

Oncofetal Chondroitin Sulfate Glycosaminoglycans Are Key Players in Integrin Signaling and Tumor Cell Motility

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Abstract

Many tumors express proteoglycans modified with oncofetal chondroitin sulfate glycosaminoglycan chains (ofCS), which are normally restricted to the placenta. However, the role of ofCS in cancer is largely unknown. The function of ofCS in cancer was analyzed using the recombinant ofCS-binding VAR2CSA protein (rVAR2) derived from the malaria parasite, *Plasmodium falciparum*. We demonstrate that ofCS plays a key role in tumor cell motility by affecting canonical integrin signaling pathways. Binding of rVAR2 to tumor cells inhibited the interaction of cells with extracellular matrix (ECM) components, which correlated with decreased phosphorylation of Src kinase. Moreover, rVAR2 binding decreased migration, invasion, and anchorage-independent growth of tumor cells *in vitro*. Mass spectrometry of ofCS-modified proteoglycan complexes affinity purified from tumor cell lines on rVAR2 columns revealed an overrepresentation of proteins involved in cell motility and integrin signaling, such as integ-

rin- β 1 (ITGB1) and integrin- α 4 (ITGA4). Saturating concentrations of rVAR2 inhibited downstream integrin signaling, which was mimicked by knockdown of the core chondroitin sulfate synthesis enzymes β -1,3-glucuronyltransferase 1 (B3GAT1) and chondroitin sulfate *N*-acetylgalactosaminyltransferase 1 (CSGALNACT1). The ofCS modification was highly expressed in both human and murine metastatic lesions *in situ* and preincubation or early intravenous treatment of tumor cells with rVAR2 inhibited seeding and spreading of tumor cells in mice. This was associated with a significant increase in survival of the animals. These data functionally link ofCS modifications with cancer cell motility and further highlights ofCS as a novel therapeutic cancer target.

Implications: The cancer-specific expression of ofCS aids in metastatic phenotypes and is a candidate target for therapy. *Mol Cancer Res*; 14(12): 1288–99. ©2016 AACR.

Introduction

Glycosaminoglycans are secondary carbohydrate modifications attached to proteoglycans on the cellular plasma membrane or secreted into the extracellular matrix (ECM). During embryogenesis, cell differentiation, and diseases, such as can-

cer, glycosaminoglycans display radical changes in expression and composition (1–3). Alterations in the glycosaminoglycan component of proteoglycans have been reported in cancer for more than 4 decades (4–7). As part of the cellular glycocalyx, glycosaminoglycans are believed to control the information flow from the ECM to signal transduction pathways stemming from the plasma membrane (8). While the function and mechanistic contribution of glycosaminoglycans in cancer are not fully understood, it is clear that they act as key regulators of the malignant phenotype (9).

Most cancer cells express a distinct chondroitin sulfate glycosaminoglycan epitope that is normally restricted to trophoblastic cells in the placenta (10). These oncofetal chondroitin sulfate (ofCS) chains, previously termed placental-type chondroitin sulfate, are expressed on chondroitin sulfate-modified proteoglycans (CSPG) of tumor and tumor-infiltrated stromal cells across multiple types of malignancies, indicating a possible broad functional importance of ofCS for the disease pathology (10). CSPGs have been associated with proliferation, migration, invasion, angiogenesis, and metastasis (11–15). In these processes, CSPGs act either alone or in concert with membrane components such as integrins, receptor tyrosine kinases (RTK), or metalloproteases to aid cellular attachment, migration, and invasion (16–19). One well-described function of chondroitin sulfate on proteoglycans is to capture growth

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factors and cytokines and present them to adjacent receptors in the membrane. As such, chondroitin sulfate can act as a scaffold or reservoir for sustained proliferative and oncogenic signaling (20–24). Glycosaminoglycans are made up of repeated disaccharide units making up long, linear polymers. Chondroitin sulfate consists of alternating glucuronic acid (GlcA) and *N*-acetyl-D-galactosamine (GalNAc) residues (24). While the base structure is simple, heterogeneity is achieved through secondary modification of the chondroitin sulfate carbohydrate backbone, such as alternate sulfation of hydroxyl groups (25). Erythrocytes infected with malaria parasites expressing the VAR2CSA protein adhere to chondroitin sulfate only in the placenta, despite the fact that chondroitin sulfate is present in most organs of the human host (24, 26–28). This suggests that chondroitin sulfate in the placenta is distinct from chondroitin sulfate present in other organs. The interaction between malarial VAR2CSA and the placenta is the key molecular event underlying placental malaria (26, 28).

The ofCS motif on cancer and placental cells can be detected using a 72-kDa recombinant fragment of the *Plasmodium falciparum* VAR2CSA protein (rVAR2; refs/ 10, 29). VAR2CSA is a large complex multidomain protein (28). The 72-kDa functional chondroitin sulfate-binding domain ID1-ID2a has an unprecedented high specificity and affinity for 4-*O*-sulfated chondroitin sulfate (C4S; refs. 29, 30).

Using rVAR2 as an ofCS-binding reagent, we have investigated the role of ofCS in human cancer. We report that ofCS is required for cellular attachment, migration, and invasion of tumor cells *in vitro* and *in vivo*. Furthermore, we identify a number of proteins that are modified or associated with ofCS in human tumor cells including components of the integrin complexes. Our study confirms a pivotal role for ofCS in integrin-mediated signaling and supports current efforts using rVAR2 as a broad therapeutic targeting reagent against ofCS in cancer.

Materials and Methods

Reagents and cell culture

Recombinant proteins were expressed in SHuffle T7 Express Competent *Escherichia coli* (NEB) and purified using HisTrap columns from GE Healthcare followed by size exclusion chromatography, as previously described (10). Purified monomeric proteins were validated by SDS-PAGE. Purified chondroitin sulfate A (CSA) was obtained from Sigma. Anti-V5-FITC antibodies were obtained from Invitrogen. Most cell lines were obtained from ATCC and grown in their suggested growth media with 1× penicillin and streptomycin cocktail. The Myla2059 Lymphoma cell lines were donated by Niels Ødum at the University of Copenhagen (Copenhagen, Denmark). Mice for animal studies were acquired from Taconic Biosciences.

ECM-binding assay

Cells were grown in 10-cm dishes to about 70% confluency. The cells were then serum-starved in the presence of 450 nmol/L rVAR2, rDBL4 (a non-ofCS-binding domain of the VAR2CSA protein) or PBS for 18 to 24 hours. The cells were collected using Cellstripper, counted, and adjusted to 0.2×10^6 cell/mL in serum-free media containing inhibitor as above. One hundred microliters was added to wells in a 96-well plate coated with fibronectin (10 µg/mL, Sigma), collagen-I (23 µg/mL, Sigma), collagen IV (23 µg/mL, Sigma), or uncoated plastic. Plastic blocked with BSA

was included as a negative control. All samples were run in triplicates. Following a 25-minute incubation, the adherent cells were stained with methylene blue in methanol for 10 minutes. The plates were washed in water and dried. The color was dissolved in 0.2 mol/L sodium citrate in 50% ethanol and absorbance was read at 650 nm.

Scratch assay

Cells were seeded into 6-well plates and allowed to grow to confluency. The cells were then washed in PBS and serum-starved for 24 hours in the presence of 450 nmol/L rVAR2, rDBL4, or PBS. CSA (400 µg/mL; Sigma) was used to outcompete rVAR2 effect. A scratch was made in the cell monolayer with a 20-µL pipette tip. The cells were washed in PBS and serum-free media containing the inhibitors was added. Pictures were taken at 0, 19, 30, and 46 hours at 2 fixed points per sample.

For the siRNA experiments, MG63 cells were transfected with siRNAs (Qiagen; 50 nmol/L final) against CSGALNACT1, using RNAiMAX (Invitrogen). Scratch was made 48 hours after transfection.

