Cancer Research

CAV1 Inhibits Metastatic Potential in Melanomas through Suppression of the Integrin/Src/FAK Signaling Pathway

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Abstract

Caveolin-1 (CAV1) is the main structural component of caveolae, which are plasma membrane invaginations that participate in vesicular trafficking and signal transduction events. Although evidence describing the function of CAV1 in several cancer types has recently accumulated, its role in melanoma tumor formation and progression remains poorly explored. Here, by using B16F10 melanoma cells as an experimental system, we directly explore the function of CAV1 in melanoma tumor growth and metastasis. We first show that CAV1 expression promotes proliferation, whereas it suppresses migration and invasion of B16F10 cells *in vitro*. When orthotopically implanted in the skin of mice, B16F10 cells expressing CAV1 form tumors that are similar in size to their control counterparts. An experimental metastasis assay shows that CAV1 expression suppresses the ability of B16F10 cells to form lung metastases in C57Bl/6 syngeneic mice. Additionally, CAV1 protein and mRNA levels are found to be significantly reduced in human metastatic melanoma cell lines and human tissue from metastatic lesions. Finally, we show that following integrin activation, B16F10 cells expressing CAV1 display reduced expression levels and activity of FAK and Src proteins. Furthermore, CAV1 expression markedly reduces the expression of integrin β_3 in B16F10 melanoma cells. In summary, our findings provide experimental evidence that CAV1 may function as an antimetastatic gene in malignant melanoma. *Cancer Res; 70(19); 7489–99.* ©*2010 AACR.*

Introduction

Malignant melanoma remains among the most life threatening of all cancers, and its incidence has been increasing dramatically in the last decades. Despite great progress in understanding the genetics and biochemistry of malignant melanoma, patients with metastatic disease have very few treatment options available. The establishment of metastases in distant organs of the body is a stepwise process that begins with the invasion of the dermis surrounding the primary tumor and ends with the colonization of ectopic sites (1). Each of the steps of the metastatic cascade is rate limiting. Thus, identifying novel mechanisms and factors regulating melanoma progression may be critical for the development of new therapeutics in this type of cancer.

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Initially identified by electron microscopy (2), caveolae are 50 to 100 nm large plasma membrane invaginations morphologically distinct from the classic clathrin-coated vesicles (3). Three different caveolin genes (*CAV1, CAV2*, and *CAV3*) encode for the structural components of these organelles (4, 5). CAV1 is the best studied of the three caveolins, and it is considered a multifunctional scaffold protein able to bind and regulate the activity of numerous signaling molecules within caveolae (6). Due to the multitude of interacting proteins described, CAV1 has been implicated in the modulation of several cancer-associated phenotypes, including cell proliferation, death, and transformation (4). Aside from data derived from cell culture experiments, there are several lines of clinical and genetic evidence implicating CAV1 as a tumor suppressor *in vivo*. First, CAV1 has been found to be downregulated and/or mutated in a number of human tumors, including mammary adenocarcinomas and squamous cell carcinomas (7, 8). Second, the generation of CAV1 knockout (KO) mice has allowed for the validation of the hypothesis that CAV1 may behave as a tumor suppressor. Although CAV1 KO mice do not develop spontaneous tumors, they are more susceptible to carcinogen [7,12-dimethylbenz(*a*) anthracene]– and oncogene-induced cancer in skin and mammary tissues, respectively (9, 10). However, the idea that CAV1 may be a "general" tumor suppressor has been recently challenged by reports showing that CAV1 expression is cancer type and/or stage dependent (11). CAV1 is upregulated in the bladder, esophagus, thyroid (papillary subtype), and prostate carcinomas, and this upregulation seems to be associated with multidrug resistance and/or metastasis (12, 13).

The role of CAV1 in malignant melanoma, however, remains poorly understood. Several groups have reported conflicting results for the role of CAV1 in melanoma transformation, migration, and invasion (14, 15). Furthermore, the role of CAV1 in melanoma tumor formation and metastasis remains to be determined. Here, to gain better insight into the function of CAV1 in melanoma progression, we used B16F10 melanoma cells as an experimental system to directly explore the function of CAV1 in melanoma tumor growth and metastasis.

In the current study, we show that CAV1 expression inhibits the motility of B16F10 melanoma cells *in vitro* and their ability to form lung metastases *in vivo*. These results were consistent with reduced CAV1 expression in a panel of human metastatic melanoma cell lines and metastatic lesions of human patients. Finally, recombinant CAV1 expression in B16F10 cells was sufficient to suppress the expression and activity of Src and FAK proteins following integrin engagement. In summary, these data underscore the importance of CAV1 as a new antimetastatic gene in malignant melanoma.

Materials and Methods

Materials

Antibodies and their sources were as follows: p-FAK(Y397) and p-Src(Y418) were from Invitrogen. Cyclin D1, cyclin A, Bcl-2, integrin α_5, integrin β_1, and CAV1(N-20) were from Santa Cruz Biotechnology. FAK Flotillin-1 and CAV1 were from BD. Src, integrin α_6, and integrin α_V (Ab1930) were from Millipore. Integrin β_3, AKT, and p-AKT(S473) were from Cell Signaling. β-Tubulin was from Sigma; S-100b was from Affinity BioReagents; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Fitzgerald.

Mice experiments

Orthotopic injections were performed by intradermally injecting 10^6 B16F10 cells, whereas i.v. injections of 10^5 cells were used to assay for experimental metastasis in 3- to 4-month-old C57Bl6/J female mice (16, 17). All *in vivo* studies were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. Detailed descriptions are available in Supplementary Methods.

Cell lines

B16F0, B16F10, A-375, WM-115, SK-MEL-28, SK-MEL-5, WM-266-4, WM-35, and normal human epidermal melanocytes (NHEM) were cultured according to the manufacturer's instructions [American Type Culture Collection (ATCC), Coriell, and Science Cell Research Laboratories]. ATCC and Coriell routinely perform DNA profiling to authenticate their cell lines. For all the *in vitro* and *in vivo* experiments, only early passages of these cells (passages 5–6) were used.

Retrovirus infection

pBabe-Puro and pBabe-CAV1-Puro retrovectors were used to stably transduce melanoma cells (18).

Western blots

Melanoma cells were sonicated and lysed in a modified radioimmunoprecipitation assay buffer and processed for Western blot analysis as we previously described (19).

