ROMA TRE
UNIVERSITÀ DEGLI STUDI

DOCTORAL SCHOOL OF BIOLOGY

Section "*Biomolecular and Cellular Sciences (SBC)***"**

XXV CYCLE

CAV1 Protein in Skin Cancer Pathogenesis

Ruolo della Caveolina-1 nella Patogenesi del Cancro della Pelle

Dr. Franco Capozza

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A.Y. 2011-2012

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ABSTRACT

Caveolins are a class of oligomeric plasma membrane proteins that function to compartmentalize signaling molecules that are involved in several signal transduction processes. Several lines of *in vitro* and *in vivo* evidence suggest that **Caveolin-1 (Cav1)** is implicated in the pathogenesis of oncogenic cell transformation, tumorigenesis, and metastasis. Although previous studies indicate that Cav1 expression is modulated during both melanoma and non melanoma skin cancers, strong experimental evidence that describes the function of Caveolin proteins in skin carcinogenesis (tumor growth and metastasis) is still lacking. Therefore, the work discussed herein aims to examine this issue by determining Cav1 regulated mechanisms in both melanoma cells and/or the surrounding micro-environment that may affect primary tumor growth and metastatic dissemination. We will also describe mechanisms by which Cav1 expression may affect skin carcinogenesis in a two stage carcinogenesis protocol and in a murine model of cutaneous squamous cell carcinoma (cSCC).

In melanoma, Cav1 is demonstrated to suppress the metastatic ability of melanoma cells by inhibiting signaling along the Integrin/Src/FAK pathway. Accordingly, Cav1 expression is shown to be significantly reduced in human metastatic lesions indicating that it may function in late stage melanomas. To determine possible functions of Cav1 in the melanoma microenvironment, we used Cav1 KO mice to determine whether loss of stromal Cav1 may affect the growth and the metastatic ability of B16F10 melanoma cells. Our findings demonstrating that loss of stromal Cav1 has a tumor promoting effects in primary melanoma while having a suppressive function for lung metastasis, illustrate the ability of this protein to affect different biological processes in a tissue specific manner. Furthermore, in non melanoma skin cancer, Cav1 is demonstrated to suppress benign tumorigenesis and inhibit epidermal proliferation both in primary keratinocytes *in vitro* and promoter-treated epidermis *in vivo*. In addition, Cav1 functions to suppress proliferation, invasion, and metastasis in a murine model of cSCC, attributed in part to its ability to inhibit signaling along the Ras/Erk/AP-1 pathway.

In summary, the work described herein provides evidence that Cav1 may function as a suppressor of tumor progression stages in both melanoma and non melanoma skin cancers and is therefore a possible biomarker for tumor aggression and is a potential target for therapeutic intervention in skin cancer.

RIASSUNTO

Le caveoline costituiscono una classe di proteine di membrana oligomeriche responsabili della regolazione di molecole coinvolte in vari processi di trasduzione del segnale. Diversi studi *in vitro* e *in vivo* suggeriscono che la Caveolina-1 (Cav1) sia implicata nella trasformazione oncogenica cellulare, nella tumorigenesi e nei processi metastatici. Sebbene precedenti studi indichino che l'espressione della Cav1 sia modulata nel melanoma e in altri tipi di tumori cutanei, ad oggi mancano chiare evidenze sperimentali che descrivano la precisa funzione delle caveoline nel processo di carcinogenesi della pelle (crescita tumorale e metastasi). Perciò il presente lavoro si propone di esaminare questa questione, determinando i meccanismi Cav1-regolati coinvolti sia nella crescita dei tumori primari sia nella diffusione metastatica. Nel corso del progetto di Dottorato tali studi sono stati affrontati esaminando la funzione di Cav1 sia nelle cellule tumorali che nello stroma circostante. In questo lavoro sono anche descritti i meccanismi mediante i quali l'espressione di Cav1 influenza la carcinogenesi in topi sottoposti a trattamento di induzione di cancro della pelle secondo il *two-stage carcinogenesis protocol*, che implica l'uso di un iniziatore (DMBA) e di un promotore (TPA). E' stato inoltre valutato l'effetto dell'espressione di Cav1 in una linea cellulare che forma *in vivo* tumori a cellule a squamose (cSCC).
Nel melanoma si dimostra che Cav1 sopprime la capacità metastatica delle cellule, inibendo il *pathway* di segnalazione Integrina/Src/FAK. In accordo con questi risultati, l'espressione di Cav1 è significativamente ridotta in lesioni metastatiche umane, indicando che la proteina possa svolgere un ruolo in stadi avvanzati di melanoma. Per determinare le possibili funzioni di Cav1 nel microambiente del melanoma, abbiamo utilizzato topi $Cav1^{-/-}$, dimostrando che la perdita di Cav1 nello stroma esercita un effetto promotore sulla crescita di tumori primari della pelle, mentre produce un effetto inibitore sulla capacità metastatica delle cellule di melanoma B16F10. I dati raccolti dimostrano come questa proteina influenzi il fenotipo tumorale in modo tessuto-specifico. Inoltre, in tipi di cancro della pelle diversi dal melanoma, dimostriamo che Cav1 si comporta da soppressore di tumori benigni della pelle, inibendo la proliferazione sia dei cheratinociti primari *in vitro* che quella dell'epidermide precedentemente trattata con un agente promotore. Infine, lo studio di un modello murino di cSCC ha permesso di attribuire a Cav1 una funzione inibitoria nei riguardi della proliferazione, invasione e metastasi, realizzata attraverso l'inibizione del *pathway* Ras/Erk/AP-1.
In conclusione, il lavoro qui presentato dimostra che Cav1 può funzionare come un soppressore tumorale in entrambi i tipi di tumore della pelle (melanoma e cSCC) e di conseguenza può essere considerato un marcatore di malignità tumorale e un possibile *target* di intervento terapeutico contro il cancro della pelle.

