Agència de Gestió d'Ajuts Universitaris i de Recerca

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# Memòria justificativa de recerca de les beques predoctorals per a la formació de personal investigador (FI)

**La memòria justificativa consta de les dues parts que venen a continuació:**
**1.- Dades bàsiques i resums**
**2.- Memòria del treball (informe científic)**

Tots els camps són obligatoris

## 1.- Dades bàsiques i resums

**Títol del projecte** ha de sintetitzar la temàtica científica del vostre document.
Progresión tumoral y búsqueda de nuevas dianas terapéuticas en la familia de tumores del sarcoma de Ewing: función biológica de la caveolina-1

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**Paraules clau:** cal que esmenteu cinc conceptes que defineixin el contingut de la vostra memòria.
Biología molecular, Sarcoma de Ewing, Caveolina 1, Migración, Invasión, Metástasis

### Data de presentació de la justificació

31/01/2012

Nom i cognoms, i signatura del beneficiari/ària

Vist i plau del/de la director/a del projecte

**Resum en la llengua del projecte** (màxim 300 paraules)

El sarcoma de Ewing es el segundo tumor óseo infantil más frecuente y presenta una alta incidencia de enfermedad metastática. Este tipo de tumores presentan una traslocación génica característica que da origen a una proteína de fusión, normalmente EWS/FLI1. Esta proteína de fusión actúa como factor de transcripción aberrante regulando la expresión de diferentes genes implicados en la iniciación, mantenimiento y progresión del tumor. Nuestro grupo describió como uno de estos genes diana a la caveolina 1 (CAV1) describiendo además su papel determinante en el fenotipo maligno del sarcoma de Ewing, en la tumorigénesis y en la resistencia a apoptosis inducida por quimioterapia. Para investigar el papel concreto de CAV1 en el proceso metastático de este sarcoma, creamos un modelo de baja expresión de CAV1 en líneas celulares de sarcoma de Ewing y determinamos cambios en su capacidad migratoria, invasiva y metastática. En los ensayos in vitro hallamos una menor capacidad migratoria de las células knockdown de CAV1 y una reducción en la expresión de MMP9 y en la actividad de MMP2. La regulación de la actividad de MMP2 parece estar relacionada con la posible regulación que ejerce CAV1 en la función de MT1-MMP, proteína fundamental para la activación de MMP2. Por otro lado, en este estudio proponemos que CAV1 promueve la expresión de MMP9 tanto transcripcionalmente, regulando la vía de señalización ERK1/2, como a nivel post-transcripcional regulando la vía RSK1/rpS6. Además, en los ensayos de metástasis experimental in vivo las células knockdown de CAV1 presentaron una menor incidencia de metástasis pulmonar, hecho que correlacionó con una disminución en la expresión de SPARC, una proteína de adhesión importante en procesos metastáticos. En resumen, nuestros resultados evidencian la importancia de CAV1 en el proceso metastático del sarcoma de Ewing.

**Resum en anglès**(màxim 300 paraules)
Ewing's sarcoma is the second most common bone tumor in childhood and occurs with a high incidence of metastatic disease. Such tumors have a characteristic gene translocation that gives rise to a fusion protein, most commonly EWS/FLI1. This fusion protein acts as an aberrant transcription factor regulating the expression of different target genes involved in the initiation, maintenance and progression of the tumor. Our group described caveolin 1 (CAV1) as one of these target genes, describing its role in the malignant phenotype, tumorigenicity and resistance to chemotherapy-induced apoptosis of Ewing's sarcoma cell lines. To investigate the specific role of CAV1 in the metastatic process of this sarcoma, we produced a model of low expression of CAV1 in cell lines of Ewing's sarcoma. Then, we measured changes in their migratory capacity, invasiveness and metastatic potential. In vitro, we found a lower migratory capability of CAV1 knockdown cells and a reduction in MMP9 expression and MMP2 activity. The regulation of MMP2 activity seems to be related to the possible regulation that CAV1 exerts on the function of MT1-MMP, an essential protein for the activation of MMP2. On the other hand, we suggest that CAV1 promotes the expression of MMP9 both, transcriptionaly through a signaling pathway regulating ERK1/2, and post-transcriptionaly by regulating the pathway RSK1/rpS6. In addition, experimental metastasis assays in vivo showed that, CAV1 knockdown cells had a lower incidence of pulmonary metastasis, a fact that correlated with a decrease in the expression of SPARC, a major adhesion protein in metastatic processes. In summary, our results demonstrate the importance of CAV1 in the metastatic process on Ewing's sarcoma tumors.

**2.- Memòria del treball** (informe científic sense limitació de paraules). Pot incloure altres fitxers de qualsevol mena, no més grans de 10 MB cadascun d'ells.

## INTRODUCCIÓN

La familia de tumores de sarcoma de Ewing (ESFT), incluye un grupo heterogéneo de neoplasias formadas por células redondas de pequeño tamaño poco diferenciadas, o por células más grandes con diferentes grados de diferenciación neuroectodermal (Ewing, 1921). El sarcoma de Ewing es el segundo tumor óseo más frecuente, afectando fundamentalmente a niños y adolescentes. La frecuencia del sarcoma de Ewing es de 1-3 por millón y año en el hemisferio occidental, con una ligera predominancia en hombres. Puede afectar a cualquier hueso, con mayor incidencia en pelvis. Además un 15% de los casos aparecen en tejidos blandos, como riñón y vejiga entre otros. Actualmente el tratamiento consiste en una combinación de cirugía, radiación y quimioterapia. Sin embargo, la tasa de supervivencia sigue siendo baja, en pacientes con enfermedad localizada es de un 50% a los 5 años y se reduce mucho en pacientes con enfermedad metastática o con recaída temprana (Karosas, 2010). Lo que hace necesaria una mayor investigación en la búsqueda de dianas terapeúticas que posibiliten nuevos tratamientos más específicos.

Estos tumores están caracterizados por la presencia de una translocación cromosómica específica, en un 90 % de los casos, entre el gen *EWS* y un miembro de la familia de factores de transcripción *ETS*, fundamentalmente *FLI1.* La translocación más común, t (11; 22) (q24; q12), que implica el extremo N-terminal de EWS y el extremo C-terminal de FLI1, da como

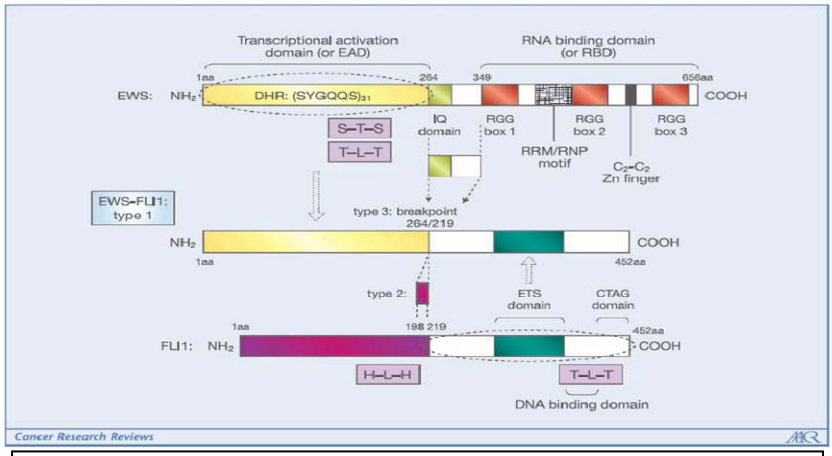


**Figura 1.** Traslocación t (11; 22) (q24; q12) que origina la proteína de fusión EWS/FLI1, responsable de la patogénesis del Sarcoma de Ewing. Hay tres tipos diferentes según los puntos de ruptura que se produzcan durante la traslocación, por

resultado la formación de la proteína de fusión EWS/FLI1 (Mackintosh, 2010) (Fig.1). Esta proteína de fusión se comporta como un factor de transcripción aberrante que contribuye a la patogénesis de ESFT mediante la modulación de la expresión de diferentes genes diana

(Delattre, 1992; Janknecht, 2005; Kauer, 2009; Hancock & Lessnick, 2008). De este modo EWS-FLI1 controla diferentes rutas características de la tumorigénesis y la progresión tumoral, como son promover la proliferación celular y supervivencia (activando, entre otros, a IGF1, MYC y NKX2.2) o evitar la inhibición del crecimiento, la senescencia y la apoptosis (inhibiendo p21, p57kip, TGFBR2 y IGFBP3). (Mackintosh, 2010)

Una característica importante de estos tumores es su gran capacidad para metastatizar, de hecho, aproximadamente un tercio de ellos se presenta con enfermedad metastática al momento del diagnóstico, lo que reduce la tasa de supervivencia al 30% en los pacientes con metástasis pulmonar y al 10% en pacientes con metástasis ósea o en médula ósea (Karosas, 2010). A pesar del gran número de genes diana de EWS/FLI1 descritos hasta la actualidad, se sabe muy poco acerca de cuáles de estos genes están implicados en el proceso metastático (Guan, 2008; Richter, 2009; Wang, 2010). Uno de estos genes fue descrito por nuestro grupo, la caveolina-1 (*CAV1*) (Tirado, 2006).

Las caveolas son invaginaciones en la membrana plasmática implicadas en diferentes procesos como endocitosis, exocitosis, homeostasis del colesterol y la regulación de diferentes vías de transducción de señales (Hansen & Nichols, 2010; Bastiani & Parton, 2010). Unos de los componentes estructurales fundamentales de estos microdominios de membrana son las caveolinas. Se conocen 3 miembros de esta familia, CAV1 y 2 que se expresan ubicuamente y caveolina-3 que solo se expresa en músculo (Smart, 1999). CAV1 es el único miembro de la familia que es imprescindible para la formación de las caveolas (Drab, 2001).Por otro lado, se ha descubierto recientemente el papel fundamental de otra familia de proteínas en la estructura y función de las caveolas, las proteínas cavin (Liu & Pilch, 2008). Las caveolas concentran un gran número de moléculas que juegan un papel importante en diferentes vías de señalización, como por ejemplo, Src tirosina kinasas, H-ras, subunidades de proteína G, isoformas de la proteína kinasa C, diversos receptores de tirosina kinasas, etc. (Parton & Simons, 2007). Cabe destacar que la mayoría de estas moléculas interactúan directamente con CAV1 a través de un dominio de unión a proteínas denominado "*caveolin-scaffolding domain* (CSD)" (Couet, 1997) (Fig.2).

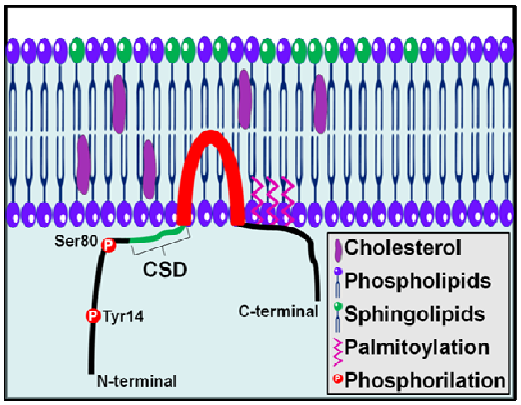


**Figura 2.** Estructura de la Caveolina 1. Se muestran los dos principales lugares de regulación por fosforilación, el *caveolin-scaffolding domain* (CSD) y las modificaciones por palmitoilación.

CAV1 y las caveolas, además de las funciones mencionadas, están implicadas en la migración celular, el control del ciclo celular, la polaridad celular y la transformación celular (Williams & Lisanti, 2005; Grande-García & del Pozo, 2008; Parton & Simons, 2007).

Tanto la pérdida como la sobreexpresión de CAV1 han sido relacionadas con el desarrollo tumorigénico y la progresión metastática

(Williams y Lisanti, 2005). En base a su baja expresión en diferentes tipos de tumores, tales como los de ovario, colon, pulmón y mama, se ha propuesto un papel supresor (Quest, 2008). Además, experimentos con células en cultivo y modelos animales han demostrado que la CAV1 funciona como un supresor de tumores en cáncer de mama (Williams, 2004). Por el contrario, niveles elevados de CAV1 han sido asociados con el aumento de la progresión tumoral y un incremento del potencial metastático en cáncer de próstata, esófago y vesícula (Burgermeister, 2008). Este aparente papel dual de la CAV1 en la progresión tumoral se podría explicar si se tiene en cuenta que CAV1 es una proteína *scaffolding*, por lo que se une a diferentes proteínas. La identidad de estas proteínas y el contexto celular sería el que determinaría el papel supresor o promotor de CAV1 en la progresión tumoral (Burgermeister, 2008) (Fig.3).

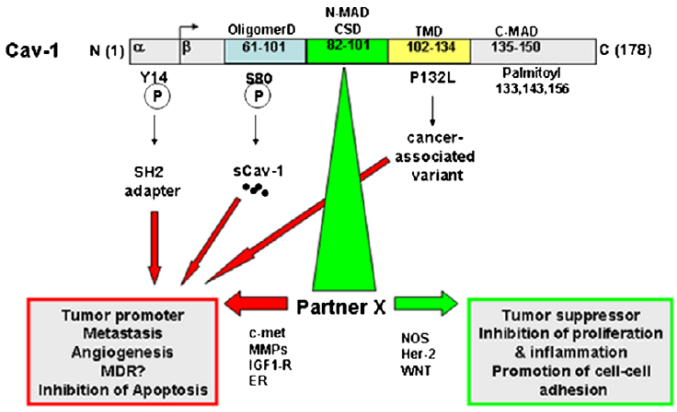


**Figura 3.** El papel dual de CAV1 en la progresión tumoral se podría explicar por las proteínas a las que se une por el CSD. Imagen extraída de Burgermeister et al, 2008.

## OBJETIVO

A CAV1 se le atribuyen funciones en migración celular y está asociada a metástasis en tumores como el melanoma (Fellicetti, 2009). Teniendo en cuenta que es una diana directa del oncogén EWS/FLI1 y que juega un papel importante en la metástasis (Tirado, 2006), CAV1 podría jugar un papel crucial en este proceso en el sarcoma de Ewing. Por lo que el objetivo de esta tesis es averiguar si CAV1 influye en el proceso metastático del sarcoma de Ewing, así como el mecanismo concreto por el que lo realiza.