Protein fractionation and Triton X-100 solubility assay

Triton X-100 solubility assay was performed as previously described (18). Cytoplasm and membrane proteins were extracted using a commercially available kit (Pierce Biotechnology).

Growth curves, cell cycle analysis, and proliferation assay

Growth curves were generated by seeding 2×10^3 cells/cm^2 in triplicate. Cells were dissociated and counted with a hemacytometer at 1, 2, 3, and 4 days after seeding. Cell cycle analysis was conducted by flow cytometry analysis of propidium iodide–stained cells (20). DNA synthesis in cells was directly analyzed by [^{3}H]thymidine incorporation assay (21). Cell proliferation was also estimated by immunostaining cells with the proliferation marker Ki67 (Abcam).

Immunofluorescence

Cells were grown on glass coverslips and double immunostained for CAV1 and CAV2 as previously described (18). Slides were mounted with the Pro-Long Gold antifade reagent (Molecular Probes) and imaged by confocal microscopy (LSM 510 META Confocal; Zeiss).

Tissue scan melanoma panel and quantitative reverse transcriptase-PCR

As previously described (22), a commercial panel of human cDNAs, obtained from normal human skin tissue and from human melanoma metastatic lesions (stages III and IV), was purchased from OriGene Technologies (MERT501). Quantitative reverse transcriptase-PCR (qRT-PCR) was performed using ready-to-use CAV1 and RPL13a primers/SYBR master mixes (SA-Biosciences). Quantitative expression data were acquired using ABI-Prism 7900HT Sequence Detection System (Applied Biosystems), and results were analyzed by the ΔΔCt method (23).

Immunohistochemistry of tissue sections

A tissue microarray of paraffin-embedded human melanoma tissue samples were purchased from U.S. Biomax (Mel207; 69 cases/207 cores) and was stained for CAV1(N-20) using standard immunohistochemical techniques (9). An expert dermatopathologist carefully analyzed and blindly scored the tissue cores for semiquantitative analysis of immunoreactivity. Detailed descriptions are available in Supplementary Methods.

Migration and invasion assays

Cells (5×10^4) suspended in 0.5 mL of serum-free medium (SFM) containing 0.1% bovine serum albumin (BSA; Sigma) were added to the wells of an 8-μm-pore polycarbonate membrane, either coated with (for chemoinvasion assays) or without (for chemotaxis assays) Matrigel (Transwells;

BD Biosciences). Serum-free NIH3T3 conditioned medium (48 hours) was used as a chemoattractant. After 6 hours, the cells that had migrated were stained and counted as previously described by others (17). For studies using Src and FAK inhibitors, SKI-606 (Selleck), PF-573,228 (Tocris Bioscience), or DMSO were placed in both the upper and lower chambers.

Adhesion/suspension assays

Integrin engagement was performed as described before (24). After being maintained in SFM containing 0.1% BSA for 18 hours, cells were dissociated, suspended in medium containing 0.1% BSA, and replated on fibronectin (FN)-coated plates (BD) for 1 hour at 37°C. Cells were either lysed immediately or following the addition of complete medium [10% fetal bovine serum (FBS)] for 10 minutes. Alternatively, following 18 hours of serum starvation, cells were dissociated and left in suspension for 1 hour, and then processed for Western blot analysis.

Statistical analysis

Results are represented as means ± SEM. Statistical analyses were performed using the Prism 4.0 Program (GraphPad Software, Inc.).

Results

CAV1 protein is correctly targeted to the plasma membrane of B16F10 melanoma cells

Lack of CAV1 expression has been described in several metastatic melanoma cell lines including B16F10 cells (15, 25, 26). Western blot analysis showed that a high expression level of CAV1 was achieved in B16F10 cells transduced with pBabeCAV1. CAV2 expression was not affected by CAV1 expression in B16F10 melanoma cells. Identical results were obtained with the low metastatic B16F0 melanoma cell line (Fig. 1A). To determine the subcellular localization of CAV1 and CAV2, we next performed confocal microscopy on pBabe and pBabeCAV1 transduced cells. Serial optical images (z sections) of pBabe and pBabeCAV1 B16F10 melanoma cells double immunostained with CAV1 and CAV2 antibodies showed that recombinant CAV1 is correctly targeted to the plasma membrane of B16F10 cells. As expected, CAV2 colocalized with CAV1 at the plasma membrane, despite the fact that a large portion of CAV2 also colocalized intracellularly (perinuclear) with CAV1 (Fig. 1B). These results were further confirmed by the observation that the CAV1/CAV2 complex

