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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 has recently accumulated describing the function of CAV1 in several cancer types, its role in melanoma tumor formation and progression remains poorly explored. Here, by employing 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, while 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 demonstrates 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 demonstrate 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 β3 in B16F10 melanoma cells. In summary, our findings provide experimental evidence that CAV1 may function as an antimetastatic gene in malignant melanoma.

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INTRODUCTION

Malignant Melanoma remains among the most life threatening of all cancers, and its incidence has been rising 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.

Initially identified by electron microscopy (2), Caveolae are 50-100nm large plasma membrane invaginations morphologically distinct from the classical clathrin coated vesicles (3). Three different Caveolin genes (*CAV1*, *2*, and *3*) 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 down-regulated and/or mutated in a number of human tumors including mammary adenocarcinomas and squamous cell carcinomas (7) (8). Second, the generation of CAV1 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 (DMBA)-and oncogene-induced cancer in skin and mammary tissue, 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

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cancer type and/or stage dependent (11). CAV1 is upregulated in 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, instead, 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 employed 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 the 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.

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MATERIALS AND METHODS

Materials. Antibodies and their sources were as follows: p-FAK(Y397) and p-Src(Y418) were from Invitrogen. CyclinD1, CyclinA , Bcl-2 , Integrin α5, Integrin β1, and CAV1(N-20) were from Santa Cruz Biotechnology. FAK, and CAV1 were from BD. Src, Integrin α6, Integrin αV (Ab1930) were from Millipore. Integrin β3, AKT, and p-AKT(S473) were from Cell Signaling. β-Tubulin was from Sigma, S-100b was from Affinity BioReagents, and GAPDH was from Fitzgerald.

Mice Experiments. *Orthotopic Injections* were performed by intradermally injecting 10^6 B16F10 cells, whereas intravenous (IV) injections of 10^5 cells were used to assay for *Experimental Metastasis* in 3-4-mo-old C57Bl6/J female mice (16) (17). All *in vivo* studies were approved by the IACUC 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 manufacturer's instructions (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 RIPA 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).

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Growth Curves, Cell Cycle Analysis, and Proliferation assay. *Growth curves* were generated by seeding 2 x 10^3 cells/cm^2 in triplicate. 1, 2, 3, and 4 days after seeding cells were dissociated and cell number counted with hemacytometer. *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 qRT-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 & IV), was purchased from OriGene Technologies (MERT501). 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 (IHC) of Tissue Sections. A tissue microarray (TMA) of paraffin embedded human melanoma tissue samples were purchased from US Biomax (Mel207; 69 cases/207 cores) and was stained for CAV1(N-20) using standard IHC techniques (9). An expert dermatopathologist carefully analyzed and blindly scored the tissues cores for semiquantitative analysis of immunoreactivity. Detailed descriptions are available in *Supplementary Methods*.

Migration and Invasion assays. 5x10^4 cells suspended in 0.5 ml of SFM containing 0.1% BSA (Sigma) were added to the wells of 8µm pore polycarbonate membrane, either coated with (for chemoinvasion assays) or without (for chemotaxis assays) Matrigel (Transwells; BD

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Biosciences). Serum-free NIH3T3 conditioned medium (48h) was used as a chemoattractant. After 6h, 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 serum-free medium (SFM) containing 0.1% BSA for 18h, cells were dissociated, suspended in medium containing 0.1% BSA, and replated on Fibronectin (FN)-coated plates (BD) for 1h at 37 ^{o}C. Cells were either lysed immediately or lysed following the addition of complete medium (10% FBS) for 10 minutes. Alternatively, following 18h serum starvation, cells were dissociated and left in suspension for 1h, and then processed for Western Blot analysis.

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

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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 (25) (26) (15). 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. 1*A*). 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 1*B*). These results were further confirmed by the observation that the CAV1/CAV2 complex was enriched in the membrane fraction and in the Triton X-100 insoluble fraction of B16F10 cells expressing CAV1 (Fig. 1*C*, 1*D*). Thus, these results provide evidence that the CAV1/CAV2 complex is correctly targeted to the plasma membrane of B16F10 cells following the re-expression 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 vs. 98 ±7 cpm/mg in pBabeB16F10) (Fig. 2*A* and 2*B*). FACS analysis of asynchronously growing cells showed a significantly increased percentage of B16F10pBabeCAV1 cells in the S and G$_2$M phases of the cell cycle (Fig. 2*C*, Supplemental

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Table 1). CAV1 expression in B16F10 cells was also associated with increased CyclinD1 and CyclinA expression and increased Ki67 positivity as determined by Western Blot and immunoflurescence analysis (Fig. 2*D*). These results demonstrate a pro-proliferative 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 3-4-month-old C57Bl/6 female mice. 18 days after injections, the determination of tumor size and weight revealed that tumor growth was not significantly different between B16F10pBabe and B16F10pBabeCAV1 (Fig. 4*A*). Additionally, lungs dissected from both groups of mice did not show any spontaneous metastasis formation. To

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assess whether CAV1 expression was able to affect the metastatic potential of B16F10 melanoma cells, 10^5 B16F10pBabe and B16F10pBabeCAV1 cells were IV injected in 3-4 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 to the B16F10pBabe injected animals (94%) (Fig. 4*B*; Supplementary Table 2). 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 to the B16F10pBabe injected mice (Fig. 4*C*, *D*). Consistent with the ability of CAV1 to reduce the motility of B16F10 cells *in vitro*, these results demonstrate 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 to primary melanoma derived cell lines (WM35, WM115). Interestingly, primary human melanocytes displayed complete absence of CAV1 expression (Fig. 5*A*). To validate the significance of the expression pattern observed in melanoma cell lines, we next determined CAV1 expression by IHC 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 absence (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. 5*B*). In cores that stained positive, CAV1 was