## RESULTADOS

### CAV1 modula la capacidad del sarcoma de Ewing para metastatizar.

Con el objetivo de averiguar el papel de CAV1 en el proceso metastático del sarcoma de Ewing creamos un modelo *knockdown* por transfección estable con shRNA en dos líneas celulares de Sarcoma de Ewing (A673 y TC252) y caracterizamos su capacidad migratoria mediante ensayos *in vitro: wound-healing assay* y ensayo de migración en cámara de Boyden; e invasiva mediante ensayo de invasión en cámara de Boyden con matrigel. Los resultados mostraron una menor capacidad migratoria en las células con baja expresión de CAV1. Al realizar los experimentos de metastasis experimental *in vivo* en ratones inmunodeprimidos Balb/c, estas células también tuvieron una menor capacidad de colonizar los pulmones, tejido principal de metástasis en el sarcoma de Ewing. Lo que evidenciaría la importancia de la CAV1 en este proceso.

Las metaloproteinasas (MMPs) son enzimas proteolíticas importantes en los procesos de migración, invasión y metástasis, por lo que buscamos alguna relación entre éstas y CAV1. Mediante zymograma de gelatina, observamos que las líneas con baja expresión de CAV1 presentan una menor cantidad de MMP9 y menor activación de MMP2, sugiriendo que la expresión de CAV1 es necesaria para la producción de MMP9 y la activación de MMP2. Estos datos podrían explicar, al menos en parte, la menor capacidad migratoria de las células con baja expresión de CAV1. Obtuvimos el mismo resultado al repetir el experimento de invasión con las células parentales tratadas con un inhibidor de MMP2 y 9. Para elucidar si el efecto de CAV1 era sobre la secrección de MMP9 o sobre su expresión, comprobamos por RT-PCR que en los clones con baja expresión de CAV1 disminuía la cantidad de mRNA de MMP9, sugiriendo una regulación a nivel transcripcional. Respecto a la activación de la MMP2, hay dos responsables que podrían estar siendo regulados por CAV1, el inhibidor de MMPs TIMP-2 y la metaloproteinasa de membrana MT1-MMP (MMP14). Ninguna presentó niveles de expresión alterados en las células con baja expresión de CAV1, pero por microscopía confocal observamos un cambio en la distribución de la MT1-MMP, por lo que CAV1 podría estar implicada en el transporte y localización de ésta a la membrana plasmática y, por lo tanto, en su función.

La colonización del pulmón por parte de las células metastáticas se explica por una serie de genes de virulencia metastática, entre los que destaca SPARC (*secreted protein acidic and rich in cysteine*), cuyos niveles de expresión se encuentran reducidos en las líneas con baja expresión de CAV1, lo que sugiere una regulación indirecta a nivel transcripcional por parte de CAV1. Previamente, en el grupo se había establecido un modelo de inhibición de SPARC por shRNA en la línea A673 que utilizamos para comprobar el efecto de esta proteína en la migración, invasión y metástasis. Los experimentos de migración e invasión *in vitro* demostraron una capacidad migratoria e invasiva menor por parte de las células con

baja expresión de SPARC, sin embargo el efecto era menor que en el caso de las células con bajos niveles de CAV1. Además, las células con bajos niveles de SPARC fueron incapaces de invadir los pulmones en los experimentos *in vivo*. También comprobamos el efecto de SPARC sobre las MMPs por zymografía, del mismo modo que con CAV1, la activación de MMP2 se redujo en las células tranfectadas con el shRNA anti-SPARC, sin embargo, los niveles de MMP9 no estaban alterados.

Todos estos resultados han quedado reflejados en una publicación de la que la estudiante es co-autora (Sainz-Jaspeado, 2010). (Ver anexo).

**Establecimiento de un nuevo modelo de baja expresión de CAV1.**

En las diferentes líneas celulares establecidas de sarcoma de Ewing encontramos dos tipos diferentes de migración: colectiva, que es la que presentan las líneas utilizadas en el estudio anterior, e individual. Por este motivo, decidimos establecer el modelo de inhibición de CAV1 por shRNA también en una línea con este último tipo de migración, A4573. En este modelo observamos también un efecto inhibidor de la capacidad migratoria e invasiva *in vitro* al inhibir la expresión de CAV1, pero el efecto era menor que en los otros modelos establecidos. Lo que sugeriría que el mecanismo por el que la CAV1 interfiere en estos procesos celulares podría diferir de una línea a otra.

**Mecanismo de regulación de MMP9 por CAV1.**

Una vez descrita la implicación de CAV1 en la expresión de MMP9, el objetivo principal es describir el mecanismo concreto. La relación de la vía de las MAPK con la expresión de MMP9 y la regulación de la invasión ha sido sugerida con anterioridad (Beliveau, 2010). Esta vía resulta una buena candidata, ya que la línea A673 contiene la mutación activadora V599E de BRAF (Davies, 2002) por lo que esta ruta está alterada. De hecho, la línea A673 presenta una mayor fosforilación de ERK1/2 (*RAS/extracellular signal regulated kinase 1 y 2*). Independientemente de la presencia de la mutación, la fosoforilación de ERK1/2 se reduce al inhibir la expresión de CAV1 en diferentes líneas de Sarcoma de Ewing (A673 y TC252).

Se ha descrito que la regulación de MMP9 por ERK1/2 podría ser por medio del factor de transcripción NFkB (Li, 2010), por lo que comprobamos el efecto de la falta de CAV1 en la activación de este factor. El tratamiento del modelo de A673 con TNFα (*Tumor Necrosis Factor α*) provoca su activación y traslocación al núcleo, sin embargo, no encontramos diferencias significativas entre los clones de baja expresión de CAV1 y los controles, lo que sugiere que la ruta CAV1➔ERK1/2➔NFkB no está implicada en la regulación de la expresión de MMP9.

Comparando el perfil proteico de los modelos A673 y A4573 mediante un array de anticuerpos (*PathScan® RTK Signaling Antibody Array Kit.* Cell Signalling) observamos que la

activación del receptor RET (*REarranged during Transfection*) y de la proteína ribosomal S6 se reducían en ausencia de expresión de CAV1. Estos resultados fueron validados por inmunoprecipitación en el caso de RET, y por western blot en el caso de rpS6. La fosforilación de rp6S puede producirse por parte de la p90 ribosomal S6 kinasa-1 (RSK1) en respuesta a la activación de la vía Ras/MAPK (Roux, 2007). Todas las líneas de sarcoma de Ewing comprobadas por western blot expresan RSK1. Por lo que CAV1 podría estar regulando esta vía, ya que observamos pérdida de fosforilación tanto de ERK1/2 como de rpS6 en los clones *knockdown* de CAV1. Además, al utilizar un inhibidor específico (U0126) de MEK, el principal activador de ERK1/2, observamos una reducción tanto en la fosforilación de RSK1 como de rpS6. En este experimento también detectamos una pérdida de expresión de MMP9 a nivel transcripcional y comprobamos la consecuente disminución de la actividad gelatinasa por zymograma. Todo esto parece indicar que ésta es la vía por la cúal CAV1 regularía la expresión de MMP9.

**Implicación de la Tyr14 en el papel pro-metastático de CAV1.**

La fosforilación de CAV1 en la tyr14 genera un lugar de unión a proteínas con dominio SH2 implicadas en la estimulación de la proliferación celular y está relacionada con la regulación de la dinámica de las adhesiones focales, proceso muy importante para la migración celular (Joshi, 2008). Por esto, quisimos comprobar cuáles de los efectos sobre la capacidad metastática que observamos al inhibir la expresión de CAV1 son dependientes de esta fosforilación. Para ello producimos un mutante Y14F (pCDNA-CAV1-Y14F) que está descrito que actúa como dominante negativo interfiriendo con las funciones normales de CAV1 endógena y que atenúa la invasión de la matriz extracelular y la actividad de las metaloproteinasas en células H1299 de cancer pulmonar (Shatz, 2010). Transfectamos con este plásmido la línea A673, obteniendo tres clones en los que detectamos mayor presencia de CAV1 exógena. Al realizar el experimento de migración *in vitro* mediante cámara de Boyden observamos una reducción en la capacidad migratoria comparable a la obtenida en el modelo de baja expresión de CAV1. Sin embargo, no detectamos ni disminución en la fosforilación de ERK1/2 ni reducción en la actividad de MMP9 por zymografía o reducción de la expresión a nivel transcripcional por RT-PCR.

**Quimiotaxis por SDF1, papel del eje CXCR4/SDF1.**

SDF1 (*Stromal Derived Factor 1*) o CXCL12 es una quimiokina que se une al receptor asociado a proteína-G CXCR4, lo que produce la activación de una cascada de señalización que da lugar a procesos de regulación de la transcripción, supervivencia celular, proliferación y quimiotaxis. Esta vía ha sido relacionada con diferentes aspectos de la progresión tumoral como angiogénesis, metástasis y supervivencia (Teicher & Fricker, 2010). Los principales lugares de metástasis del sarcoma de Ewing son los pulmones y la médula ósea y en ambos

tejidos se ha descrito la expresión de SDF1. Además, se ha relacionado a CXCR4 con metástasis en este sarcoma (Bennani-Baiti, 2010) y en cáncer de mama se ha descrito la relación de este receptor con CAV1 (Altenburg & Siddiqui, 2009). Por lo que nuestro grupo intenta profundizar en esta interacción y su implicación en la colonización de tejidos como pulmón y médula ósea durante el proceso metastático de sarcoma de Ewing.

Tras comprobar los niveles de expresión de ligando y receptor en diferentes líneas celulares, realizamos experimentos de exposición a SDF1 en ausencia de FBS (suero fetal bovino), sin embargo, los resultados no han sido concluyentes, por lo que, de momento hemos dejado esta línea de investigación.

## DISCUSIÓN Y EXPERIMENTOS PREVISTOS

**CAV1 modula la capacidad del sarcoma de Ewing para metastatizar.**

Las metaloproteinasas están implicadas en la remodelación de la matriz extracelular y han sido relacionadas con metástasis en diversos estudios (revisado en Yilmaz & Christofori, 2010; Chiang & Massagué, 2008). La MMP2 y la MMP9 han sido relacionadas con la agresividad en diferentes tumores, incluidos los sarcomas (Benassi, 2001). Nuestros datos revelan que CAV1 regula estas dos MMPs de manera diferencial. Las MMPs pueden estar reguladas a diferentes niveles: transcripcional, post-transcripcional o a nivel de la activación del pro-enzima (Clark, 2008). En nuestro modelo, CAV1 parece regular la transcripción de MMP9 de un modo indirecto, es decir, CAV1 regula una ruta de señalización, que es el objeto de estudio de esta tesis, que está afectando a la expresión de MMP9.

Los resultados en nuestro modelo muestran además que CAV1 regula la activación de MMP2. En este proceso de activación, TIMP2 se une a pro-MMP2 y a MT-MMP1 permitiendo que otra molécula de MT1-MMP active catalíticamente a MMP2 (Kessenbrock, 2010). Nuestros resultados no parecen indicar una regulación de CAV1 sobre TIMP-2, al menos a nivel transcripcional, sin embargo, encontramos cierto grado de co-localización de CAV1 y MT1-MMP y un cambio de la distribución celular de esta última en ausencia de CAV1. Esto sugiere que la regulación de la activación de MMP2 por parte de CAV1 es por medio de la regulación de la actividad de MT1-MMP. Esto concordaría con un estudio previo donde CAV1 ha sido relacionada con MT1-MMP y con la formación de invadopodia y degradación de la matriz extracelular en cáncer de mama (Yamaguchi, 2009). De nuestros resultados podemos deducir que CAV1 promueve la invasión de las células de sarcoma de Ewing mediante la regulación de la producción de MMP9 y la activación de MMP2.

Uno de los principales factores de mal prognosis es la presencia de enfermedad metastática. Aproximadamente un 30% de los pacientes de sarcoma de Ewing presentan metástasis detectables al momento del diagnóstico, siendo el tejido más común el pulmón, con un 50% de los casos (Balamuth, 2010). Se ha descrito que la especificidad de tejido de las metástasis de los diferentes tipos de tumores puede estar determinado por la expresión de una serie de genes concretos que promueven la selección de las células metastáticas (Nguyen, 2009). En el caso del sarcoma de Ewing no hay datos sobre genes que determinen la metástasis pulmonar, pero en el caso de cancer de mama se ha descrito SPARC como uno de esos genes determinantes (Minn, 2005). En nuestro modelo encontramos que CAV1 está implicada en la regulación de la expresión de SPARC. Al hacer el modelo *knockdown* de SPARC observamos una reducción de la capacidad migratoria e invasiva, pero el efecto era menor que en el caso del modelo de baja expresión de CAV1, lo que podría explicarse por la mayor producción de MMP9 de las células *knockdown* de SPARC respecto a las células *knockdown* de CAV1. Estos resultados sugerirían que CAV1 influye en la migración e

invasión por otros mecanismos diferentes a la regulación de SPARC. En los experimentos de metástasis experimental realizados con el modelo *knockdown* de SPARC, no se observó colonización de pulmón por ninguno de los clones de baja expresión, lo que pondría de manifiesto la importancia de esta proteína de adhesión para el establecimiento de las células metastáticas en este tejido. El mecanismo por el cual SPARC ejerce este efecto no está determinado, por lo que se necesita profundizar más en la investigación en este ámbito.

**Mecanismo de regulación de MMP9 por CAV1**

Una vez descartada la vía de NFkB como posible regulador transcripcional de MMP9, al no encontrar diferencias significativas en su activación por TNFα entre los clones con baja expresión de CAV1 y los controles, realizamos un *screening* de receptores Tyr-kinasa y de algunas proteínas importantes de señalización para averiguar si la falta de CAV1 afectaba a su fosforilación. Efectuamos un array de anticuerpos con diferentes modelos de baja expresión de CAV1 en condiciones basales y los resultados más destacables fueron que el receptor RET y la proteína ribosomal S6 estaban menos fosforilados en ausencia de CAV1.