INTRODUCTION

Caveolae: initial discovery. Caveolae were originally identified (1950s) by electron microscopy in endothelial and epithelial cells. They have been described as 50-100nm flask-shaped invaginations of the plasma membrane morphologically distinct from the more electron dense and larger "clathrin coated pits" (1, 2). However, further investigations have led scientists to the discovery of alternative forms of caveolae that are present intracellularly in the form of grape like clusters, rosettes and/or elongated tubules (3, 4). These structures have been prevalently identified at the plasma membrane of numerous well differentiated tissues and cell types including adipocytes, endothelial cells, Type 1 pneumocytes, smooth and

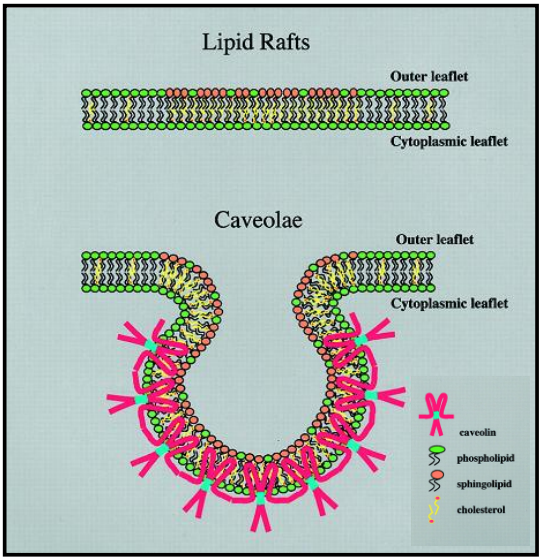

Fig. 1. Detailed organization of lipid rafts and caveolae. Lipid rafts at the plasma membrane are enriched in cholesterol and sphingolipids. Upon integration into lipid rafts, Cav1 monomers assemble into discrete homo-oligomers containing ~14-16 individual Cav1 molecules able to form caveolae invaginations.

skeletal muscles. Interestingly, neurons and lymphocytes lack these invaginations (5, 6). According to earlier views, the plasma membrane was considered a "fluid mosaic" where integral membrane proteins were thought to diffuse freely thought the phospholipids of the plasma membrane (7). A more contemporary view, describes a more discrete distribution for these proteins that are clustered within special microdomains of the plasma membrane termed lipid rafts. These lipid rafts are believed to form in the Golgi via the aggregation of cholesterol and sphingolipids (glycosphingolipids and sphingomielin) and then are delivered to the plasma membrane. These lipid rafts are also enriched in several resident proteins including glycophosphatidylinositol (GPI)-linked proteins (8, 9). Their atypical lipid composition confers resistance to solubilization to mild nonionic detergents such Triton X-100 at 4 ^{o}C (10). These properties constitute the base for the

biochemical identification, purification and characterization of lipid rafts and caveolae. Although caveolae and lipid rafts have similar biochemical properties, the presence of Caveolin proteins renders Caveolae distinct microdomains of the plasma membrane (11).

Caveolin-1: initial discovery. Caveolin-1 (Cav1) was initially identified as one of the phosphorylated substrate in Rous sarcoma virus transformed fibroblasts (12) (13). Interestingly, a separate group identified the VIP-21 (vesicular integral protein of 21 kDa), an integral protein component of *trans*-Golgi-derived transport vesicles (14). It turned out, the caveolin sequence was identical to that of VIP-21, thereby showing that the same protein could possibly serve as a structural component of plasma membrane caveolae, as well as have roles in oncogenesis and vesicular trafficking, all at the same time (15). In **Fig. 1** a schematic on the current view of lipid rafts and caveolae is shown. Briefly, upon integration of the Caveolin-1 into lipid rafts, adjacent CAV1 homo-oligomers (containing 14-16 individual caveolin molecules are thought to pack side-by-side within caveolae membranes thereby providing the structural backbone for caveolae invagination.

Caveolin genes. Three members of the caveolin (*CAV*) gene family have been identified **(Fig. 2)**. Caveolin was the first gene discovered and is composed of three exons that are highly conserved in sequence and structure across species. Caveolin-2 was discovered when micro-sequencing of purified adipocyte caveolae membrane domains revealed a strikingly similarity to caveolin-1, differing in several key conserved residues (16). Caveolin-3 was cloned from a cDNA library using a caveolin-1-related sequence found immediately downstream of the rat oxytocin receptor (17). In addition to these two new members of the caveolin gene family, it was also found that both caveolin-1 and -2 have multiple isoforms. Caveolin-1 has two isoforms, termed α and β, with the α-isoform consisting of residues 1–178 and the β-isoform containing residues 32–178, resulting in a protein 3 kDa smaller in size (18). The β-isoform was originally thought to derive from an alternate translation initiation site occurring at a methionine in position 32. However, later on the existence of two mRNA isoforms was reported using an RNAase protection assay (19). This was later confirmed by another group which detected two mouse caveolin-1 mRNA variants. The full length variant generated mostly caveolin-1α while the 5-variant, lacking the first exon, generated exclusively caveolin-1β (20). The authors confirmed that both α and β caveolin-1 isoforms are produced when a caveolin-1 cDNA construct, having no 5-untranslated region (UTR), is transfected into mammalian cells. They further showed that in vivo expression of the full length and 5'-variant mRNAs correlated with expression of the α and β isoforms, respectively (21). While the exact functional significance of these distinct isoforms remains unclear, studies have suggested that the caveolin-1α isoform is localized predominantly to deeply

invaginated caveolae and can more efficiently drive the formation of caveolae than the β-isoform (20). Cav2 is usually coexpressed with Cav1 in most differentiated cell types with the exception of skeletal muscle. Three Caveolin-2 isoforms have been identified, the full-length caveolin-2 (α), and two truncated Caveolin-2 variants, termed -2β and -2γ. However, it remains unknown the functional significance of the Cav-2 isoforms (22). The caveolin proteins share significant homology. Human Caveolin-2 is ~58% similar to human Caveolin-1, while Caveolin-3 is 85% similar to Caveolin-1. A short stretch of eight amino acids has been identified (FEDVIAEP) that constitutes the "caveolin signature sequence," a motif that is identical between all three caveolin proteins. The pattern of expression of Cav3, instead, is distinct from that of Cav1 and Cav2 that are not co-expressed in differentiated cardiac and muscle cells (23). Interestingly, reconstitution experiments in fibroblasts have demonstrated that Cav1 expression is required for Cav2 to be correctly targeted to the plasma membrane/Caveolae domains of cells (23). The Cav-3 protein is muscle specific and, similarly to Cav1, is known to form high-molecular-mass homo-oligomeric complexes and is sufficient to drive Caveolae formation in cells lacking Caveolins (23).

Membrane topology and post-translational modifications of Cav1. Initial studies have identified Cav1 as an integral membrane protein, as it was found to be resistant to sodium carbonate and high salt concentration extraction. In addition, further studies have shown evidence that Cav1 has an unusual topology with both the NH_2 and COOH termini of the protein facing the cytoplasm with a connecting hairpin hydrophobic intramembrane domain (residues 103-134) (**Fig. 1**). This structure is consistent with the posttranslational modifications of Caveolin such as phosphorylation and palmitoylation that can only occur in the cytoplasmic side of the plasma membrane. One of the most important properties of this unusual protein is its ability to homo- or hetero-oligomerize (with Cav2) that may be one of the possible mechanisms driving Caveolae formation. In fact, Cav1 after being synthesized as an integral membrane protein in the endoplasmatic reticulum (ER) goes through a first stage of homo- or hetero-oligomerization in the ER forming oligomers containing 14-16 individual Caveolin monomers. A further stage of oligomerization between Cav1 oligomers is described to happpen at the level of the *trans*-Golgi, where several Cav1 oligomers self-associate via C-terminal domain (TD domain, **Fig. 2**) and interact with cholesterol to form an extensive networks of Cav1 proteins that may be able to drive the invagination of Caveolae at the plasma membrane. Interestingly, deletion mutagenesis analysis has demonstrated that the hydrophobic residues constituting the transmembrane domain (TM 102-134) is not necessary for membrane attachment. Two other regions instead termed N-MAD (82-101) and C-MAD (135-150) are required to target Cav1 to Caveolae membrane and *cis* Golgi complex respectively. In fact, when fused to the C-MAD of CAV1, the GFP protein was resistant to extraction