Boyden chamber invasion and migration assays

The cells were grown to 70% confluency. They were then serum-starved in the presence of 450 nmol/L rVAR2 or rDBL4 for 24 hours. The cells were dislodged with Cellstripper and counted 3 times. Then, 100,000 cells were added to each insert of a Boyden chamber plate (Chemi-Con). Separate kits were used for migration and invasion. Invasion kit included membranes coated in basement membrane extract. Media with or without chemoattractant were added to the lower well. Plates were then incubated for 18 to 36 hours at 37°C. The number of migrating cells was determined by a fluorescent probe and compared to a standard curve.

Identification of ofCS-conjugated CSPGs

Column-based pull down. Membrane proteins were extracted with EBC lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, 2.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1% CHAPS, and a protease inhibitor cocktail; Roche). The lysate was loaded onto a Hitrap NHS HP column (GE) containing immobilized rVAR2 or rContrl (rDBL4) control protein. The column was washed in lysis buffer as well as lysis buffer containing 250 mmol/L NaCl. Bound protein was eluted with 0.5 mol/L NaCl in lysis buffer and Upconcentrated on a Vivaspin Column (MWCO; 10,000 kDa). Protein samples, dissolved in SDS loading buffer, and a high-molecular-weight marker (LC5699, Life Technologies) were loaded onto a NuPAGE Tris-acetate gel (Life Technologies). Proteins were subsequently transferred to a nitrocellulose membrane overnight at 4°C at 75 mA. The membranes were stained with anti-CSPG4 antibody (LHM2, Abcam) or antibodies against the $\alpha 4$ -, $\alpha 5$ -, or $\beta 1$ -integrin subunits. The staining was developed in ECL and scanned.

Bead-based pull down. Membrane proteins were extracted in EBC lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, 2.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1% CHAPS, and a protease inhibitor cocktail; Roche). Biotinylated rVAR2 protein was added to the lysate and the mix was incubated overnight at 4°C. The rVAR2 and bound protein were pulled down on streptavidin dynabeads (Invitrogen).

Proteomics. The pulled-down material was dissolved in non-reducing LDS loading buffer (Invitrogen), reduced in 1 mmol/L dithiothreitol (DTT), and alkylated with 5.5 mmol/L iodoacetamide. The samples were then run 1 cm into Bis-Tris gels and stained with Coomassie Blue. The protein was cut out, washed, and in-gel digested with trypsin. The resulting peptides were captured and washed using C18 resin. The peptides were sequenced using a Fusion Orbitrap Mass Spectrometer. Sample analysis and hit verification were performed using the MaxQuant software. All samples were verified against control samples being either a control protein coupled columns or empty beads. For the Ingenuity Pathways Analysis (IPA), proteins that were found to be significantly different between rVAR2 and rControl were analyzed using the IPA software (Qiagen) against their involvement in cellular function and disease.

Proximity ligation analysis

The proximity ligation analysis (PLA) experiment was done according to the manufacturer's guidelines (Sigma). In short, adherent cells were fixed in 4% paraformaldehyde. The cells were blocked in 1% BSA and 5% FBS in PBS. The cells were then stained with primary antibodies together with rVAR2 or rDBL4 overnight in these concentrations: rVAR2 (50 nmol/L), anti-integrin- α 4 (MAB16983; 1:100), anti-integrin- α 5 (H-104, sc-10729; 1:50), anti-NG2 (LHM 2, ab20156; 1:400), anti-panCD44 (2C5, BBA10; 1:400) anti-integrin- β 1 (EP1041Y, ab52971; 1:200), and anti-integrin- β 1 antibody (4B7R, sc-9970; 1:50). Cells were washed in Wash Buffer A (DUO82047) between incubations. An anti-V5 (mouse or rabbit) antibody was used for rVAR2 detection. The cells were then stained with Duolink In Situ PLA Probe Anti-Mouse MINUS (DUO92004) and Duolink In Situ PLA Probe Anti-Rabbit PLUS (DUO92002) diluted in Antibody Diluent (DUO82008). The cells were then treated with the ligation solution, followed by incubation with the amplification solution. Both reagents were provided with the kit Duolink In Situ Detection Reagents Orange (DUO92007). The cells were washed with Wash Buffer B (DUO82048). Slides were mounted using Duolink In Situ Mounting Medium with DAPI (DUO82040). Results were analyzed under a Nikon C1 confocal microscope with a 60 \times oil objective. A total of 75 to 100 cells were imaged per sample. The images were analyzed using the BlobFinder software (version 3.2.).

Flow cytometry-binding analysis

Cells were grown to 70% to 80% confluency and harvested using Cellstripper. Cells (200,000) were added to each well in a 96-well plate. All incubations were in PBS containing 2% FBS. Cells were incubated with protein (400 nmol/L–25 nmol/L) for 30 minutes at 4 $^{\circ}$ C. Cells were washed 2 times and incubated with secondary antibody (anti-V5-FITC) for 30 minutes at 4 $^{\circ}$ C, washed 2 times, and analyzed in a FACSCalibur (BD Biosciences) for FL-1 signal intensity. Results were analyzed using the FlowJo software.

Signaling stimulation assays

Cells were seeded into 6-well plates and allowed to adhere overnight. The cells were washed in PBS and serum-starved in the presence of 450 nmol/L rVAR2 or rDBL4 control for 18 to 24 hours. The cells were stimulated with 1% to 3% FBS, 5 μ g/mL fibronectin (Sigma), or 10 to 80 μ g/mL fibronectin CS1 peptide (GeneArt) for the given timepoints. The cells were put on ice, washed 3 times in PBS, and lysed in EBC lysis buffer, containing 0.5% NP40 and phosphatase and protease inhibitor cocktails

(Roche). The samples were balanced on protein concentrations (Bradford assay). The samples were run in Western blotting and probed for the indicated phosphoproteins. For total protein determination, the membranes were stripped and reprobed with antibodies for the indicated proteins.

For the siRNA experiments, cells were transfected with siRNAs (Qiagen; 10 nmol/L final) against B3GAT1 and CSGALNACT1, using RNAiMAX (Invitrogen) and analyzed for rVAR2 binding by flow cytometry and for mRNA expression by RT-PCR, following 72-hour exposure. The evaluation of intracellular signaling in these cells was performed as described above.

The adhesion signaling experiments were performed as follows. The cells were grown to 70% confluency in 10-cm dishes. They were serum-starved 18 to 24 hours prior to the assay in the presence of 450 nmol/L rVAR2 or rDBL4. The cells were dislodged in Cellstripper, counted, and seeded into the wells of a 6-well plate for 120 minutes. Cell lysates were collected and analyzed as described above.

Antibodies used were: α -phospho-Erk1/2 (thr202/tyr204; Cell Signaling, 9101), α -Erk 1/2 (Cell Signaling, 9102), α -Src (Cell Signaling, 2108), α -phospho-Src (Tyr416; Cell Signaling, 2101), α -Akt (Cell Signaling, 9272), α -phospho-Akt (Thr308; Cell Signaling, 2965), α -FAK (Cell signaling, 3285), α -phospho-P130Cas (Tyr410; Cell Signaling, 4011), and α -p130Cas (Santa Cruz, sc-20029).

Tissue samples and immunohistochemistry

A tissue microarray (TMA) containing 38 patients with primary human pancreatic cancer and corresponding metastatic tissues, as well as control normal pancreas, was obtained from the UNMC Rapid Autopsy Pancreas (RAP) program and stained using the Ventana Discovery platform. Sectioned, paraffin-embedded TMA was stained with 500 picomolar V5-tagged recombinant VAR2CSA (rVAR2) without antigen retrieval followed by 1:700 monoclonal anti-V5 step and an anti-mouse-HRP detection step. Mounted and stained TMA was subsequently scored for membranous staining intensity on a 0–3 scale. Score 2 reflects a staining intensity equal to that of placenta (included as a positive control in each staining run). Expression is considered "low" when ofCS expression is present only in cellular or stromal compartment with intensity score 1 and considered "high" when ofCS expression is present either in cellular or stromal compartment or both with intensity score 2 or 3.

