Caveolin-1 (CAV1) Is a Target of EWS/FLI-1 and a Key Determinant of the Oncogenic Phenotype and Tumorigenicity of Ewing’s Sarcoma Cells

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Abstract

Tumors of the Ewing’s sarcoma family (ESFT), such as Ewing’s sarcoma (EWS) and primitive neuroectodermal tumors (PNET), are highly aggressive malignancies predominantly affecting children and young adults. ESFT express chimeric transcription factors encoded by hybrid genes fusing the EWS gene with several ETS genes, most commonly FLI-1. EWS/FLI-1 proteins are responsible for the malignant phenotype of ESFT, but only few of their transcriptional targets are known. Using antisense and short hairpin RNA–mediated gene expression knockdown, array analyses, chromatin immunoprecipitation methods, and reexpression studies, we show that caveolin-1 (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 EWS cell–derived tumors in nude mice xenografts, indicating that CAV1 promotes the malignant phenotype in EWS carcino genesis. Reexpression of CAV1 or E-cadherin in CAV1 knockdown EWS cells rescued the oncogenic phenotype of the original EWS 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. Overall, these data identify CAV1 as a key determinant of the tumorigenicity of ESFT and imply that targeting CAV1 may allow the development of new molecular therapeutic strategies for ESFT patients. (Cancer Res 2006; 66(20): 9937-47)

Introduction

Ewing’s sarcoma (EWS), a highly aggressive malignancy of soft tissues and predominantly bone, is the second most common bone tumor in children and young adults (1). Survival rates for patients with primary tumors are about 60%. However, 20% to 30% of cases are already metastatic at diagnosis, with survival rates below 20% (2). Over 90% of EWS cases express chimeric proteins encoded by hybrid genes generated by translocations involving the EWS and FLI-1 genes. These EWS/FLI-1 genes are heterogeneous with regard to the location of the translocation junction, resulting in different breakpoints (3). Regardless of the fusion type, all EWS/FLI-1 proteins act as aberrant transcription factors that promote oncogenesis by modulation of multiple target genes (4). Although several EWS/FLI-1 targets [i.e., transforming growth factor-βRII (TGFβRII), Id2, p21, and PTPL1] have been already described (5–8), direct targets specific to the most aggressive tumors of the Ewing’s sarcoma family (ESFT) have not been yet discovered. Identification of such targets is crucial to better understand the disease, improve treatment options and, ultimately, enhance patient survival.

Caveolae are plasma membrane invaginations that regulate several intracellular signaling pathways. The defining components of caveolae are 21- to 25-kDa proteins termed caveolins. Caveolin-1 (CAV1) and caveolin-2 (CAV2) are ubiquitously expressed. Caveolin-3 (CAV3) is only expressed in muscle tissue (9). CAV1 is the only member of the family shown to be required for caveolae formation (10). Roles in tumor development and metastatic progression have been attributed to both CAV1 loss and overexpression. A negative regulatory role has been proposed on the basis of the reduced CAV1 levels detected in cancers such as ovarian, colon, lung, and breast carcinomas and various sarcomas (11–14). Experiments with cultured cells and animal models showed that CAV1 acts as a tumor suppressor in breast cancer and other tumors (14, 15). In contrast, high CAV1 levels were associated with enhanced tumor progression and metastatic activity in kidney, bladder, esophagus, and prostate tumors (14, 16, 17). Recent studies showed a biphasic expression of CAV1 in primary versus metastatic oral, kidney, and pancreatic tumors (14, 18). CAV1 function in tumor development and metastasis is determined by intrinsic factors specific to different cell, tissue, and tumor types and/or malignant progression stages (11–18).

Studies of the putative CAV1 involvement in ESFT pathobiology have not been reported to date. Based on results from experiments using antisense oligonucleotides, retroviral-mediated RNA interference, gene array–based analyses, and reexpression studies, we now report for the first time the identification of CAV1 as a new transcriptional target of EWS/FLI-1. We show that suppression of CAV1 expression down-regulates the malignant phenotype of EWS cells in vitro and their tumorigenicity in nude mice. CAV1 knockdown up-regulates Snail and concomitantly decreases E-cadherin. The fact that reexpression in CAV1 knockdown cells of either CAV1 or E-cadherin rescued the oncogenic phenotype of unmanipulated EWS cells strongly supports a role for CAV1 as an...
important determinant of ESFT tumorigenicity and suggests that targeting CAV1 may allow the development of new molecular therapeutic strategies for ESFT patients.