El receptor RET fue uno de los primeros receptores Tyr-kinasa de los que se descubrió un papel en la neoplasia. Mutaciones en este proto-oncogen están asociadas al cancer medular tiroideo. La activación de RET precisa de la formación de un complejo multimérico con un receptor tipo a de la familia GDNF (*glial cell line–derived neurotrophic factor*) y uno de sus ligandos GFL (*GDNF family ligands*). Estos complejos se forman en los *lipids rafts*, microdominios de membrana ricos en esterol y esfingolípidos cuya regulación está asociada a CAV1. La activación de RET crea lugares de unión a los que se unen diferentes proteínas (SHC, IRS1/2, FRS2, PKCα) capaces de activar diferentes vías de transducción de señales, entre ellas la de ERK1/2 (revisado en Phay, 2010). Además, RET presenta un motivo de unión a CAV1 en su dominio tyr-kinasa intracelular y se expresa en diferentes líneas de Sarcoma de Ewing, lo que le hace un buen candidato a ser uno de los receptores que activa la vía por la que CAV1 regula la expresión de MMP9.

La proteína ribosomal S6 (rpS6) forma parte de la subunidad ribosomal 40S y está descrita como el principal sustrato de S6K1 (proteina ribosomal S6 kinasa-1), que es una de las dianas de mTOR. Lo que sugiere que esta vía podría estar implicada en la regulación de la expresión de MMP9. Sin embargo, no encontramos alterados los niveles de p4E-BP1, la otra diana principal de mTOR, lo que descarta esta vía. Se ha

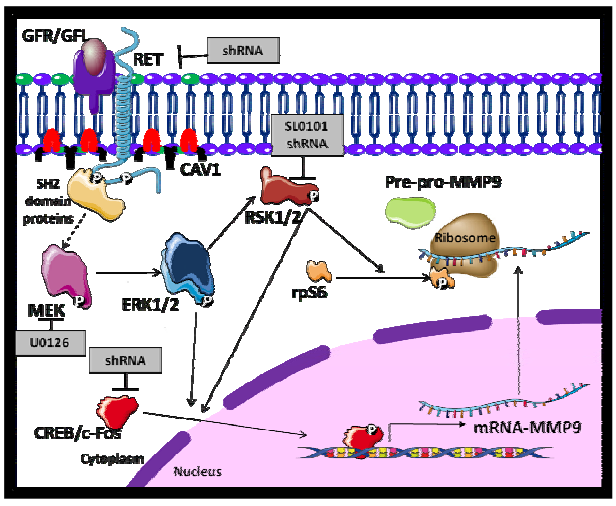


**Figura.4** Posible vía de regulación de MMP9 y métodos de inhibición sugeridos.

descrito la fosforilación de rp6S por parte de la p90 ribosomal S6 kinasa-1 (RSK1) en la Ser235/236 en respuesta a la activación de la vía Ras/MAPK. Esta fosforilación se asocia a la regulación de la afinidad de rpS6 al complejo 7-metilguanosin 5' del mRNA, lo que contribuye al ensamblaje del complejo de iniciación de la traducción (Roux, 2007). Según nuestros resultados esta parece ser la vía de regulación de MMP9 por parte de CAV1, ya que al inhibir específicamente la activación de ERK1/2, se reproducen la pérdida de fosforilación de estas proteínas y la reducción de la expresión de MMP) observadas en el modelo *knockdown* de CAV1.

Otro de los sustratos de RSK1 es el factor de transcripción CREB (Anjum & Blenis, 2008), del que se ha demostrado su participación en la regulación de la expresión de MMP9, por lo que comprobamos la expresión de CREB en las líneas de sarcoma de Ewing y su fosforilación. A pesar de estar presente en las líneas analizadas, no observamos cambios en la fosoforilación en los clones de baja expresión de CAV1, por lo que no parece ser éste el factor de transcripción implicado. Otro factor implicado en la transcripción de MMP9 es c-fos (Crowe & Brown, 1999). Este factor puede ser activado por medio de ERK1/2 (O'Donell, 2012), lo que lo convierte en un buen candidato, por lo que analizaremos su expresión y fosforilación en las líneas de Sarcoma de Ewing y en los modelos *knockdown* de CAV1 establecidos.

Así pues, creemos que CAV1 puede estar afectando a MMP9 a diferentes niveles: por medio de RSK a nivel traduccional mediante la regulación de la activación de rpS6 y/o a nivel transcripcional por medio de ERK1/2 que activaría a un factor de transcripción de MMP9 como puede ser c-fos.

Por lo tanto, la linea principal de esta tesis es continuar dilucidando los diferentes pasos de esta posible vía (Fig.4), y la implicación de CAV1, mediante la inhibición de cada uno de los componentes por inhibidores químicos específicos o por inhibición de su expresión por shRNA (Fig.4).

**Implicación de la Tyr14 en el papel pro-metastático de CAV1.**

La fosforilación de CAV1 en la tyrosina 14 (Tyr14) ha sido identificada como regulador de la actividad de Rho durante el proceso migración direccional (Grande-Garcia, 2007). Además estabiliza a FAK (*Focal Adhesion Kinase*) dentro de las adhesiones focales (FA), limitando el intercambio de FAK entre las FAs y el citosol, lo que lleva al reclutamiento de efectores que promueven la renovación de las Fas, favoreciendo así la migración celular (Goetz, 2008).

En nuestro modelo, quisimos averiguar el papel concreto que ejercía esta fosforilación en el efecto de la CAV1 observado en los procesos de migración, invasión y metástasis. Para ello establecimos un modelo con la forma mutada no fosforilable de CAV1. De acuerdo con los resultados obtenidos hasta ahora, la Tyr14 es esencial en el papel de CAV1 como

promotora de la migración celular. Sin embargo, no detectamos una reducción ni de la fosforilación de ERK1/2 ni de la actividad metaloproteinasa, lo que sugeriría que el papel de esta Tyr en el proceso de degradación de la matriz no es esencial. Estos resultados aún deben ser verificados por otros métodos como el ensayo de invasión en cámara de Boyden con matrigel, además de comprobar el efecto *in vivo* de esta mutación no fosforilable en el modelo de metástasis experimental.

**Modelo de metástasis espontáneas *in vivo***.

Para poder validar los efectos observados in vitro queremos establecer un modelo de metástasis espontánea en ratones inmunodeprimidos Balb/c para corroborar los efectos de la falta de CAV1 en el proceso metastático del sarcoma de Ewing. Con el fin de ser poco invasivos y mantener con vida al animal, transfectaremos establemente las células con un vector luciferasa [enhanced luc2(pGL4)] y estudiaremos el desarrollo tumoral y su progresión a metástasis mediante el sistema de imagen IVISLumina XG disponible en nuestra institución. Utilizaremos 2 aproximaciones:

a) Inyección subcutánea de $5x10^6$células. Esta aproximación está en curso, aunque sin estar las células transfectadas por luciferasa, por lo que no podemos hacer el seguimiento de la progresión metastática

b) Inyección ortotópica en costilla de $0.5x10^6$ células; esta aproximación ha sido demostrada por el grupo de la Dra. Andrea Hayes-Jordan (MD Anderson Cancer Center, Houston). En la actualidad hemos iniciado una colaboración con la Dra. Hayes-Jordan para que la estudiante aprenda la metodología quirúrgica necesaria en su laboratorio (pendiente de financiación BE-DGR 2011). Con esta aproximación se consigue una incidencia del 40% de metástasis a pulmón (Wang, 2010) y la utilizaremos en caso de que nuestra aproximación por inyección subcutánea no produjese metástasis espontáneas como prevemos.

## CONCLUSIONES

1. La inhibición de la expresión de CAV1 reduce la migración y la capacidad invasiva de las lineas de sarcoma de Ewing *in vitro*.

2. La inhibición de la expresión de CAV1 reduce y retrasa la formación de metástasis pulmonar en el modelo experimental.

3. CAV1 participa en la regulación de la expresión de MMP9 y en la activación de MMP2, dos proteínas importantes en el proceso invasivo.

4. SPARC actúa *downstream* CAV1 para regular la capacidad invasiva de las células de sarcoma de Ewing.

5. La regulación transcripcional de MMP9 por parte de CAV1 no se realiza a través del factor de transcripición NFkB.

6. Hay indicios de que la regulación de MMP9 por CAV1 se produzca mediante el mecanismo descrito.

## PUBLICACIONES

Parte de los resultados aquí expuestos han sido publicados en revistas científicas internacionales gracias a la financiación recibida por la estudiante predoctoral durante estos tres años.

Ver ANEXO

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## ANEXO

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*Angiogenesis, Metastasis, and the Cellular Microenvironment*

Molecular Cancer Research

# Caveolin-1 Modulates the Ability of Ewing's Sarcoma to Metastasize

Miguel Sáinz-Jaspeado[1], Laura Lagares-Tena[1], Jaime Lasheras[1], Fariba Navid[3], Carlos Rodriguez-Galindo[3], Silvia Mateo-Lozano[2], Vicente Notario[4], Xavier Sanjuan[1], Xavier Garcia del Muro[1], Àngels Fabra[1], and Oscar M. Tirado[1]

## Abstract

Metastasis is the final stage of tumor progression and is thought to be responsible for up to 90% of deaths associated with solid tumors. Caveolin-1 (CAV1) regulates multiple cancer-associated processes related to malignant tumor progression. In the present study, we tested the hypothesis that CAV1 modulates the metastatic ability of cells from the Ewing's sarcoma family of tumors (ESFT). First, we analyzed the expression of CAV1 by immunostaining a tissue microarray containing 43 paraffin-embedded ESFT tumors with known EWS translocations. Even though no evidence was found for a significant association between CAV1 expression and stage, size or tumor site, all metastatic samples (10 of 10) had significantly high CAV1 expression, suggesting that high CAV1 content could positively contribute to enhance ESFT metastasis. To determine the effect of CAV1 on the migratory and invasive capabilities of ESFT cells, we knocked down CAV1 expression in TC252 and A673 cells by stably transfecting a previously validated shRNA construct. *In vitro*, migration and invasion assays showed that for both cell lines, CAV1 knocked-down cells migrated and invaded significantly less ($P \leq 0.01$) than control cells. Moreover, control A673 cells introduced into BALB/c nude mice by tail vein injection strongly colonized the lungs. In contrast, animals injected with CAV1 knocked-down cells showed either no incidence of metastasis or developed lung metastases after a significant delay ($P < 0.0001$). Finally, we show that the molecular mechanisms by which CAV1 carries out its key role in regulating ESFT metastasis involve matrix metalloproteinase production and activation as well as the control of the expression of SPARC, a known determinant of lung colonization. *Mol Cancer Res; 8(11); OF1–12. ©2010 AACR.*

## Introduction

Metastasis is the final stage in tumor progression and is thought to be responsible for up to 90% of deaths associated with solid tumors. This multifaceted process consists of a series of steps whereby cancer cells enter the circulation, disseminate to distal capillary beds, enter a parenchyma by extravasation, adapt to the new host microenvironment, and eventually grow into lethal tumor colonies in distal organs (1). Much has been learned about the processes that initiate and sustain general tumor growth; however, the mechanisms that enable metastasis remain largely unknown.

The Ewing's sarcoma family of tumors (ESFT) includes aggressive bone-associated malignancies that affect the pediatric population. ESFT are characterized by early metastases, and metastatic spread is commonly hematogenous. Nearly all patients with ESFT already have micrometastases at diagnosis, resulting in a >95% relapse rate when treated locally and a 40% relapse rate after systemic chemotherapy. Therefore, knowledge of key regulators of circulating or dormant metastatic tumor cells after traditional therapy is needed. Most ESFT harbor a reciprocal translocation, t(11;22)(q24;q12), which links a strong transcriptional activation domain from the EWS to the ETS DNA-binding domain of FLI-1 (2). The *EWS/FLI-1* fusion is required for Ewing's sarcoma oncogenesis, as inhibition of its function results in the loss of transformation of ESFT cells (3-5). Thus, enhancing our current knowledge of EWS/FLI-1 function is critical to understanding ESFT development.

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Caveolin-1 (CAV1) was previously identified as a metastasis-associated gene that is a transcriptional target of EWS/FLI-1 as well as an important determinant of ESFT malignant phenotype, tumorigenicity, and resistance to chemotherapy-induced apoptosis of ESFT cells (6, 7). CAV1 is a multifunctional scaffolding protein with multiple binding partners that associates with cell surface caveolae. CAV1 regulates multiple cancer-associated processes including cellular transformation, tumor growth, cell death and survival, multidrug resistance, angiogenesis, cell migration, and metastasis (8). Clinical studies have revealed that CAV1 upregulation is associated with poor prognosis and the occurrence of metastasis in several human cancers (9). Moreover, it was recently reported that CAV1 regulates cell migration and invasion in human breast cancer cells (10).

A better understanding of the complex biology of ESFT may lead to the successful development of biologically targeted therapies. As the regulatory pathways responsible for transformation, growth, and metastasis of ESFT become well defined, the efficacy of therapeutic intervention will improve. Therefore, because the possible involvement of CAV1 in the ability of ESFT to metastasize has not been established to date, we tested the hypothesis that CAV1 modulates the metastatic ability of ESFT cells. Our results show that CAV1 controls migration and invasion in ESFT cells in culture by mechanisms involving the production and activation of metalloproteinases as well as lung colonization in nude mice by regulating SPARC expression levels. These data conclusively show that CAV1 plays a key role in ESFT metastasis.

## Materials and Methods

### Tissue samples

Forty-three tumor samples were procured from the archives of the St. Jude Children's Research Hospital Pathology Department and referring institutions. A minimum of three representative cores from ESFT tumors were used to construct a tissue microarray (TMA) using a manual arrayer (Beecher Instruments). All tumor samples were fixed in formalin, embedded in paraffin, and had not undergone decalcification. Cores from various normal paraffin-embedded tissues were scattered in each of the TMA blocks as controls. Samples from two patients in the TMA were lost during immunohistochemical procedures; therefore, Table 1 only shows results from 41 patients.

### Immunohistochemistry

Expression of CAV1 in human Ewing's sarcoma and lung metastasis specimens was analyzed using immunohistochemical techniques done essentially as previously described (6, 7). CAV1 was detected with a 1:2,000 dilution of a rabbit polyclonal antibody (from BD Biosciences). Protein expression levels were coded as 0, 1 (low expression), or 2, and 3 (high expression) according to the level of staining (Fig. 1). Matrix metalloproteinase 9 (MMP9) was detected with a 1:100 dilution of a mouse monoclonal antibody (Abcam).