Immunocytochemistry

We obtained formalin-fixed, paraffin-embedded (FFPE) slides of metastatic lesions in murine allografts, created by injecting C57BL/6 mice with 4T1 mammary tumor cells in the left cardiac ventricle, in the animal model (published in ref. 10). The slides were deparaffinized and stained with rVAR2-Alexa488. The staining of ofCS was visualized using confocal microscopy.

Animal studies

The methodologies described were reviewed and approved by the Institutional Animal Care Committee (IACC) at the University of British Columbia (Vancouver, BC, Canada) and the animal experiments inspectorate at the University of Copenhagen before conducting the study. During the study, the care, housing, and use of animals were performed in accordance with the Canadian Council on Animal Care Guidelines and the Danish animal experiments inspectorate guidelines.

For the Lewis lung carcinoma seeding model, 5- to 6-week-old C57black/6 female mice were maintained under isoflurane anesthesia and 5×10^5 Lewis luciferase cells suspended in 100 μ L of 100 nmol/L of rVAR2 or saline solution were injected into the left ventricle under ultrasound guidance using a 30-gauge needle. The location of the tip of the needle in the left ventricle was confirmed by pulsatile blood flow in the hub of the needle. Animals were monitored until 7 weeks after injection using IVIS imaging system. Metastasis sites were collected at day of sacrifice and fixed in formalin for pathology studies. Mice were sacrificed when they reached the predefined humane end point.

For the B16 melanoma model, 5×10^5 B16-F10GP cells in 100 μ L PBS were injected into the right flank of C57BL/6 mice. The animals were randomized into 2 groups of 10 mice. One group was treated by intravenous injection of 100 μ g rVAR2 at days 0, 6, and 9. The control group was treated with equal volume PBS. Tumor size was monitored by manual measurements using a caliper-measuring tool, taking measurements at the 2 longest perpendicular axes in the x/y plane of each tumor. Tumor volume was calculated according to the standard formula: volume = $xy^2 \times 0.5236$ (31).

Statistical methods

Correlations between clinicopathologic parameters and ofCS expression were analyzed by the Fisher exact test. $P < 0.05$ was

considered statistically significant. Statistical analyses were performed with GraphPad Prism (version 6, GraphPad Software, Inc.). Survival of mice was analyzed by Kaplan–Meier survival plot. Statistical significance was determined with Prism GraphPad version 6.0 using the log-rank (Mantel–Cox) test (χ^2 : 3.84; $P < 0.05$).

Results

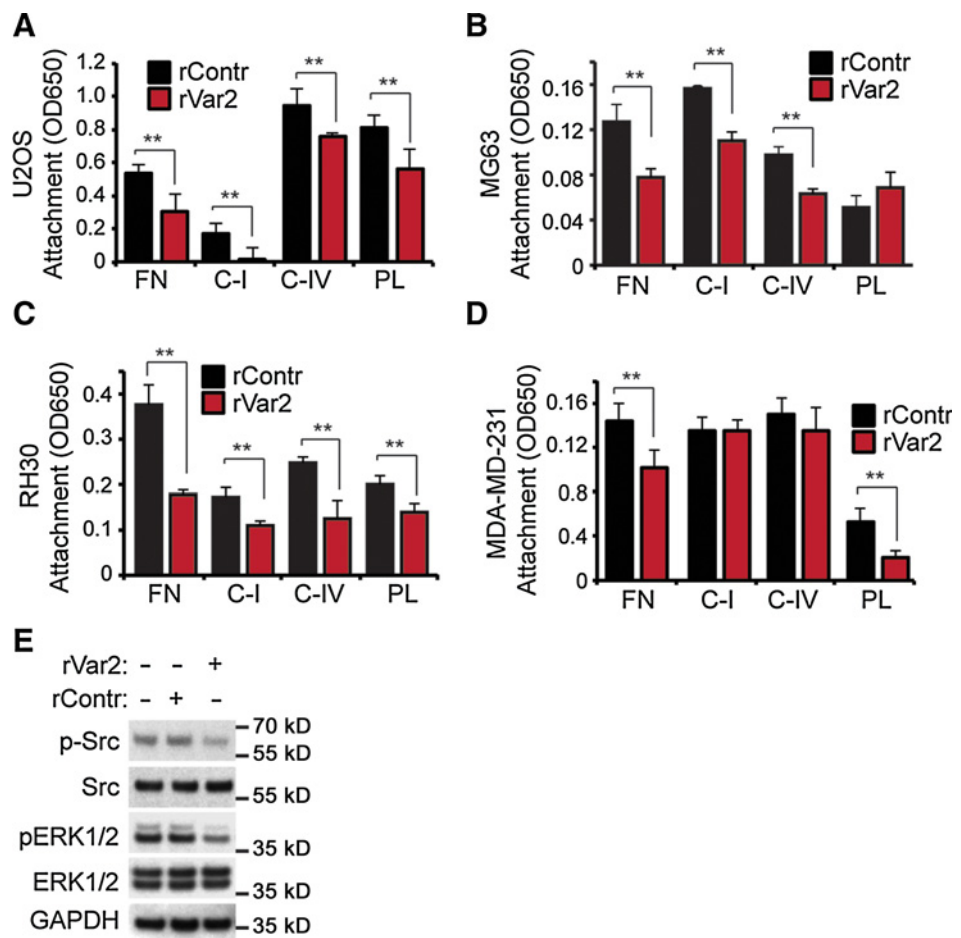
rVAR2 blocks cellular adhesion to ECM components

We have shown that placental and tumor cells share the expression of a placental-type chondroitin sulfate now designated ofCS (10). The expression of this modification in human cancers suggests a pivotal role for ofCS in malignant disease. On the basis of the preliminary observation that cellular attachment of tumor cells to normal culture flask plastic surfaces was impaired after rVAR2 incubation, we hypothesized that ofCS may play a role in cell motility. To test our hypothesis, we investigated whether rVAR2 targeting of ofCS on U2OS and MG63 osteosarcoma cells would block cellular adhesion to plastic (PL), fibronectin (FN), and collagens I (C-I) and IV (C-IV). While different binding preferences for individual ECM components could be observed, both cell lines displayed a significant reduction in adhesion to fibronectin, collagen I, and collagen IV (Fig. 1A and B). The same was true for cell lines representing other cancers, such as RH30 rhabdomyosarcoma cells (Fig. 1C) and MDA-MB-231 breast

Figure 1.

rVAR2 impedes attachment and detachment mechanics in tumor cells.

A, U2OS cells were preincubated with rVAR2 and tested for their capacity to adhere to fibronectin (FN), collagen I (C-I), collagen IV (C-IV), and plastic (PL). Adherent cells were stained with methylene blue and quantified by reading absorbance at 650 nm. **B**, MG63 cells were preincubated with rVAR2 and tested for their capacity to adhere to fibronectin (FN), collagen I (C-I), collagen IV (C-IV), and plastic (PL). Adherent cells were stained with methylene blue and quantified by reading absorbance at 650 nm. **C**, RH30 cells were preincubated with rVAR2 and tested for their capacity to adhere to fibronectin (FN), collagen I (C-I), collagen IV (C-IV), and plastic (PL). Adherent cells were stained with methylene blue and quantified by reading absorbance at 650 nm. **D**, MDA-MB-231 cells were preincubated with rVAR2 and tested for their capacity to adhere to fibronectin (FN), collagen I (C-I), collagen IV (C-IV), and plastic (PL). Adherent cells were stained with methylene blue and quantified by reading absorbance at 650 nm. **E**, U2OS cells were preincubated with rVAR2 or rContrl and allowed to adhere to plastic. The phosphorylation of Src and Erk1/2 kinases in response to the adhesion event was monitored by Western blotting.



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cancer cells (Fig. 1D). MDA-MB-231 was not inhibited in binding to collagen, suggesting another mode of binding in this cell type (Fig. 1D).