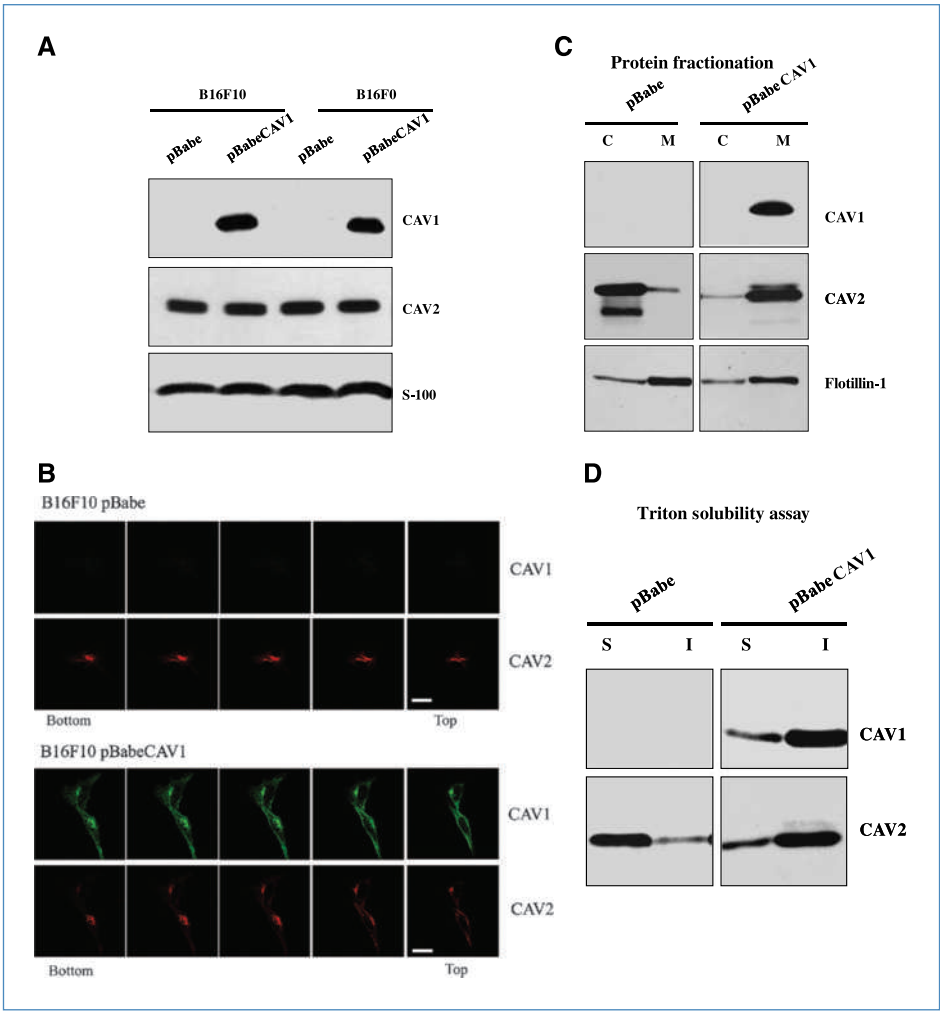

Figure 1. Absence of *CAV1* expression in B16F0 (weakly metastatic) and B16F10 (highly metastatic) melanoma cell lines. A, immunoblotting of retrovirally transduced pBabe and pBabeCAV1 B16F10 and B16F0 cells for CAV1 and CAV2. S-100 immunoblot is shown as a loading control. Note the absence of CAV1 expression in both pBabe transduced B16F0 and B16F10 cells. B, confocal microscopy. Serial optical images (z sections) of pBabe and pBabeCAV1 B16F10 melanoma cells double immunostained with CAV1 and CAV2 antibodies show correct targeting of CAV1 to the plasma membrane. CAV2 extensively colocalizes with CAV1 in B16F10pBabeCAV1 cells (scale bar, 20 μm). C, immunoblot analysis of cytoplasmic (C) and membrane (M) fractions reveals that both CAV1 and CAV2 are enriched in the membrane fraction of B16F10pBabeCAV1 cells. Immunoblot for the membrane protein Flotillin-1 is also displayed. D, immunoblot analysis of Triton X-100–soluble (S) and Triton X-100–insoluble (I) fractions reveals that CAV1 is enriched in the Triton X-100–insoluble fraction. Note that CAV1 expression renders CAV2 Triton X-100 insoluble in B16F10 cells.