### Cell culture and stable transfections

A673 and TC252 cell lines (gifts from Dr. Heinrik Kovar, Children's Cancer Research Institute, Kindrespitalgasse, Vienna, Austria) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). All cell lines were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Exponentially growing cells within two sequential passages were used for all experiments. Cells were transfected using Fugene (Roche) following the protocols of the manufacturer. Transfected cells were selected with neomycin (0.4 mg/mL, ShCAV1; Invitrogen) and with puromycin (0.5 μg/mL, ShSPARC; Sigma; ref. 11) for 14 days, and antibiotic-resistant pools and individual colonies were isolated for further analysis and maintained in the presence of neomycin (0.2 mg/mL) and puromycin (0.1 μg/mL), respectively.

### Reverse transcription-PCR

Total RNA (3 μg), extracted using the RNeasy Mini Kit (Qiagen), was used for cDNA synthesis with SuperScript II Reverse Transcriptase (Invitrogen). Amplifications of SPARC, MMP2, MMP9, TIMP2, and β-actin were carried out using specific primers (Supplementary Table) designed using the Oligo 6.0 software (National Bioscience). For each set of primers, the number of cycles was adjusted so that the reaction end points fell within the exponential phase of product amplification, thus providing a semiquantitative estimate of relative mRNA abundance. Reverse transcription-PCR determinations were carried out at least twice for each relevant transcript.

### Western blot

ESFT cells were lysed with radioimmunoprecipitation assay buffer containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL aprotinin, and 10 mg/mL leupeptin) and the lysates were centrifuged at 13,000 × *g*, at 4°C, for 30 minutes. The protein content of the supernatants was determined with the BCA assay system (Pierce). Lysate aliquots (50 μg) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% skimmed milk in PBS containing 0.2% Tween 20 at room temperature for 1 hour, membranes were incubated overnight at 4°C with the appropriate primary antibody (CAV1 from BD, SPARC from Abcam, and FLI-1 from Santa Cruz Biotechnology). Blots were then incubated at room temperature for 1 hour with a horseradish peroxidase–conjugated secondary antibody (1:2,000) and the peroxidase activity was detected by enhanced chemiluminescence (Pierce) following the instructions of the manufacturer. Immunodetection of β-actin was used as a loading reference.

### Migration assays

***Wound-healing assay.*** Cells cultured to 95% confluence were scratched with a 10-μL pipette tip. Wounds were

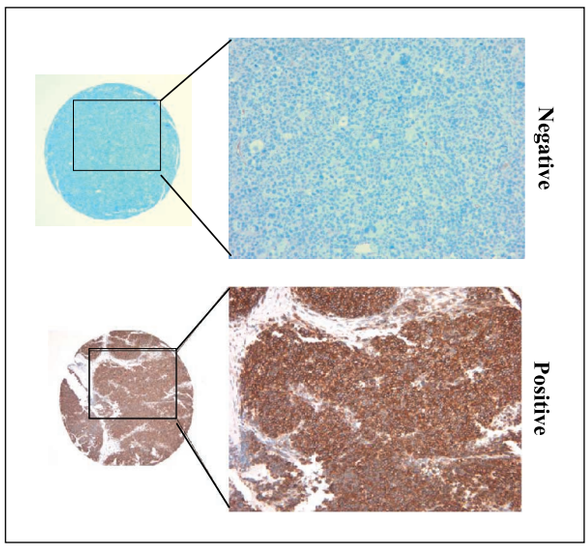


**FIGURE 1.** Example of negative and positive CAV1 staining of a human ESFT patient sample from the TMA (magnification, ×40; insert, ×400).

photographed immediately and followed by imaging at later time points (the ensuing wound closure was photographed using an OLYMPUS Inverted Microscope). The nude area was measured and the percentage of wound closure was calculated.

***Transwell migration assay.*** Cells ($2.0 \times 10^5$) in 100 μL of serum-free medium were added to the top chamber of 8-μm Costar polycarbonate transwells, while 500 μL of complete medium was added to the bottom chamber. After allowing migration for various times (37°C, 100% humidity, 5% $CO_2$ in air), cells on the upper membrane surface were removed and migrant cells on the membrane underside were fixed using 70% ethanol, stained using 0.1% crystal violet solution (Invitrogen), and visualized and counted under the microscope. Data were presented as the average number of migrating cells in eight high-power fields (200×). Each experiment was done in triplicate, and then the data were averaged for statistical analysis.

**Invasion assays**

Matrigel-coated, sterile 8-μm polycarbonate filters (BD PharMingen) were rehydrated as described above. The lower chambers of the 24-well plate were filled with 0.5 mL of complete medium, then 0.5 mL of serum-free RPMI 1640 containing $2.0 \times 10^5$ tumor cells was added to the transwell chambers, and the plate was incubated at 37°C in 5% $CO_2$ humidified atmosphere for 48 hours. Further staining and recording of results was done as mentioned above (see Migration assays). The specific MMP2/9 inhibitor [*N*-phenylsulfonyl-hydroxamic acid derivative (2R-[(4-biphenylsulfonyl)amino]-*N*-hydroxy-3-phenylpropinamide); 10 μmol/L] was purchased from Calbiochem.

**Gelatin zymography**

Metalloproteinase activity was analyzed by gelatin zymography. In brief, conditioned medium was concentrated to one-third of its original volume and electrophoresed on SDS polyacrylamide gels (10%) containing 120 μg/mL of gelatin. After electrophoresis, the gel was incubated for 60 minutes in 2.5% Triton X-100 and overnight at 37°C in 50 mmol/L of Tris (pH 8.0)/5 mmol/L $CaCl_2$. Then, the gels were stained with 0.1% Coomassie brilliant blue and destained with 10% isopropanol in 10% acetic acid, and the gelatinolytic activity was identified as transparent bands in the Coomassie brilliant blue–stained background.

**Immunofluorescence and confocal microscopy**

Cells were cultured in sterile slides (Lab-Tek Chamber Slide System, NUNC) for 24 hours and fixed with 4% formaldehyde, washed thrice in Dulbecco's PBS (D-PBS), permeabilized in 0.1% Triton for 2 minutes, blocked for 1 hour in blocking buffer (10% goat serum in D-PBS), and incubated with primary antibodies overnight. Cells were then washed thrice in D-PBS for 5 minutes each followed by a 1-hour incubation with secondary antibodies (Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit; Invitrogen). Then, cells were washed twice in D-PBS for 10 minutes and twice in distilled water for 10 minutes, and mounted in ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen). Photographs were taken with a Leica TCS SP5 spectral confocal microscope (argon, 405 diode and DPSS561 lasers) using a lambda blue 63× 1.35 numerical aperture oil objective. Images were analyzed with the MacBiophotonics ImageJ program.

**Triton X-114 extraction**

Extraction of membrane proteins was done essentially as described previously (12). An equal number of cells (~$1 \times 10^6$) from each cell line tested was cultured in 100-mm dishes for 3 days. Cells were then washed with TBS, incubated for 20 minutes on ice with 2.5% Triton X-114 detergent (Sigma) and scraped. Triton X-114 has the property of separating into aqueous and detergent (hydrophobic) phases at temperatures above 20°C, and amphiphilic integral membrane proteins remain in the detergent phase. Triton X-114 extracts were centrifuged (13,000 × *g* for 15 min at 4°C) to remove Triton X-114–insoluble material, incubated at 37°C for 5 minutes and spun at 3,000 × *g* for 2 minutes at room temperature to separate the aqueous and the hydrophobic phases. Aliquots (100 μL) of each hydrophobic phase were stored at −80°C.

**Experimental metastasis assay**

An *in vivo* experimental metastasis model was established by injection of $2 \times 10^6$ A673 cells suspended in 100 μL of saline into the tail vein of athymic nude mice (BALB/$c^{nu/nu}$) from Harlan. Animal care and procedures were followed according to the Institutional Guidelines for the Care and Use of Laboratory Animals. Mice were

fed under standard conditions with weight monitoring, sacrificed after manifestation of morbidity, and their lungs were removed for further analysis.

**Statistical analysis**

Data were analyzed for statistical significance using Student's *t* test and ANOVA. Survival curves were generated by Kaplan-Meier method and compared by using the log rank test. Unless otherwise stated, $P \leq 0.01$ was regarded as significant.

## Results

First, we analyzed the expression of CAV1 by immunostaining a TMA containing 43 paraffin-embedded ESFT tumors with known EWS translocations (13). As expected, and in agreement with previous studies, ~85% of the patients expressed CAV1. Clinicopathologic analyses revealed that CAV1 overexpression correlated significantly with metastasis and poor prognosis in several human cancers, including prostate, breast, and lung cancer (14). Although no evidence was found for a significant association between CAV1 expression and stage, size, or tumor site, all metastatic samples (10 of 10) had significantly high CAV1 expression (Table 1), suggesting that high CAV1 content could positively contribute to enhancing ESFT metastasis.

To metastasize, tumor cells need to migrate from the primary site (15). To determine the effect of CAV1 on the migratory and invasive capabilities of ESFT cells, we knocked down CAV1 expression in TC252 and A673 cells (Fig. 2A) by stably transfecting a previously validated shRNA construct (6, 7). EWS/FLI-1 protein levels were unaffected by downregulation of CAV1 (Supplementary Fig. S1), further demonstrating the specificity of the shRNA construct. *In vitro*, wound-healing (Fig. 2B) and transwell migration assays (Fig. 2C) showed that in both cell lines, CAV1 knocked-down cells migrated significantly less ($P \leq 0.01$) than mock- and vector-transfected cells. These results are consistent with previous reports that show the key role of CAV1 regulating the migratory capability of selected metastatic cell lines (10, 16).

As the acquisition of an invasive phenotype by cancer cells is a critical step for tumor progression (15), we also examined the effects of CAV1 downregulation on the invasive ability of CAV1 knocked-down ESFT cells derived from both cell lines. Relative to control cells, as shown in Fig. 3A, downregulation of CAV1 correlated significantly ($P \leq 0.01$) with a reduction in the invasiveness of A673 and TC252 cells. Early events in cancer cell invasion and metastasis involve the proteolytic degradation of extracellular matrix components. MMP are zinc-containing proteolytic enzymes that cleave extracellular matrix proteins, and play important roles in tumor cell migration and invasion. Along with other family members, MMP9 and MMP2 are able to degrade the main components of the basement membranes and play a key role in metastasis (17). To determine whether ESFT invasion was associated with MMP activation, conditioned media from both model cell cultures were analyzed by zymography with gelatin as the substrate (Fig. 3B). The proteolytic activities of MMP9 and MMP2 were present in conditioned medium from all cell lines. Interestingly, the band detected at 100 kDa (corresponding to the pro-MMP9 precursor form that became active during zymography), was found to be substantially decreased in all CAV1 knocked-down cell lines. Furthermore, a faster migrating gelatinolytic form of MMP2 was present only in the control cells, but was undetectable in CAV1 knocked-down cells, suggesting that CAV1 expression is required for MMP9 production and MMP2 activation. The involvement of MMPs on the invasive ability of Ewing's sarcoma cells was further confirmed by using a known MMP2/9 inhibitor [*N*-phenylsulfonyl-hydroxamic acid derivative (2R-[(4-biphenylsulfonyl)amino]-*N*-hydroxy-3-phenylpropinamide)], which induced a significant reduction in the invasiveness of A673 and TC252 cells (Supplementary Fig. S2). MMP9 production had been shown to be regulated at the transcriptional level in other tumor types (18). To determine whether the MMP9 decrease was associated with a downregulation of its mRNA levels, we compared the expression of MMP9, MMP2, and TIMP2 in cells derived from both CAV1 silencing models by reverse transcription-PCR. As shown in Fig. 3C, only MMP9 mRNA was downregulated as a consequence of CAV1 knockdown, suggesting that CAV1 regulates MMP9 expression differently from the way it affects other MMPs.

Activation of the secreted pro-MMP2 is mediated by a cell surface complex that consists of a homodimer of membrane type 1-MMP (MT1-MMP) and a single molecule of tissue inhibitor of metalloproteinases 2 (TIMP2; a natural

**Table 1.** Summary of TMA data with regard to CAV1 expression (negative versus positive) and clinical features

| | Stage | | Tumor size | | Tumor site | |
|---|---|---|---|---|---|---|
| | Localized | Metastatic | <8 cm | >8 cm | Axial | Ext. |
| Caveolin (*n* = 41) | | | | | | |
| Negative (0-1) | 2 | 0 | 1 | 1 | 1 | 1 |
| Positive (2-3) | 25 | 10 | 12 | 23 | 24 | 11 |
| Null/unsure | 4 | 0 | 1 | 3 | 2 | 2 |