The Src and Erk kinases are activated by cellular adhesion (32, 33). Given the effects of rVAR2 incubation on functional cell adhesion, we tested whether these pathways were affected in rVAR2-treated U2OS cells. Indeed, we observed a clear inhibition of the phosphorylation of Src and Erk (Fig. 1E), in line with the shown effect on cellular adhesion (Fig. 1A–C).

rVAR2 inhibits cellular migration, invasion, and anchorage-independent growth in cancer

We next wanted to test whether blocking ofCS function impacts cellular migration. We therefore performed scratch assays using the invasive MG63 osteosarcoma cell line. Confluent monolayers of MG63 cells were scratched and incubated with rVAR2 for 24 hours under serum starvation. We then observed and documented wound closure over time. The cells incubated with rVAR2 failed to close the wound, whereas the control cells effectively filled in the scratch (Fig. 2A and B). MG63 cells treated with siRNAs against CSGALNACT1, which is involved key enzyme involved in chondroitin sulfate synthesis, showed similar effects (Supplementary Fig. S1). The antimigratory effect of rVAR2 on the cells could be inhibited by the addition of soluble CSA to outcompete rVAR2 cell binding (Fig. 2C). Furthermore, rVAR2 significantly inhibited the migration of MG63 cells across a membrane in a Boyden chamber

assay (Fig. 2D). Next, we tested the ability of rVAR2 to block cancer cell invasion across an ECM-modified membrane in the same Boyden chamber assay. This showed that rVAR2 potently inhibited the invasive capacity of cancer cells (Fig. 2E). Finally, we tested the ability of rVAR2 to inhibit anchorage-independent growth of MG63 cells in soft agar colony growth assays (Fig. 2F). rVAR2 efficiently reduced the number of colonies formed compared with the control. The effect was shown to be ofCS specific, as addition of soluble CSA to outcompete rVAR2 cell binding efficiently rescued the colony formation. Collectively, this shows that blocking the function of ofCS with rVAR2 inhibits the metastatic potential of cancer cells.

rVAR2 interacts with ofCS-modified proteoglycans

Chondroitin sulfate is a posttranslational modification to many CSPGs within the cell membrane. Although these CSPGs differ greatly in their protein cores, they have many overlapping functions in cancer development (11–19). We previously showed that rVAR2, through ofCS chains, interacts with numerous cancer-associated proteoglycans including CSPG4 and CD44, based on overexpression of membrane receptors in HEK cells (10). In continuation of this work, we wanted to explore the significance of ofCS targeting in terms of the number and diversity of CSPG targets in different cancers. To do this, we selected 5 cell lines derived from different cancer types: melanoma (C32), T-cell lymphoma (Myla2059), prostate cancer (PC3), osteosarcoma (U2OS),

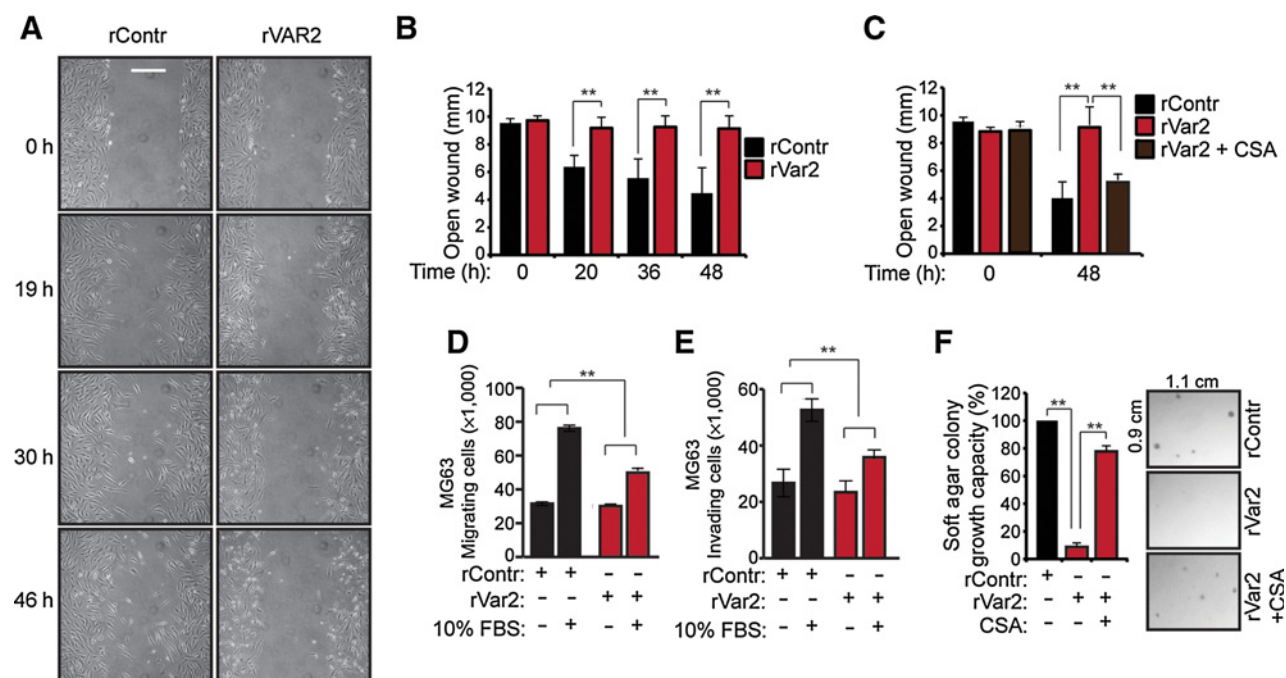


Figure 2. Chondroitin sulfate-dependent motility and anchorage-independent growth in tumor cells. **A**, MG63 cells were tested for their ability to migrate in the presence of rVAR2 or saline in a scratch wound heal assay. The figure shows representative pictures of the analysis taken at time 0, 19, 30, and 46 hours. **B**, Column graph showing the statistical analysis of the MG63 scratch wound heal assay. **C**, Scratch wound heal assay was repeated with rVAR2 treatment in the presence of CSA to illustrate ofCS specificity. **D**, MG63 cells were tested for their ability to migrate across a membrane in a Boyden chamber assay in the presence of rVAR2 or rContr. **E**, MG63 cells were tested for their ability to invade across an ECM-modified membrane in a Boyden chamber assay in the presence of rVAR2 or rContr. **F**, MG63 cells were tested for their ability to elicit anchorage-independent growth in the presence of rVAR2, rContr, or rVAR2 + CSA in a soft agar colony formation assay.

Table 1. List of known CSPGs identified in the rVAR2 pull-down screen

Protein name	Gene	Source
Cell surface CSPGs		
Chondroitin sulfate proteoglycan 4	<i>CSPG4</i>	U2OS, C32, RH30
Amyloid-like protein 2	<i>APLP2</i>	U2OS, PC3, Myla2059, C32
Syndecan-1	<i>SDC1</i>	U2OS, PC3, C32, RH30
CD44	<i>CD44</i>	U2OS, C32, Myla2059, RH30
Integrin-β1	<i>ITGB1</i>	U2OS, C32
Syndecan-4	<i>SDC4</i>	U2OS, C32, Myla2059
Glypican-1	<i>GPC1</i>	U2OS
Sushi repeat-containing protein	<i>SRPX</i>	U2OS, C32
Neuropilin-1	<i>NRP1</i>	U2OS, PC3
Sushi repeat-containing protein 2	<i>SRPX2</i>	U2OS
Syndecan 2	<i>SDC2</i>	C32
Neuropilin-2	<i>NRP2</i>	C32
Glypican-2	<i>GPC2</i>	C32
Glypican-6	<i>GPC6</i>	C32
Glypican-4	<i>GPC4</i>	C32
Endorepellin	<i>HSPG2</i>	C32
Delta-sarcoglycan	<i>SGCD</i>	C32
Agrin	<i>AGRN</i>	C32, Myla2059
Syndecan-3	<i>SDC3</i>	C32
HLA class II histocompatibility antigen gamma chain	<i>CD74</i>	C32, Myla2059
Laminin subunit alpha-4	<i>LAMA4</i>	C32
Leukocyte surface antigen CD47	<i>CD47</i>	Myla2059
Testican-1	<i>SPOCK1</i>	Myla2059
Secreted CSPGs		
Serglycin	<i>SRGN</i>	PC3, Myla2059

NOTE: Each CSPG hit is listed at either cell surface or secreted. The hits are given by name and gene. The source of these hits is given in the right column.

and rhabdomyosarcoma (RH30). We then performed pull-downs from cell extracts using rVAR2 coupled columns or biotinylated rVAR2 captured on streptavidin-coated beads. The elution extracts were then analyzed by mass spectrometry. Using this method, we identified 24 proteins previously reported to carry chondroitin sulfate (Table 1). Interestingly, all cell lines tested co-expressed different CSPGs, and several CSPGs were shared between tumor cell lines of different origin.