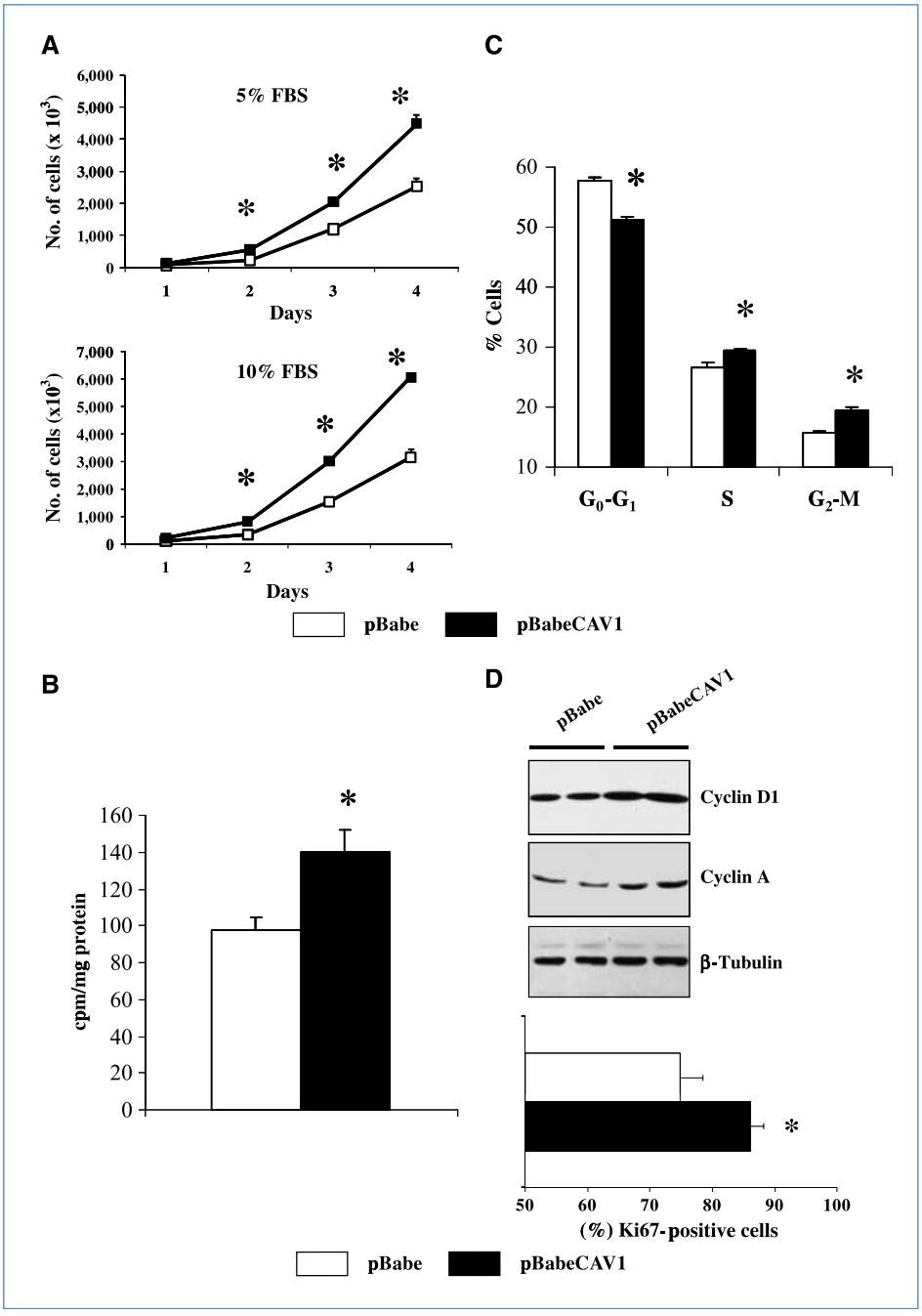

Figure 2. CAV1 expression promotes proliferation of B16F10 melanoma cells *in vitro*. A, growth curves of pBabe and pBabeCAV1 B16F10 cells grown in 5% and 10% FBS (n = 3 per group) showing that CAV1 expression increases cell growth. B, a [^{3}H] thymidine incorporation assay showing increased proliferative rate of B16F10 cells expressing CAV1 (n = 6 per group). C, FACS analysis demonstrating increased percentage of B16F10pBabeCAV1 in the S-G_2M phases of the cell cycle (n = 4 per group). D, immmunoblot analysis showing increased cyclin D1 and cyclin A expression in B16F10pBabeCAV1 cells (top). Positivity for the proliferative marker Ki67 is also displayed (bottom). Results are reported as means ± SEM (*, $P < 0.05$, as determined by two-tailed Student's t test).

was enriched in the membrane fraction and in the Triton X-100–insoluble fraction of B16F10 cells expressing CAV1 (Fig. 1C and D). Thus, these results provide evidence that the CAV1/CAV2 complex is correctly targeted to the plasma membrane of B16F10 cells following the reexpression of CAV1 by retroviral strategy.

CAV1 expression promotes proliferation of B16F10 melanoma cells *in vitro*

Given the role of CAV1 in regulating proliferation and cell cycle progression (27), we next performed a proliferation assay and cell cycle analysis. Interestingly, growth curves (in 5% and 10% FBS) and [^{3}H]thymidine incorporation assay showed enhanced cell growth and increased DNA synthesis in B16F10pBabeCAV1 cells (140 ± 13 versus 98 ± 7 cpm/mg in pBabeB16F10; Fig. 2A and B). Fluorescence-activated cell sorting (FACS) analysis of asynchronously growing cells showed a significantly increased percentage of B16F10pBabe-CAV1 cells in the S and G_2M phases of the cell cycle (Fig. 2C; Supplementary Table S1). CAV1 expression in B16F10 cells was also associated with increased cyclin D1 and cyclin A expression and increased Ki67 positivity as determined by

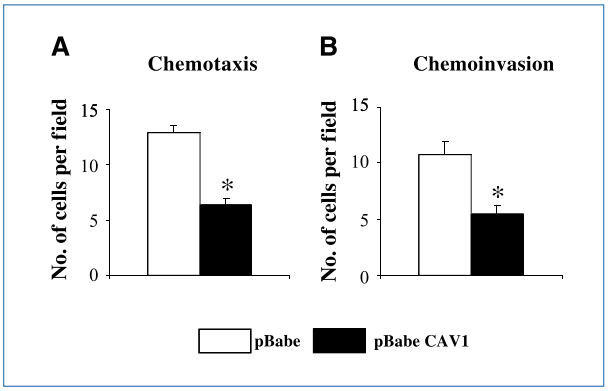

Figure 3. CAV1 expression decreases migration and invasion of B16F10 melanoma cells *in vitro*. Chemotaxis (A) and chemoinvasion (B) were performed by seeding 5×10^4 pBabe and pBabeCAV1 B16F10 cells in the upper wells of Matrigel-coated (for chemoinvasion) or uncoated (for chemotaxis) transwell chambers in SFM containing 0.1% BSA. Serum-free conditioned medium (48 h) from cultures of NIH3T3 cells was used as chemoattractant in the lower wells. After 6 h, the cells that had migrated to the underside of the membrane were washed with PBS, stained with crystal violet, and counted. Data represent the average of three independent experiments. Five fields per sample were counted. Results are reported as means ± SEM (*, $P < 0.05$, as determined by two-tailed Student's *t* test).

Western blot and immunofluorescence analysis (Fig. 2D). These results show a proproliferative role for CAV1 in the B16F10 melanoma cell line.

CAV1 expression decreases migration and invasion of B16F10 melanoma cells *in vitro*

Migration and invasion through a basement membrane are hallmarks of malignancy. To determine whether CAV1 expression affects these properties, pBabe and pBabeCAV1 B16F10 cells were subjected to migration (chemotaxis) and chemoinvasion assays. Specifically, we observed a roughly 2-fold reduction in the capacity of pBabeCAV1 B16F10 cells to migrate through the polycarbonate membrane of transwell chambers when NIH3T3 serum-free conditioned medium was used as a chemoattractant. Moreover, when cells were subjected to chemoinvasion assays, we observed a reduced capacity (roughly 2-fold reduction) of pBabeCAV1 B16F10 cells to invade through Matrigel-coated transwell chambers when NIH3T3 conditioned medium was used as a chemoattractant (Fig. 3). These results, along with the results from our proliferation assays, suggest that CAV1 inhibits migration and invasion while maintaining a positive effect on cell cycle progression in B16F10 melanoma cells.

CAV1 expression dramatically reduces the metastatic potential of B16F10 cells *in vivo* without affecting primary tumor growth

To determine the effect of CAV1 expression on B16F10 tumor growth *in vivo*, 10^6 pBabe and pBabeCAV1 B16F10 melanoma cells were orthotopically (intradermally) implanted in the skin of 3- to 4-month-old C57Bl/6 female mice. Eighteen days after injections, the determination of tumor size and weight revealed that tumor growth was not significantly different between B16F10pBabe and B16F10pBabeCAV1 (Fig. 4A). Additionally, lungs dissected from both groups of mice did not show any spontaneous metastasis formation. To assess whether CAV1 expression was able to affect the metastatic potential of B16F10 melanoma cells, 10^5 B16F10pBabe and B16F10pBabeCAV1 cells were i.v. injected in 3- to 4-month-old C57Bl/6 female mice (experimental lung metastasis). After 18 days, examination of lungs revealed that the incidence of metastasis was significantly reduced in the B16F10pBabeCAV1-injected mice (42%) compared with the B16F10pBabe-injected animals (94%; Fig. 4B; Supplementary Table S2). Strikingly, the B16F10pBabeCAV1-injected mice that showed metastasis formation displayed a significant reduction (roughly 3.5-fold) in the number of visible metastases per lung compared with the B16F10pBabe-injected mice (Fig. 4C and D). Consistent with the ability of CAV1 to reduce the motility of B16F10 cells *in vitro*, these results show that CAV1 expression suppresses the metastatic potential of B16F10 cells without affecting primary tumor growth *in vivo*.

