerably enhanced aggrecanolysis mediated by both ADAMTSs and MMPs, as BC-3 and BC-14 immunoreactive products were increased compared with samples obtained without treatment with the cytokine (Fig. 4b). However, presence of luteolin caused a marked reduction of BC-3 immunoreactivity meanwhile release of high molecular weight aggrecan species was still clearly detected by BC-14. These results indicate that MMP-mediated aggrecanolysis is less affected by the presence of the flavonoid. Altogether, these results show that luteolin can act as an aggrecanase inhibitor, and provide novel evidence of how this flavonoid may modulate ADAMTSs gene expression.

Inhibition of glycosaminoglycans release by luteolin

To evaluate whether luteolin could affect GAG release from cartilage explant, the flavonoid was added to the explant cultures medium of murine femoral head cartilages stimulated with IL-1α and retinoic acid. Quantification of GAG release from cartilage explants indicates that the presence of luteolin significantly reduces the appearance of these degradation products (*, p value <0.05 by Student's T test) in the case of samples stimulated with IL-1α and retinoic acid (Fig. 5a). Thus, luteolin is indeed diminishing the aggrecanolysis mediated by aggrecanases but not by MMPs (Fig. 5b), thus reinforcing the inhibitory effect of luteolin on these enzymes. For unstimulated samples, the flavonoid slightly increases GAG release (not significant, Fig. 5a). This effect, however, is not statistically significant and cannot be attributable to neither aggrecanases nor MMPs since no products of their degradation were detected (Fig. 5b). Nevertheless, it cannot be ruled out that other GAG-degrading enzymes are also affected by the presence of the flavonoid.

Discussion

Depletion of aggrecan is a critical event in OA and RA which is mainly caused by uncontrolled proteolytic activities. In this regard, it is well characterized that MMPs cleave aggrecan at the N^{341}–^{342}F bond within the IGD domain, hence these metalloproteases emerged as potential therapeutic targets to block disease progression [5]. However, in 1991, Sandy et al. [22] found that the main cleavage in the aggrecan core protein occurred at E^{373}–^{374}A within the IGD domain. This important finding indicated

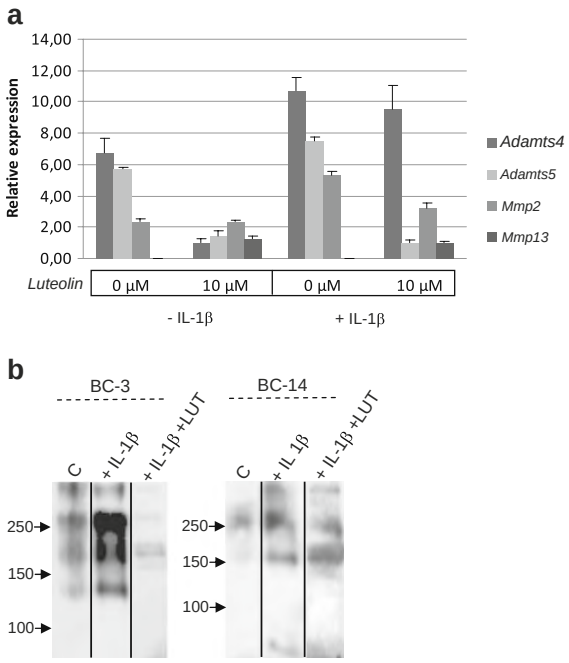

Fig. 4 Inhibitory effect of luteolin on proteoglycan degradation in ATDC5 cells. **a** Analysis of aggrecanases, *Mmp2*, and *Mmp13* expression in ATDC5 cells in presence or absence of luteolin at the indicated concentration. *+IL-1β* indicates that cells were treated with this cytokine and *−IL-1β* that cells were left untreated. No *Mmp7* expression was found in these assays. **b** Western-blot analysis of the ^{374}ARGSVI and ^{342}FFGVG immunoreactive fragments found in the media of ATDC5 cells and detected by the BC-3 and BC-14 antibodies. *+IL-1β* indicates presence of the cytokine, and *+IL-1β +LUT* indicates presence of both cytokine and luteolin. *C* indicates absence of both IL-1β and luteolin

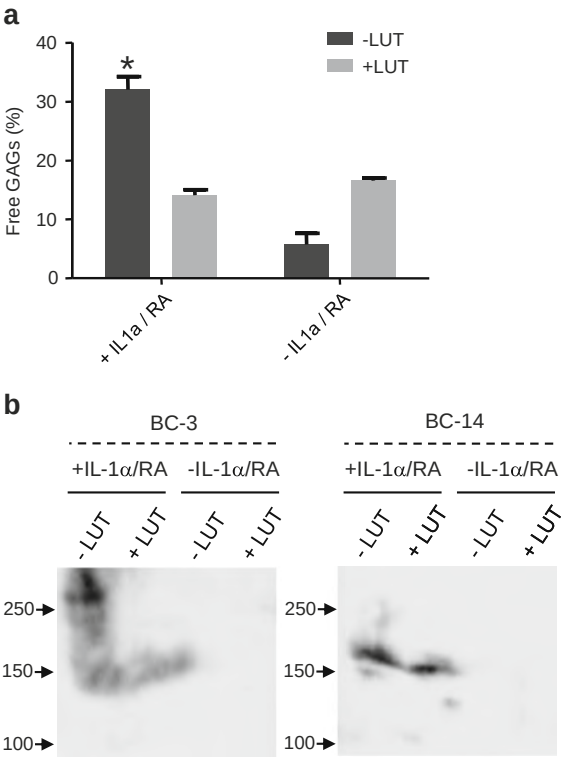

Fig. 5 Inhibition of cartilage glycosaminoglycans release by luteolin. **a** Quantification of GAGs release was carried out using the dimethylmethylene blue assay. Cartilage explants were stimulated with IL-1α and retinoic acid (+IL-1α/retinoic acid), or were left unstimulated (−IL-1α/retinoic acid). *+LUT* indicates presence of luteolin. (*p value <0.05 by Student's T test). **b** Western-blot analysis of the aggrecan fragments generated by ADAMTS (*BC-3*) or MMPs (*BC-14*) activity in cartilage explants stimulated (+IL-1α/retinoic acid) or not (−IL-1α/retinoic acid), with IL-1α and retinoic acid in presence (*+LUT*) or absence (*−LUT*) of luteolin

that proteolytic enzymes other than MMPs might be involved in pathological aggrecan degradation and therefore this proteolytic activity was referred as aggrecanase. Two ADAMTS metaloproteases, ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2), were identified as the main enzymes responsible for the cleavage at this E^{373}–^{374}A bond [5]. Functional implication of ADAMTS-5 in arthritis has been further demonstrated by the phenotypic characterization of *Adamts5*-deficient mouse, which has revealed that this metalloprotease is the main responsible for cartilage degradation in vivo [8].