Cancer-associated CSPG complexes are involved in cell motility

The combined pull down and mass spectrometric analysis identified many ofCS CSPGs across diverse tumor types. Furthermore, we noted that several known CSPG-associated partners were among the identified proteins. To gain insights into the CSPG-related proteome and its impact on cellular function, we subjected the full list of pulldown hits to IPA. This revealed an involvement of CSPG complexes in cellular motility and metastasis (Fig. 3A and B).

Several integrin subunits were consistently among the most significant hits in the pulldown analysis (Fig. 3C). To verify this, we investigated the interaction between rVAR2 staining and integrin-β1, integrin-α4, and the heterodimeric integrin-α5β1 complex by colocalization in immunofluorescence (Fig. 3D), coprecipitation using an rVAR2-coupled column (Fig. 3E; Supplementary Fig. S2A), and PLA (refs. 34, 35; Fig. 3F and G; Supplementary Fig. S2B and S2C). This suggested a strong interaction between rVAR2-targeted ofCS and integrin-β1, integrin-α4, and integrin-α5β1.

ofCS is involved in integrin-related intracellular signaling

Several studies have shown that targeting the CSPG4 core protein on diverse cancer cell types has a direct effect on integ-

rin-related intracellular signaling through effectors such as FAK, Src, and Erk1/2 (15, 36, 37). To test whether targeting of the ofCS part of these CSPG/integrin complexes had a similar effect, we incubated the osteosarcoma cell line, U2OS, with rVAR2 and analyzed the intracellular response to FBS stimulation. We used 450 nmol/L rVAR2 as this concentration effectively saturated the rVAR2-binding sites on the cells, shown by FACS-binding analysis (Fig. 4A). A non-chondroitin sulfate-binding part of VAR2CSA, rDBL4, was used as a recombinant protein negative control (rContr). This showed that phosphorylation of Src and P130Cas was indeed inhibited in response to FBS stimulation (Fig. 4B). There was an effect of rVAR2 on the signaling prior to FBS stimulation as well.

To test whether these effects were isolated to U2OS cells, we performed the same analysis on MG63 osteosarcoma (Fig. 4C) and MDA-MB-231 breast cancer cells (Fig. 4D). The latter has previously been tested in an anti-CSPG4 targeting strategy (15). Again, we saw that phosphorylation of Src was inhibited (Fig. 4C and D).

We have previously shown that siRNA-mediated knockdown of the enzymes B3GAT1 and CSGALNACT1, which are involved in chondroitin sulfate biosynthesis, reduces binding of rVAR2 to the cell surface (10). To test whether the effects seen on intracellular signaling were due to inhibition of the ofCS-binding epitope, we tested the effects of B3GAT1 and CSGALNACT1 knockdown in our FBS stimulation assay (Fig. 4E and F). This showed inhibition of phosphorylation of Src and P130Cas with knockdown of both B3GAT1 and CSGALNACT1. This supports our hypothesis that the rVAR2 ofCS-binding epitope is involved in integrin-mediated signaling in cancer.

While stimulation with FBS revealed inhibition of several integrin related effector proteins, such broad stimulation may show effects through other signaling pathways converging on the same effectors. To narrow down the source of stimulation, we wanted to investigate the specific cellular activation of integrin complexes, with a ligand such as fibronectin. Fibronectin is a large complex molecule containing binding sites for numerous cell surface receptors and ECM components. The integrin subunits of various types can interact with several of these sites both with and without CSPGs (38–42). However, one region of fibronectin, called the CS1 region, has been shown to support integrin-α4β1 binding only when it is in complex with a CSPG (43). We therefore tested whether rVAR2 would inhibit cellular activation in response to stimulation with recombinant CS1 peptide, which has been shown to stimulate the phosphorylation of FAK (44). Accordingly, rVAR2 blocked phosphorylation of FAK (Y397) in response to stimulation with the CS1 peptide on U2OS cells (Fig. 4G). This work shows the importance of ofCS in integrin-mediated signaling.

ofCS is expressed in metastasis

We have shown the implication of ofCS in integrin-mediated cellular function and in cellular adhesion, migration, and invasion, pointing to a potential role for ofCS in metastasis. To investigate the presence of ofCS at metastatic sites, we stained metastatic lesions in murine allografts from C57BL/6 mice injected with 4T1 mammary tumor cells in the left cardiac ventricle (10), using rVAR2-Alexa488. The analysis showed that ofCS was highly and specifically expressed in liver and bone metastasis

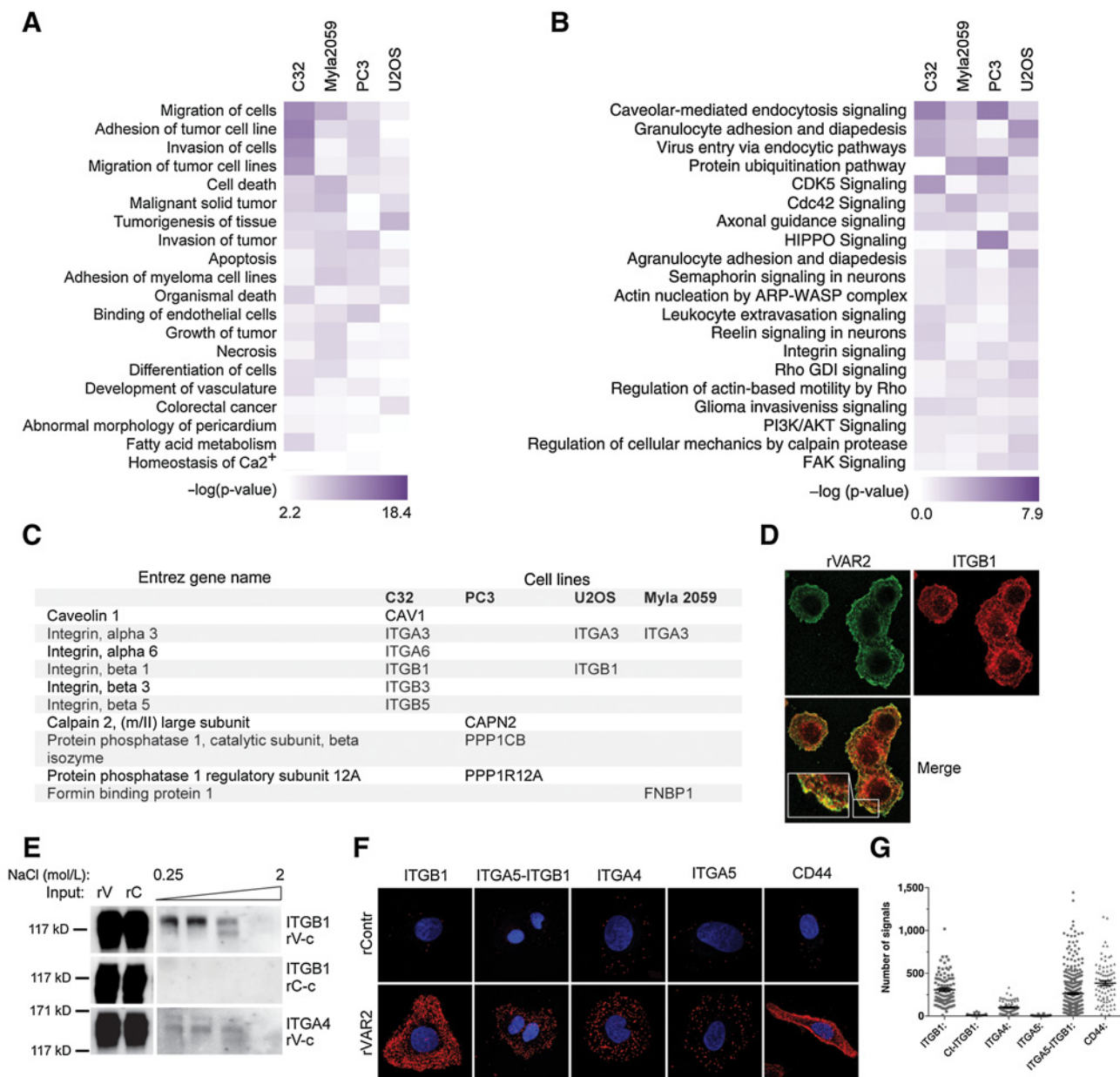


Figure 3.