CAV1 expression is reduced in human metastatic melanoma cell lines and human tissue samples derived from metastatic lesions

Because CAV1 expression had no effect on the growth of B16F10-derived tumors, we next wanted to determine CAV1 expression levels in a panel of primary and metastatic melanoma-derived cell lines. Immunoblot analysis revealed that CAV1 expression was significantly reduced in metastatic melanoma cell lines (SK-MEL-28, A-375, SK-MEL-5, WM-266-4) compared with primary melanoma–derived cell lines (WM35, WM115). Interestingly, primary human melanocytes displayed a complete absence of CAV1 expression (Fig. 5A). To validate the significance of the expression pattern observed in melanoma cell lines, we next determined CAV1 expression by immunohistochemistry in normal skin, primary melanoma samples, and metastatic lesions from 69 melanoma patients (207 tissue cores). CAV1 immunoreactivity scores revealed that ~90% of the metastatic lesions showed absent (scored as 0) or weak (scored as 1) CAV1 staining. In contrast, we observed that only 30% of the primary melanoma samples showed absent or weak CAV1 staining (Fig. 5B). In cores that stained positive, CAV1 was observed to localize in the cytoplasm and at the plasma membrane of melanoma cells (Fig. 5C, center). In the skin, CAV1 immunostaining was observed in the keratinocytes of the basal cell layer as we have previously described (Fig. 5C, left; ref. 9). To further analyze the extent of CAV1 alterations in melanoma progression, we determined CAV1 expression by qRT-PCR on cDNA obtained from stage III ($n = 20$) and stage IV ($n = 19$) metastatic lesions. Analysis of CAV1 mRNA levels revealed that CAV1 expression was significantly reduced in stage IV metastases compared with stage III metastases (Fig. 5D, left). In addition, when CAV1 mRNA levels for both stages III and IV metastatic lesions were combined, they were significantly reduced (~2-fold reduction) when compared with CAV1 mRNA levels in

normal skin (Fig. 5D, right). Taken together, these findings suggest that CAV1 loss is a late event in melanoma progression, and they imply that CAV1 may be involved in regulating mechanisms that affect the metastatic process.

CAV1 expression suppresses the integrin/Src/FAK pathway following integrin engagement in B16F10 melanoma cells

A large body of experimental evidence has described integrin–extracellular matrix (ECM) interactions as being critical to acquire metastatic competence in melanoma (28, 29). CAV1 has often been described as an important component in the regulation of the integrin/Src/FAK pathway in both normal and tumor cell lines (26, 30, 31). To determine whether the integrin/Src/FAK pathway may be altered by CAV1 expression, we plated B16F10pBabe and B16F10pBabeCAV1 cells on FN-coated plates and then cultured them in the presence or absence of serum. Remarkably, in the absence of serum, B16F10pBabeCAV1 showed a significant reduction in FAK(Y397) and Src(Y418) activation when compared with B16F10pBabe cells due at least in part to a reduction in total levels of FAK and Src proteins (Fig. 6A, left). In the presence of serum, B16F10pBabeCAV1 corroborated the reduced FAK and Src activity seen in serum-free conditions. These results, however, were not associated with reduced expression of FAK/Src proteins, suggesting that CAV1 interferes directly with integrin signaling (Fig. 6A, right). We next determined the expression of several integrin subunits that have been associated with FAK/Src signaling and melanoma metastasis (32, 33). Interestingly, in both the presence and absence of serum, B16F10pBabeCAV1 plated on FN displayed a