The identification of ADAMTS-4 and ADAMTS-5 as pathological aggrecanases has increased the interest in the discovery and/or design of specific inhibitors [5]. Synthetic hydroxamic acid-derived compounds such as ilomastat are potent ADAMTSs inhibitors though their zinc-chelating ability. These products were originally designed to block MMPs to avoid the uncontrolled activity of these metalloproteases in tumorigenesis [23]. However, their use in anticancer therapies resulted disappointing since different MMPs show protective roles in tumor progression [24] and because their lack of specificity implies that other tumor-protective metalloproteases are also affected [25–27]. Similarly, the use of these broad spectrum inhibitors would not be suitable in the case of arthritic diseases. Under pathological conditions, a proinflammatory environment would predominate, and ADAMTS-4 and ADAMTS-5 would deteriorate the cartilage by degrading its components. On the other side, MMPs would be necessary for normal tissue remodeling. Hence, the inhibition of ADAMTSs would prevent cartilage degradation, while blocking MMPs would affect normal cartilage turnover. Consequently, new inhibitors aimed to selectively block ADAMTS activities are necessary. In this regard, a possibility would consist in targeting the exosite interactions of these enzymes. In fact, different studies have shown that aggrecan cleavage depends on the numerous

interactions occurring between the core enzyme outside the active site and the substrate [28]. Thus, calcium pentosan polysulfate (CaPPS) inhibits ADAMTS-4 aggrecanase activity without affecting mRNA expression in chondrocytes treated with IL-1 or retinoic acid [29]. Chondroprotective role of CaPPS could occur through its binding with the carboxy-terminal domain of the protease, avoiding a proper interaction with the substrate. Similarly, glucosamine and manosamine also inhibit aggrecan degradation in chondrocyte cultures and it has been suggested that these compounds prevent mechanical changes occurring to the cartilage following IL-1 treatment [30].

In this work, we have explored whether different nutraceutical compounds could have an inhibitory effect on ADAMTS-4 and -5 activities as numerous natural products have proven to exert anti-inflammatory effects in vitro and in vivo. For instance, it has been recently shown that licochalcone A, a major phenolic constituent of licorice, decreases levels of proinflammatory cytokines in mouse models [31]. We first performed a screening to identify natural products able to target ADAMTS-4 and ADAMTS-5 activity. These products included piceatannol, a polyphenol with ability to act as an ADAMTS-4 inhibitor [32]. However, our results indicated that luteolin has the highest capacity to block these enzymes among all products assayed, whereas degradation mediated by MMPs was less affected by this flavonoid. In this regard, it has been previously reported that a MMP-2 form including the catalytic domain with the fibronectin-like repeats can be inhibited by luteolin [33]. Nevertheless, in this work, we have employed an entire form of the protein, including all ancillary domains and requiring activation to perform its catalytic activity. Differences in the level of inhibition obtained between these two MMP-2 forms could indicate the relevance of the ancillary domains in the recognition of the substrate and consequently for the catalytic process. Luteolin also possesses anti-inflammatory activity, which is mediated by inhibiting the production of cytokines such as IL-8, IL-15, or TGF-β in synovial fibroblasts [34], and also by blocking COX-2, iNOS, LOX [12], and IL-6 [13] activities. However, to our knowledge, it had not been yet examined whether luteolin could also act as an aggrecanase inhibitor. In this work, we have shown that this flavonoid inhibits degradation of recombinant aggrecan and its release by chondrocytic cells in vitro, and prevents, ex vivo, the release of glycosaminoglycans from murine femoral head cartilages treated with IL-1α and retinoic acid. Additionally, we have demonstrated that its inhibitory function is more effective on ADAMTSs than on MMPs activity. Finally, we have provided evidence that luteolin can modulate *Adamts* gene expression, an observation which is consistent with previous findings on nobiletin, a polymethoxy flavonoid that shows a high structural similarity with luteolin [11].

In conclusion, luteolin is a novel and potential alternative in anti-arthritic therapies, due to its specificity over aggrecanases and to its natural product condition. Thus, the action of this flavonoid would be mainly restricted to the undesired degradation of aggrecan, and its presence in components of normal diet guarantees the possibility of oral administration and non-toxicity at low doses [12]. Moreover, these properties could be improved through the design of new molecules chemically derived from luteolin, which could represent new therapeutic candidates for arthritic diseases [35].

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IX. Otros trabajos relacionados con la Tesis Doctoral

Durante el desarrollo de la presente Tesis Doctoral he tenido la oportunidad de participar en otras líneas de investigación, como la centrada en la caracterización de la familia de serín-proteasas denominadas poliserasas. Asimismo, he podido colaborar con otros grupos en proyectos relacionados con la investigación oncológica. Estos trabajos han dado lugar a las siguientes publicaciones:

Artículo 9: Santiago Cal, Juan R. Peinado, María Llamazares, Víctor Quesada, **Ángela Moncada-Pazos**, Cecilia Garabaya y Carlos López-Otín. "Identification and characterization of human polyserase-3, a novel protein with tandem serine-protease domains in the same polypeptide chain".*BMC Biochemistry*. 27; 7:9 (2006).

Artículo 10: Santiago Cal, **Ángela Moncada-Pazos** y Carlos López-Otín. "Expanding the complexity of the human degradome: polyserases and their tandem serine protease domains". *Frontiers in Bioscience* 12: 4661-4668 (2007).

Artículo 11: **Ángela Moncada-Pazos**, Santiago Cal y Carlos López-Otín. "Polyserases". *Handbook of Proteolytic Enzymes*, 3ra edición. Editores: A. J. Barrett, N. D. Rawlings and J. F. Woessner. Editorial Academic Press (Londres); 2011. En prensa.

Artículo 12: Ester Lara, Vincenzo Calvanese, Covadonga Huidobro, Agustin F Fernández, **Ángela Moncada-Pazos**, Álvaro J Obaya, Oscar Aguilera, José Manuel González-Sancho, Laura Sánchez, Aurora Astudillo, Alberto Muñoz, Carlos López-Otín, Manel Esteller y Mario F Fraga "Epigenetic repression of ROR2 has a Wnt-mediated, pro-tumourigenic role in colon cancer". *Molecular Cancer* 9: 170 (2010).

DISCUSIÓN

La investigación del universo proteolítico ha experimentado un crecimiento explosivo en las últimas décadas. Lejos de agotarse, los nuevos conocimientos han expandido las fronteras de este campo de estudio y aún hoy es habitual la identificación de una nueva proteasa o incluso de una nueva clase catalítica, o la descripción de una función oculta para una proteasa ya conocida. Este efecto sinérgico se alimenta en parte de la relevancia funcional que estos enzimas han resultado tener. Los eventos proteolíticos son indispensables para la homeostasis de los seres vivos y abarcan desde tareas básicas como los procesos de digestión y catabolismo de proteínas, a otras mucho más sutiles como la amplificación del repertorio proteico a nivel post-traduccional mediante la ejecución de reacciones específicas de procesamiento proteolítico. La dependencia proteolítica es tal que nos expone a situaciones patológicas cuando se producen alteraciones en la actividad de estos enzimas. Por este motivo, es necesario incorporar al estudio degradómico una dimensión más aplicada, que tenga como fin comprender el papel de las proteasas en la enfermedad y trasladar los descubrimientos a la clínica. Paralelamente, en años recientes hemos asistido a una revolución tecnológica en la que se han solapado las eras genómica, transcriptómica y proteómica, a la vez que se ha abierto el camino para la epigenómica o los microRNAs. Es en este escenario en el que se ha desarrollado la presente Tesis Doctoral, con la que hemos pretendido profundizar en el conocimiento fisiológico y patológico de algunas proteasas de especial interés para nuestro laboratorio.