with Triton X-100 and show a predominantly Golgi like distribution (24). When fused to the N-MAD domain of Cav1, instead, the GFP displayed a predominantly Caveolae localization. Further analysis identified the minimal sequence (KYWFYR) in the N-MAD domain sufficient to confer membrane localization to GFP protein but not to Caveolae (24). As we will discuss below, the N-MAD region, also called the Caveolin Scaffolding Domain (CSD), is the region of the Caveolin protein believed to directly interact and modulate the activity of several signaling molecules within Caveolae microdomains. Interestingly, Cav1 undergo a series of post-translational modifications that are important in defining its function in several biological processes. Caveolin-1 is palmitoylated on three Cysteines (133,143,156) located in the C-terminus of the protein (25) and these post-translational modifications appear not necessary for caveolar targeting of the protein. However, studies using palmitoylation mutants of the Cav1 protein show that palmitoylation at Cys-156 is required to facilitate the coupling of Cav1 and the c-Src tyrosine kinase within Caveolae (26, 27). Other studies have suggested that palmitoylation may be a mechanism to stabilize Cav1 oligomers and to couple Cav1 with others lipid modified signaling proteins such as Abl, Cbl, Src, Gα subunits, Ras related GTPases, eNos and to cholesterol (28) (26). Caveolin has also been described undergoing phosphorylation on Tyr 14 **(Y14)** (12) and on Ser-80 (**S80**) (29). The role of Y14 phosphorylation has only partially been clarified. It occurs in response to oxidative (30) and osmotic stress (31) and also in response to growth factor and hormonal stimulation (26). In addition, recent studies demonstrate the importance of Y14 phosphorylation in regulating migration/invasion and Caveolin mediated endocytosis in several cell types (32) (33). Furthermore, Cav1 resulted to be phosporylated on S80 and it seems important in regulating ER localization and in regulating the secretory pathway. Mutation of Serine 80 to Ala impairs the ability of Cav1 to be secreted in certain cell types (29) (34).

Vesicular Trafficking. Based on their structural features as plasma membrane invaginations, Caveolae were initially proposed to function in potocytosis (35), a kind of receptor mediated endocytosis that involves the selective uptake of small molecules. With the development of new more sophisticated techniques it appears clear that caveolae take part in other vesicular trafficking processes such as transcytosis and endocytosis that constitute an alternative endocytic pathway to clathrin-coated pits for trafficking to Golgi, ER or lysosome. Several lines of experimental evidence have demonstrated the existence of caveolae mediated endocytosis. First, caveolae endocytosis is significantly slower than clatrin coated pits endocytosis. Second, the phosphatase inhibitor okadaic acid stimulates caveola-mediated endocytosis but inhibits the formation of clathrin-coated vesicles. Third, the sterol binding agent filipin has little or no effect on clathrin-mediated endocytosis, yet inhibits the internalization of caveolae. Finally,

activators of PKC, which do not inhibit clathrin-mediated endocytosis, prevent the formation of caveolar invaginations (36). In *transcytosis* Caveolae transport proteins from the luminal side of endothelial cells to the interstitial side of the endothelial cell layer. Elegant experiments were performed using Caveolar proteins specific antibodies clearly demonstrating the movement of caveolar proteins across the endothelial cells from the luminal side to the interstizial side (37). In *endocytosis,* Caveolae have been shown to mediate the intracellular trafficking of molecules to the ER and Golgi either directly or through an intracelluar organelle termed Caveosome or VVO. Macromolecules such as Cholera toxin, folic acid and albumin are internalized by a Caveolae mediated endocytosis to intracellular compartments that are negative for endosomal markers (EEA1 and TfR). Although the exact process mediating caveolar endocytosis remains unknown, several recent findings suggest that the molecular machinery involved in the generation of caveolar vescicles is the same used for numerous other vesicular transport processes that requires, dynamin, VAMP, SNAP-25, the SNARE complex, and GTP hydrolysis (36) (8). Interestingly, several pathogens, including viruses, bacteria, fungi, parasites and even prions can use caveolar endocytosis as a mean to bypass the classical clathrin mediated endocytosis and as a consequence avoid the degradative compartment constituted by the endosome-lysosome vescicular system. The intracellular trafficking of simian virus 40 is perhaps the most well studied infectious agent that selectively uses caveolae to enter the cells. After its initial binding, and following its accumulation in the "caveosome," SV40 is delivered directly to the ER. In doing so, SV40 avoids the inactivation that would have occurred by using the clathrin coated pits pathway (38) (39). Interestingly, other viruses have been shown to utilize the folate receptors as a cofactor to entry into the cells (40). Importantly, caveolae as a common mechanism to gain entry into cells avoiding the intracellular degradative compartments may be used as a strategy to deliver therapeutic agents (41).

Lipid homeostasis. Since their discoveries Caveolae/Caveolins have a strict interrelationship with cholesterol and with its homeostasis. Initially described as a cholesterol binding proteins, Caveolins and Caveolae are sensitive to agents such as filipin and nystatin that can reduce Caveolar structures, intracellular localization and their stability (42). Additionally, cholesterol transcriptionally regulates Cav1 expression through two steroid regulatory binding elements in the Cav1 promoter. This results in decreased Cav1 mRNA in cells depleted of cholesterol (43). Thus, Cav1 expression and its intracellular distribution are clearly dependent upon cellular cholesterol levels. Other findings suggest that Caveolin can regulate cholesterol homeostasis by modulating its cellular influx or efflux (44). Interestingly, Cav1 and Cav2 have been found associated with lipid droplets, metabolically active organelles serving for lipid storage functions (45).