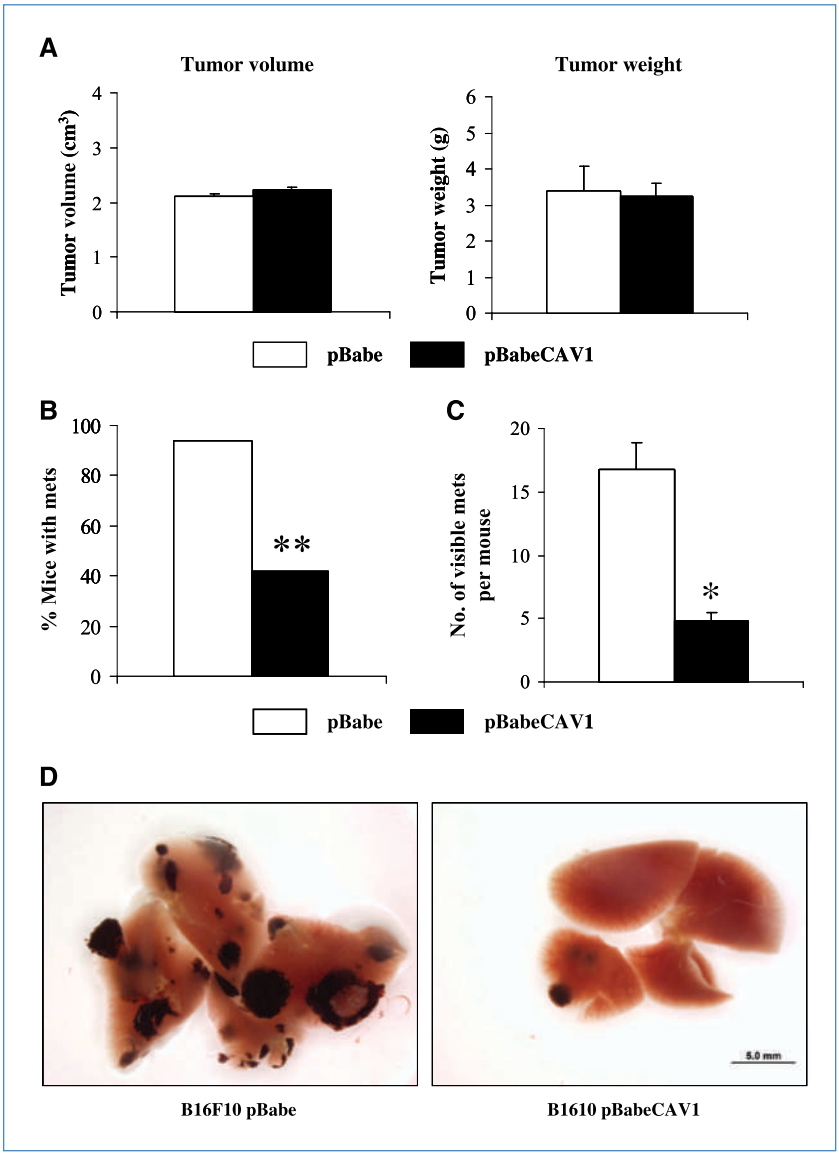

Figure 4. CAV1 expression dramatically reduces the metastatic potential of B16F10 melanoma cells without affecting primary tumor growth. A, tumor growth. Effect of CAV1 expression on B16F10 tumor growth *in vivo* ($n \geq 7$ per group). B and C, experimental lung metastasis assay. Effect of CAV1 expression on the metastatic ability of B16F10 cells represented as incidence (B) and number of visible metastases per lung (C; $n \geq 18$ per group). Note that CAV1 expression significantly reduces the ability of B16F10 melanoma cells to form lung metastasis in C57Bl/6 mice (**, $P = 0.0014$, as determined by two-tailed Fisher's exact test). D, representative images of lung lobes dissected from mice i.v. injected with pBabe and pBabeCAV1 B16F10 cells. Results are reported as means ± SEM (*, $P < 0.05$, as determined by two-tailed Student's *t* test).

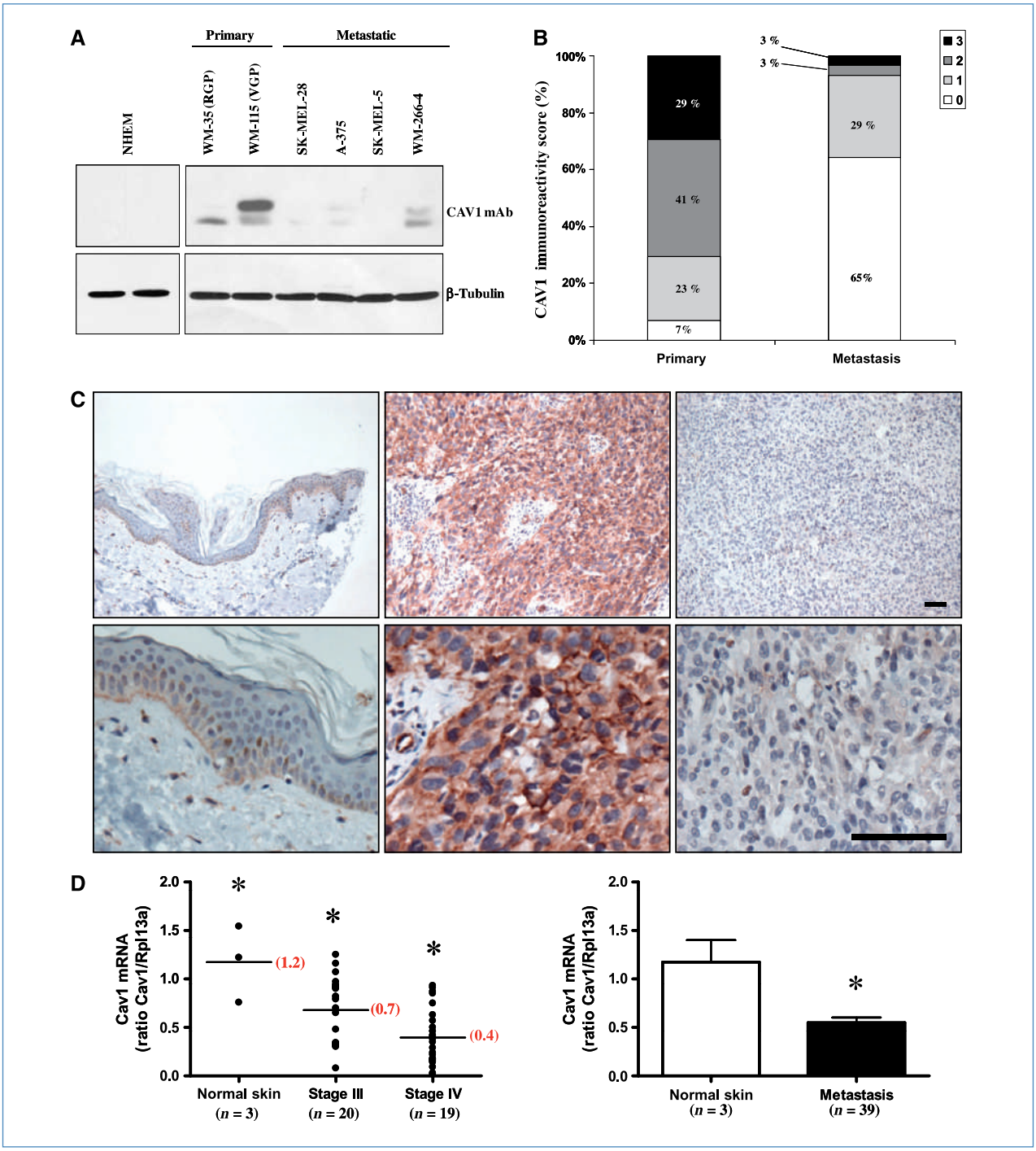

Figure 5. CAV1 expression is reduced in human metastatic melanoma cell lines and human tissue samples derived from metastatic lesions. A, immunoblot analysis showing CAV1 expression in primary human melanoma cell lines (WM35, WM115) compared with a reduced or absent CAV1 expression in primary melanocytes (NHEM) and metastatic melanoma cell lines (SK-MEL-28, A-375, SK-MEL-5, WM-266-4). β-Tubulin is used as a loading control. B, distribution of CAV1 immunoreactivity scores in primary melanoma and metastatic lesions (tissue microarray, 69 cases, 207 tissue cores) showing that primary tumors display significantly high immunoreactivity scores compared with metastatic lesions ($P \leq 0.0001$, as determined by χ^2 test). C, representative CAV1 immunostaining in normal skin (left), primary melanoma (center), and metastasis tissue sections (right). Note the intense CAV1 staining in primary melanoma and in the basal cell layer of normal skin in contrast to reduced or absent CAV1 staining in metastases (scale bar, 50 μm). D, relative expression levels of CAV1 mRNA were determined in normal human skin and stage III and stage IV metastatic lesions. CAV1 mRNA expression levels were normalized to RPL13a mRNA, and samples (closed circles) were grouped according to stage and averaged (solid lines). Note that CAV1 mRNA expression in stage IV metastases is significantly reduced compared with CAV1 mRNA in stage III and normal skin (*, $P < 0.05$ between groups, as determined by Tukey's multiple comparisons test and two-tailed Student's *t* test).