En concreto, nuestro trabajo se ha centrado en el estudio de miembros de la familia de adamalisinas. Esta elección responde en principio a razones históricas, pues numerosos integrantes de esta familia, y más específicamente diversas ADAMTSs, se clonaron previamente en nuestro laboratorio. Además, la información disponible sobre muchos de estos enzimas al iniciar esta Tesis era prácticamente nula o muy limitada. No obstante, hay que señalar que las adamalisinas constituyen un objetivo de estudio complicado. Por ello hemos intentado tener siempre presente la complejidad de estas proteínas, que son a la vez moléculas de adhesión y proteasas **(I)** en todos los análisis que hemos llevado a cabo. Esta dualidad

hace de ellas elementos multifacéticos con comportamientos que en ocasiones varían radicalmente según el contexto. Por este motivo, optamos por metodologías globales y modelos de estudio acordes, con especial preferencia por los sistemas *in vivo*.

Basándonos en estas premisas, nos ocupamos en primer lugar de estudiar el papel de la metaloproteasa ADAMTS-12 en el desarrollo tumoral **(II, III)**. Los escasos datos previos sobre esta proteína incluían su detección en líneas tumorales de diverso origen así como en cánceres gastrointestinales, de ahí que pudiese existir alguna relación entre ADAMTS-12 y la progresión neoplásica. Para abordar esta cuestión, empleamos en primer lugar el modelo celular MDCK **(II)**. Esta línea celular no tumoral deriva de riñón canino y muestra un carácter típicamente epitelial. Sin embargo, tras el tratamiento con determinados factores como el HGF, estas células experimentan un cambio morfológico y un aumento de la movilidad característicos de transiciones epitelio-mesénquima, adoptando finalmente un fenotipo tumoral. En nuestro caso, los clones seleccionados que producían elevadas cantidades de ADAMTS-12 eran refractarios al cambio fenotípico propio de esta línea, tanto al crecer en placa como al hacerlo en matrices tridimensionales de colágeno, en las que las células control tienden a formar extensiones y túbulos. A nivel molecular, la transformación observada va acompañada de un incremento en los filamentos de vimentina y una disminución de la presencia de cadherina E, que tampoco se reflejaban en presencia de ADAMTS-12. Contrariamente, experimentos similares realizados con esta misma línea sobreexpresando ciertas MMPs, provocaban un incremento de sus características migratorias y mesenquimales (Kadono y col., 1998; Kang y col., 2000; Soulie y col., 2005). Es presumible que en estos casos los enzimas actúen degradando componentes de la matriz extracelular, requiriendo para ello la acción del dominio metaloproteasa aunque también de otros como el hemopexina (Hotary y col., 2000; Wang y col., 2004). Sin embargo, los efectos observados en el caso de ADAMTS-12 son completamente independientes de la actividad metaloproteasa, pues al llevar a cabo los mismos ensayos con células que producían una forma catalíticamente inactiva de ADAMTS-12, el resultado no variaba. En cambio,

al trabajar con un mutante de la proteína que carecía de los dominios TSP-1 finales, las células pasaban a comportarse como las células control. Resultados análogos se obtuvieron al emplear las células endoteliales bovinas BAE-1 cultivadas en una matriz de colágeno. Así, al evaluar su capacidad de formación de túbulos, ésta se veía reducida notablemente en presencia de ADAMTS-12, pero no de la forma de ADAMTS-12 sin parte de los dominios TSP-1 **(II)**.

Complementariamente, detectamos que los efectos fenotípicos desencadenados por ADAMTS-12 iban acompañados por cambios en la señalización a través de la ruta Ras-MAPK (*Mitogen Activated Protein Kinase*) **(II)**. Mientras que en las células control se producía una activación de esta ruta, ésta se veía marcadamente reducida en los clones que sobreexpresaban ADAMTS-12, los cuales mostraban una llamativa disminución en los niveles de ERK-fosforilado. Estas observaciones concuerdan con los cambios morfológicos descritos, pues la activación de esta ruta de señalización es el motor necesario y suficiente en las células MDCK para promover los fenómenos de migración y tubulogénesis (Hellman y col., 2005; Liu y col., 2007). Por lo tanto, la presencia de ADAMTS-12 inhibiría los cambios pro-tumorales de estas células impidiendo la activación de esta ruta, y más concretamente este proceso estaría mediado por los dominios TSP-1 finales de la proteína. Esta hipótesis resulta razonable si tenemos en cuenta que otras proteínas relacionadas con ADAMTS-12 también bloquean la activación de ERK: ADAMTS-1 de *X. laevis* (Suga y col., 2006) y ADAMTS-15 humana **(IV)**. En ambos casos, la inhibición requiere de las regiones carboxilo terminal de estas proteasas, las cuales incluyen varias repeticiones TSP-1. No obstante, en ninguno de estos casos se ha clarificado el mecanismo por el cual estos dominios podrían activar dicha ruta. En este sentido, apuntamos a la participación del receptor de superficie celular CD36 como uno de los posibles mediadores. Estudios previos habían implicado al receptor CD36 en fenómenos de inhibición de la migración de células endoteliales y de la angiogénesis mediante unión a trombospondina-1 y trombospondina-2 (Dawson y col., 1997; Febbraio y col., 2001; Primo y col., 2005; Koch y col., 2011). A la luz de estas observaciones, un mecanismo que

explicaría los resultados obtenidos para ADAMTS-12, incluiría la interacción entre la región carboxilo terminal de esta proteína y CD36, dando lugar a una modulación negativa de la ruta Ras-MAPK y en última instancia, impidiendo los cambios celulares en respuesta a HGF.

Considerando los resultados obtenidos, decidimos explorar el posible efecto supresor tumoral de ADAMTS-12 *in vivo* **(II)**. Para ello, llevamos a cabo una primera aproximación estudiando el crecimiento en ratones inmunodeficientes SCID de la línea de cáncer de pulmón A549 transfectada con ADAMTS-12. Las diferencias observadas resultaron esclarecedoras: la sobreexpresión de la metaloproteasa por parte de las células tumorales era suficiente para reducir significativamente la progresión de los tumores, retrasando el tiempo hasta su aparición y el volumen que éstos alcanzaban. En conjunto, y a falta de la descripción de un mecanismo molecular concreto, nuestros datos señalaban un papel anti-tumoral para ADAMTS-12 **(II)**.