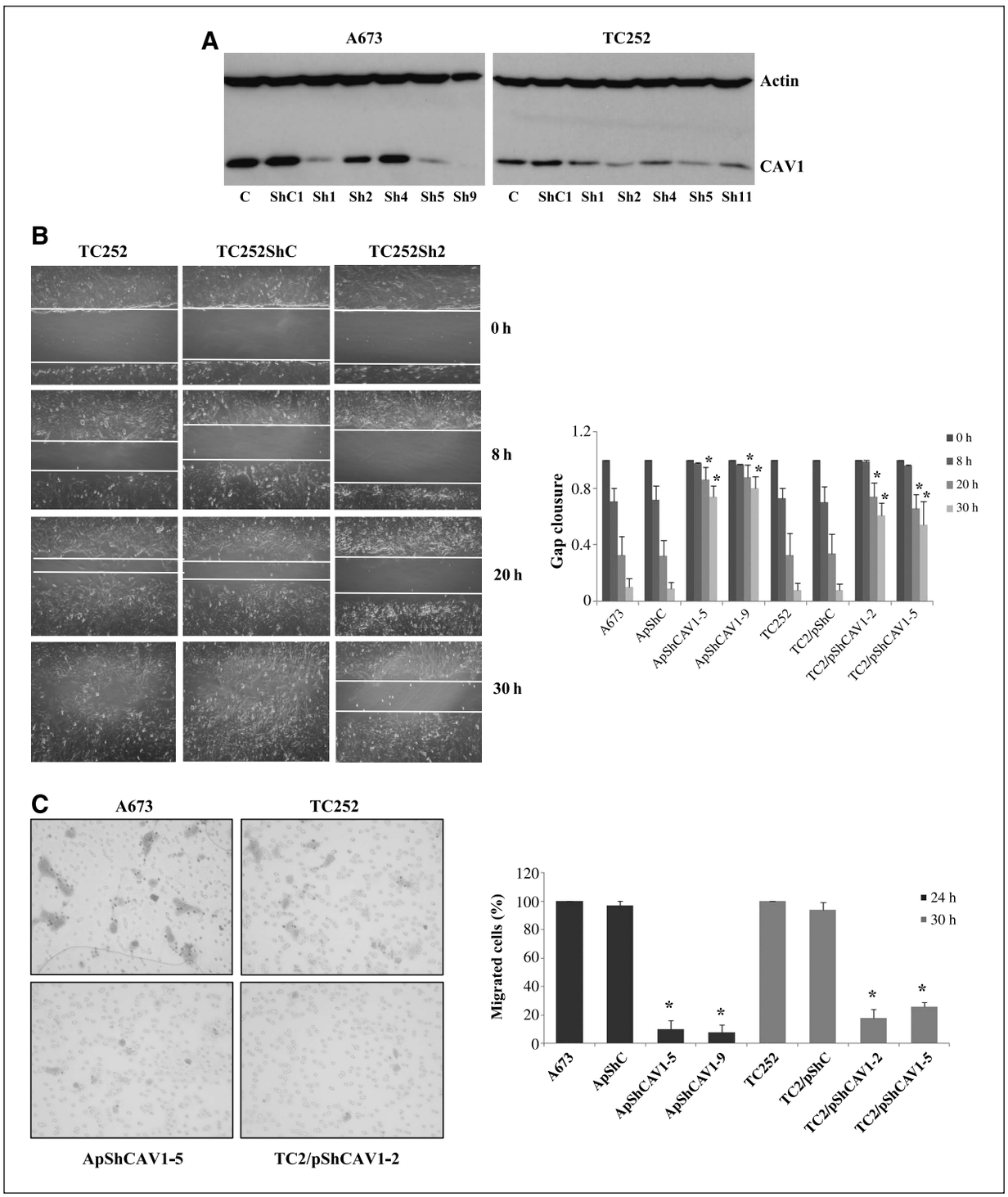


**FIGURE 2.** CAV1 downregulation reduces the migration of ESFT cells. A, immunoblot showing substantially reduced CAV1 levels in individual clones of A673 and TC252 cells stably expressing *CAV1* shRNA compared with untransfected and vector-transfected cells. β-Actin was the loading control. B, wound-healing assay carried out with cells derived from the TC252 CAV1 silencing model. Phase contrast microscope images (×100) were taken at the indicated times after monolayer scratching (left); quantification of wound-healing assays with A673- and TC252-derived cells (right) with vertical bars representing mean values of gap closure from three independent experiments, each done in triplicate; bars, SD (*, $P \leq 0.01$). C, photomicrographs (×40) showing differences in transwell migration among the cells indicated (left); quantification of transwell cell migration (right), with vertical bars representing mean percentages of the number of migrated cells from three independent experiments each done in triplicate; bars, SD (*, $P \leq 0.01$).

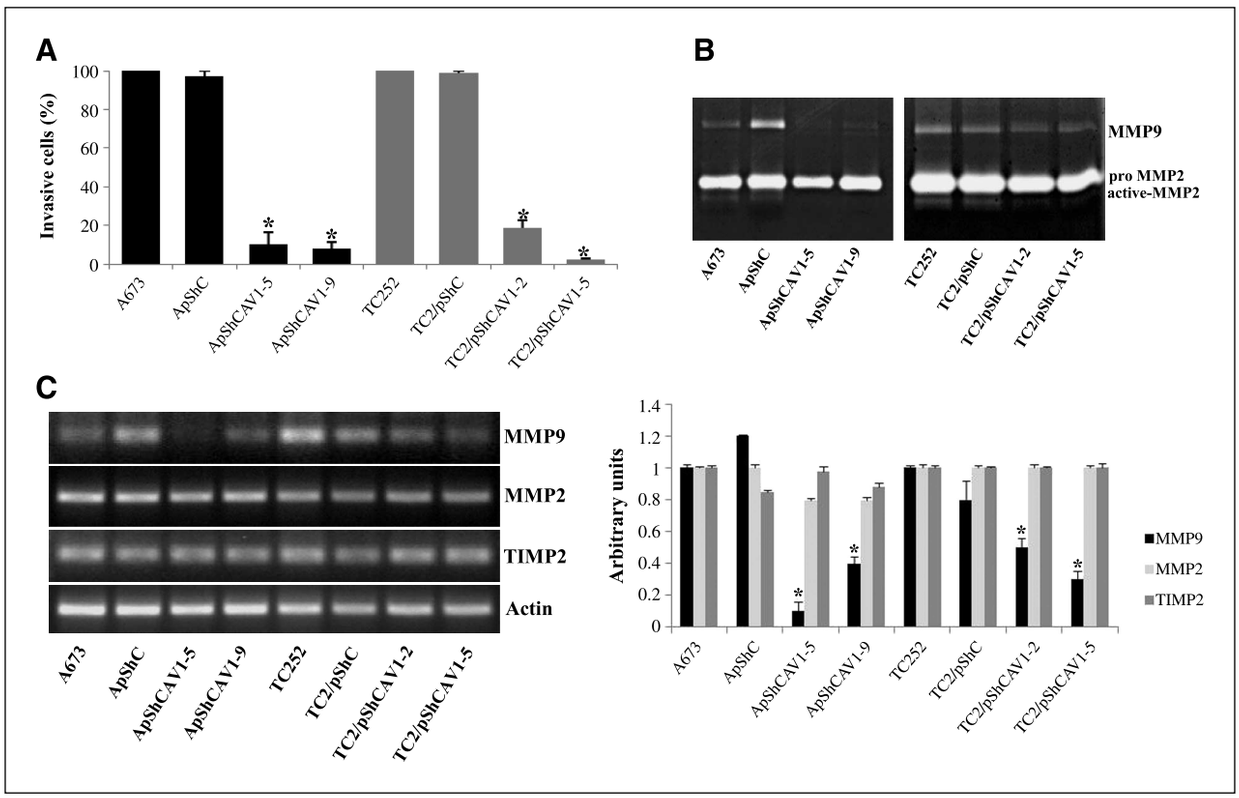


**FIGURE 3.** CAV1 downregulation reduces the invasive ability of ESFT cells. A, invasion of cells through 8 μm Matrigel-coated polycarbonate pores was determined using Boyden chamber assays. Vertical bars represent mean percentages of invading cells from three independent experiments each done in triplicate; bars, SD (*, $P \leq 0.01$). B, gelatin zymogram measuring activation of MMP9 and MMP2 related to CAV1 expression in the same cells in A. C, MMP9, MMP2, and TIMP2 mRNA levels were analyzed by semiquantitative reverse transcription-PCR (left). β-Actin was used as the loading reference. Histogram bars (right) represent mean densitometry values from two independent experiments; bars, SD (*, $P \leq 0.05$).

inhibitor of MMPs; refs. 19, 20). Moreover, CAV1 had been shown to be required for invadopodia formation and extracellular matrix degradation through regulation of MT1-MMP function in human breast cancer cells (21). Ewing's sarcoma cells had been shown to express MT1-MMP (22). Consequently, zymography from isolated membrane extracts (12) in the A673 model showed that membrane-bound MMP2 was mostly observed in control, but barely present in low CAV1 cells (Supplementary Fig. S3), suggestive of MT1-MMP activation in control cells but not in low CAV1 cells. Furthermore, confocal microscopy analysis showed that the decrease of MT1-MMP activity correlated with a diffuse redistribution of this protein in low CAV1 cells (Fig. 4). We were unable to observe wide colocalization between CAV1 and MT1-MMP. However, in control cells, these two proteins colocalized in some structures at the bottom of the cell, suggestive of invadopodia (Supplementary Fig. S4).

Based on the *in vitro* results, we asked whether downregulation of CAV1 in ESFT cells might affect their metastatic potential in an experimental metastasis assay *in vivo*. The cell lines derived from the A673 CAV1 silencing model were injected into the tail vein of BALB/c nude mice. Animals were sacrificed after manifestation of morbidity. In agreement with a previous report using A673 cells as a model of metastasis (23), our parental and vector-transfected A673 cells strongly colonized the lungs. On the contrary, animals injected with CAV1 knocked-down cells either showed essentially no incidence of metastasis to any organ, or showed lung colonization only after a significant delay. Consequently, there were highly significant ($P = 0.0001$) differences in survival among the experimental groups of mice (Fig. 5A). Macroscopically, metastatic foci were quantified showing significant differences between control and low CAV1 cell–derived lungs (Fig. 5B and C). Histologic analysis of paraffin-embedded lungs clearly evidenced several lung metastases in mice injected with control cells in comparison with the lungs of animals injected with A/ShCAV1-5 and A/ShCAV1-9 cells (Fig. 5D). Although previous studies with A673 cells also reported metastasis to sites other than the lungs, only lung metastases developed in our experimental system. This may be explained by their use of a different immunodeficient mouse strain, which has recently been shown to allow ESFT cells to colonize other organs in addition to the lungs (24). Immunohistochemical analyses of paraffin-embedded metastatic lung tumors showed either diminished or no detectable CAV1 expression in A/ShCAV1-derived lung

tumors from mice sacrificed throughout the experiment compared with the highly positive staining of parental or control-derived lung tumors from mice sacrificed during the 3rd week (Fig. 5E). In agreement with the *in vitro* results, MMP9 expression appeared mostly in controls. These results further showed the validity of the shRNA construct used in the present study and strongly support the hypothesis that CAV1 is necessary for ESFT cells to metastasize.

The ability of ESFT cells to enter into the bloodstream (intravasation) and to exit from capillary beds into the parenchyma of an organ (extravasation) might be explained by the regulation that CAV1 exerts over MMPs. However, the ability of these cells to colonize the lungs may also be driven by the so-called metastasis virulence genes (25). Among a subset of these genes, we focused in SPARC (secreted protein acidic and rich in cysteine), a matricellular glycoprotein that mediates interactions between cells and their microenvironment as both CAV1 and SPARC have been found to be determinants of metastasis in basal-like breast carcinomas and melanomas (26, 27). In addition, neither the expression of SPARC in ESFT nor the mechanistic interaction between the SPARC and CAV1 proteins have been reported to date. Therefore, we tested whether SPARC mRNA and protein expression were affected by CAV1 knockdown. Results showed that SPARC mRNA and protein levels were indeed downregulated in CAV1 knockdown cells (Fig. 6A and B), suggesting that CAV1 regulates SPARC expression at the transcriptional level. SPARC has been found to induce migration and invasion in different types of cancer (28). Consequently, to explore whether SPARC collaborated with CAV1 inducing such processes, we knocked down SPARC in A673 cells by stably transfecting a validated (11) specific shRNA construct (Fig. 6C) without affecting CAV1 expression (Supplementary Fig. S5) and analyzed its effects on migration, invasion and lung colonization. Even though wound-healing

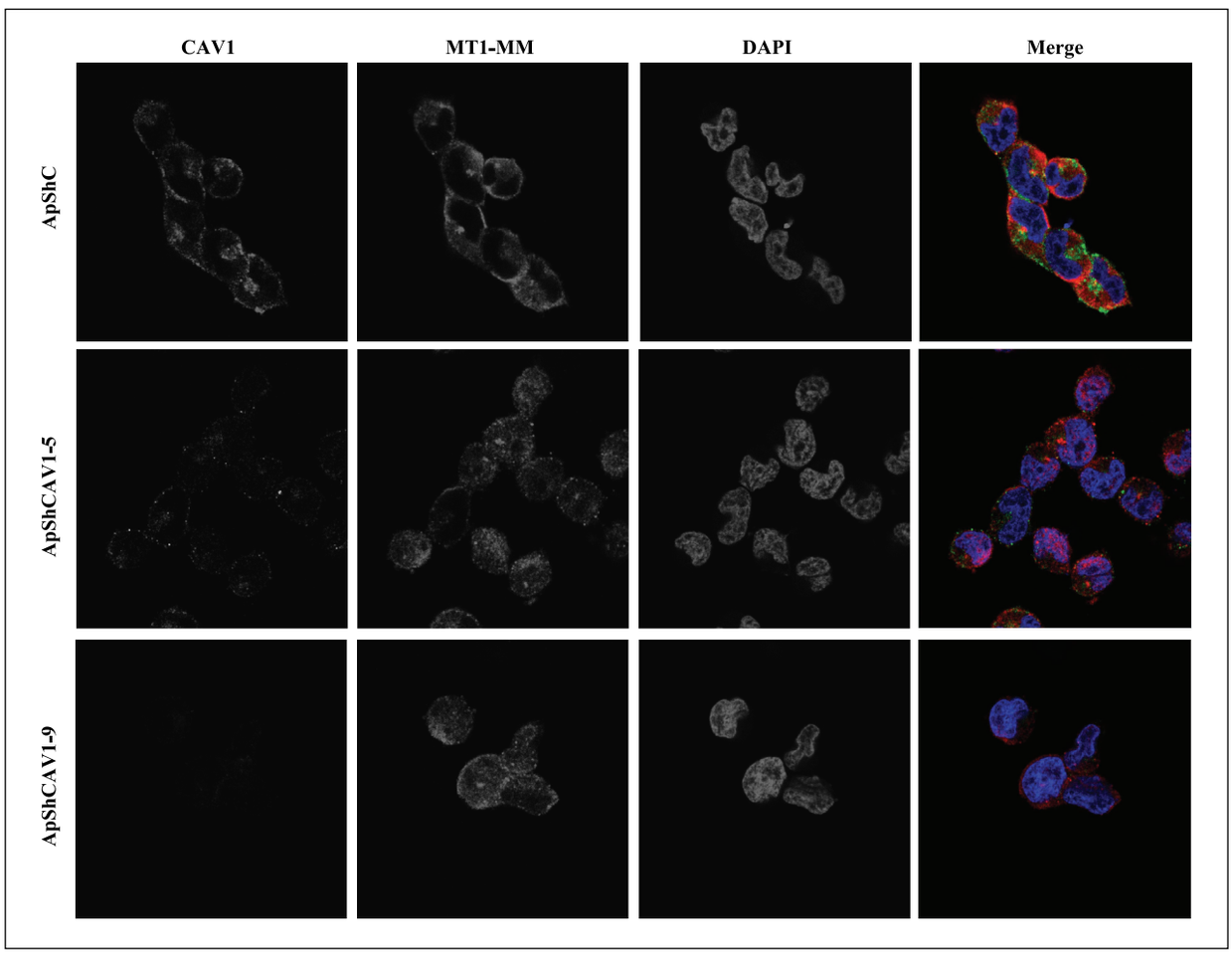


**FIGURE 4.** Localization of CAV1 and MT1-MMP in cells derived from the A673 CAV1 silencing model. Exponentially growing, A673-derived cells were immunostained with anti-CAV1 antibody using an Alexa Fluor 488 goat anti-mouse secondary antibody (CAV1), anti–MT1-MMP antibody using an Alexa Fluor 594 goat anti-rabbit secondary antibody (MT1-MMP) or stained with 4′,6-diamidino-2-phenylindole to identify the nuclear compartment. Merging of the three images (Merge) showed the relocation of MT1-MMP from the cell membrane to a more diffusely staining compartment in CAV1 knocked-down cells (magnification, ×630).