Materials and Methods

Cell lines, survival assays, general methods, and reagents. EWS cell lines were cultured as described (19–21). HTC-116, HT-29, PC-3, and RD cells were maintained in either RPMI 1640 (HTC-116, HT-29, and RD) or DMEM (PC-3) supplemented with penicillin, streptomycin, and 10% fetal bovine serum (all from Ambrx Corporation, Carlsbad, CA), at 37 °C in a humidified atmosphere of 5% CO2 in air. Cell survival was measured by using the trypan blue exclusion assay as described (19, 20). Antisense (ATCCGTGAGCCGCTTTTCTCCT) and the sequence scrambled control (GTTATCTTACCTCAGCAGCTG) oligonucleotides (ESAS2 and ESSC2 in ref. 22) from Bio-Synthesis, Inc. (Lewisville, TX), contained two phosphorothioate modified bonds from each end. Oligonucleotides were directly added to cell cultures, without any carriers, and maintained at 10 μmol/L in the medium for 48 hours. The hemagglutinin (HA)–tagged Snail cDNA vector was a generous gift from Dr. A. Cano (Universidad Autonoma de Madrid/Consejo Superior de Investigaciones Cientificas, Madrid, Spain), and the wild-type and dominant-negative E-cadherin expression vectors were generous gifts from Dr. S.M. Troyanovsky (Washington University, St. Louis, MO). EWS/FLI-1–targeted, pSR-based retroviral short hairpin RNA (shRNA) vectors (EF4, EF22, and EF30) were described previously (21). The pCMV-EWS/FLI-1 expression construct was a generous gift from Dr. Dennis K. Watson (Medical University of South Carolina, Charleston, SC).

General immunologic techniques. Anti-HA-tag antibody was from Cell Signaling Technology, Inc. (Beverly, MA). CAV1, FLI-1 (COOH-terminal specific), and EWS (NH2-terminal specific) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLI-1 (NH2-terminal-specific) was from Calbiochem/EMD Biosciences, Inc. (San Diego, CA), and monoclonal anti-E-cadherin antibody was from BD Biosciences (San Jose, CA). Anti–β-actin (Abcam Ltd., Cambridge, England) detection was used for loading normalization. Western blot analyses were done as described (19, 20). All immunofluorescence and immunohistochemical techniques were essentially as described (20).

Chromatin immunoprecipitation assays. The ChIP-IT kit (Active Motif, Inc., Carlsbad, CA) was used following manufacturer’s protocols, after cross-linking with 1% formaldehyde for 15 minutes. Chromatin was sheared by sonication on ice, and agarose gel electrophoresis was used to select for chromatin fragments with an average size of <500 bp. Primers GAGCTCTTGGAAGATGCGCC (upper) and GCCTACCTCCAGTACCGC (lower) were used for PCR amplification of input DNA and DNA precipitated with anti-FLI-1-N, anti-FLI-1-C, or anti-EWS antibodies, or with a nonspecific control IgG. These primers were designed to amplify a 234-bp fragment containing the ETS-binding site (EBS) within the CAV1 promoter (positions −216 to +18).

CAV1 knockout with antisense and shRNA expression constructs and reexpression experiments. To generate the pBK-ASCAV1 antisense CAV1 expression vector, a human CAV1 (Invitrogen) NotI/Sall fragment encompassing its coding sequences was inserted into the Sall/NotI sites of pBK-CMV (Invitrogen). Validated SureSilencing human CAV1 shRNA and control plasmids were from SuperArray Bioscience Corp. (Frederick, MD). EWS cells were transfected using LipofectAMINE (Invitrogen) following manufacturer’s protocols. Transfected cells were selected with neomycin (0.8 mg/mL) 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). The sense CAV1 construct, pBK-CMV-CAV1, including the entire CAV1 open reading frame but lacking the 3′ untranslated region, was used for the reexpression of CAV1 in A4573 cells expressing a shRNA construct targeted to the CAV1 3′ sequences. The pBK-CMV-CAV1 vector and pCHC6-hygro, a plasmid that provides hygromycin resistance, were cotransfected (at a 50:1 molar ratio) into A4574/shCav1 cells using LipofectAMINE. Transfected cells were selected for 2 weeks with hygromycin (100 μg/mL) in medium containing the neomycin maintenance dose (as above), and resistant cellular pools and individual colonies were isolated for further analysis and maintained in the presence of neomycin (as above) and hygromycin (50 μg/mL). Similar conditions were used for E-cadherin reexpression experiments.

Gene arrays. Total RNAs (2 μg) from cells treated with scrambled or ASATG oligonucleotides were processed using the SuperArray Ampolabeling Linear Polymerase Reaction kit. Array analyses were independently replicated twice for each experiment. Hybridizations were done on GEArray Q Series (+HS-007, Human Metastasis; see http://www.superarray.com for functional gene grouping, and Supplementary Table S1) according to manufacturer’s protocols. Relative gene expression levels from each replicated array were determined by densitometry, normalized for loading with β-actin, and analyzed with the GEArray Expression Analysis Suite (SuperArray) software. Arrays for the analysis of EWS/FLI-1 shRNA effect on gene expression were carried out and analyzed as previously described (21).

Electron microscopy. General procedures were as described (23). Preparations were examined and photographed using a Hitachi H-7600 instrument (Hitachi High Technologies America, Inc., Pleasanton, CA).