The chondroitin sulfate-modified tumor cells proteome is enriched for proteins involved in motility. **A**, Network analysis of pulled down proteins using the IPA software. Proteins that were found to be significantly different between rVAR2 and rControl in the pull-down analysis were analyzed. The top 20 diseases and biofunctions involved are shown in a heatmap. We generated the heatmap by comparing independent analyses of rVAR2 pull downs in different cell lines. **B**, IPA heatmap analysis of canonical pathways most significantly enriched in proteins pulled-down with rVAR2. **C**, List of the significant rVAR2 pulled-down proteins involved in the integrin signaling. **D**, Colocalization analysis between ofCS [rVAR2 stain (green)] and ITGB1 antibody stain (red). **E**, Column-based pull down of integrin subunits using rVAR2 (rV-c) from U2OS cells. Figure shows Western blot analysis of eluates in increasing NaCl concentration. An rContr coupled column is used as negative control (rC-c). **F**, PLA analysis of colocalization between ofCS (rVAR2 stain) and integrin subunits. CD44 is used as the positive control. **G**, Quantification of the PLA analysis.

(Fig. 5A). A stain for the Ki-67 proliferation marker showed that the rVAR2-staining metastatic cancer cells were rapidly proliferating (Fig. 5A).

To investigate the presence of ofCS in metastatic sites in human tumors, we stained pancreatic cancer specimens collected in the UNMC RAP program from primary tumor

tissue as well as tissue from metastatic sites in different organs. As in the murine model, rVAR2 showed strong staining of the primary tumor as well as all metastatic sites, with little staining of normal pancreatic tissue (Fig. 5B and C). Notably, high ofCS expression was unrelated to all annotated clinical parameters (Supplementary Table S1). These data

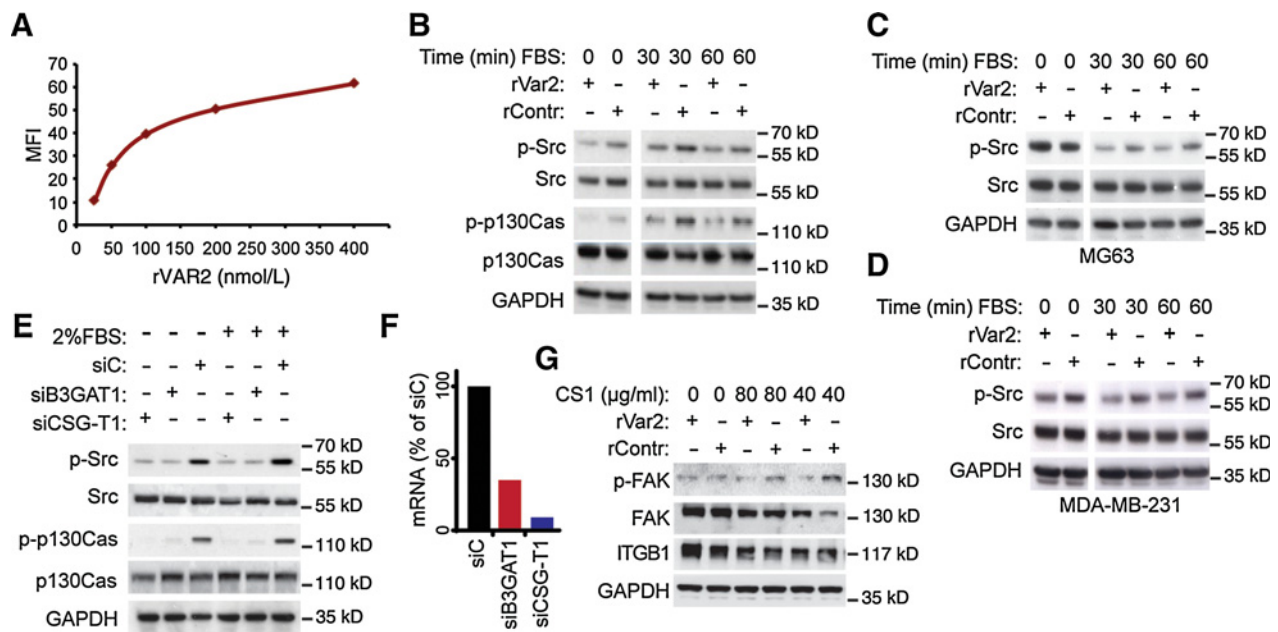


Figure 4. A role for ofCS in signaling downstream integrin-β1. **A**, Titration of rVAR2 staining of U2OS cells in flow cytometric analysis. **B**, Analysis of downstream integrin signaling in rVAR2- or rContr-treated U2OS cells in response to FBS stimulation. Figure shows effect on Src and p130Cas phosphorylation. **C**, Analysis of downstream integrin signaling in rVAR2- or rContr-treated MG63 cells in response to FBS stimulation. Figure shows effect on Src. **D**, Analysis of downstream integrin signaling in rVAR2- or rContr-treated MDA-MB-231 cells in response to FBS stimulation. Figure shows effect on Src. **E**, Analysis of downstream integrin signaling in U2OS cells treated with siRNA (72 hours) against B3GAT1 and CSGALNACT1 (CSGT-1). A scrambled siRNA is used as control. Figure shows effect on Src and p130Cas phosphorylation. **F**, RT-PCR analysis of cells treated with siRNA against B3GAT1 and CSGALNACT1 for the analysis shown in **E**. **G**, Analysis of FAK phosphorylation in rVAR2- or rContr-treated U2OS cells in response to stimulation with the CS1 fibronectin peptide.

support a role for ofCS in both primary and metastatic tumor compartments.

rVAR2 adhesion to ofCS inhibits metastasis

Having shown that ofCS is present in metastatic tumors, we wanted to investigate the effect of rVAR2 treatment on metastasis formation *in vivo*. First, we tested the effect of intravenous administration of rVAR2 on the implementation of B16 melanoma tumors in subcutaneous allotransplanted mice. After B16 cell inoculation, the mice were randomized into 2 groups (*n* = 10 each) and treated with rVAR2 at days 0, 6, and 9. The control group was treated with saline. The results showed that 100% of mice in the saline group developed tumors within 12 days whereas tumor growth in the mice treated with rVAR2 at days 0, 6, and 9 was significantly delayed (Fig. 6A; Supplementary Fig. S3, *P* = 0.008178). These data show that rVAR2 binding to ofCSA on the cell surface of B16 melanoma cells inhibits tumor implantation.