Nuestro siguiente paso en la caracterización de ADAMTS-12 en el cáncer se centró en la búsqueda de mecanismos que controlasen su expresión en las células tumorales **(III)**. Muchos genes supresores tumorales se ven sometidos a fenómenos de silenciamiento a través de hipermetilación de sus promotores. La existencia de una isla CpG en la región promotora de *ADAMTS12* sugería que este gen podía estar controlado epigenéticamente. En este sentido, cabe destacar que otros miembros de la familia ADAMTS están también sujetos a esta forma de represión, como *ADAMTS8* (Dunn y col., 2004), *ADAMTS9* (Zhang y col., 2010) y *ADAMTS18* (Jin y col., 2007; Li y col., 2010). Los análisis epigenéticos de *ADAMTS12* mostraron que este gen está expuesto a elevados niveles de metilación en tumores de colon y en líneas celulares cancerígenas de diverso origen. No obstante, y de manera llamativa, descubrimos que esta hipermetilación no correlacionaba con un silenciamiento en la expresión de la proteasa en las muestras de pacientes con cáncer de colon. De hecho, los niveles de expresión resultaban notablemente más elevados en el tejido tumoral en comparación con el tejido sano, aun siendo indetectables en las líneas tumorales ensayadas **(III)**. Esta situación aparentemente contradictoria es explicable si advertimos que los tumores son entidades formadas por la agregación de múltiples

componentes, que además de las células tumorales encierran un estroma propio del órgano que las alberga. Así pues, células presentes en el estroma distintas de las células tumorales podrían expresar *ADAMTS12*. Los estudios histopatológicos confirmaron esta hipótesis: en los tumores de colon coexistiría una represión epigenética de *ADAMTS12* en las células tumorales junto con una sobreexpresión del mismo gen en células circundantes, concretamente en miofibroblastos y específicamente en estadios tumorales bien diferenciados. Estos resultados describen unas circunstancias originales que no se habían propuesto previamente, pero que podrían ser comunes a otras proteínas relacionadas, ya que algunos miembros de la familia ADAMTS (incluyendo *ADAMTS12*) se han detectado sobreexpresados en los fibroblastos del estroma de tumores de mama (Porter y col., 2004). Asimismo, estos hallazgos ponen de manifiesto que la detección de niveles elevados de expresión de ciertas metaloproteasas en cáncer no se traduce forzosamente en un comportamiento pro-tumoral, pues de igual manera podría tratarse de respuestas del estroma con naturaleza protectora. No es descartable, pues, que otras metaloproteasas identificadas clásicamente como agentes pro-tumorales por su incremento en tumores, enmascaren en realidad contextos semejantes al de ADAMTS-12. Esta posibilidad ha de contemplarse en los futuros diseños terapéuticos pues, como hemos mencionado, el fracaso de los ensayos clínicos con inhibidores de metaloproteasas de amplio espectro recae posiblemente en el bloqueo inespecífico de proteasas con efectos beneficiosos (López-Otín y Matrisian, 2007).

Con el fin de analizar con mayor precisión la situación descrita, ideamos un modelo celular en el que co-cultivamos células tumorales y fibroblastos, ambos procedentes de colon **(III)**. Este modelo nos permitió describir la concomitancia de dos procesos: por un lado la sobreexpresión de *ADAMTS12* en los fibroblastos y como consecuencia de la proximidad de las células tumorales, y por otro el incremento en la apoptosis de las células tumorales debido a la presencia de los fibroblastos. Si bien estas observaciones no establecen ninguna relación causal, son perfectamente acordes con el modelo propuesto. Finalmente, mediante el empleo de

anticuerpos bloqueantes de TGFβ1 determinamos que este factor podría ser uno de los responsables de estimular la producción de ADAMTS-12, una idea reforzada por estudios previos de nuestro grupo que ya habían identificado a TGFβ1 como uno de los principales inductores de esta proteasa (Cal y col., 2001).

ADAMTS-12 fue la primera adamalisina para la que describimos propiedades anti-tumorales. Otro miembro del grupo candidato a sumarse a la lista de proteasas protectoras en cáncer, fue ADAMTS-15, que al igual que ADAMTS-12, se identificó en nuestro laboratorio (Cal y col., 2002). En el caso de *ADAMTS15* existían indicios previos que apuntaban a las alteraciones en este gen como un suceso frecuente en cáncer de colon (Sjoblom y col., 2006). Por este motivo, y para conocer el posible papel de ADAMTS-15 en el cáncer, realizamos un análisis mutacional en un conjunto más amplio de tumores colorrectales de pacientes, así como diversos ensayos funcionales para evaluar la verdadera relevancia de estos acontecimientos en sistemas *in vitro* e *in vivo* **(IV)**. En concreto, llevamos a cabo la secuenciación de todos los exones del gen *ADAMTS15* en 50 muestras de pacientes y en 6 líneas celulares de cáncer de colon; como resultado, identificamos 4 nuevas mutaciones, que se suman a las 2 referidas anteriormente. Si bien uno de los cambios hallados era un cambio sinónimo, el resto de las mutaciones causaban alteraciones en la pauta de lectura o consistían en cambios puntuales que se predecían *in silico* como deletéreos para la función proteica. En conclusión, juzgamos que las mutaciones en el gen *ADAMTS15* no constituían eventos estocásticos aislados, sino que conferirían alguna ventaja selectiva al tumor, posiblemente a través de la pérdida de función de ADAMTS-15.

Encaminados a valorar tal hipótesis, efectuamos diferentes ensayos funcionales **(IV)**. Al valorar la capacidad de crecimiento e invasión celular *in vitro*, observamos que la sobreexpresión de *ADAMTS15* causaba una disminución en estas propiedades. En congruencia con estos resultados, el silenciamiento de su expresión daba lugar a un efecto inverso, al igual que la producción de una de las proteínas mutantes descritas a partir del análisis de mutaciones. Estos cambios se producían conjuntamente con cambios

fenotípicos y moleculares: la presencia de ADAMTS-15 era pareja a una apariencia celular de carácter epitelial y a una menor activación de ERK, mientras que su silenciamiento o la producción de la proteína mutante daban lugar a una fenotipo mesenquimal y a un incremento en los niveles de ERK fosforilado. En este sentido, es especialmente informativo el hecho de que la mutación ensayada generaba una proteína que perdía los dos dominios TSP-1 finales **(IV)**. La situación es por tanto análoga a la de ADAMTS-12, reforzando la idea de que la región terminal de estas proteínas que incluye varios dominios TSP-1 puede modular negativamente la ruta Ras-MAPK, la cual está asociada a procesos de diferenciación como la transición epitelio mesénquima. De hecho, esta ruta se ha señalado como una posible diana para intervenciones terapéuticas (Montagut y Settleman, 2009). Curiosamente, detectamos también que la pérdida de los dominios TSP-1 finales era causa suficiente para modificar la localización de la proteína que de situarse asociada al entorno pericelular pasaba a ser mayoritariamente soluble.