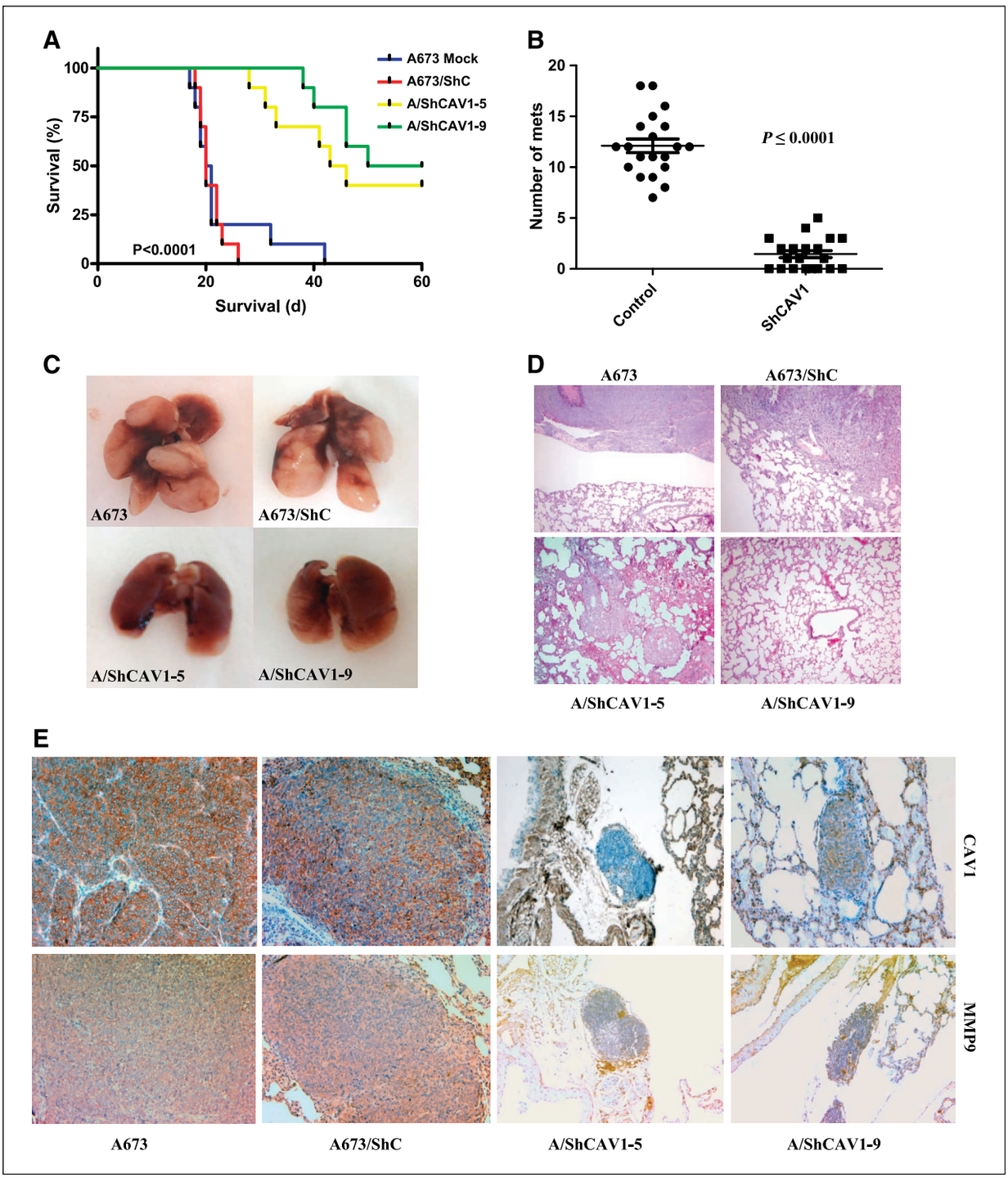


**FIGURE 5.** CAV1 downregulation reduces metastasis in nude mice. A, survival of mice bearing ESFT cells using a mouse lung metastasis model. Kaplan-Meier survival curves of nude mice bearing tumors initiated by injection of mock-transfected A673 cells (A673 Mock), control vector transfected cells (A673/ShC), or CAV1 knocked-down (A/ShCAV1-5 and A/ShCAV1-9) cells ($n$ = 10 for each cell line). B, quantification of lung metastases derived from control (A673 Mock and A673/ShC) and CAV1 knocked-down (ShCAV1) cells (A/ShCAV1-5 and A/ShCAV1-9) C, representative lungs excised from animals injected with A673-derived cells. Control mice were sacrificed during the 3rd wk whereas animals injected with cells expressing shCAV1s were sacrificed during the 8th week. D, H&E from corresponding paraffin-embedded lungs (×200). E, CAV1 and MMP9 staining of metastasis on lung sections from mice injected with control vector transfected A673 cells (A673/ShC) sacrificed on day 24 and ShCAV1 transfected cells (A/ShCAV1) sacrificed on day 60 (magnification, ×100).

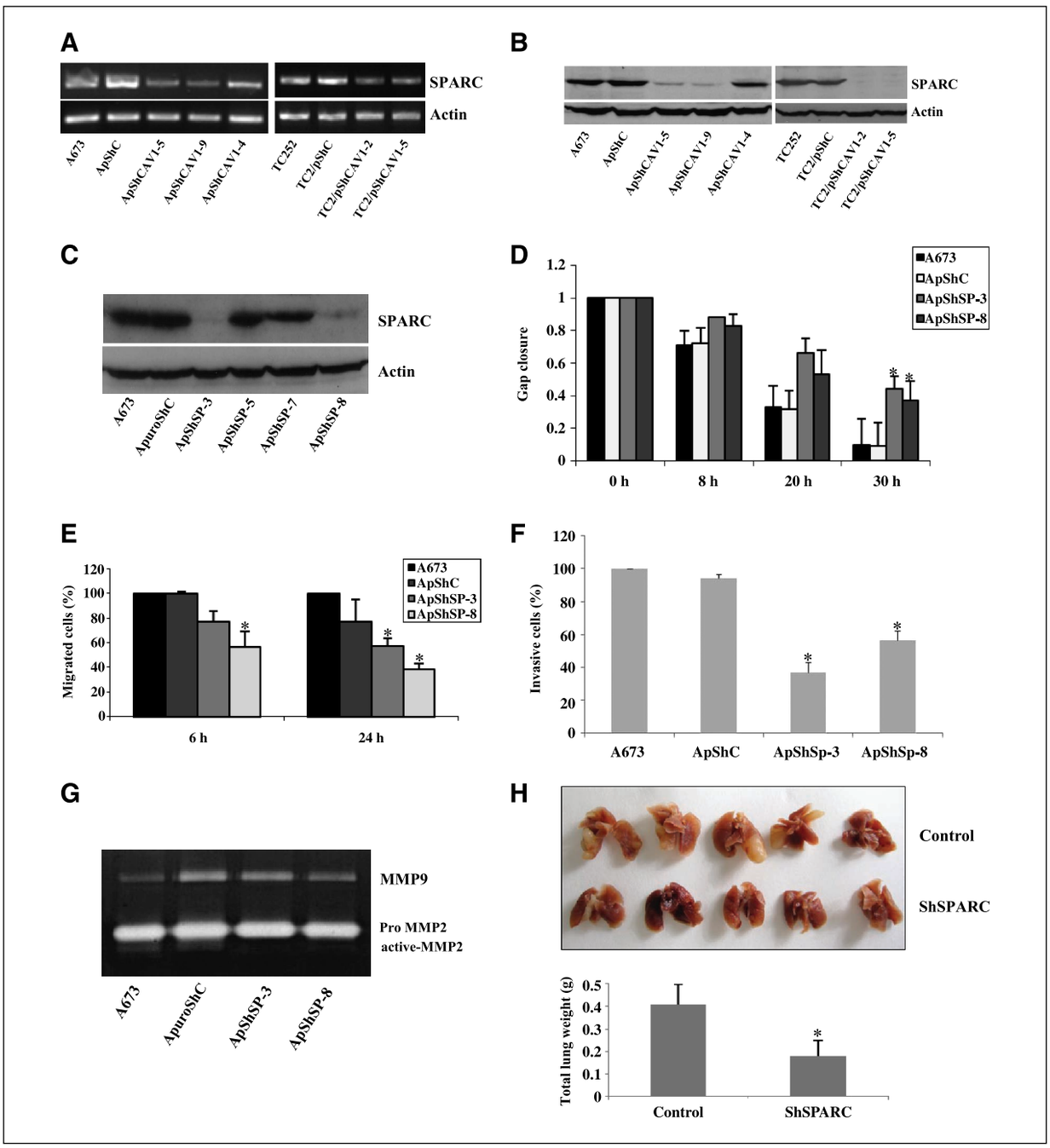


**FIGURE 6.** SPARC acts downstream of CAV1 to regulate ESFT cell invasiveness. A, SPARC mRNA levels were analyzed by semiquantitative reverse transcription-PCR in individual clones of A673 and TC252 cells stably expressing CAV1 shRNAs were compared with those in untransfected cells and in cells transfected with control vector DNA. B, SPARC protein levels in cells derived from the A673 and TC252 CAV1 silencing models. C, immunoblot showing substantially reduced SPARC levels in individual clones of A673 cells stably expressing SPARC shRNA compared with untransfected cells and cells transfected with control vector DNA. Wherever applicable, β-actin was used as the loading control. D, quantification of wound-healing assays, where vertical bars represent mean values of gap closure from three independent experiments each done in triplicate; bars, SD (*, $P \leq 0.05$). E, quantification of transwell cell migration where vertical bars represent mean percentages of the number of migrated cells from three independent experiments each done in triplicate; bars, SD (*, $P \leq 0.05$). F, invasion of cells through 8 μm Matrigel-coated polycarbonate pores was determined using Boyden chamber assays. Vertical bars represent mean percentages of invading cells from three independent experiments each done in triplicate; bars, SD (*, $P \leq 0.05$). G, gelatin zymogram measuring activation of MMP9 and MMP2 related to SPARC expression in the same cells in D. H, representative lungs excised after 15 d from mice injected with either cells transfected with control vector and from ShSPARC-transfected cells (top). Quantification of lung weight (bottom) from mice injected with control or SPARC knocked-down cells (*, $P \leq 0.001$).

(Fig. 6D), transwell migration (Fig. 6E), and invasion assays (Fig. 6F) showed that SPARC knocked-down cells migrated and invaded significantly less ($P \leq 0.05$) than control cells, the effect of SPARC downregulation did not recapitulate the extent of the inhibitory effects induced by CAV1 knockdown. Conditioned medium from SPARC knocked-down cell cultures were analyzed by zymography (Fig. 6G). Similar to what happened in CAV1 knocked-down cells, MMP2 activation was undetectable in SPARC knocked-down cells. In contrast, SPARC downregulation did not provoke a decrease of MMP9, suggesting that SPARC knocked-down cells invaded better than CAV1 knocked-down cells in part because MMP9 was unaffected and thus remained active despite the low cellular levels of SPARC. Lastly, experimental metastasis assays showed that in contrast with control cells, SPARC knocked-down cells were unable to colonize the lungs (Fig. 6H), suggesting that SPARC is necessary for lung metastasis by Ewing's sarcoma cells.

## Discussion

CAV1 regulates multiple cancer-associated cellular processes and its expression is a marker that predicts poor cancer patient prognosis (14). In our TMA, all metastatic samples had significantly high CAV1 expressions. This is in agreement with several publications reporting that certain genes expressed in primary tumors were also found in metastases from the same patient, suggesting that the metastatic potential of human tumors is encoded in the bulk of the primary tumors (29).

Migrating cells display sequential changes in morphology characterized by protrusion of lamellipodia and filopodia at the leading edge, attachment to the substratum, forward flow of cytosol, focal adhesion loosening, and retraction of the rear of the cell (30). CAV1 drives the formation of plasma membrane caveolae and anchors them to the actin cytoskeleton, modulates cell interaction with the extracellular matrix, pulls together and regulates signaling molecules, and transports cholesterol. Through these functions, CAV1 could play an important role in cell movement by controlling cell membrane composition and membrane surface expansion, polarization of signaling molecules, and/or cytoskeleton remodeling (31). Therefore, our laboratory is currently investigating whether CAV1 controls ESFT cell migration directly, acting on the migrating protrusions or indirectly behaving as functional platforms that regulate the organization, coordination, and activation status of signaling complexes (receptors, transducers, and effectors) involved in cell migration.

Our data represent the first description of CAV1 differently regulating MMP9 and MMP2 to promote invasion in ESFT. The regulation of MMP expression and activity occurs at many levels, including gene transcription, posttranscriptional processing, and proenzyme activation (32). Our results show that CAV1 regulates MMP9 by indirectly modulating its expression at the transcriptional level, while it regulates MMP2 by promoting the activation of its proenzyme form. Intriguingly, CAV1 knockdown induced the downregulation of MMP9 but not MMP2 or TIMP2. Multiple cytokines and growth factors are able to induce MMP expression, although the tissue specificity of the diverse family members is mainly achieved by the combination of different transcriptional control mechanisms. The integration of multiple signaling pathways facilitates the strict spatiotemporal control of MMP transcriptional activity (33). Therefore, in our model, CAV1 could be regulating specific signaling pathways that affect MMP9 expression independently of other MMPs.