Caveolae, Caveolins and Signal Transduction Mechanisms. Another important feature of Caveolins and Caveolae biology was the discovery that biochemically purified Caveolae are enriched in signaling molecules such as Src-like-tyrosine-kinases and heterotrimeric G-proteins. These initial observations led the authors to the "Caveolae signaling hypothesis"that describes Caveolae as hubs that may serve to specifically compartmentalize certain signaling molecules and thereby rapidly

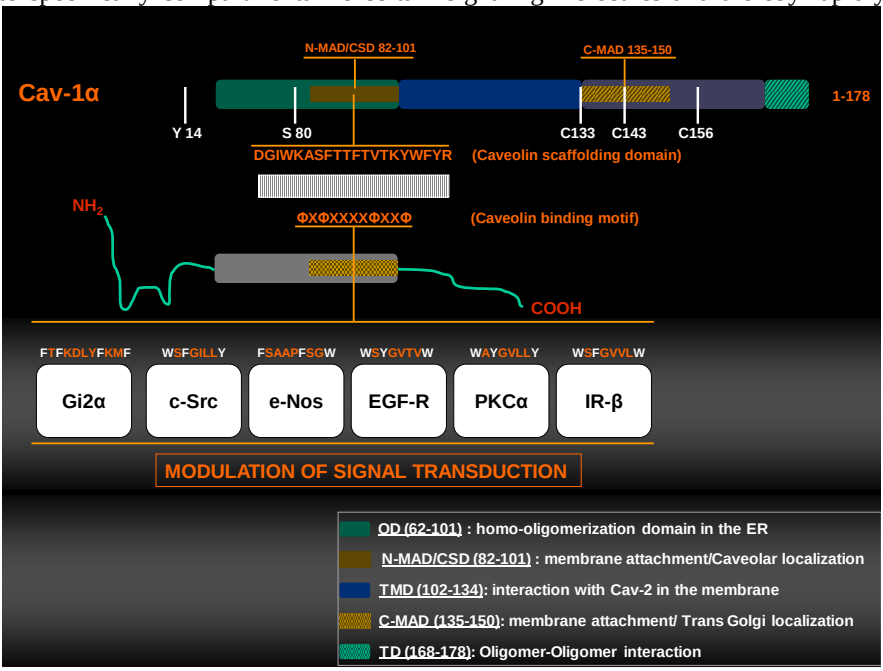

Fig. 2. The sequence of the caveolin-scaffolding domain and the caveolin binding motif sequences of several proteins are shown. In most cases, this caveolin interaction is inhibitory, leading to inactivation of the signaling pathway and thereby modulation of downstream signal trusduction.

modulating signal transduction events and the cross-talk between different signaling pathways (46) (47). This hypothesis was confirmed by studies demonstrating that the activity of G-protein-alpha subunit could be suppressed by a peptide mimicking the Caveolin scaffolding domain (CSD; 82-101) of Cav1. Similar results were obtained with other signaling proteins such as Src/Fyn, EGF-R, Neu, PKC, and PKA (48). With the proteomic analysis of purified Caveolae it has been possible to identify a wide assortment of proteins that are localized to these structures in several different tissues and cell types. For instance proteomic analysis of purified caveolae from lung tissue identified a prevalence of cytoplasmatic signaling molecules (Src-like kinases and heterotrimeric G protein subunits), including the small GTPases Rap 1, Rap 2 and cytoskeletal elements,

such as monomeric G-actin and myosin regulatory light chain and several transmembrane proteins were identified, including CD36 and RAGE, as well as an abundance of GPI-linked proteins (10). Importantly, extremely similar results were obtained during the proteomic analysis of Caveolae purified from other cell types (49). These and other studies led to the development of the idea that caveolae are actively engaged in the compartmentalization of various signaling molecules and thereby behaving as active signaling organelles in the cell. There is now evidence that the region termed the caveolin scaffolding domain (CSD) seems to be responsible for many of the biological functions attributed to Cav1 by interacting with a sequence called the Caveolin binding domain (CBD) identified in several Cav1 binding proteins including tyrosine/serine threonine kinases and eNos. Site-directed mutagenesis performed to modify the caveolin binding sequance within the Caveolin binding domain of eNos, identifiyed the following motifs that are always found in Caveolin interacting proteins: *ΦXΦXXXXΦ*, *ΦXXXXΦXXΦ*, *ΦXΦXXXXΦXXΦ*, where *Φ* is an aromatic residue (Phe, Tyr, or Trp) and *X* is any amino acid (36) (50). Except few exceptions, the current view is that upon binding to signaling molecule with the CSD, Cav1 leads to inhibition of downstream signaling and thereby functioning as a negative regulator of signal transduction (**Fig.2**). Given the ability of many of these molecules to be involved in cellular transformation (i.e. H-Ras, MAPK, PDGFR, EGFR) when hyperactivated, it is reasonable to speculate that Cav1 may behave as tumor suppressor gene *in vivo*.

Caveolin1: role in cancer pathogenesis. The initial discovery that Cav1 is a major phosphorylation substrate in Rous sarcoma virus transformed cells indicated it was a potential target in the process of oncogenesis (12). Indeed, subsequent experiments showed that both Cav1 mRNA and protein levels decrease when NIH3T3 cells are transformed by a variety of oncogenes, including H-RasG12V, polyoma virus mTAg, and $p210^{bcr\text{-}abl}$ (51) (52). Transformation with H-RasG12V was further shown to alter transcription factor binding at the Cav1 promoter and decreases transcriptional activity (53). Additionally, the Cav1 promoter contains p53-responsive elements and the p53 tumor suppressor protein increases Cav1 mRNA and protein levels (54). Finally, the Cav1 promoter has c-myc-repressive elements and expression of the c-myc oncogene decreases Cav1 mRNA levels (55). Because one of the hallmarks of transformation is the downregulation of tumor suppressor proteins (56), the down-regulation of Caveolin-1 at the mRNA and protein levels in response to a broad range of oncogenic stimuli provided evidence that this protein could have a function in cancer. Evidence for the tumor suppressive function of Cav1 comes from the observation that Cav1 is lost in human cancers through gene deletion, misregulation, or mutation. Fragile sites are chromosomal loci sensitive to replication stress, and they are frequently involved in chromosomal rearrangements in human disease (57). The q31 region of Chromosome 7, encompassing the fragile site FRA7G at 7q31.2, is frequently lost