To analyze the impact of rVAR2-ofCS binding on metastatic spread, luciferase-positive Lewis lung carcinoma cells were pre-incubated with 100 nmol/L of rVAR2 or saline and injected into the left cardiac ventricle of mice (*n* = 7 each). In the control arm, 42% (*n* = 3) of the mice formed metastases as visualized by bioluminescence imaging, whereas no metastases were found in any of the rVAR2-treated mice (*P* < 0.05; Fig. 6B and C). Of the mice in the rVAR2 arm, 100% were alive after 45 days. One mouse in the saline group died unexplainably without signs of metastasis and is therefore excluded from the survival graph. With this in

mind, 50% of the saline control group were dead by day 45 days (*n* = 3; *P* < 0.05; Fig. 6D). At the experimental endpoint, autopsy and immunohistochemical (IHC) analysis were performed. The control mice treated with PBS had visible metastases in different organs, including kidney and ovary, which stained strongly for ofCS using rVAR2-alexa 488 (Fig. 6E). No metastases were found in the lung, liver, and kidney of the rVAR2-treated mice (Fig. 6F). This shows that interfering with ofCS on cancer cells significantly (*P* < 0.05) inhibits the seeding of cancer cells *in vivo*.

Discussion

We have recently shown that tumor and placental cells carry a common ofCS secondary modification that can be targeted by the malarial VAR2CSA protein (10). The binding of rVAR2 to cancer is nearly universal and highly specific with minimal to absent binding in normal tissue compartments, except for placenta. As such, rVAR2 can potentially be utilized in various diagnostic and therapeutic settings (10).

Chondroitin sulfate in cancer is well described (14, 15, 45–51). The roles of chondroitin sulfate and CSPGs have been described in many cellular functions including proliferation, migration, invasion, metastasis, angiogenesis, and capture of growth factors, cytokines, and chemokines (11–15, 20–24). A well-described function of chondroitin sulfate, and of many of the distinct CSPGs, is their interaction with, and potentiation of integrin function (18, 39, 40, 43, 52). In this article, we investigated the link between ofCS modification and cellular function in cancer.

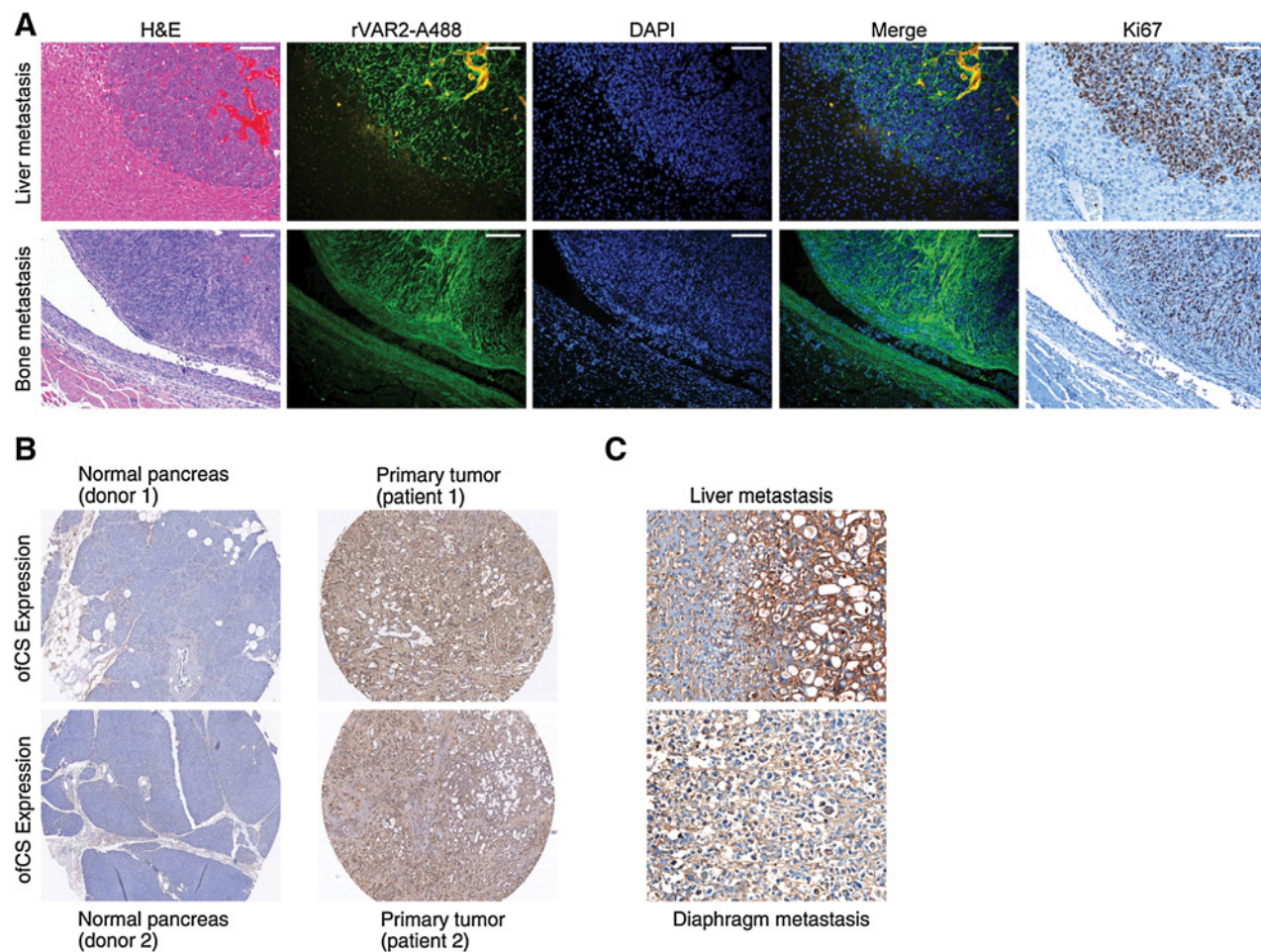


Figure 5.

Expression of oncofetal CS in metastatic lesions. **A**, Hematoxylin and eosin (H&E) images (left), immunofluorescent (rVAR2-Alexa 488, DAPI and merged; middle), and Ki-67 cell proliferative marker staining (right) images of murine liver and bone metastases derived from Balb/C mice inoculated with 4T1 murine mammary cancer cells by intracardiac injection. The scale bars represent 100 μ m. **B**, IHC analysis of ofCS, stained with rVAR2-V5 using anti-V5-HRP antibody, in human normal pancreas (left), pancreatic cancer tissues (right). **C**, Liver and diaphragm metastases.

There are more than 50 known proteoglycans in human tissues. They are found in the cell membranes, in the ECM, excreted into the body fluids, or kept in intracellular granules (24, 53). We recently tested rVAR2 binding in a functional screen where 3,500 cell surface receptors were overexpressed in HEK cells. We identified 17 positive binding partners of which some were known proteoglycans (10). Overexpression of receptors in a non-cancer cell line is unlikely to be fully representative of how the receptors are modified during expression in cancer cells. To more directly analyze proteins associated with ofCS, we performed co-precipitation from tumor cell extracts using rVAR2. Here, we identified 24 known CSPGs as well as known CSPG-associated proteins, including integrin subunits (Table 1). Furthermore, the analysis identified several proteins not previously described in relation to CSPGs (data not shown). The co-precipitated proteins included both cell membrane CSPGs and secreted serglycin. Several CSPGs were coprecipitated from each cell type and some were common among tumor cell lines of diverse origin. The broad presence of ofCS on so many CSPGs emphasizes the significance of ofCS

substitution in cancer rather than the expression of a specific CSPG. This promotes ofCS-modified CSPG as potential candidate targets in anticancer therapy. Our results are refined by the current knowledge of chondroitin sulfate-carrying proteoglycans. The part-time glycosylation status of many proteoglycans, meaning that they are not always glycosaminoglycan-modified, does however make CSPGs difficult to identify. It is therefore possible that proteoglycans not previously associated with a chondroitin sulfate chain are modified with ofCS during transformation to fuel proliferation and tumor cell motility. A thorough and detailed analysis into glycosaminoglycan displacement at the proteome level is needed.