Reverse transcription-PCR. RT and PCR product analysis and sequencing were as described (19, 20). Primers ACAAGGAAACAACAAAAGG (upper) and ATCCGGATGCAAGAAGG (lower) were used for amplification of CAV1; for E-cadherin, CTCAACCCACACTCCTCTCCT (upper) and GCCCTGCTCTCTGGCCCCCTAGT (lower) were used for amplification of E-cadherin. TcPaul (lowerm) and AAGCAGGAGCAAGGACC (lower) were used for amplification of TGFβ (TGFβ/β2) and human β-actin as were described (19, 20). 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.

Site-directed mutagenesis and luciferase reporter assays. The reporter plasmid pGL3-cavFL, carrying luciferase under transcriptional control of the human, full-length CAV1 promoter was a gift from Dr. Vijay Shah (Mayo Clinic College of Medicine, Rochester, MN). Two similar constructs containing either a G → T mutation (pGL3-mut1Cav) or a C → T mutation (pGL3-mut2Cav) within the ETS-binding site present in the human CAV1 promoter (position −140; Fig. 2A) were generated using the QuickChange Multi Site-Directed Mutagenesis system (Stratagene Corp., La Jolla, CA) and each of the mutant primers (both from Bio-Synthesis) GGCCGACACCGTCCGGCCCAACCCTGAG (for the pGL3-mut1Cav construct) or GGCACAGACGCGGGCAAGC (for the pGL3-mut2Cav construct). The expected mutations (in bold and underlined above) were verified by sequencing. For gene reporter assays, cells were plated in six-well plates, in triplicate, and cotransfected with 4 μg of either pGL3-cavFL, pGL3-mut1Cav, pGL3-mut2Cav, or the pGL3-basic, promoterless vector, plus 5 ng of the pRL-SV40 vector, with LipofectAMINE 2000 (Invitrogen). Luciferase activity in cell lysates was determined with a Dual-Luciferase Reporter System (Promega Corp., Madison, WI) by luminometry, and data were normalized to renilla luciferase activity and total protein concentration.

Anchorage-independent growth and tumorigenicity assays. Soft agar assays were done as described (19). Tumorigenicity assays were carried out essentially as described (20), under protocols approved by the Georgetown University Animal Care and Use Committee. Immunodeficient, athymic nude (BALB/c nu/nu) mice (from the U.S. National Cancer Institute) were injected s.c. into the right posterior flank with 4 × 106 pBK-CMV– or AS10/C0–Cav1–transfected cells in 100 μL of Matrigel basement membrane matrix (BD Biosciences). Tumor growth was monitored daily for 20 days.

Statistical analysis. Unless otherwise indicated, RT-PCR and Western blot analyses were repeated at least thrice. Data from densitometric quantification analyses were expressed as mean ± SD. For these and other assays involving statistical analysis, ANOVA or Student’s t tests were used to assess the significance of differences between groups or individual variables, respectively. P < 0.01 was regarded as significant.
Results

Consistent down-regulation of CAV1 by EWS/FLI-1 knockdown in EWS cells. To identify EWS/FLI-1 targets related to invasion/metastasis, we initially studied A4573 EWS cells, which carry a type 3 translocation (24) and, having a high proliferation rate, represent a good model for aggressive EWS. Our strategy involved two steps: antisense oligonucleotide treatment to knockdown EWS/FLI-1 expression and comparative array analysis of subsequent expression changes of genes related to invasion and metastasis. Exposure of A4573 cells, for 48 hours, to a 25-mer antisense oligonucleotide (ASATG) targeting the EWS/FLI-1 mRNA translation initiation region (22) reduced EWS/FLI-1 mRNA and protein by about 50% and 65%, respectively, relative to control cells treated with control scrambled oligonucleotide (Fig. 1A), which had no detectable effect. Up-regulation of TGFβR2, a known target transcriptionally repressed by EWS/FLI-1 (5), validated the functional effectiveness of ASATG treatment (Fig. 1A). Comparative analyses between scrambled and ASATG-treated cells using cDNA arrays containing 96 metastasis-associated genes (Fig. 1B) identified CAV1 as the gene showing the greatest extent of down-regulation (~2.5-fold) after ASATG treatment (complete data and a comparison with previous reports on EWS/FLI-1 expression profiling are included in Supplementary Table S1). Results on CAV1 down-regulation were validated by RT-PCR and Western blot analyses. Relative to scrambled controls, ASATG treatment substantially reduced CAV1 mRNA (~2.5-fold) and, more remarkably, CAV1 protein (~5-fold; Fig. 1C).

Because ASATG targeted the translation initiation region of EWS/FLI-1, it could also down-regulate the expression of the normal EWS allele present in EWS cells. To ascertain that CAV1 down-regulation was not a consequence of EWS down-regulation (~1.8-fold; data not shown) or a nonspecific effect of either the antisense technique or the A4573 cells, we explored the effect on CAV1 expression of EWS/FLI-1 knockdown with retroviral constructs expressing translocation type-specific shRNAs in various other EWS cell lines, by examining data from microarray analyses done previously (21) with the Affimetrix HG-U133A GeneChip (Affymetrix, Inc., Santa Clara, CA). Results (Fig. 1D) showed that, relative to cells expressing either translocation type mismatched shRNA constructs or the empty pSR vector, CAV1 was consistently down-regulated (1.6- to 3.5-fold) by EWS/FLI-1 knockdown in all EWS cell lines tested.