in human cancers, including breast and prostate cancers as well as head and neck squamous cell carcinomas (58) (57) (59) (60) (61) Significantly, work by Engelman and colleagues mapped the location of both human *CAV1* and *CAV2* genes to Chromosome 7q31.1 (62) (63), and this discovery led to the hypothesis that *CAV1* and *CAV2* are potential tumor suppressor genes that contribute to tumor development when this region is lost in human cancers (53, 62). Methylation of the *CAV1* gene promoter provides a second mechanism by which expression of this protein is lost in human cancers. In addition to mapping the chromosomal location of *CAV1* and *CAV2*, Engelman and colleagues found methylation of CpG islands in the *CAV1* promoter region in human breast cancer cell lines (53). Subsequent research found similar results in breast cancer tissue, small cell lung cancer cell lines, a human ovarian cell line and ovarian adenocarcinoma tissue, and human prostate cell lines and prostate tumor samples (64) (65, 66) (67). This work suggests *CAV1* expression can be lost in human cancer not only through gene deletion, but also through alterations to promoter methylation status and transcriptional repression. Finally, a third mechanism of functional Cav1 loss in human cancer is through mutation. Hayashi and colleagues examined the *CAV1* gene sequence in primary human breast cancer tissue and discovered a mutation—P132L—in 16% of the tumors. The mutation is found mainly in invasive carcinomas, and expression of this mutant protein is able to transform NIH3T3 cells (68). Interestingly, further work on this mutation showed expression of Cav1-P132L in mammary epithelial cells expressing endogenous WT Cav1 results in the retention of both mutated and WT protein in the Golgi, demonstrating that P132L is a dominant negative mutation (69). In addition to these specific mechanisms of Caveolin-1 down-regulation, Cav1 mRNA or protein expression has been examined in specific cancers with varying results. Multiple studies have consistently shown that Cav1 expression is lost or decreased in: 1) ovarian carcinoma, demonstrated at both the mRNA and protein levels in cell lines and tissue, 2) lung carcinoma, demonstrated in some studies for several types of lung cancer and in others as being more common in small cell lung cancer, and 3) breast cancer, shown to correlate with tumor size and grade. For some cancers, Cav1 expression seems to be related to metastatic, but not primary, tumor growth. For example, in head and neck squamous cell carcinoma, Cav1 expression is lost in metastatic tumors and lymph node metastases. It should be noted that Cav1 is not a universal tumor-suppressor gene. Indeed, its role in oncogenesis has been shown to be context-dependent, and Cav1 is up-regulated in the development and progression of certain cancers. For example, in bladder carcinoma, studies have shown that Cav1 expression in not observed in normal epithelium but is associated with higher-grade tumors. In addition, Cav1 has a growth-promoting role in prostate cancer. In the prostate, phosphorylation of Cav1 at serine-80 causes it to be secreted by cancer cells; in human patients with advanced disease, secreted Cav1 can act in an autocrine/paracrine manner to promote prostate cancer cell

growth (70). Consequently, Cav1 is up-regulated in both primary and metastatic prostate cancers, where it correlates with Gleason Score and lymph node involvement (71) (70) (72). Obviously, there are tissue-specific differences in the role of Cav1 in cancer development. However, the loss of Cav1 expression in several human cancers through gene deletion, transcriptional repression, or mutation indicates an anti-oncogenic function for this protein in many contexts, which is further supported by examination of the effects of gene deletion in a mouse model.

The Caveolin-1 knock-out mouse and tumorigenesis. The generation of the *Cav1* KO mouse model allowed for the examination of the effect of complete Cav1 loss on numerous phenotypes. These mice were developed in parallel by two different groups (73) (74). Given the previously-discussed importance of Cav1 in caveolar biogenesis (75), it is not surprising that both groups report loss of caveolae structures in Cav1-expressing tissues (i.e caveolae are still apparent in tissues which predominantly express Caveolin-3). Interestingly, in *Cav1* KO cells Cav2 expression is significantly reduced demonstrating that Cav1 is necessary for the stabilization of his binding partmner (76). *Cav1* KO mice show a roughly 90% decrease in Cav2 protein levels in Cav1- expressing tissues, which is rescued by proteasome inhibition (74). Due to this phenomenon, *Cav1* KO mice are essentially double knock-outs for both Caveolins-1 and 2. *Cav1* KO mice display numerous pathologies, including lung hypercellularity and thickening of the alveolar septa, increased eNOS activation resulting in vascular abnormalities, and metabolic defects that include decreased fat accumulation and insulin resistance (74). Given the proposed tumor suppressive function of Cav1, *Cav1* mice null have been used extensively in studies of cancer biology and tumorigenesis. *Cav1* KO mice do not develop spontaneous tumors throughout the course of their approximately 500 day lifespan (77). The first evidence that loss of Cav1 contributes to *in vivo* oncogenesis was provided by Capozza and colleagues (78). When C57BL/6 WT and *Cav1* KO mice were subjected to chronic topical treatments of the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), KO mice displayed an increase in epidermal hyperplasia, tumor incidence, tumor multiplicity, and tumor size along with hyperactivation of the Erk1/2 MAPK pathway (78). In addition, *Cav1* KO mice display hyperproliferative defects. Specifically, mouse embryonic fibroblasts (MEFs) isolated from these mice show an increase in proliferation, and their lungs show evidence of hypercellularity (74). In addition, these mice display mammary epithelial hyperplasia by 6 weeks of age and increased lobular development with acini formation by 9 months of age (69). Crossing *Cav1* KO mice to a model of spontaneous breast tumor development (MMTVPyMT) revealed that *Cav1* KO mice develop mammary lesions approximately 5 weeks earlier and show a roughly 2-fold increase in tumor multiplicity when compared to WT mice. In addition, lesions from *Cav1* KO mice

were of a higher 20 histological grade than WT lesions, and KO mice had a greater incidence of pulmonary metastasis (79). Recently, much attention has been given to the function of Caveolin-1 in the tumor microenvironment, specifically the stromal compartment surrounding epithelial tumor cells. Interestingly, loss of Cav1 has been shown to affect benign tissue stroma in aged *Cav1* KO mice. Yang and colleagues showed that the stromal compartments of various organs, including the lung and liver, are disorganized in KO mice, and this pathology results in defects in epithelial cell growth and differentiation (80). Examination of the effect of Cav1 loss in cancer-associated stroma revealed that the injection of breast cancer cells into the *Cav1* KO mammary fat pad increases tumor growth in comparison to injection in the WT fat pad, indicating the KO fat pad is more permissive to malignant growth (81) (82). Similarly, injection of lung carcinoma cells into *Cav1* KO mice increases angiogenesis and tumor growth in comparison to cells injected in WT mice (83). Different groups have reported that loss of stromal Cav1 in human breast cancers correlates with poor prognosis, suggesting that Cav1 loss in the stroma could be a biomarker for an aggressive cancer microenvironment (84). However, these findings are controversial, as other researchers assert that the disorganized stroma displayed by the *Cav1* KO mouse is actually an inhibitory microenvironment for invasion and metastasis (85). The potential function of Caveolin-1 in the tumor microenvironment is interesting but contentious, and most work still focuses on the role of this protein in the epithelial component of the tumor. As detailed above, the Caveolin-1 knock-out mouse has been used extensively to study the effect of Cav1 loss on tumor development and progression through the examination of both epithelial cancer cells and their associated stroma. These studies demonstrate that the *Cav1* KO mouse does not develop spontaneous tumors. Given this phenomenon, it has been proposed that while Cav1 loss is not sufficient in and of itself to transform normal cells, its loss in conjunction with an oncogenic stimulus exacerbates tumor development, growth, and progression (86). Further support of this hypothesis is the extensive research describing the role of Cav1 in the modification of several cancer-associated phenotypes, i.e. proliferation and survival, anchorage-independent growth, and invasion (87).