The interaction of CSPGs, including CSPG4, CD44, and the syndecans, with integrin subunits are well described (38–40, 52, 54). Furthermore, it has been shown that the $\alpha 5 \beta 1$ complex can be modified with a chondroitin sulfate chain itself (55, 56). In line with this, $\alpha 4$ - and $\beta 1$ -integrins were co-precipitated with rVAR2. The association of ofCS with the integrin complexes was further verified using PLA that allows for colocalization analysis at the resolution of a single molecule (35). This

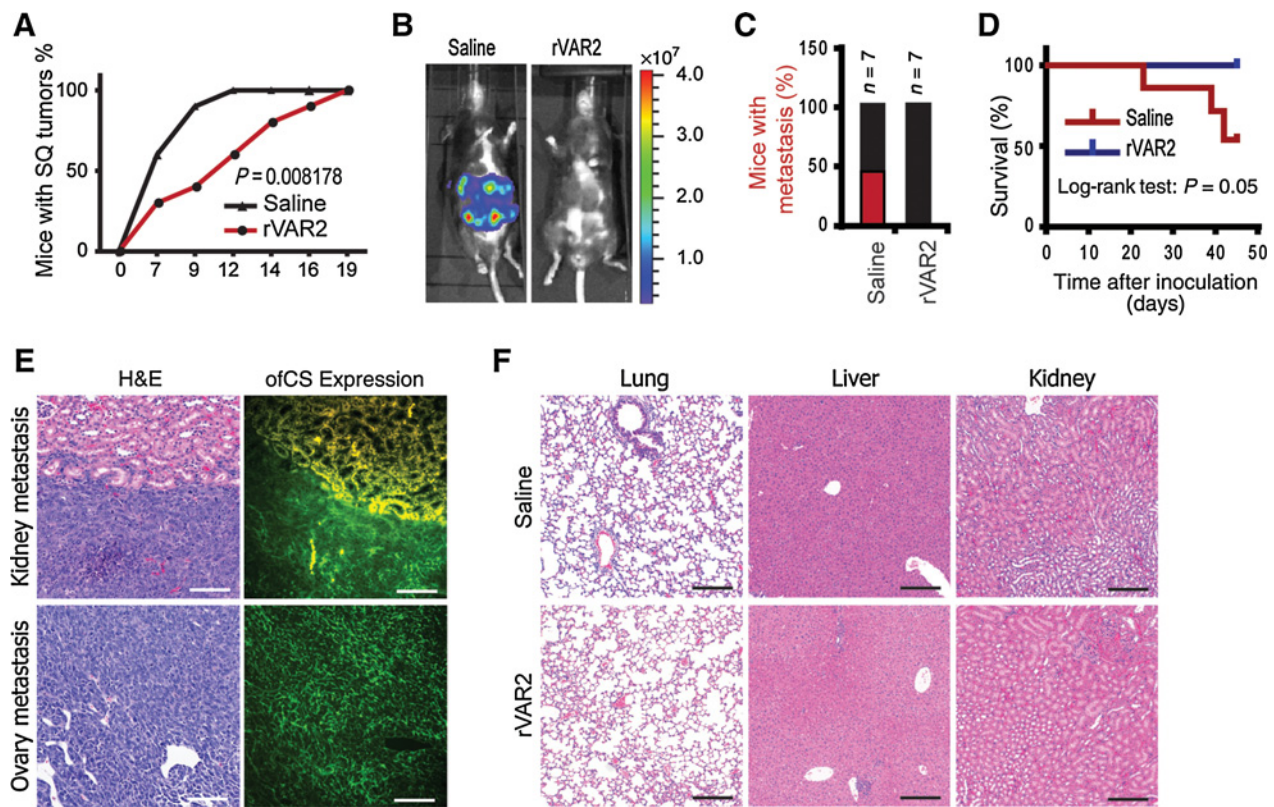


Figure 6.

Inhibition of tumor initiation and metastatic spread by rVAR2. **A**, Mice were inoculated with subcutaneous B16 tumors and randomized into 2 groups ($n = 10$) and treated with rVAR2 at days (0, 6, 9). The control group was treated with saline. Figure shows tumor size progression over time (days). **B**, Two groups of C57black/6 mice were inoculated with murine Lewis lung cancer cells preincubated with control (saline) or rVAR2. Representative luciferase images 39 days after inoculation. **C**, Percentage of C57black/6 mice carrying Lewis cell metastasis (luciferase-positive) in saline and rVAR2 group; $n = 7$. **D**, Kaplan–Meier plot of disease-free survival ($P = 0.05$). **E**, Metastatic kidney and ovary from control group stained with hematoxylin and eosin (H&E) and immunofluorescence using rVAR2-Alexa488 (right). **F**, Lung, liver, and kidney tissue from control group (top) and the rVAR2 group (bottom) were stained with H&E. The white scale bars represent 100 μm ; the black scale bars represent 200 μm .

method confirmed a strong correlation between integrin- $\alpha 4$, - $\beta 1$, and - $\alpha 5\beta 1$ and rVAR2 cell binding. These data confirm the findings of others (18, 39, 40, 43) and emphasize the involvement of CSPGs in tumor-associated integrin signaling.

Several articles have shown that targeting the CSPG4 protein core with monoclonal antibodies has an effect on integrin-related cellular function (15, 36, 37, 57). Here, we show that targeting the ofCS modification present on the proteoglycan component of the integrin complexes has similar effects. These effects include inhibition of intracellular signaling through Src, FAK, P130Cas, and Erk in stimulation and adhesion experiments. The mode of action could be 2-fold. On one hand, a bulky protein bound to ofCS chains could prevent formation of the CSPG-integrin complexes at the cell surface, whereas on the other hand, shielding of the ofCS epitope could abrogate its cancer-promoting effects. We have previously shown that knockdown of key enzymes in the chondroitin sulfate biosynthesis pathway reduces rVAR2 binding, suggesting that these enzymes are involved in producing the distinct ofCS epitope (10). In the present study, we show that targeting the same enzymes has a similar effect on integrin-related signaling, which suggests that the ofCS epitope is indeed crucial for these events to take place. It is likely that targeting the ofCS modification rather than the protein component of proteoglycans

such as CSPG4 will present a more universal and efficacious treatment strategy.

We have presented evidence for a function of ofCS in cellular adhesion, migration, and invasion. These are all driver functions of metastatic disease. Indeed we found that ofCS was present in both the primary and the metastatic lesions of human pancreatic cancer.

Finally, we wanted to see whether targeting ofCS with rVAR2 would interfere with tumor settlement *in vivo*. For this purpose, we established 2 animal models exploring 2 essential events of metastatic spread; cell settlement and tumor implementation. Preincubating Lewis lung carcinoma cells with rVAR2 strongly inhibited settlement in distant organs and significantly prolonged lifespan of the treated mice. The same was evident for treatment of subcutaneous B16 melanoma tumors at time of implementation. This aligns with our data showing that rVAR2 inhibits cellular attachment, migration, invasion, and integrin function. It also confirms what others have shown in targeting specific CSPG components of cancer cells using monoclonal antibodies (15).

Taken together, these data demonstrate the involvement of ofCS chains in cancer cell growth and motility, promoting ofCS as a candidate target for therapy.

Disclosure of Potential Conflicts of Interest

S. Lee is the Research Assistant at Vancouver Prostate Centre. A. Salanti is the CEO of and has Ownership Interest (including patents) in VAR2Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.M. Clausen, M.A. Pereira, N.A. Nakouzi, H.Z. Oo, M.Ø. Agerbæk, S. Lee, M.S. Ørum-Madsen, A.R. Kristensen, P.M. Grandgenett, J. L. Grem, M.A. Hollingsworth, P.J. Holst
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.M. Clausen, M.A. Pereira, N.A. Nakouzi, H.Z. Oo, A. El-Naggar, P.M. Grandgenett, T. Theander
Writing, review, and/or revision of the manuscript: T.M. Clausen, M.A. Pereira, H.Z. Oo, A. El-Naggar, P.M. Grandgenett, P.J. Holst, T. Theander, P.H.B. Sorensen, M. Daugaard, A. Salanti
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.Z. Oo, M.A. Hollingsworth, A. Salanti
Study supervision: T.M. Clausen, M. Daugaard, A. Salanti
Other (conducted motility studies, analyzed and interpreted the data, and provided figures): A. El-Naggar

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