The consistent CAV1 down-regulation caused by EWS/FLI-1 knockdown with two different methods, in multiple EWS cell lines,
strongly suggested that CAV1 could be an EWS/FLI-1 transcrip-
tional target. To explore whether EWS/FLI-1 participates in the
regulation of the CAV1 promoter, we did gene reporter assays after
transfection of A4573 cells with a construct carrying luciferase
under the transcriptional control of the full-length CAV1 promoter
(pGL3-cavFL) and maintaining the cells in the presence or absence
of the ASATG oligonucleotide or the scrambled control. Results
(Fig. 1E) showed that the CAV1 promoter was functional in A4573,
and that the increase in luciferase levels (~4-fold) relative to
control pGL3-basic–transfected cells was not significantly affected
by treatment with the scrambled control, whereas luciferase
activity was only increased by about 1.7-fold in cells treated with
ASATG. The significant ($P < 0.01$) difference between CAV1
promoter activity in untreated or scrambled-treated cells versus
ASATG-treated cells further implicated EWS/FLI-1 as a regulator
of CAV1 expression in EWS cells.

CAV1 is a direct target of EWS/FLI-1. Reasoning that
regulating CAV1 expression in EWS cells may be a function
common to EWS/FLI-1 proteins carrying distinct fusion subtypes,
we used chromatin immunoprecipitation to investigate the
possible binding of EWS/FLI-1 proteins in vivo to EBS(s) present
within the human CAV1 promoter, using EWS cell lines (TC-71, SK-
ES-1, and A4573) representative of the main three EWS/FLI-1
junction subtypes (19). Analysis of possible EBS(s) in the human
CAV1 promoter (Genbank accession no. AF095591; ref. 25) with the
MatInspector software (26), identified a putative EBS at position
−140. Interestingly, this site (GTCC; Fig. 2A) is a nonconsensus
EBS (27) that retains the invariant TCC ETS-binding core (28) and
was shown to have prominent ETS-binding activity in the human
HNP-defensin-1 promoter (29). Using a set of primers that
amplified a fragment between positions −216 and +18, encom-
passing the EBS (Fig. 2A), and an antibody that recognized the
COOH terminus of the FLI-1 protein, results showed that EWS/
FLI-1 proteins did bind in vivo to the EBS within the CAV1
promoter in all cell lines tested (Fig. 2A). Furthermore, the fact
that similar results were obtained when immunoprecipitations
were carried out with an antibody against the NH2 terminus of the EWS
protein, but not when an antibody against the NH2 terminus of
FLI-1 (not present in the EWS/FLI-1 hybrid proteins) or a
nonspecific immunoglobulin (IgG) control were used, strongly
supported the notion that CAV1 expression is regulated by EWS/
FLI-1. The specificity of these results was further strengthened by

![Figure 2.](image-url)

**Figure 2.** CAV1 is a direct target of EWS/FLI-1. A, CAV1 promoter region
completes a nonconsensus EBS (black rectangle). The nucleotide sequence
of the main motif is framed, and the invariant
core is underlined in capitals (bases
mutated in B and C). Small arrows show the
position and coordinates of PCR primers
used for chromatin immunoprecipitation
analysis (right). Immunoprecipitation of
soluble chromatin from indicated cells was
done with antibodies shown (against NH2-
or COOH-terminal peptides) or a nonspecific
immunoglobulin (IgG). Final DNA extracts
and total input DNA, as positive control,
were PCR amplified with the same primers.
B, relative to luciferase expression levels
driven by the wild-type promoter
(pGL3-Cav1), mutations of the EWS/FLI-1
binding site within the CAV1 promoter
significantly reduced its activity to luciferase
levels approaching those in negative controls
(pGL3-basic), in the three EWS cell lines
indicated.* $P < 0.001$ (Student’s $t$ test).
C, relative luciferase activity in RD cells
cotransfected with EWS/FLI-1 and wild-type
or mutated constructs of the CAV1
promoter. Only the wild-type promoter could
be progressively activated over time by
introduction of EWS/FLI-1 in cells that do not
express the oncoprotein, whereas the
luciferase activity levels driven by the
mutated forms of the promoter were
indistinguishable from the controls.
$. $P < 0.001$ versus cells without EWS/FLI-1.
the fact that no PCR products were obtained in chromatin immunoprecipitation experiments with a primer set designed to amplify a 160-bp nonoverlapping upstream CAV1 gene fragment (positions −576 to −736) lacking an EBS (Supplementary Fig. S1).