For MMP2 activation, TIMP2 binds to the catalytic domain of one of the MT1-MMP molecules in the dimer and to the HPX domain of pro-MMP2, thereby facilitating cleavage and activation of pro-MMP2 by the second (TIMP2-free) MT1-MMP molecule of the dimer (20). Recently, it was shown that CAV1 is required for invadopodia formation and extracellular matrix degradation in human breast cancer cells, demonstrating that CAV1 is an essential regulator of MT1-MMP function (21). Accordingly, our results strongly suggest that CAV1 mediates MMP2 activation through regulation of MT1-MMP activity. However, and contrary to our results, CAV1 has been shown to inhibit invasion and MMP expression in pancreatic cancer (34). Also, in breast cancer cell lines, CAV1 has been shown to inhibit invasion, MMP activation, and metastasis *in vivo* (35). These opposite results could be explained because ESFT is not an epithelial cancer as is the case of pancreatic and mammary tumors, indicating that depending on the tissue type, CAV1 may exert opposite functions. In agreement with our results, in melanoma (another mesenchymal tumor), CAV1 promotes invasion by regulating MMP expression (36), underscoring the importance of the tissue of origin as a determinant of protein function.

As in many other tumors, metastatic disease is a poor prognostic factor for overall survival of patients with ESFT. Furthermore, this family of tumors has a great propensity to metastasize to the lungs (37). Accordingly, our experimental metastasis model showed preferential lung colonization of A673 cells in nude mice. Very recently, it has been suggested that the organ specificity of metastatic cells is determined by unique infiltrative and colonization functions which are dependent on specific gene sets (1). There is no data about genes that determine lung colonization in ESFT. Nevertheless, we focused on SPARC because it has been described as a determinant of lung colonization in metastatic breast tumors (11) and it has been shown to induce the production and activation of several MMPs (38).

Similar to MMP9, SPARC mRNA was affected by CAV1 knockdown, suggesting that in ESFT, expression of both genes might be regulated by CAV1. However, not all ESFT cells expressed SPARC (data not shown), this might be explained by the fact that SPARC expression in tumor cells is highly regulated by mechanisms other than transcription (38). Therefore, in some ESFT cells, SPARC expression could be regulated epigenetically, posttranscriptionally, or by miRNAs. In our model, SPARC knockdown resulted in a significant reduction in the migratory and invasive capabilities of A673 cells. However, the effects

induced by SPARC downregulation never recapitulated the extent of the inhibitory actions provoked by CAV1 knockdown, suggesting that CAV1 mediates migration and invasion in part by indirectly regulating the expression of SPARC, but also by regulating other still unidentified proteins. These observations are in agreement with the situation reported in melanomas, in which both proteins have been shown to promote metastatic disease (27, 36). Zymography analysis showed that as in CAV1 knockdown cells, MMP2 activity was reduced as a consequence of SPARC knockdown, in contrast, MMP9 production was unaffected explaining why SPARC knockdown did not induce the same level of invasive inhibition than CAV1 knockdown. Similar to CAV1, the activity of SPARC is context- and cell type–dependent. In fact, SPARC has shown seemingly contradictory effects on tumor progression in both clinical correlative studies and in animal models (39). Therefore, the activity of SPARC related to invasion might be different in ESFT than in other tumor types. Interestingly, compared with control cells, none of SPARC knocked-down cells were able to colonize the lungs, suggesting that SPARC, as an adhesion protein, may indeed be necessary for ESFT cells to adhere to the lung epithelia. Alternatively, the hypothesis that SPARC plays an important role in the fusion of tumor cells with bone marrow–derived cells in the circulation has been proposed a new explanation for its role in metastasis (40). Nevertheless, in ESFT, whether SPARC promotes metastasis through one mechanism or the other requires further investigation.

Overall, our study shows that CAV1 plays a key role in regulating the ability of ESFT cells to migrate, invade the extracellular matrix, and colonize distant organs such as the lungs. This is the first study to evaluate the role of CAV1 on ESFT metastasis. Our results add relevance to the key roles that CAV1 plays in ESFT biology by controlling tumorigenicity (6), resistance to chemotherapy-induced apoptosis (7), and metastatic ability.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Caveolin-1 in sarcomas: friend or foe?

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## ABSTRACT:

**Sarcomas represent a heterogeneous group of tumors with a complex and difficult reproducible classification. Their pathogenesis is poorly understood and there are few effective treatment options for advanced disease. Caveolin-1 is a multifunctional scaffolding protein with multiple binding partners that regulates multiple cancer-associated processes including cellular transformation, tumor growth, cell death and survival, multidrug resistance, angiogenesis, cell migration and metastasis. However, ambiguous roles have been ascribed to caveolin-1 in signal transduction and cancer, including sarcomas. In particular, evidence indicating that caveolin-1 function is cell context dependent has been repeatedly reported. Caveolin-1 appears to act as a tumor suppressor protein at early stages of cancer progression. In contrast, a growing body of evidence indicates that caveolin-1 is up-regulated in several multidrug-resistant and metastatic cancer cell lines and human tumor specimens. This review is focused on the role of caveolin-1 in several soft tissue and bone sarcomas and discusses the use of this protein as a potential diagnostic and prognostic marker and as a therapeutic target.**

## INTRODUCTION

Sarcomas constitute a heterogeneous category of neoplasms composed mostly of uncommon tumors of different histology, biology, and outcome. According to its molecular features, soft-tissue and bone sarcomas can be classified in two big groups: associated to specific genetic alterations or without specific molecular patterns [1]. One third of sarcomas have well defined genetic alterations. The identification of these genetic patterns has supposed a revolution since some of them have diagnosis, prognosis and therapeutic implications [2]. They are further divided in two particular categories: sarcomas with specific chromosomal translocations and sarcomas with specific gene mutations [3].

About 15-20% of sarcomas are associated with specific chromosomal translocations involving, most commonly, a member of the TET family (EWS, FUS or TAF15) and a transcription factor. These translocations are thought to happen early in carcinogenesis, promoting some of the processes that finally lead to the appearance of cancer cells [4]. Single gene mutations in certain group of sarcomas encode proteins leading to tumor formation. Usually, the product derived from the mutated gene is a transmembrane tyrosine kinase receptor constitutively activated in a ligand-independent manner. This activation triggers intracellular pathways that finally lead to carcinogenesis [5].

About two thirds of sarcomas lack specific genetic alterations. Adult sarcomas frequently belong to this group and complex karyotypes are often found instead of chromosomal translocations. In this category of sarcomas, p53 inactivation seems to be an early and common event in carcinogenesis. Anomalies in the retinoblastoma pathway are also frequently found but no specific patterns of abnormalities can be described in this subgroup of tumors [6]. Thus, tumors with the same diagnosis present different molecular patterns and different chromosomal aberrations depending on each case.

## CAVEOLIN-1

Caveolin-1 (CAV1) belongs to a family of proteins named caveolins. There are three members: CAV1, 2 and 3, which can form homo- and hetero-oligomeric complexes mediated by domains in the N- and C-terminal domains [7]. CAV1 and 2 are ubiquitously expressed in a variety of cell types such as endothelial cells, pneumocytes, adipocytes and fibroblasts [8]. Expression of CAV2 nearly always mirrors that of CAV1. This may be, in part, due to requirement of CAV1 to transport CAV2 to the plasma membrane where it can be incorporated into caveolae [9]. In contrast, expression of CAV3 is restricted to striated muscle cells and is a component of the sarcoplasmic reticulum of skeletal, cardiac and smooth muscle [10, 11]. CAV1 is the major structural protein in caveolae; small invaginations within the plasma membrane. Caveolae are involved in signal transduction, wherein CAV1 acts as a scaffold to organize multiple molecular complexes regulating a variety of cellular events, for a complete review see [12]. However, CAV1 might be present on flat plasma membrane or/and on different organelles (Figure 1). Whether its role is different at such diverse locations remains to be elucidated. Proteins that associate with CAV1 contain the canonical caveolin-1 binding domain, ϕxϕxxxϕ or ϕxxxxϕxxϕ (where ϕ= Trp, Phe or Tyr). Interaction with a large majority of these proteins occurs through the caveolin scaffolding domain (CSD) (Figure 1). It is the ability to interact with numerous proteins that makes CAV1 a keystone in signaling by organizing signaling complexes at the inner plasma membrane [12].

CAV1 has been shown to possess an ambiguous role in cancer and to act both as a tumor suppressor or promoter. Both activities had been described for CAV1 in breast, oral, colon, lung, uterus and thyroid cancer. This apparent contradiction may be explained by different interacting partners during cancer progression [13], and it is proposed that *in vivo*, CAV1 plays a tissue and stage specific modulatory role in cancer [14].

## CAV1 IN SARCOMAS

Because CAV1 is most abundantly expressed in terminally differentiated mesenchymal cells such as

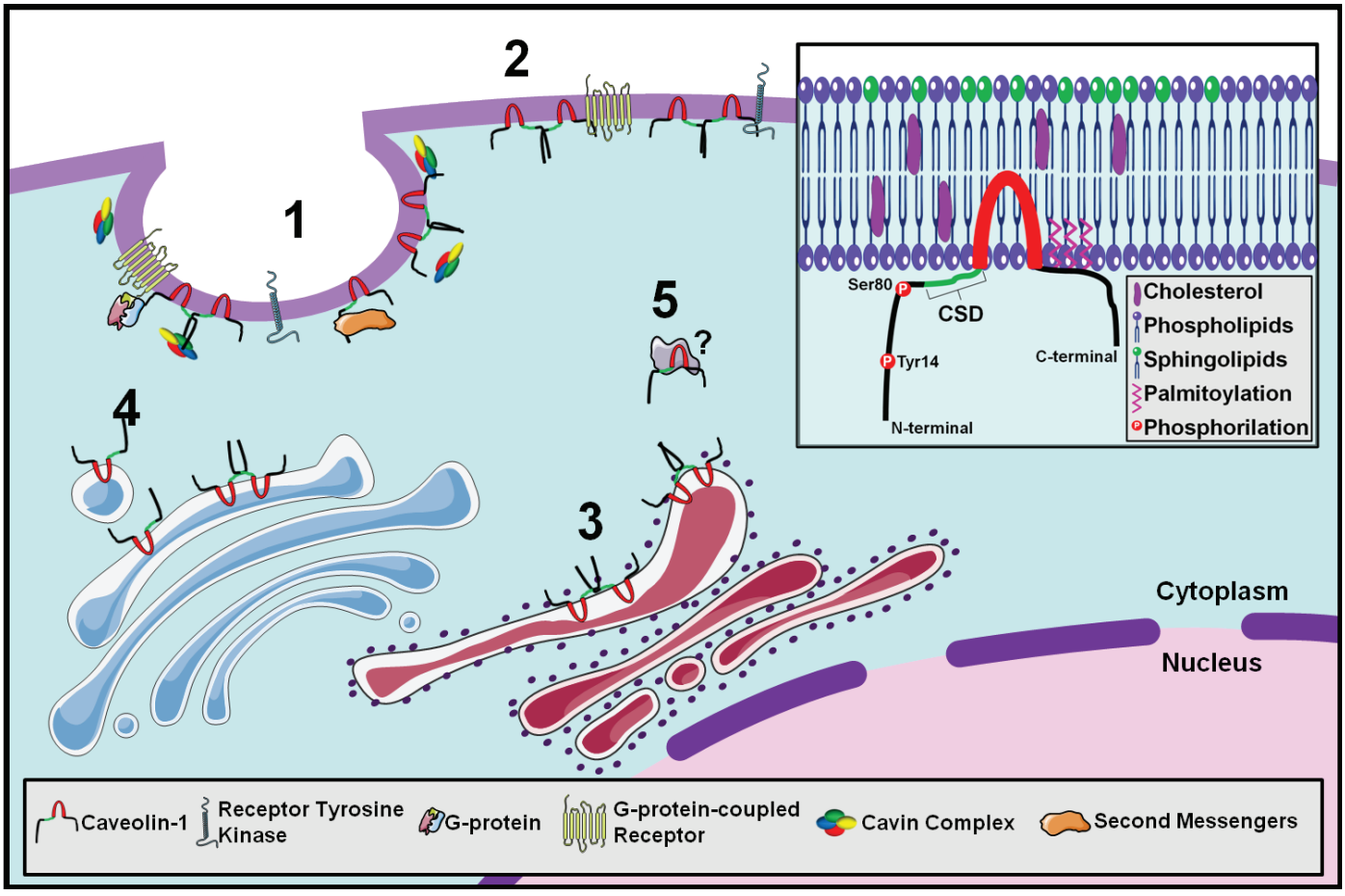


**FIGURE 1: CAV1 localization options inside the cell.** **1** In caveolae complexed with cavins interacting with tyrosine-kinase or G-protein coupled receptors. **2** Out of caveolae interacting with the same receptors. **3** At the endoplasmic reticulum synthesis of CAV1. **4** From the plasma membrane to the golgi apparatus caveolae-dependent endocytic functions. **5** In the cytoplasm interacting with still unknown proteins. **Inset** Structure and membrane topology of CAV1 showing phosphorylation sites, the caveolin scaffolding domain (CSD), and the transmembrane domain.