En resumen, si integramos todos los datos obtenidos, parece verosímil un modelo según el cual distintas ADAMTSs como ADAMTS-1, -12 o -15 frenarían acontecimientos pro-tumorales al impedir la activación de la ruta Ras-MAPK. En este sistema, serían necesarios los dominios TSP-1 pero no su actividad catalítica. Este efecto podría tener lugar directamente mediante, por ejemplo, el secuestro de factores que activen dicha ruta o actuando a través de receptores como CD36, pero también indirectamente al modificarse la localización de la proteasa e imposibilitando con ello el acceso a estas moléculas en la superficie. En este sentido, hay que resaltar que los procesamientos proteolíticos que afectan a ADAMTS-1 y -12 podrían ser también relevantes. Así, ya se han documentado casos en los que ciertas ADAMTSs son capaces de unirse a diversos factores de crecimiento (Luque y col., 2003; Guo y col., 2010), aunque estas propiedades se modifican drásticamente cuando se trata de las formas procesadas, como ocurre con ADAMTS-1 (Liu y col., 2006b). Adicionalmente, llevamos a cabo un ensayo con ratones SCID para valorar la relevancia *in vivo* de ADAMTS-15 en la progresión tumoral **(IV)**. Los datos obtenidos revelaron que el silenciamiento

de su expresión fomentaba un marcado aumento del crecimiento tumoral. Finalmente, el análisis de los niveles de la proteína en muestras de tumores de colon humanos de distintos estadios apuntaba al papel de esta metaloproteasa como un marcador de buen pronóstico, pues los niveles eran mayores en tejido normal y se reducían en las fases más avanzadas. En conclusión, la pérdida de función de ADAMTS-12 y ADAMTS-15 parece conferir ventajas para la progresión tumoral, existiendo rutas comunes implicadas en el mecanismo por el que ambas proteasas desarrollan sus funciones protectoras. No obstante, esta pérdida de función se produce de manera distinta en ambos casos. Mientras que la hipermetilación resulta ser la estrategia principal en el gen *ADAMTS12*, son las mutaciones el motor fundamental de inactivación funcional en *ADAMTS15*.

Concluida esta etapa del trabajo, y en estrecha conexión con el mismo, decidimos participar en un amplio proyecto dirigido al análisis mutacional de la familia ADAM en melanoma **(V)**. De manera general, nos propusimos la identificación de miembros de esta familia como posibles dianas de modificaciones genéticas en este tipo de tumor, así como la posterior asignación de un significado funcional a las hipotéticas mutaciones. Estudios previos ya habían localizado mutaciones en ADAMs en tumores de diverso origen, lo que sugería la importancia de las mismas. No obstante, nuestro estudio en colaboración con la Dra. Y. Samuels del NIH-USA, supone el primero de carácter exhaustivo y sistemático en un número elevado de muestras (79) y acompañado de un análisis funcional. El tipo de tumor investigado fue el melanoma, en el que la base de datos COSMIC (*Catalogue Of Somatic Mutations In Cancer*, www.sanger.ac.uk/genetics/CGP/cosmic) y el trabajo precedente de Pleasance y colaboradores ya habían descrito algunas mutaciones en ADAMs (Pleasance y col., 2010). Las cifras obtenidas eran elocuentes: un 34% de los tumores analizados mostraba mutaciones en genes de esta familia y en concreto los dos genes más mutados, *ADAM7* y *ADAM29* estaban afectados en un 12,7% y 15,2%, respectivamente, del total de muestras estudiadas. Por ello, se seleccionaron estos dos genes para efectuar ensayos funcionales en modelos celulares. Resulta llamativo el hecho de que ambos genes codifican proteínas sin

actividad catalítica, al no conservar todos los residuos necesarios para la misma. Por este motivo, enfocamos los consiguientes ensayos funcionales a la evaluación de sus capacidades en procesos de adhesión y migración celular **(V)**. Los resultados mostraron que varias de las mutaciones identificadas en *ADAM7* disminuían la unión a sustratos habituales de la matriz extracelular como laminina y colágeno tipo IV en comparación con las células que expresaban la proteína normal. Además, la sobreexpresión de *ADAM7* producía una reducción en la migración, mientras que algunas de las mutaciones incrementaban este fenómeno. Estos datos sugerían, en conjunto, una posible pérdida de función para este gen. La expresión de *ADAM29*, por el contrario, provocaba una disminución de la adhesión, en tanto que las mutaciones ensayadas daban lugar, en términos generales, a un acrecentamiento de la misma. Tales resultados no hacen sino subrayar la necesidad de considerar individualmente a cada una de estas proteasas. Al mismo tiempo, resaltan la trascendencia de los modelos celulares que contribuyen a desvelar, a partir de conclusiones semejantes de los análisis mutacionales, consecuencias muy dispares a nivel funcional.

Las adamalisinas son, por lo tanto, una familia compleja de proteínas relevantes durante la progresión tumoral. En algunos casos, los datos obtenidos sugieren un papel pro-tumoral, mientras que en otros casos los resultados experimentales apuntan a una función protectora de estos enzimas. Esta dualidad no se presenta únicamente entre unos y otros miembros, sino que en ocasiones afecta a una misma proteína en función de, por ejemplo, su estado proteolítico. Los modelos celulares contribuyen sustancialmente a desentrañar el papel de cada una de ellas, pero para intentar profundizar en este aspecto, optamos por la generación de modelos murinos deficientes en las proteasas de interés. Así, procedimos a la obtención y caracterización de una colonia de ratones carentes de Adamts-12 **(VI)**.

Los animales generados fueron capaces de desarrollarse y reproducirse normalmente, por lo que esta metaloproteasa no sería vital para los mismos. Teniendo en cuenta los nexos previos entre ADAMTS-12, cáncer y angiogénesis, decidimos evaluar su relevancia en estos contextos. A través

de distintas aproximaciones, concluimos que la presencia de Adamts-12 se traducía en un efecto angioinhibidor y en una atenuación del crecimiento tumoral. Cabe destacar que en los modelos estudiados, la expresión de *Adamts12* acaecía no en las células tumorales sino en el tejido huésped y a continuación del trasplante de células malignas. En concreto, nuestros resultados mostraban expresión a cargo de los fibroblastos murinos, lo cual está en perfecta consonancia con los obtenidos previamente. Datos similares publicados recientemente también refuerzan la idea de que son los fibroblastos del estroma la fuente de esta metaloproteasa en respuesta a la progresión tumoral (Wang y col., 2011a). Por otro lado, el efecto protector de Adamts-12 frente a la excesiva angiogénesis era, aparentemente, exclusivo de las condiciones tumorales, pues su participación resultaba prescindible durante la angiogénesis fisiológica en el desarrollo de los ratones y en el modelo de vascularización de la coroides **(VI)**. Una interpretación acorde con los nuevos descubrimientos sería que la intervención de ADAMTS-12 tiene lugar, *in vivo*, bajo determinados estímulos propios de un ambiente como el tumoral. En estos contextos priman señales particulares como el TGFβ que estimularían la producción de esta proteasa. En los fibroblastos, este evento podría formar parte del proceso de activación para dar lugar a los llamados miofibroblastos, un tipo celular distintivo de situaciones reactivas como la cicatrización de heridas o la tumorigénesis, y que se caracteriza por la producción de actina de músculo liso, factores de crecimiento específicos y proteasas (Mueller y Fusenig, 2004; Allen y Louise Jones, 2011). De hecho, uno de los orígenes propuestos para este tipo celular es la activación de los fibroblastos normales en respuesta a señales como TGFβ (Kalluri y Zeisberg, 2006). En este sentido, cabe destacar que la expresión de *Adamts12* en los ratones silvestres se detectó en tejidos sometidos a remodelación constante, como por ejemplo las glándulas mamarias, el útero, el ovario o los nódulos linfáticos, en los que también estarían presentes fibroblastos activados **(VI)**.