Skin biology and function. The integumentary system, comprised of the skin and hair, is the largest organ system of the human body, accounting for roughly 15 percent of total body weight. Mammalian skin consists of the dermal and epidermal layers (**Fig. 3**). The underlying dermis, composed mainly of fibroblasts and connective tissue, functions as the support system for the epidermis. The epidermis is a stratified epithelium that is constantly undergoing renewal as

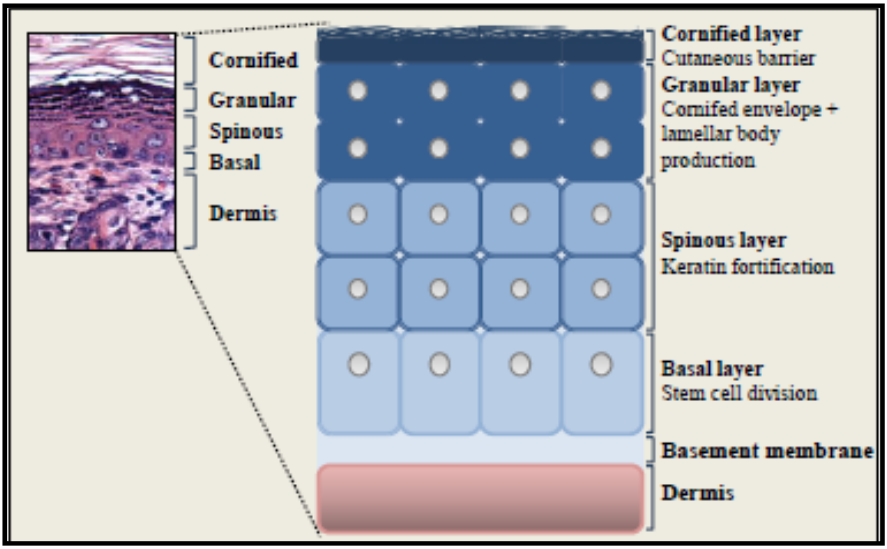

Fig. 3. **Skin and epidermal morphology.** The two main layers of the skin, dermis and epidermis separated by the basement membrane. Keratinocytes in the basal layer divide and undergo terminal differentiation to form a layer of enucleated, dead cells in a matrix of lipids, called the cornified layer.

outside cells are sloughed off and replaced by cells from the lower layers. Epidermal homeostasis is maintained by actively-dividing stem cell populations in both the hair follicles and the basal layer (88) (89) (90). Keratinocytes are the major cell type of the epidermis, and they undergo a process of terminal differentiation and cell death that culminates in the creation of an environmental barrier. Stem cell division in the basal layer of the epidermis produces new keratinocytes; these cells lose their ability to adhere to the basement membrane between the dermis and the epidermis, causing the keratinocytes to undergo differentiation. The cells exit the cell cycle and are pushed from the basal layer into the upper layers of the epidermis. In the spinous layers, the cells fortify their keratin filament network. In the granular layers, keratins are bundled into larger units called macrofibrils, lipids are produced in epidermal lamellar bodies, and cornified envelope proteins are deposited beneath the plasma membrane. In the final steps of terminal differentiation, the cell membrane disintegrates, triggering

the cross-linking of the cornified envelope proteins into a rigid layer surrounding the keratin macrofibrils. Lipids are then expelled from the lamellar bodies into the intercellular space, creating a waterproof, durable layer of dead squames in a bed of lipids called the cornified layer (**Fig. 3**). As the body's major barrier, the skin is subjected to constant damage from environmental stresses, including chemicals, toxins, and ultraviolet radiation, and accordingly is a common site for tumorigenesis (88).

Skin cancer characteristics. Malignancies that develop in the epidermis are broadly termed skin cancer and are categorized into two groups: malignant melanoma and non-melanoma skin cancer. *Malignant melanoma* (MM) originates in the melanocytes localized at the dermal-epidermal junction. MM is the least common type of skin cancer, but it is also the deadliest due to its higher rate of metastasis. Transformation of skin melanocytes to cutaneous melanoma is a multistep process also called melanomagenesis (91)**.** The first steps, considered as benign, are associated with the formation of a nevus and the radial growth phase (RGP). In a nevus, melanocytes are clustered and have lost their appropriate contact with keratinocytes. During RGP, melanocytes tend to proliferate superficially to the basement membrane of the epidermis. During the next stage, the vertical growth phase (VGP), the cells bypass senescence to proliferate actively in a vertical manner in the dermis, crossing the basement membrane. At this stage, cells migrate and become clearly invasive. The last stage is the acquisition by the cells of metastatic characteristics: the cells are able to enter the bloodstream or lymphatic vessels from which they colonize tissues and organs. In addition, melanomagenesis is accompanied by dysregulation of various signaling pathways which ultimately affect several cancer associated phenotypes such cell cycle, invasion, migration, tumor growth and metastatic dissemination. Several genetic and epigenetic abnormalities have been detected in melanomas that results in inactivation of tumor suppressor genes and hyperactivation of oncogenes, the inhibition of apoptosis, changes in morphology and migration capacity, and also modification of DNA repair enzyme activities. Two of the most common genetic alterations found in melanoma are the loss/inactivation of the tumor suppressor $p16^{Ink4a}$ and the activating mutations in N-Ras and B-Raf (>50%). Additional alterations in terms of signaling may accumulate at later stages of melanoma progression (91)**.** *Non-melanoma-skin-cancer (NMSC)* is the most prevalently-diagnosed malignancy in the United States and estimated that roughly 45 percent of Americans that live to age 65 will have NMSC at least once in their lives (92). There are two types of non-melanoma skin cancer: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC is the most common type of skin cancer and comprises a group of tumors that can vary phenotypically, but primarily contain undifferentiated epidermal basal cells and mutations that constitutively activate the Hh pathway are found in the vast majority of these lesions (93).

Cutaneous squamous cell carcinoma (cSCC) is the second-most common form of skin cancer, with an estimated 700,000 cases diagnosed yearly in the U.S. (92). While BCCs are largely the product of a single molecular alteration, the development of cSCCs is considered a multi-stage process in which an accumulation of genetic events in necessary (94). As such, precursor lesions have been identified for cSCC. These include actinic keratoses (AK), a pre-cancerous lesion with a 5-10% chance of developing into cSCC, and Bowen's disease (BD), a non-invasive tumor that precedes invasive cancer and is referred to as carcinoma *in situ* (95) (96). Another potential precursor lesion is keratoacanthoma (KA), a low grade lesion with a rapid early growth phase that is histologically very similar to a well differentiated SCC (97). Squamous cell carcinomas are the result of abnormal proliferation of the more-differentiated squamous cells of the epidermis. However, the actual cell of origin for SCC most likely resides in the basal epidermal layer, and the squamous phenotype is a consequence of what and when specific mutations are acquired along the progression to malignancy (98) (99). The outcome of this multi-step process is a squamous cell carcinoma that, unlike BCC, has the potential for metastatic dissemination. Given the potential to metastasize, extensive research has examined the characteristics of the primary tumor that would indicate a potential for tumor progression and metastatic dissemination. Given their prevalence and potential for metastasis, much research is needed to identifying the factors that contribute to cSCC development.