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observed to localize in the cytoplasm and at the plasma membrane of melanoma cells (Fig. 5*C, center*). In the skin, instead, CAV1 immunostaining was observed in the keratinocytes of the basal cell layer as we have previously described (Fig. 5*C, left*) (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 to Stage III metastases (Fig. 5*D***,** *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 to CAV1 mRNA levels in normal skin (Fig. 5*D***,** *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 affecting 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-ECM interactions as being critical to acquire metastatic competence in melanoma (28) (29). CAV1 has often been described as an important component necessary to regulate the Integrin/Src/FAK pathway in both normal and tumor cell lines (30, 31) (26). In order 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 to B16F10pBabe cells due at least in part to a reduction in total levels of FAK and Src proteins (Fig. 6*A***,** *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 the integrin signaling (Fig. 6*A*, *right*). We

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next determined the expression of several integrin subunits that have been associated with FAK/Src signaling and melanoma metastasis (32) (33). Interestingly, in both presence and absence of serum, B16F10pBabeCAV1 plated on FN displayed a dramatic reduction in the expression of integrin β3. Expression of integrin α5 was also reduced in B16F10pBabeCAV1 but only in presence of serum. No changes were observed in the expression of β1, αV, and α6 integrins (Fig. 6*A*). 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. 6*B*). Given the role of CAV1 in regulating cell proliferation (27, 34), we next wanted to determine whether pathways involved in melanoma cells proliferation were altered by CAV1 expression after integrin activation. Immunoblot analysis revealed reduced expression of CyclinD1 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, CyclinD1, and total Akt proteins (Fig. 6***C*****,** *bottom)*. These results were consistent with the proliferation rates of B16F10pBabe and B16F10pBabeCAV1 grown on FN for 12h (Fig. 6C, *top*). Interestingly, Bcl-2 expression was also found increased in human metastatic melanoma cell lines compared to primary cell lines indicating an inverse correlation with CAV1 expression (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 (Fig. S2). Taken together, these findings suggest that in 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 demonstrate that CAV1's ability to suppress the Integrin/Src/Fak pathway is serum independent.

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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 demonstrated that introduction of CAV1 using 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. The over-expression 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 in comparison to normal human melanocytes, while 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 β3 and reduced expression and activity of FAK and Src proteins following integrin activation. Thus, here we demonstrate for the first time that CAV1 may be functioning to suppress metastasis in malignant melanoma.

The role of CAV1 in regulating 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, while its loss is a key factor in metastatic progression. Thus, it appears evident that CAV1 has a biphasic expression pattern in melanoma, in which it is being upregulated in primary tumors compared to melanocytes and ultimately lost in melanoma metastasis.

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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 demonstrated that Extracellular Matrix (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 cell's actin cytoskeleton, and these interactions occur at focal adhesions (FAs)(28). Multiple structural and signaling molecules have been shown to localize to focal adhesions, 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 demonstrated 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 that show 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) (39-41) (42). Additionally, CAV1 expression in B16F10 cells resulted in a dramatic reduction in the expression of integrin β3 and integrin α5, 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

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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]thymindine 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 demonstrate 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 β3 and to reduce the overall expression and activity of Src and FAK, two proteins critical in regulating FA dynamics.

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FIGURE LEGENDS

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 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 demonstrate correct targeting of CAV1 to the plasma membrane. CAV2 extensively co-localizes with CAV1 in B16F10pBabeCAV1 cells (Scale Bar, 20μm). **(C)** Immunoblot analysis of cytoplasmatic (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 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.

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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 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 % of B16F10pBabeCAV1 in the S/G_2M phases of the cell cycle (*n*=4, *per* group). **(D)** Immmunoblot analysis showing increased CyclinD1 and CyclinA 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).

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Figure 3. CAV1 expression decreases migration and invasion of B16F10 melanoma cells *in vitro*. Chemotaxis **(A)** and Chemoinvasion **(B)** were performed by seeding 5x10^4 pBabe and pBabeCAV1 B16F10 cells in the upper wells of matrigel coated (for chemoinvasion) or uncoated (for chemotaxis) transwell chambers in serum-free medium containing 0.1% BSA. Serum-free conditioned medium (48h) from cultures of NIH3T3 cells was used as chemoattractant in the lower wells. After 6h, 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).

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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* ≥7, *per* group). (**B, 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* ≥ 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 a two tailed Fisher's Exact Test). **(D)** Representative images of lung lobes dissected from mice IV 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).

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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 to 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 loading control. (**B)** Distribution of CAV1 immunoreactivity scores in primary melanoma and metastatic lesions (tissue microarray, 69 cases, 207 tissue cores) shows that primary tumors display significantly high immunoreactivity scores compared to metastatic lesions (*P*•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, 50μm). **(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 to 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).

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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 18h and were then re-plated on FN coated plates for 1h in serum free medium and either lysed *(left)* or pulsed for 10' with 10% FBS containing medium and lysed (*right*). Western Blot analysis with antibodies directed against FAK, pFAK(Y397), Src, pSrc(Y418), and αV, β3, α6, α5, β1 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 β3 integrin are significantly reduced in B16F10pBabeCAV1 cells compared to 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 demonstrates 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 12h in SFM or 10% FBS (*n* ≥6, *per* group) shows 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 CAV1 expression reduces Bcl-2 and CyclinD1 in SFM while their expression is increased in FBS containing medium.

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