To further show that EWS/FLI-1 proteins indeed play a role in controlling CAV1 promoter activity, we did gene reporter assays using constructs carrying luciferase under the transcriptional control of either the full-length (pGL3-CavFL) wild-type CAV1 promoter (30) or versions of the same promoter in which we altered the EBS by site-directed mutagenesis at two different positions (Fig. 2A): a G → T at the nucleotide immediately flanking the ETS-binding invariant core (pGL3-mut1Cav) and a C → T within the core itself (pGL3-mut2Cav). These constructs, and the pGL3-basic negative control, were transiently transfected into TC-71, SK-ES-1, and A4573 cells, and luciferase levels were determined. Results (Fig. 2B) showed that the CAV1 promoter was functional in the three cell lines, and that its activity was significantly reduced (~2.1- to 3.5-fold) in the three cell types (P ≤ 0.001 in all cases) by introduction of each of the single mutations within the putative EWS/FLI-1 binding site. In additional experiments, we used RD rhabdomyosarcoma cells to perform gene reporter assays by cotransfecting the same CAV1 promoter constructs described above with a cytomegalovirus (CMV)-based EWS/FLI-1 expression construct. Interference from endogenous factors is greatly minimized in RD cells because they do not carry EWS/FLI-1 translocations and express nearly undetectable CAV1 levels (refs. 31, 32; data not shown). Results (Fig. 2C) showed increased luciferase activity (up to about 7-fold, 48 hours posttransfection; P ≤ 0.001 versus all other cases) only in cells cotransfected with the wild-type CAV1 promoter and EWS/FLI-1. Luciferase levels in cells cotransfected with either of the CAV1 promoter mutants were not significantly different from those in cells cotransfected with wild-type CAV1 promoter and empty CMV vector.

Taken together, these data not only provided unambiguous evidence supporting that CAV1 is a direct transcriptional target of EWS/FLI-1 but also predicted that CAV1 would be expressed at high levels in ESFT cell lines and tumor samples. Indeed, expression analyses showed that ESFT cell lines (Fig. 3A, lanes 1-5) expressed substantially greater CAV1 levels than prostate cancer cell lines (Fig. 3A, lanes 6 and 7), generally considered as examples of high CAV1 expression (33). For comparison, the same EWS cell lines expressed CAV2 at very low levels (Fig. 3A), only detectable after exposures much longer than those needed to detect CAV1. Interestingly, the elevated levels of CAV1 expression in EWS cells were not affected by either their proliferation status or the induction of apoptosis, as shown by the fact that they remained invariable when A4573 cells were either cultured without serum or induced to undergo apoptosis by cisplatin treatment (Supplementary Fig. S2). A4573 cells was functional in the three cell lines, and that its activity was determined. Results (Fig. 2B) showed that the CAV1 promoter was functional in the three cell lines, and that its activity was significantly reduced (~2.1- to 3.5-fold) in the three cell types (P ≤ 0.001 in all cases) by introduction of each of the single mutations within the putative EWS/FLI-1 binding site. In additional experiments, we used RD rhabdomyosarcoma cells to perform gene reporter assays by cotransfecting the same CAV1 promoter constructs described above with a cytomegalovirus (CMV)-based EWS/FLI-1 expression construct. Interference from endogenous factors is greatly minimized in RD cells because they do not carry EWS/FLI-1 translocations and express nearly undetectable CAV1 levels (refs. 31, 32; data not shown). Results (Fig. 2C) showed increased luciferase activity (up to about 7-fold, 48 hours posttransfection; P ≤ 0.001 versus all other cases) only in cells cotransfected with the wild-type CAV1 promoter and EWS/FLI-1. Luciferase levels in cells cotransfected with either of the CAV1 promoter mutants were not significantly different from those in cells cotransfected with wild-type CAV1 promoter and empty CMV vector.

CAV1 knockdown inhibits anchorage-independent growth and tumorigenicity of EWS cells. To investigate a possible role for CAV1 in EWS-FLI-1–induced oncogenesis, A4573 cells, which express the highest CAV1 levels among the ESFT cell lines tested (Fig. 3A, lane 3), were stably transfected with an antisense CAV1 cDNA construct. Antisense efficacy was determined by Western analysis of extracts of several individual transfected clones and one cellular pool isolated after antibiotic selection (Fig. 4A), to rule out possible clonal variability confounders. Relative to untransfected cells and to cells transfected with empty vector (pBK-CMV), all transfected cells showed substantially reduced CAV1 expression (>80% inhibition), and clone 10 (AS10/CAV1), with CAV1 reduced about 90% (Fig. 4A, lane 3), was selected for further characterization. In vitro, although there were no
significant differences in growth and cell cycle distribution between AS10/CAV1 cells and pBK-CMV–transfected cells (Supplementary Fig. S2). CAV1 down-regulation resulted in remarkable morphologic changes (Fig. 4B). Whereas, similar to untransfected A4573 cells, control pBK-CMV–transfected (A4573/pBK-CMV) cells grew in dense clusters with small cells piling up into three-dimensional structures, A4573/AS10/CAV1 and A4573/ASPOOL/CAV1 antisense-transfected cells seemed strikingly more flat and growing in loosely packed monolayers of more refractile cells, but retained the A4573 original size (Fig. 4B). Furthermore, CAV1 down-regulation inhibited anchorage-independent growth of A4573 cells, causing a significant (∼90%, \( P < 0.01 \)) reduction in the number of colonies growing in soft-agar cultures (Fig. 4C). Similar morphologic changes and inhibition of anchorage-independent growth were observed with individual clones and pooled populations of all cell lines tested (data from similar experiments with SK-ES-1 cells are shown in Supplementary Fig. S3). The specific dependence of these phenotypic changes on the loss of CAV1 was shown by the fact that essentially identical results were obtained when CAV1 knockdown was achieved by transfection of A4573 cells with shRNA vectors targeting two different CAV1 regions. Stable expression of each shRNA vector substantially reduced CAV1 levels (Fig. 5A), caused a nearly complete disappearance of caveolae from the cells, as shown by electron microscopy (Fig. 5B), resulted in morphologic changes similar to those described above (Fig. 5C), and significantly inhibited (∼89%, \( P < 0.01 \)) soft agar growth (Fig. 5D). These results showed that the observed effects were indeed due to CAV1 down-regulation, and confirmed that CAV1 plays an important role controlling the morphology and some neoplastic traits of EWS cells.