Cav1 expression and function in normal skin. Upon barrier disruption through tape stripping, *Cav1* KO mice display increased raft formation and accelerated barrier reacquisition due to increased lamellar body (LB) secretion and also experimental epidermal hyperplasia due to reduced terminal differentiation (100). Similarly, wound healing studies demonstrated that cutaneous wounds in *Cav1* KO mice heal more quickly than those of wild-type (WT) mice (101). Studies in primary human keratinocytes have demonstrated that Cav1 mRNA and protein levels increase dramatically in the later stages of keratinocyte differentiation, further supporting a role for Cav1 in this process (102). In addition, loss of Cav1 confers a hyperproliferative phenotype to basal keratinocytes indicating an enhanced ability to re-populate a wounded site with keratinocytes (100) (103). In addition to functioning in re-epithelization of the epidermis, Cav1 also plays a role in intercellular junctions in the skin. Caveolin-1 has been implicated in the regulation of three different types of junctions in the epidermis: adherens, desmosomes, and gap junctions (104) (105) (100) (106). These studies indicate that Cav1 has a significant function in maintaining structure and homeostasis in the skin. For instance, Connexin-43 (Cx43) is highly expressed in the epidermis where it co-localizes with Cav1 in keratinocytes. Loss of this association between Cav1 and Cx43 renders keratinocytes more susceptible to transformation (107). Thus, loss Cav1 expression decreases intercellular adhesion through adherens and

desmosomes and communication through gap junctions. In combination with the function of Cav1 in the epidermal barrier, these studies indicate that Cav1 has a significant function in maintaining structure and homeostasis in the skin, and suggest that its loss could confer sensitivity to the development of skin disease.

Cav1 and skin cancer. The role of Cav1 in melanoma progression remains not very well defined. Overexpression of Cav1 in a metastatic melanoma cell line SKMEL28 resulted in inhibition of their proliferation and motility. The reduced motility of these cells was attributed to the reduced levels of phosphorylated levels of p130Cas and paxillin in Cav1 expressing cells. In addition, Cav1 expressing cells displayed a diffused staining for Gangliosides GD3 compared to their wild type counterpart that showed a more localized staining to the leading edge of melanoma cells, suggesting that Cav1 expression may inhibit or at least attenuate the malignant properties of this cell line. Alonso and colleagues used a melanoma tissue microarray of 165 samples in which they used a panel of 39 different antibodies for cell cycle proteins, apoptosis, transcription factors and other proteins. Interestingly, this panel of antibodies included Cav1 whose expression was significantly diminished in advanced stages of melanomas relative to nevus, suggesting for the first time that Cav1 could be used as a biomarker to predict melanoma progression and disease outcome. While these results from earlier investigations suggest a tumor suppressive function for Cav1 in malignant melanoma, more recent *in vitro* studies proposed a tumor promoting role for Cav1. Sargiacomo and colleagues observed increased proliferation and increased motility in melanoma cells expressing Cav1. These authors attributed this phenotype to the ability of Cav1 to hyperactivate the FGFR/Src/Rho GTPases signaling pathway which is critical in regulating cell cycle progression and several aspects of cell motility (108) (109). In another recent article, Del Pozo and colleagues reported that in a melanoma cell line the presence of Cav1 was required for the internalization of the lipid raft protein Rac1 in suspended cells, resulting in the inhibition of downstream effectors proteins (110). Several lines of experimental evidence suggest that tumor growth results from key bidirectional interactions between cancer cells and their surrounding stroma (111). Although the role of stromal Cav1 has been recently investigated in breast cancer (112) the function of Cav1 in melanoma microenvironment and consequent tumor development remains largely unexplored. As described above, Cav1 loss-of-function in the skin may contribute to hyperproliferative disorders of the epidermis by promoting pro-proliferative signals or by affecting the differentiation stage of epidermal keratinocytes (107) (113) (100). Interestingly, in addition to showing a decreased expression in hyperproliferative diseases of the skin, Langlois and colleagues demonstrated that Cav1 expression is significantly reduced in both basal and squamous cell carcinomas of the skin suggesting that Cav1 may have a function in the pathogenesis of skin cancer. Accordingly, Capozza and colleagues showed that

Cav1 KO mice are more susceptible to skin tumor development following chronic treatment with the carcinogen (DMBA) (78). *Cav1* KO mice had increased epidermal hyperplasia and tumor incidence, multiplicity, and size, attributed to enhanced growth signaling, including cyclin D1 and activated Erk1/2, in the expanded epidermal proliferative compartment. Similarly, Roelandt and colleagues showed that the epidermis of *Cav1* KO mice had increased baseline and experimentally-induced proliferating cell nuclear antigen (PCNA) incorporation (100). Collectively, this work indicates that loss of Cav1 may confer a malignant advantage in the context of skin cancer development.

OBJECTIVES

As the main protein component of the membrane lipid rafts caveolae, Cav1 functions as a modulator of cellular signal transduction through both internalization and interaction with various signaling molecules. By means of its scaffolding domain, Cav1 compartmentalizes signaling proteins, often resulting in the suppression of their signaling function. Given its multi-functional nature, Cav1 has been implicated in the pathogenesis of several human diseases, including cancer. Its role seems to be context-specific and dependent on the type of tissue being examined. In several cancers, Cav1 expression is lost through mutation, gene misregulation, or unknown mechanisms. In addition, Cav1 is a negative regulator of many cancer associated phenotypes, including proliferation, anchorage independent growth, and invasion. Published data from us and from other investigators indicates that Cav1 expression is modulated during both melanoma and non melanoma skin cancers. Several lines of *in vitro* and *in vivo* evidence suggest that Cav1 is implicated in the pathogenesis of oncogenic cell transformation, tumorigenesis, and metastasis. However, while Cav1 does not appear to have a direct role in tumor initiation, it does synergize with other oncogenes and tumor suppressors to modulate the transformed/tumorigenic phenotype. Recent published data from us and others indicates that Cav1 expression is modulated during both melanoma and non melanoma skin cancers. However, very little work has examined the contribution of Cav1 to specific stages of skin carcinogenesis, namely initiation, promotion of growth, and progression to malignancy and metastasis. *Therefore, the work discussed herein aims to address this issue by determining mechanisms by which modulation of Cav1 protein expression in both melanoma cells and/or the surrounding micro-environment may affect melanoma tumor growth and metastatic dissemination. We will also describe mechanisms by which Cav1 expression may affect skin carcinogenesis in a to two stage carcinogenesis protocol and in a murine model of cutaneous squamous cell carcinoma (cSCC.)* In summary, the work described herein provides evidence that Cav1 may function as suppressor of tumor progression stages in both melanoma and non melanoma skin cancers and is therefore a biomarker for tumor aggression and is a potential target for therapeutic intervention in skin cancer.