Estos nuevos descubrimientos aportaron luz acerca de la función fisiológica de ADAMTS-12, aunque no sobre el mecanismo que la posibilita. Lo mismo que ha ocurrido con otras metaloproteasas, para las que la identificación de nuevos sustratos ha permitido explicar sus propiedades más

allá de la simple destrucción de la matriz extracelular (Overall y Blobel, 2007), es posible que ADAMTS-12 actúe digiriendo alguna molécula aún no identificada. Sin embargo, la actividad catalítica de esta proteasa resultó ser dispensable también en la inhibición de la angiogénesis. Una vez más, estos datos señalan a los otros dominios de la proteína, incluyendo los TSP-1, como los determinantes de sus acciones angioinhibidoras y anti-tumorales.

Tras evaluar el papel de ADAMTS-12 en la angiogénesis *in vivo*, nos propusimos emplear el mismo modelo murino para investigar su contribución a otros procesos, y más en concreto a la respuesta inflamatoria **(VII)**. Estos estudios nos han llevado a concluir que la ausencia de esta metaloproteasa se traduce en una respuesta inflamatoria incrementada. En este trabajo prestamos especial interés al modelo de colitis, pues los datos previos ya habían establecido una participación de ADAMTS-12 en este tejido. No obstante, la implicación de Adamts-12 en los ratones parece ser un fenómeno común a toda clase de procesos inflamatorios con independencia del tejido protagonista, pues se manifestó también en modelos de sepsis y pancreatitis. Las diferencias sintomatológicas entre los dos genotipos se reflejaron también en los estudios moleculares. A partir de muestras de colon de animales afectados y control, llevamos a cabo un análisis global de las diferencias en términos de expresión y de abundancia proteica. Estos estudios constituyen una herramienta adecuada para la búsqueda de rutas celulares alteradas o de posibles sustratos. Ambas aproximaciones sirvieron para confirmar la mayor inflamación en los animales deficientes en *Adamts12*, pues numerosas moléculas típicamente inflamatorias, como S100A8, S100A9 o IL-6, se hallaban elevadas en estos animales. Entre todas ellas, cabe destacar a las proteínas de unión a calcio S100A8 y S100A9, que se encontraban aumentadas tanto a nivel transcripcional como proteico y que son importantes marcadores de inflamación aguda y crónica (Gebhardt y col., 2006). Ambas proteínas interaccionan formando un heterodímero denominado calprotectina o calgranulina, cuya presencia en heces se utiliza hoy en día en la clínica como un marcador de enfermedades inflamatorias intestinales (Lewis, 2011).

Aunque estos datos refuerzan la observación de que la ausencia de Adamts-12 causa una intensificación de la inflamación, no aportan información adicional acerca del posible mecanismo molecular por el que actúa, pues serían también efecto y no causa de la misma. En este sentido, un indicio que nos ha acercado al mecanismo subyacente fue la marcada neutrofilia que mostraban los tejidos de ratones *Adamts12*$^{-/-}$ **(VII)**. La presencia prolongada o excesiva de neutrófilos puede explicar, al menos parcialmente, los síntomas observados, pues estas células son fuente de diversas moléculas que provocan daño tisular y promueven la inflamación (Kessenbrock y col., 2011; Lonkar y Dedon, 2011). En el patrón normal de inflamación local, estos leucocitos acudirían al punto afectado siguiendo señales quimiotácticas, se extravasarían a través de los vasos adyacentes y procederían a su desgranulación y a sus acciones citotóxicas. Finalmente, el proceso culminaría con la activación de rutas apoptóticas y la fagocitosis de los neutrófilos por parte de macrófagos. La vida de los neutrófilos está por lo tanto estrictamente regulada. Cuando se produce un retraso en su apoptosis o en su eliminación, se promueve la generación de una situación inflamatoria crónica, en la que sus acciones perdurarían en el tiempo (Filep y El Kebir, 2009). Con el propósito de evaluar estas posibilidades, efectuamos un estudio de los niveles de apoptosis en función de la presencia de ADAMTS-12 **(VII)**. Los resultados mostraron que la apoptosis se veía intensificada en presencia de la metaloproteasa. ¿Por qué vía podría actuar ADAMTS-12 para inducir apoptosis en los neutrófilos? Existen abundantes datos que describen la interacción de trombospondinas y de ADAMTSs con receptores de la superficie celular como CD36 y CD47 (Jimenez y col., 2000; Davis y col., 2009; Xing y col., 2009; Koch y col., 2011), que a su vez están ligados a fenómenos de muerte celular programada de neutrófilos y a su eliminación mediante macrófagos y monocitos (Lawrence y col., 2009; Mikolajczyk y col., 2009). Según esto, es posible que los dominios TSP-1 de ADAMTS-12 pudiesen interaccionar con alguna de estas moléculas promoviendo la apoptosis de neutrófilos y permitiendo con ello la resolución fisiológica de la respuesta inflamatoria. De manera llamativa, pudimos demostrar que los neutrófilos expresan ADAMTS-12 **(VII)**. En una primera aproximación, describimos que el uso de anticuerpos bloqueantes para CD36 revertía

discretamente la acción pro-apoptótica desencadenada por ADAMTS-12. Según esta hipótesis, la interacción entre CD36 y esta metaloproteasa podría ser responsable de los efectos pro-apoptóticos. Complementariamente, estudiamos la capacidad de cicatrización de heridas de los ratones deficientes en Adamts-12, con el hallazgo de que estos ratones experimentaban un retardo en el cierre de las mismas, así como en el desarrollo de nuevos anejos cutáneos (principalmente pelo y glándulas) **(VII)**.

A lo largo de todo el trabajo discutido, que supone la mayor parte de esta Tesis Doctoral, hemos procurado esclarecer la labor que desempeñan diferentes miembros de la familia de las adamalisinas, y especialmente de ADAMTS-12 y ADAMTS-15. Como resultado, hemos aportado nuevos datos sobre su papel fisiológico y patológico, con especial interés en el ámbito tumoral. Los datos recogidos describen la influencia de estos enzimas en la transición epitelio mesénquima, en la apoptosis, en la angiogénesis y en la digestión de componentes del cartílago (Figura 3).