Most importantly, in vivo experiments showed that, as early as 16 days after s.c. injection into nude mice, A4573/AS10/CAV1–derived xenografts were significantly smaller (\( P = 0.001 \)) than those induced by A4573/pBK-CMV control cells (Fig. 6A). Four days later, when tumors derived from A4573/pBK-CMV cells reached an average volume about 1,600 mm\(^3\), and mice had to be sacrificed to comply with institutional Animal Care and Use guidelines, tumors derived from A4573/AS10/CAV1 cells had reached an average volume of only 250 mm\(^3\) (Fig. 6B). Immunohistochemical analyses of paraffin-embedded tumors showed no detectable CAV1 expression in A4573/AS10/CAV1 xenografts compared with the highly positive staining of A4573/pBK-CMV–derived tumors (Fig. 6C). Altogether, these data show that CAV1 is a key positive effector, necessary the development of the transformed phenotype in EWS carcinogenesis.

**Down-regulation of CAV1 function leads to Snail up-regulation and the concomitant loss of E-cadherin expression.** The altered morphology of CAV1 knockdown cells (Fig. 4B and Fig. 5C), their inability to grow in soft agar (Fig. 4C and Fig. 5D),
and slow growth as nude mouse xenografts (Fig. 6A and B) suggested that CAV1 down-regulation could have disrupted intercellular contacts, probably by inducing the loss of E-cadherin as described for other systems (15). The loss of E-cadherin mRNA and protein in A4573/AS10/CAV1 cells was confirmed by RT-PCR and immunoblotting (Fig. 7A) and immunofluorescence analyses (Fig. 7B). Because Snail has been described as a transcriptional repressor of E-cadherin (34, 35), and CAV1 down-regulation induced Snail mRNA up-regulation in other cell types (15), we examined whether Snail mediated the CAV1 knockdown effects in EWS cells. RT-PCR assays showed that CAV1 down-regulation increased Snail mRNA expression in A4573 cells (Fig. 7C). The specific dependence of E-cadherin and Snail expression changes on CAV1 loss was shown by the fact that CAV1 knockdown with the same CAV1 shRNA vectors mentioned above yielded essentially identical results (Supplementary Fig. S4A). To show that the increased Snail expression was responsible for the E-cadherin loss, the altered morphology, and the inhibition of anchorage-independent growth of A4573/AS10/CAV1 cells, A4573 cells were transfected with a HA-tagged Snail cDNA expression vector. Snail transfection did strongly down-regulate E-cadherin (Fig. 7D), induced concomitant morphologic alterations in A4573 cells (Fig. 7E) essentially identical to those induced by CAV1 down-regulation in A4573/AS10/CAV1 cells, and blocked their anchorage-independent growth (Fig. 7F). These data clearly showed that increased expression of Snail was sufficient to recapitulate in A4573 cells a phenotype essentially identical to that promoted by CAV1 knockdown in A4573/AS10/CAV1 cells. In the context of EWS tumor progression, CAV1 may be directly or indirectly involved in maintaining Snail expression at low levels to allow the functional expression of E-cadherin. Because E-cadherin loss has been described as a frequent alteration in a variety of tumor types and has been associated with their invasive/metastatic properties (36), we sought to more firmly establish the biological significance of the pathway leading from CAV1 overexpression to maintenance of E-cadherin in EWS by transfecting A4573 cells with a dominant-negative construct to disrupt E-cadherin function and comparing its effects to those induced by CAV1 knockdown (Fig. 4B and C) or by Snail overexpression (Fig. 7E and F). Relative to A4573 cells transfected with empty vector, stable expression of a dominant-negative E-cadherin mutant (Trp156Ala; ref. 37) caused morphologic changes essentially identical (Supplementary Fig. S4B) to those induced by CAV1 down-regulation and Snail overexpression and also caused a significant (P < 0.01) decrease in the ability of A4573 cells to grow in soft agar (Supplementary Fig. S4C). Basically, the same effects were observed with SK-ES-1 and TC-71 cells (data not shown). The contribution and importance of the CAV1/Snail/
E-cadherin pathway to the malignant phenotype of EWS cells was shown by the fact that reexpression of CAV1 or E-cadherin, but not of dominant-negative E-cadherin, in A4573 cells in which CAV1 expression was previously knockdown with shRNA constructs rescued the growth pattern and morphology characteristic of the A4573 cells: cells regained expression of CAV1 or E-cadherin (Fig. 8A) and grew again in dense clusters of small cells piling up into three-dimensional structures, whereas the same cells transfected with dominant-negative E-cadherin, as a control, did not recover the original A4573 phenotypic traits (Fig. 8B). Furthermore, these reexpression experiments resulted in the rescue of the ability of A4573 cells to form colonies with high efficiency in soft agar cultures (shown for the case of E-cadherin in Fig. 8C).