smooth muscle cells, adipocytes and endothelial cells, Wiechen et al. investigated whether it was a tumor suppressor in sarcomas [15]. By immunohistochemistry they found that CAV1 expression was high in fibroblasts, smooth muscle cells, adipocytes and endothelial cells with a fine granular membranous and a diffuse cytoplasmic staining pattern. Moreover, levels of CAV1, comparable to normal mesenchymal cells, were retained in all benign mesenchymal tumors, including 5 of 5 fibromatoses, 7 of 7 leiomyomas, 4 of 4 lipomas, and 6 of 6 hemangiomas. CAV1 expression was found to be absent or strongly reduced in 3 of 3 fibrosarcomas, 17 of 20 leiomyosarcomas, 5 of 8 angiosarcomas, 15 of 18 malignant fibrous histiocytomas, and 8 of 8 synovial sarcomas. Therefore it was concluded that CAV1 is a candidate tumor suppressor gene in sarcomas [15]. Accordingly, the analysis of CAV1 in Gastrointestinal stromal tumors (GISTs) suggested that this protein may also act as a tumor suppressor [16]. The authors showed that in a sample of 108 GISTs patients 86.1% (93 patients) did not express CAV1. However, there was no correlation between the caveolin-1 expression status and any of the clinicopathologic variables, including mitosis and tumor grade. The expression of caveolin-1 was not correlated with other immunohistochemical marker proteins including, c-kit, CD34 and SMA. On the univariate analysis, CAV1 expression was not a significant predictor of the disease-free survival for GIST patients [16]. Other studies focusing in the expression of CAV1 in specific sarcomas have shown that this protein is expressed in the cytoplasm of cells from a variety of mesenchymal benign tumors, including the adipocyte of all types of lipoma and well-differentiated liposarcoma, the myocyte of angiomyolipoma, leiomyoma, and well-differentiated leiomyosarcoma [17]. The immunostaining properties were uniform among the cells of each of these lesions, and gender and age did not influence the results. However, all of the malignant mesenchymal tumors which are poorly differentiated and dedifferentiated, including leiomyosarcoma and liposarcoma, showed weak immunoreactivity or failed to stain with CAV1 [17], suggesting that loss of CAV1 might be a necessary step towards a differentiation block typical of malignancy and visible in most soft tissue sarcomas. Interestingly, we have observed high expression of CAV1 in some cell lines representative of these sarcomas, such as leiomyosarcoma and synovial sarcoma (Figure 2). Therefore, whether the expression of CAV1 in these cells correlates with the degree of differentiation and consequently it relates to any function, requires further investigation. Nevertheless, there are some functional studies about CAV1 in specific sarcomas such Ewing's sarcoma family of tumors (ESFT), Osteosarcoma and Rhabdomyosarcoma trying to figure out the clinical importance of this protein that we will review thereafter:

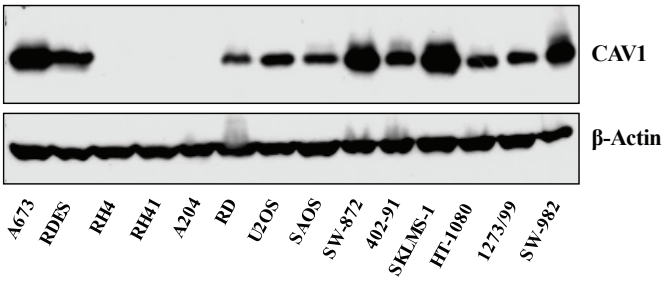


**FIGURE 2: Western Blot analysis showing CAV1 expression in different sarcoma cell lines: Ewing Sarcoma (A673, RDES); Alveolar Rhabdomyosarcoma (Rh4, RH41); Embryonal Rhabdomyosarcoma (A204, RD); Osteosarcoma (U2OS, SAOS); Liposarcoma (SW-872); Myxoid Liposarcoma (402-91); Leiomyosarcoma (SKLMS-1); Fibrosarcoma (HT-1080) ; Synovial Sarcoma (1273/99, SW-982).**

## Ewing's sarcoma family of tumors

ESFT includes aggressive bone-associated malignancies that affect the pediatric population. Nearly all ESFT patients already have micrometastases at diagnosis, resulting in a >95% relapse rate when treated locally and a 40% relapse rate after systemic chemotherapy. Most ESFT harbor a reciprocal translocation, t(11;22) (q24;q12), which links a strong transcriptional activation domain from EWS to the ETS DNA-binding domain of the transcription factor FLI-1 [18]. The *EWS/FLI-1* fusion is required for Ewing's sarcoma oncogenesis, as inhibition of its function results in the loss of transformation of ESFT cells [19-23]. CAV1 was identified as a metastasis-associated gene that is a transcriptional target of EWS/FLI-1 as well as an important determinant of ESFT malignant phenotype and tumorigenicity [24]. Using antisense and short hairpin RNA-mediated gene expression knockdown, array analyses, chromatin immunoprecipitation methods, and reexpression studies, the authors showed that CAV1 is a new direct target of EWS/FLI-1 that is overexpressed in ESFT cell lines and tumor specimens and is necessary for ESFT tumorigenesis. CAV1 knockdown led to up-regulation of Snail and the concomitant loss of E-cadherin expression. Consistently, loss of CAV1 expression inhibited the anchorage-independent growth of EWS cells and markedly reduced the growth of Ewing's sarcoma cell-derived tumors in nude mice xenografts, indicating that CAV1 promotes the malignant phenotype in Ewing's sarcoma carcinogenesis. Reexpression of CAV1 or E-cadherin in CAV1 knockdown Ewing's sarcoma cells rescued the oncogenic phenotype of the original Ewing's sarcoma cells, showing that the CAV1/Snail/E-cadherin pathway plays a central role in the expression of the oncogenic transformation functions of EWS/FLI-1 [24]. Later on, CAV1 with other 3 proteins was considered a differential diagnostic immunomarker for Ewing's sarcoma/PNET in a sample of 415 genetically confirmed cases [25].

Another study from the same authors showed that

CAV1 expression determines the sensitivity of ESFT cells to clinically relevant chemotherapeutic agents [26]. Analyses of endogenous CAV1 levels in several ESFT cells and ectopic CAV1 expression into ESFT cells expressing low endogenous CAV1 showed that the higher the CAV1 levels, the greater their resistance to drug treatment. Moreover, results from antisense- and shRNA-mediated gene expression knockdown and protein re-expression experiments demonstrated that CAV1 increases the resistance of ESFT cells to doxorubicin- and cisplatin-induced apoptosis by a mechanism involving the activating phosphorylation of PKCalpha. CAV1 knockdown in ESFT cells led to decreased phospho-PKCalpha levels and a concomitant sensitization to apoptosis, which were reversed by CAV1 re-expression. These results were recapitulated by PKCalpha knockdown and re-expression in ESFT cells in which CAV1 was previously knocked down, thus demonstrating that phospho-PKCalpha acts downstream of CAV1 to determine the sensitivity of ESFT cells to chemotherapeutic drugs. These data, along with the finding that CAV1 and phospho-PKCalpha are co-expressed in approximately 45% of ESFT specimens tested [26], implied that targeting CAV1 and/or PKCalpha may allow the development of new molecular therapeutic strategies to improve the treatment outcome for patients with ESFT.

Our group has demonstrated that CAV1 controls migration and invasion in ESFT cells in culture by mechanisms involving the production and activation of metalloproteinases as well as lung colonization in nude mice by regulating SPARC expression levels [27], adding relevance to the key roles that CAV1 plays in ESFT biology. Moreover, by ectopic expression of a Myc-tagged CAV1 protein in ESFT cells, as well as the supplementation of culture media with purified CAV1 protein followed by its intracellular localization using immunofluorescence, we showed that ESFT cells secrete CAV1. Likewise, we showed that ESFT cells are able to take up the secreted protein, and that extracellular CAV1 enhances EWS cell proliferation [28]. Whether this secreted CAV1 has roles other than proliferation remains to be elucidated.

## Osteosarcoma

Osteosarcoma (OS) is the most common primary tumor of bone, occurring predominantly in the second decade of life. High-dose cytotoxic chemotherapy and surgical resection have improved prognosis, with long-term survival for patients with localized (non-metastatic) disease approaching 70%. At presentation approximately 20% of patients have metastases and almost all patients with recurrent OS have metastatic disease, and cure rates for patients with metastatic or recurrent disease remain poor [29]. CAV1 has been shown to act as an oncosuppressor in human osteosarcoma; its down-regulation is part of osteoblast transformation and osteosarcoma progression [30]. In the study the authors did a survey of 6-year follow-up that indicated a better overall survival for osteosarcoma expressing a level of CAV1 similar to osteoblasts. Moreover, the majority of primary osteosarcoma showed significantly lower levels of CAV1 than normal osteoblasts suggesting its role as an oncosuppressor. Mechanistically, the authors showed that Met-induced osteoblast transformation was associated with CAV1 down-regulation. *In vitro*, osteosarcoma cell lines forced to overexpress CAV1 showed reduced malignancy with inhibited anchorage-independent growth, migration and invasion. *In vivo*, CAV1 overexpression abrogated the metastatic ability of osteosarcoma cells. They also showed that c-Src and c-Met tyrosine kinases, which are activated in osteosarcoma, co-localized with CAV1 and were inhibited upon CAV1 overexpression [31]. In contrast, in a recent immunohistochemical study of 61 xenotransplanted osteosarcoma tumors it was shown that CAV1 showed immunoreactivity in the majority of the tumors with no significant variation among the subtypes or subsequent passages; even in the majority of the metastatic cases. Nevertheless, the authors could not conclude that CAV1 is a marker either for good or for bad prognosis [31], suggesting that the role of CAV1 in OS requires further investigation.

## Rhabdomyosarcoma

Rhabdomyosarcoma, a neoplasm composed of skeletal myoblast-like cells, represents the most common soft tissue sarcoma in children. It can be divided into two major histological subtypes: so-called embryonal and alveolar rhabdomyosarcoma [32]. The embryonal subtype is the most common and predominates at favorable anatomic sites such as the orbit, other head and neck sites, and the genitourinary tract. The alveolar subtype occurs in both children and adults, and it is more common at extremity sites and carries an overall inferior prognosis [32]. Rhabdomyosarcoma is defined histologically as a small round blue cell tumor which expresses markers of myogenic differentiation, such as MyoD, myogenin, desmin, and actin. These myogenic markers discriminate it from other soft tissue or bone sarcomas, but late markers of myogenic differentiation are absent, and rhabdomyosarcoma cells do not form myotubes or functional muscle units [33]. Alveolar rhabdomyosarcoma is associated in the vast majority of cases with a specific balanced translocation involving chromosomes 2 and 13 [t(2;13)] or, less commonly, 1 and 13 [t(1;13)] [34, 35], each of which encodes a novel fusion protein, PAX3/FOXO1 and PAX7/FOXO1, respectively. In skeletal muscle, CAV1 and Caveolin-3 (CAV3), a member of the caveolin family expressed specifically in muscle tissue, are both expressed. In particular, CAV1 expression is restricted to satellite cells, which represent a pool of quiescent reserve elements; whereas CAV3

is expressed in myoblasts undergoing differentiation and in mature fibers [36, 11], suggesting that a timely coordinated expression of CAV1 and CAV3 contributes to skeletal muscle homeostasis. In rhabdomyosarcomas, CAV3 is considered a sensitive and specific marker of both subtypes [37]. In contrast to other sarcomas, CAV1 has been suggested to be a marker of poor differentiation for rhabdomyosarcomas [38] associating its expression to a better prognostic entity. CAV1 was predominantly expressed in the embryonal subtype and its expression was associated to an immature cell phenotype. On the other hand, most alveolar rhabdomyosarcoma tumors exhibiting advanced degree of maturation had very low levels of CAV1 suggesting that CAV1 might be a tumor suppressor in alveolar rhabdomyosarcomas. However, the number of samples used in this study was very small and further analysis using a bigger sample would be highly desirable.

## PERSPECTIVE

In the recent years it has been relatively well agreed that depending on tumor type, CAV1 can have either tumor suppressor or oncogenic effects on a cell. Many proteins contain putative CAV1 binding domains. The effect of CAV1 on tumor phenotype seems to be very heterogeneous and strongly dependent on the molecular partners interacting with this protein [13]. Several important proteins involved in cell transformation and growth have been shown to interact with CAV1 including the molecules that stimulate tumor cell invasion and cytoskeletal rearrangement such as growth factor receptors, protein kinases, heterotrimeric G-proteins and Rho GTPases [39-42]. In general, CAV1 is supposed to concentrate signaling molecules within specialized membrane domains, named caveolae [43, 44]. In several cell lines, despite the high expression levels of CAV1, caveolae are not morphologically distinguishable suggesting a non-caveolar role for CAV1. For example, CAV1 can accumulate at focal adhesions and as reported by several groups, this translocation requires CAV1 phosphorylation at Tyr14. Thus, this might represent a novel example of a caveolae-independent role of CAV1 as a molecular organizer. Also, there is no evidence supporting the existence of caveolae-like invaginations in neurons however, physical organization between CAV1 and several receptors may occur in the context of membrane microdomains [45]. Furthermore, it is now clear that cavins (a new family of proteins that form the cavin complex) (Figure 1) are indispensable for caveolae formation and function [46]. Therefore, the possible loss of cavins in tumor cells opens a new exciting window to explore caveolar and non-caveolar roles of CAV1.

In epithelial tumors the role of CAV1 in sarcomas is very contradictory; this is aggravated by the fact that functional studies are very scarce. However, as demonstrated in some bone and soft tissue sarcomas, CAV1 may have a key role in their malignant progression. From our point of view it will be of key importance to correlate caveolar and non-caveolar functions with tumor suppressor or oncogenic activities.

Depending on the role assigned to CAV1 in every sarcoma type it will be possible to use different targeting options. In those sarcomas where CAV1 was demonstrated to act as a tumor suppressor, targeted ectopic re-expression or introduction of a CSD would be a feasible option. In fact, re-expression of CAV1 has successfully reduced the tumor growth of breast cancer cells [47] and the invasive capability of pancreatic [48] and breast [49] cancer cells. Delivery of CSD peptides fused to the C-terminus of the Drosophila antennapedia (AP) homeodomain internalization sequence to subcutaneously implanted tumors inhibited tumor progression by reducing vascular permeability and mediating an indirect anti-angiogenic effect [50]. On the other hand, direct targeting of CAV1 using antisense and siRNA, or indirectly by chemical inhibition, or lowering cholesterol (disrupts caveolae) may result of great help in the cases where CAV1 acts as an oncogene. Similar to prostate cancer [51] and melanomas [52], our group successfully showed that targeting CAV1 with shRNAs reduced Ewing's sarcoma progression [27]. In multiple myeloma, proteasome inhibitors blocked VEGF-triggered CAV1 phosphorylation and expression resulting in reduced migration and survival [53]. The use of statins to inhibit cholesterol synthesis in glioma cells suppressed CAV1 expression and consequently reduced migration and survival [54]. Whether these drugs may be of use for the treatment of sarcoma patients remains unknown. However, the development of new reagents such as siRNAs, peptide and small-molecule inhibitors will define new avenues for therapies, not only for some sarcomas but for tumors that present CAV1-triggered progression. In addition, as more protein and signaling molecules are found to interact with and regulate CAV1 expression the design of novel therapies for sarcomas will evolve. These therapies may include humanized antibodies, small-molecule inhibitors and targeted siRNAs altogether with improved gene therapy delivery systems.

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