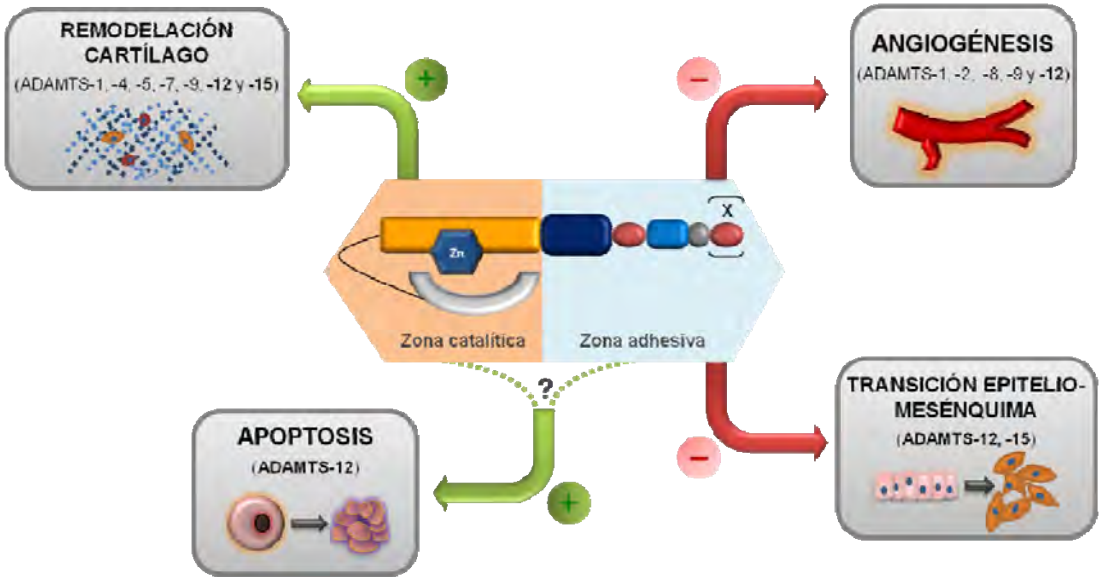

Figura 3. Esquema de procesos en los que participan ADAMTS-12 y ADAMTS-15. A través de sus dominios TSP-1, estas proteínas intervienen en la angiogénesis (ADAMTS-12) y en la transición epitelio mesénquima modulando la ruta Ras-MAPK (ADAMTS-12 y -15). En cuanto a su dominio catalítico, es capaz de degradar componentes del cartílago. Asimismo, ADAMTS-12 puede inducir apoptosis de células tumorales y de neutrófilos, aunque por un mecanismo todavía desconocido.

Los resultados obtenidos han servido para establecer un nexo entre ADAMTS-12 y ADAMTS-15 y la transición epitelio mesénquima, que como hemos visto se basaría en impedir la activación de la ruta Ras-MAPK. Asimismo, sabemos que ambos enzimas podrían estar implicados en los procesos de remodelación y degradación del cartílago a través de la digestión de agrecano (ADAMTS-12 y ADAMTS-15) y de la proteína oligomérica de la matriz del cartílago (COMP, ADAMTS-12) (Liu y col., 2006a). En el caso de ADAMTS-12, además, hemos asociado esta molécula con la angiogénesis y la apoptosis de células tumorales y neutrófilos. La nueva información disponible sobre ADAMTS-12 nos permite definirla como una proteasa con propiedades anti-tumorales. Por otro lado, su intervención en la respuesta inflamatoria, además de en el cáncer, podría ser relevante en afecciones inflamatorias. En conjunto, su presencia no resulta vital ni su pérdida de función provoca, por sí misma, enfermedad. Esto se debe posiblemente a fenómenos de compensación por proteínas funcionalmente próximas, como otras ADAMTSs, o al hecho de que su aportación biológica se da bajo ciertos estímulos y no constitutivamente. Su papel en el cáncer o en otras enfermedades habría pasado inadvertido debido a estos motivos y al hecho de que su expresión se produce no en las células tumorales sino en el estroma y porque la desregulación de su función es de origen fundamentalmente transcripcional y epigenético, y no mutacional.

Estos hallazgos, sumados a los trabajos recientes de otros grupos, nos llevan a postular que es posible que haya finalizado la era del descubrimiento de los oncogenes y genes supresores tumorales mutados en cáncer de manera recurrente y frecuente y sin embargo aún estamos lejos de comprender enteramente la causa de la mayoría de enfermedades. El reto científico actual conlleva encontrar la raíz de patologías monogénicas infrecuentes y de otras muchas habituales pero multifactoriales. Es en esta última clase en la que se incluirían el cáncer o las enfermedades inflamatorias. Muchas de estas circunstancias se caracterizan por patrones hereditarios y por lo tanto están regidas al menos en parte por un componente genético, que sólo conocemos mínimamente. En el cáncer colorrectal, por ejemplo, las mutaciones en los genes *APC*, *MLH1* y *MLH2*

únicamente explican un 5% del riesgo en familias afectadas (Houlston y Peto, 2004). El resto del riesgo, según el modelo multigénico, se debería a la combinación en un mismo genoma de varios alelos que contribuyen con pequeños incrementos, en ocasiones con un efecto directo y en otras en asociación con otras variantes y por lo tanto en función del contexto genómico completo. En la actualidad, ya no existen las barreras tecnológicas que imposibilitaban la identificación de estos genes contribuyentes de baja penetrancia, y así han surgido los estudios de asociación genómica a gran escala, que permitirán completar en gran parte los vacíos de explicación genética (Cazier y Tomlinson, 2010). Gracias a estos análisis, sabemos que existen variantes génicas con una implicación modesta pero en dos o más enfermedades de las que se desconocía podían compartir etiología (Frazer y col., 2009). Tal podría ser el caso de ADAMTS-12: este enzima intervendría discretamente en múltiples procesos, sin representar un papel esencial pero sí como integrante de ese riesgo compuesto. Esta situación sería especialmente patente en trastornos complejos y secuenciales como el cáncer (Figura 4). La contribución de los factores de susceptibilidad menores en la progresión tumoral o en cualquier otra patología no debe ser subestimada. Gracias a las nuevas técnicas, disponemos de un torrente constante de información, pero su utilidad depende del esfuerzo consiguiente para que pueda ser aplicada en la clínica. La medicina personalizada, para ser exitosa, reclama el manejo de muchos más datos simultáneamente, con el fin de optimizar los cálculos de predisposición, pronóstico y tratamiento para cada paciente.

Finalmente, y como parte de este proyecto de Tesis, intentamos acercarnos a la investigación terapéutica con la búsqueda de productos naturales con actividad inhibidora de agrecanasas **(VIII)**. Como ya mencionamos, las ADAMTSs con actividad agrecanasa, y principalmente ADAMTS-4 y ADAMTS-5, son las responsables primordiales de la degradación de agrecano en condiciones patológicas, por lo que ambos enzimas emergen como dianas idóneas en los tratamientos de artrosis y de artritis (Fosang y Little, 2008). Actualmente disponemos de potentes inhibidores de ADAMTSs como el ilomastat, si bien su actividad es

inespecífica, bloqueando la catálisis de otras metaloproteasas como las MMPs. Al igual que ocurrió en el cáncer, sería previsible que estos compuestos de amplio espectro fracasaran, al anular también la acción de proteasas beneficiosas como las MMPs encargadas de la remodelación no patológica del cartílago, y de ahí la trascendencia de obtener inhibidores que actúen selectivamente sobre las agrecanasas.