Discussion

Efficient antisense oligonucleotide-mediated EWS/FLI-1 down-regulation led to array experiments to identify possible EWS/FLI-1 targets involved in EWS invasion/metastasis. Results from these experiments and microarray analyses of other EWS cells in which EWS/FLI-1 was knockdown with junction type-specific shRNAs (21) consistently and reproducibly identified CAV1 as a possible EWS/FLI-1 target. Results agree with (Supplementary Table S1) and are supported by previous reports describing CAV1 expression changes in studies of EWS/FLI-1 gene expression profiling in other systems. EWS/FLI-1 knockdown by retroviral-mediated RNA interference down-regulated CAV1 expression in EWS cells (38). Moreover, expression of EWS/FLI-1 in cells that do not express it (mouse NIH/3T3, human neuroblastoma and rhabdomyosarcoma), increased CAV1 expression (31, 39, 40), and increased expression of CAV1 was identified as a feature differentiating EWS from other sarcomas (41) and related tumor types (32).

Because our results also agreed with reports that ETS transcription factors enhance CAV1 promoter transactivation (42), we analyzed it by chromatin immunoprecipitation assays with PCR primers that would amplify the −140 region containing its putative EBS and antibodies that should (against the EWS NH2 terminus or the FLI-1 COOH terminus) or should not (against the FLI-1 NH2 terminus or a non-specific IgG) immunoprecipitate DNA-cross-linked EWS/FLI-1. Considering the negative results obtained with PCR primers for a CAV1 gene region that would not cofractionate with the EBS-containing fragment, data from chromatin immunoprecipitation experiments strongly supported the EWS/FLI-1 binding to the CAV1 promoter EBS, which, interestingly, is a nonconsensus ETS site. However, it is important to note that ETS-binding factors can bind to sites that do not conform to their in vitro–derived high-affinity consensus sequences (27, 28), and that this particular site (GTCC) was shown to have
substantial ETS-binding activity in the human HNP-defensin-1 promoter (29). It seems possible that, as an aberrant transcription factor, EWS/FLI-1 may have greater affinity for the nonconsensus CAV1 promoter EBS. That CAV1 is a direct EWS/FLI-1 target was also strongly supported by (a) results from luciferase reporter assays demonstrating that the CAV1 promoter activity was significantly reduced by both antisense-mediated EWS/FLI-1 down-regulation (Fig. 1E) and by mutations within or immediately adjacent to the putative EWS/FLI-1 binding site (Fig. 2B), and (b) the fact that the wild-type CAV1 promoter, but not its mutated versions, was up-regulated when cotransfected with an EWS/FLI-1 expression construct into RD cells, which express negligible CAV1 levels and no EWS/FLI-1 (Fig. 2C). Together with the high CAV1 expression detected in EWS cell lines and most EWS tumor samples tested, these data provide strong evidence supporting that CAV1 is a direct EWS/FLI-1 transcriptional target, are consistent with the reported association of CAV1 expression with metastasis and poor prognosis for different cancers (14), and agree with data (31, 32, 41, 43) describing CAV1 as a potentially useful diagnostic marker for EWS.

CAV1 is a “tumor- and metastasis-modifying” gene product (14) that may act differently depending on cell/tissue context, tumor type, and/or progression stage. Previous reports showed that CAV1 acts as a tumor suppressor in cancers such as breast tumors (14), and as a tumor promoter in prostate and other tumors (17), including some soft tissue sarcomas (12). Our results demonstrating that in EWS cells (a) CAV1 knockdown promoted remarkable morphologic changes and reduced their anchorage-independent growth; (b) reexpression of CAV1 in CAV1-knockdown cells rescued their original morphology; and (c) CAV1 down-regulation significantly reduced their tumorigenicity, conclusively show that CAV1 functions to promote the malignant phenotype in EWS carcinogenesis. Although EWS cells express CAV2, its expression levels are markedly lower than those of CAV1 and were not consistently down-regulated by EWS/FLI-1 knockdown in the EWS cell lines tested. It seems unlikely that CAV2 may be an important coparticipant with CAV1 in promoting the EWS malignant phenotype.