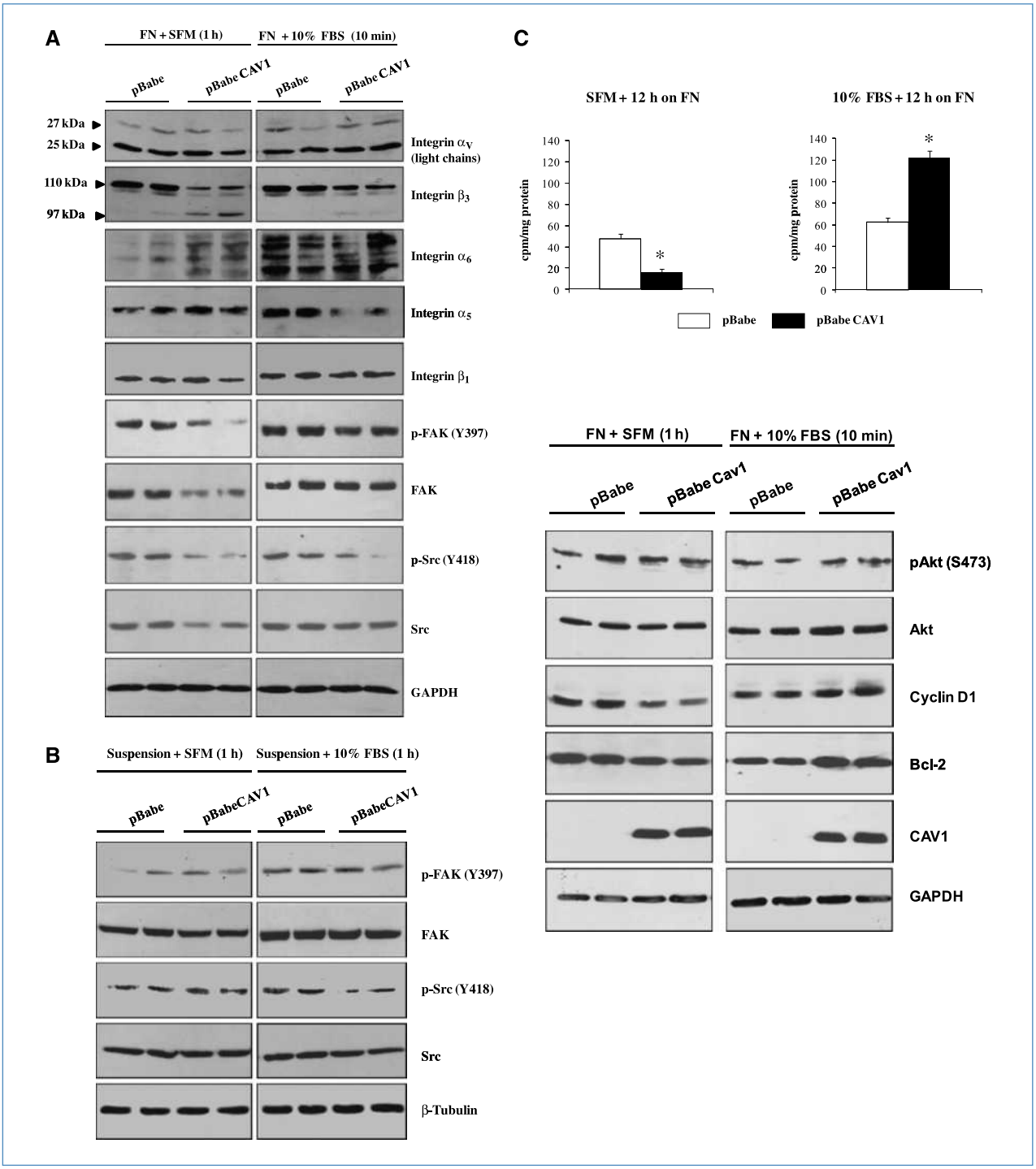

Figure 6. CAV1 suppresses the integrin/Src/FAK pathway following integrin engagement in B16F10 melanoma cells. A, pBabe and pBabeCAV1-B16F10 melanoma cells were serum starved for 18 h, replated on FN-coated plates for 1 h in SFM, and either lysed (left) or pulsed for 10 min with 10% FBS-containing medium and lysed (right). Western blot analysis with antibodies directed against FAK, pFAK(Y397); Src, pSrc(Y418); and α_V, β_3, α_6, α_5, and β_1 integrins was performed. GAPDH was used as a loading control. Note that following integrin activation in SFM, both expression levels and activity of Src and FAK proteins and levels of β_3 integrin are significantly reduced in B16F10pBabeCAV1 cells compared with B16F10pBabe cells. Inhibition of Src and FAK activity is also maintained by CAV1 expression in B16F10 cells treated with serum (right). B, immunoblot analysis of pBabe and pBabeCAV1 B16F10 cells maintained in suspension showing that CAV1 expression does not affect expression and activity of FAK and Src proteins. C, top, [^{3}H]thymidine incorporation assay of pBabe and pBabeCAV1 B16F10 cells plated on FN for 12 h in SFM or 10% FBS ($n \geq 6$ per group) showing that CAV1 promotes proliferation only in the presence of serum. C, bottom, immunoblot analysis of pBabe and pBabeCAV1 B16F10 cells plated on FN as in A, showing that CAV1 expression reduces Bcl-2 and cyclin D1 in SFM while their expression is increased in the FBS-containing medium.