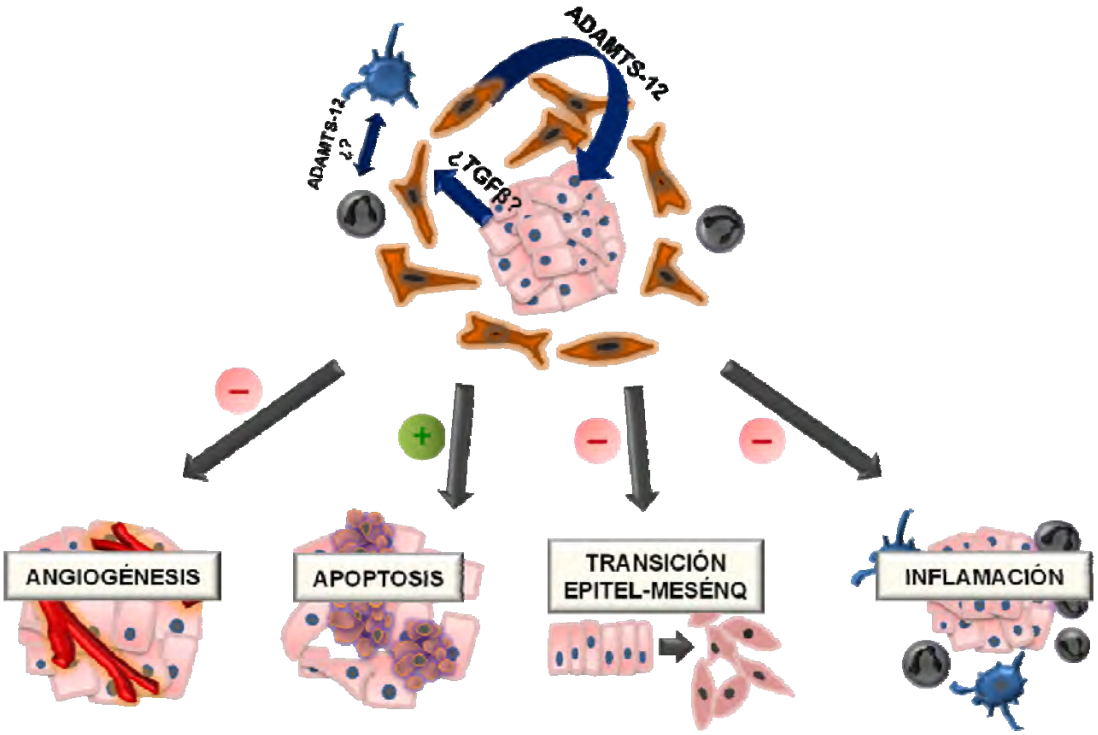

Figura 4. Modelo propuesto del papel de ADAMTS-12 en cáncer. ADAMTS-12 actuaría, potencialmente, como un supresor tumoral, controlando los procesos de inflamación, angiogénesis y los cambios que tienen lugar durante la transición epitelio mesénquima, además de promoviendo la apoptosis de las células tumorales. En el tumor, la fuente de ADAMTS-12 serían los fibroblastos activados (posiblemente estimulados por la presencia de TGFβ) y los leucocitos (neutrófilos y macrófagos).

En este sentido, hace aproximadamente una década se descubrió que algunos flavonoides tenían actividad inhibidora sobre agrecanasas (Demeule y col., 2000; Vankemmelbeke y col., 2003). Con el objetivo de identificar algún compuesto natural que bloqueara específicamente la acción de ADAMTS-4 y -5, llevamos a cabo un sondeo con numerosas moléculas de origen vegetal. De entre todas ellas, seleccionamos la luteolina para su posterior caracterización, pues era el compuesto que mostraba mayor efecto

sobre las agrecanasas y no sobre las MMPs **(VIII)**. Descubrimos que esta molécula era capaz de reducir notablemente la digestión de agrecano mediada por ADAMTSs pero no por MMPs, así como atenuaba la liberación de productos de degradación del cartílago de ratón a partir de cultivos *ex vivo* de este tejido. También describimos que la luteolina puede actuar a nivel transcripcional, disminuyendo la expresión de ambas agrecanasas. Estas actividades descritas serían además susceptibles de mejora mediante modificaciones farmacéuticas dirigidas (Cudic y col., 2009). Por otro lado, la presencia de este compuesto en alimentos habituales (Lopez-Lazaro, 2009) garantiza la viabilidad de administración oral y la ausencia de toxicidad, al menos a dosis bajas. En conjunto, la luteolina se perfila como un adecuada terapia adyuvante en el tratamiento de enfermedades articulares.

En resumen, en esta Tesis Doctoral hemos tratado de contribuir al mayor conocimiento del degradoma a través de la caracterización de diversos miembros de la familia de las adamalisinas. Hemos aportado los primeros datos funcionales de ADAMTS-12 y ADAMTS-15, que hasta ahora se incluían dentro de la categoría de ADAMTSs “huérfanas”, asignándoles propiedades antitumorales. Además, hemos descrito que los genes de otras metaloproteasas cercanas, *ADAM7* y *ADAM29*, están frecuentemente mutados en melanoma, y hemos propuesto que estas mutaciones podrían tener consecuencias opuestas funcionalmente. Los nuevos hallazgos resaltan la complejidad de estos enzimas y, en general del cáncer, que ha de ser concebido como un proceso multifactorial dinámico y consecuencia, no de la acción de elementos puntuales sino de su conjunción en sistemas. Asimismo, hemos mostrado que ADAMTS-12 podría intervenir en la respuesta inflamatoria. Por último, hemos definido la luteolina como un inhibidor específico de agrecanasas potencialmente útil en el tratamiento de las alteraciones degenerativas del cartílago.

CONCLUSIONES

1. La metaloproteasa ADAMTS-12 actúa como un supresor tumoral inhibiendo la transición epitelio mesénquima mediante bloqueo de la ruta Ras/MAPK y disminuyendo el crecimiento tumoral en ratones inmunodeficientes.

2. El gen *ADAMTS12* está silenciado en células tumorales de múltiples orígenes mediante hipermetilación de su promotor.

3. El estroma adyacente a las células tumorales de carcinomas colorrectales experimenta una inducción transcripcional de la expresión de *ADAMTS12*, que contribuye a limitar el crecimiento tumoral.

4. El gen *ADAMTS15* se comporta en cáncer de colon como un supresor tumoral sometido a inactivación por mutaciones.

5. Los genes de la familia ADAM se encuentran frecuentemente mutados en melanoma, especialmente *ADAM7* y *ADAM29*. Algunas de las mutaciones identificadas provocan cambios en la capacidad de adhesión y migración de las células tumorales de melanoma.

6. Los ratones deficientes en Adamts-12 son viables, fértiles y no manifiestan anomalías fisiológicas evidentes.

7. La ausencia de Adamts-12 en ratones incrementa la respuesta angiogénica e inflamatoria frente a diversos estímulos y favorece la progresión tumoral.

8. La luteolina inhibe específicamente la actividad agrecanasa de ADAMTS-4 y ADAMTS-5, afectando mínimamente a la de las MMPs.

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ANEXO: Autorizaciones