Smith et al. (38) recently showed that EWS/FLI-1 knockdown caused the loss of oncogenic transformation and tumorigenicity by EWS A673 cells, without affecting their growth in culture. These results strongly supported that EWS/FLI-1 functions such as stimulation of cell proliferation and induction of oncogenic transformation and tumorigenesis may be dissociated and, consequently, may be mediated by independent sets of EWS/FLI-1 targets. The fact that EWS/FLI-1 knockdown results in CAV1 down-regulation regardless of its effect on cell growth strongly suggests that CAV1 belongs in the category of EWS/FLI-1 targets related to EWS oncogenic transformation. EWS/FLI-1 knockdown in A673 EWS cells down-regulated CAV1

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**Figure 7.** Inhibition of tumorigenicity by CAV1 down-regulation correlates with up-regulation of Snail and the concomitant loss of E-cadherin. A, RT-PCR and Western blot analysis showing that, relative to control A4573/pBK-CMV cells, the expression of E-cadherin mRNA and protein are down-regulated in A4573/AS10/CAV1 cells. β-Actin was used as the loading control. B, immunofluorescence microscopy images of E-cadherin (Texas Red) and 4’,6-diamidino-2-phenylindole (DAPI)–stained nuclei (blue) in A4573/pBK-CMV (three-dimensional aggregates) and A4573/AS10/CAV1 (singly attached) cells. C, RT-PCR analysis indicates that Snail mRNA is markedly up-regulated in A4573/AS10/CAV1 cells relative to control A4573/pBK-CMV cells. β-Actin was used as the loading control. D, Western blot analysis indicates that stable transfection of HA-tagged Snail cDNA into A4573 cells (A/Snail) results in substantial down-regulation of E-cadherin protein levels, relative to the levels observed in cells transfected with empty vector (A/pcDNA). β-Actin was used as the loading control. E, phase-contrast micrographs of empty vector (A/pcDNA) and Snail-transfected (A/Snail) A4573 cells show that Snail expression recapitulates the morphology and growth pattern (shown in Fig. 4 and Fig. 5C) induced by antisense-mediated down-regulation of CAV1 in A4573/AS10/CAV1 cells. F, analysis of colony formation data from triplicate cultures of A4573 transfected with empty vector (A/pcDNA) or with the Snail expression construct (A/Snail) plated in soft agar and giving rise to colonies larger than 0.5 mm in diameter. *, P < 0.001, differences observed were statistically significant.
without affecting their growth in culture (38), whereas EWS/FLI-1 knockdown consistently down-regulated CAV1 in all EWS cells tested by us, although their proliferation was slowed down to different degrees. The absence of CAV1 expression changes when cells were grown without serum or induced to undergo apoptosis (Supplementary Fig. S2) also support the dissociation between CAV1 expression and proliferation status in EWS cells.

Our results show the contribution of the CAV1/Snail/E-cadherin pathway to the malignant phenotype of EWS cells. The observations that (a) EWS cells displayed essentially identical phenotypic characteristics after either CAV1 knockdown, Snail overexpression, or E-cadherin down-regulation and, in particular, (b) these phenotypic traits could be reversed by reexpression of CAV1 or E-cadherin in cells in which CAV1 expression was down-regulated to those typical of unmanipulated A4573 cells, are consistent with a coordinated regulation of CAV1, Snail, and E-cadherin in EWS cells and agree with reports indicating that CAV1 down-regulation up-regulates Snail mRNA in other cell types (15), and that Snail is a direct repressor of E-cadherin expression (34, 35). Consistent with the CAV1/Snail/E-cadherin pathway working downstream of EWS/FLI-1 in EWS cells, EWS/FLI-1 expression was not altered by ectopic changes in the expression of any individual pathway component. However, our finding that EWS cells express E-cadherin contrasts with the frequent E-cadherin loss reported for other tumors (36). Nevertheless, simultaneous CAV1 and E-cadherin expression has been described also in some tumor cell lines and solid tumors (44, 45), and E-cadherin down-regulation correlated with diminished cell-cell adhesion, cell proliferation, and tumor growth in mice (46). Our finding that E-cadherin expression is not lost in EWS cells places EWS closer to synovial sarcomas (45) and agrees with the recent description of a novel tumor progression pathway not involving E-cadherin loss (47). Overall, our results show that E-cadherin expression in EWS cells is dependent on CAV1 and necessary for tumor development and strongly suggest that intercellular contacts are important in EWS tumorigenesis.

By identifying CAV1 as a direct EWS/FLI-1 target necessary for EWS tumorigenicity, our findings also recognize CAV1 as a
suitable target for developing new ESFT therapies that, because normal cells express much lower CAV1 levels, may provide an improved treatment scenario with greater specificity and efficacy.

Acknowledgments

References


Caveolin-1 (CAV1) Is a Target of EWS/FLI-1 and a Key Determinant of the Oncogenic Phenotype and Tumorigenicity of Ewing's Sarcoma Cells

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