dramatic reduction in the expression of integrin β_3. Expression of integrin α_5 was also reduced in B16F10pBabeCAV1 but only in the presence of serum. No changes were observed in the expression of β_1, α_V, and α_6 integrins (Fig. 6A). Expression and activity of Src and FAK proteins were unchanged in cells maintained in suspension (with and without serum), demonstrating that CAV1 inhibits the integrin/Src/Fak pathway in an adhesion-dependent manner (Fig. 6B). Given the role of CAV1 in regulating cell proliferation (27, 34), we next wanted to determine whether pathways involved in melanoma cell proliferation were altered by CAV1 expression after integrin activation. Immunoblot analysis revealed reduced expression of cyclin D1 and Bcl-2 proteins in B16F10pBabeCAV1 cells plated on FN in the absence of serum. Following addition of serum, B16F10pBabeCAV1 cells displayed increased levels of Bcl-2, cyclin D1, and total Akt proteins (Fig. 6C, bottom). These results were consistent with the proliferation rates of B16F10pBabe and B16F10pBabeCAV1 grown on FN for 12 hours (Fig. 6C, top). Interestingly, Bcl-2 expression was also found increased in human metastatic melanoma cell lines compared with primary cell lines, indicating an inverse correlation with CAV1 expression (Supplementary Fig. S1). The importance of the integrin/Src/Fak pathway in regulating motility was further proved by the ability of Src (bosutinib) and FAK (PF-573,228) inhibitors to significantly reduce the migration and invasion of B16F10 cells (Supplementary Fig. S2). Taken together, these findings suggest that in the absence of a proliferative stimulus (serum), CAV1 has a negative effect on the proliferative pathways of B16F10 cells following integrin/Src/FAK pathway activation. In contrast, in the presence of a proliferative stimulus, CAV1 expression activates pathways that promote proliferation after integrin activation. Nevertheless, our results show that the ability of CAV1 to suppress the integrin/Src/Fak pathway is serum independent.

Discussion

In the present study, we have established the function of CAV1 in melanoma tumor cell growth and metastasis using both the murine B16F10 melanoma cell line and human melanoma tissue samples. For the first time, we provide *in vivo* evidence that CAV1 may be functioning as a repressor of metastasis in malignant melanoma. We first showed that introduction of CAV1 using a retroviral strategy was sufficient to achieve high protein expression levels in B16F10 cells, and both CAV1 and CAV2 were correctly targeted to the plasma membrane. Overexpression of CAV1 resulted in an increase in cell proliferation *in vitro*, but did not affect primary tumor growth *in vivo*. Conversely, CAV1 expression decreased migration and invasion *in vitro* while suppressing the ability of these cells to metastasize *in vivo*. These results translated to human cancer cell lines and melanoma tissue. Primary melanoma tissue samples and cell lines showed significant CAV1 expression compared with normal human melanocytes, whereas metastatic cell lines and tissue samples showed complete loss or a striking reduction in CAV1 levels. Finally, we show that B16F10 cells expressing CAV1 displayed decreased expression of integrin β_3 and reduced expression and activity of FAK and Src proteins following integrin activation. Thus, here we show for the first time that CAV1 may be functioning to suppress metastasis in malignant melanoma.

The role of CAV1 in regulating the critical aspects of melanomagenesis has not previously been addressed. Our results showing CAV1 expression in primary human melanoma tumors and cell lines versus its reduced expression in metastatic tissues and cell lines may indicate that CAV1 expression contributes to primary tumor growth, whereas its loss is a key factor in metastatic progression. Thus, it seems evident that CAV1 has a biphasic expression pattern in melanoma, in which it is being upregulated in primary tumors compared with melanocytes and ultimately lost in melanoma metastasis.

Given that both our mouse and human tissue data indicate that CAV1 behaves as a "metastasis suppressor gene" in malignant melanoma, we next sought to examine possible mechanisms for this observed phenotype. A large body of experimental *in vivo* and *in vitro* evidence has shown that ECM-cell interactions are critical to acquire the metastatic phenotype. Integrins are families of surface heterodimeric molecules that regulate adhesion of different ECM components such as collagen and fibronectin to the actin cytoskeleton of the cell, and these interactions occur at focal adhesions (FA; ref. 28). Multiple structural and signaling molecules have been shown to localize to FAs, and FAK and Src seem to play key roles in regulating the dynamics of these structures in terms of signaling and protein-protein interaction (35). Interestingly, CAV1 has been shown to functionally interact with components of the FA complex. CAV1 has also been shown to localize to FAs and regulate the dynamics of these structures following integrin activation (26, 31). However, it seems that the function of CAV1 in regulating the integrin/Src/FAK pathway is cell type specific, behaving as a suppressor or an enhancer of this pathway activity depending on cell context (31, 36, 37).

Because of these considerations, we investigated the role of CAV1 in regulating the activity and the expression of FAK and Src proteins following integrin activation in B16F10 cells. Our results, showing a significant reduction in the activities and expression of both Src and FAK in B16F10 cells expressing CAV1, are consistent with their reduced motility *in vitro* and with their reduced metastatic potential *in vivo*. Our results are in agreement with studies showing that reduction of activity and/or expression of FAK protein in melanoma cells suppresses their motility and their ability to form metastases *in vivo* (38–42). Additionally, CAV1 expression in B16F10 cells resulted in a dramatic reduction in the expression of integrin β_3 and integrin α_5, two molecules often implicated in regulating the motility and metastatic ability of melanoma cells (32, 33, 43). In addition to alterations in integrin/Src/FAK signaling, integrin engagement in these cells also affects proliferative and apoptotic pathways. When plated on FN in the absence of serum, B16F10 cells expressing CAV1 had reduced levels of cyclin D1 and of the antiapoptotic protein Bcl-2, an effect that is reversed when the cells were incubated with medium containing serum. This suggests that CAV1 promotes proliferative

and suppresses apoptotic pathways only in the presence of a proliferative stimulus (i.e., serum), a result supported by increased [^{3}H]thymidine incorporation only in complete medium. Uncoupling of signals regulating proliferation and migration have been observed before (44, 45) and may indicate a contextual effect of CAV1 on mechanisms regulating proliferation and migration. To our knowledge, this is the first study linking the ability of CAV1 to promote cell proliferation and suppress metastatic potential in melanoma with alterations in integrin/Src/FAK signaling.

In conclusion, we show that CAV1 expression promotes B16F10 melanoma cell proliferation while dramatically suppressing their ability to form metastases *in vivo*. In human tissue, CAV1 expression is maintained in primary melanoma tumors but is reduced or lost in a large proportion of metastatic lesions. Mechanistically, this phenotype was associated with the ability of CAV1 to decrease the expression of integrin β_3 and to reduce the overall expression and activity of Src and FAK, two proteins critical in regulating FA dynamics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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