Chapter 1

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

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

Molecular and Cellular Pathobiology

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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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-0900

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).

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

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 $\Delta\Delta$Ct 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;

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

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

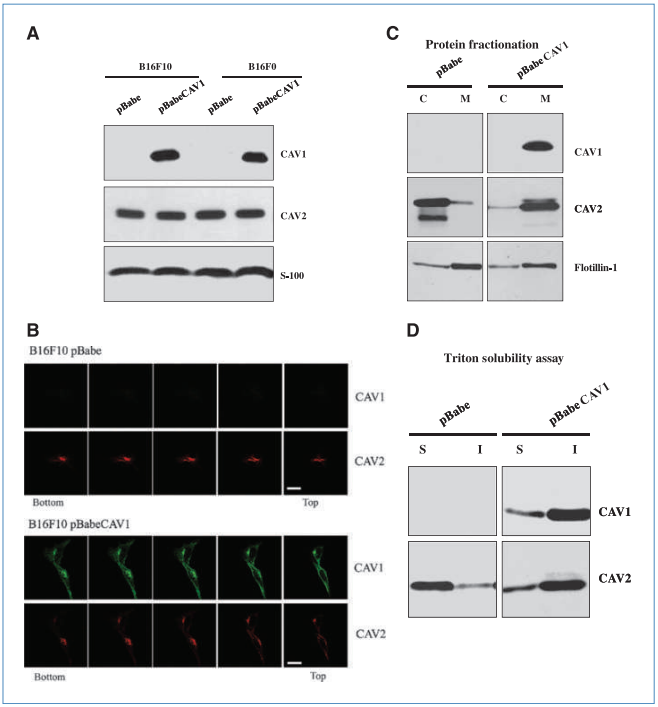

Figure 1. Absence of *CAV1* expression in B16F0 (weakly metastatic) and B16F10 (highly metastatic) melanoma cell lines. A, immunoblotting of retrovirally transduced pBabe and pBabeCAV1 B16F10 and B16F0 cells for CAV1 and CAV2. S-100 immunoblot is shown as a loading control. Note the absence of CAV1 expression 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.

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

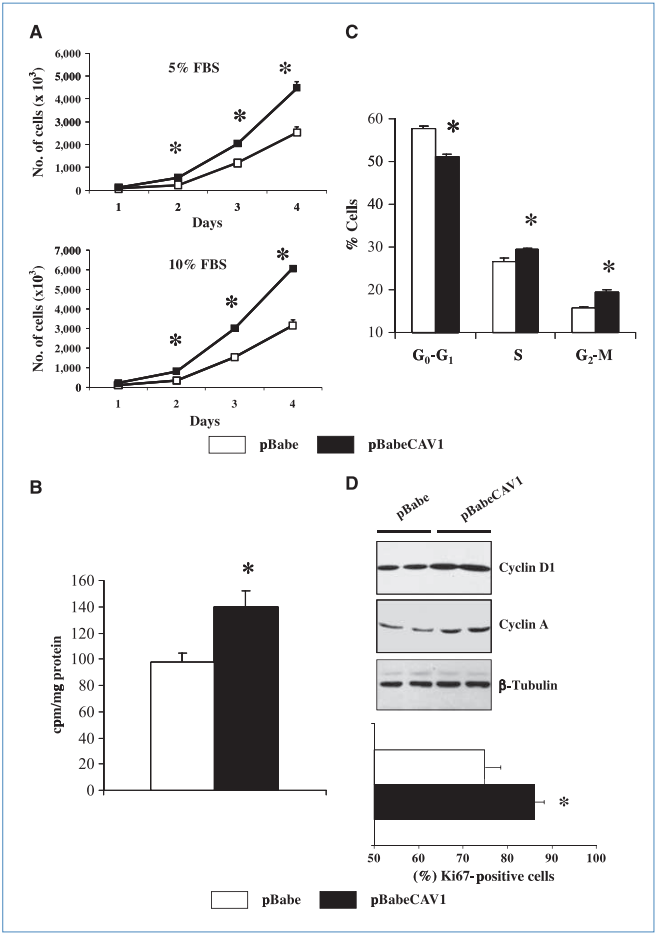

Figure 2. CAV1 expression promotes proliferation of B16F10 melanoma cells *in vitro*. A, growth curves of pBabe and pBabeCAV1 B16F10 cells grown in 5% and 10% FBS (n = 3 per group) showing that CAV1 expression increases cell growth. B, a [^{3}H] thymidine incorporation assay showing increased proliferative 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

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

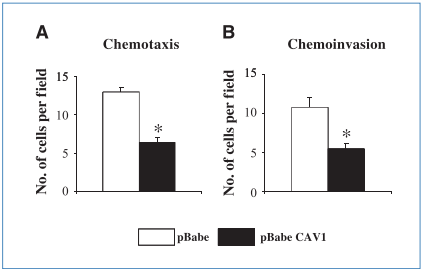

Figure 3. CAV1 expression decreases migration and invasion of B16F10 melanoma cells *in vitro*. Chemotaxis (A) and chemoinvasion (B) were performed by seeding 5×10^4 pBabe and pBabeCAV1 B16F10 cells in the upper wells of Matrigel-coated (for chemoinvasion) or uncoated (for chemotaxis) transwell chambers 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

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

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

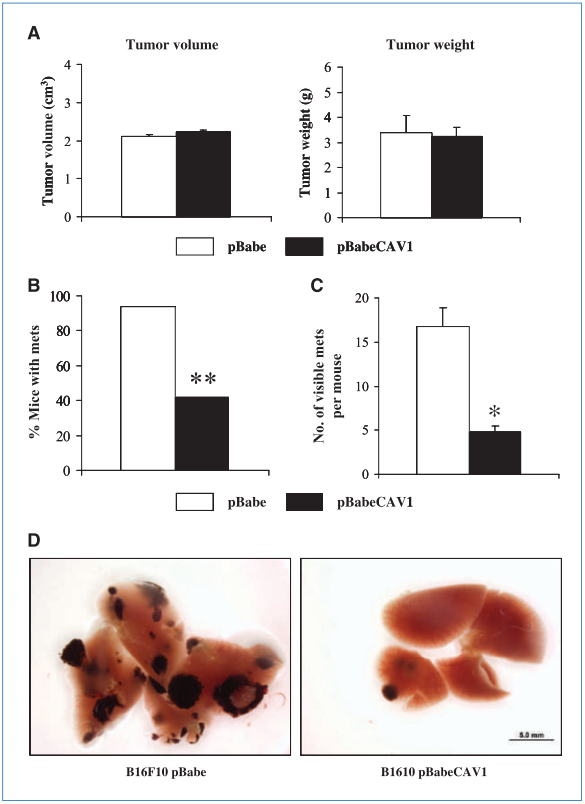

Figure 4. CAV1 expression dramatically reduces the metastatic potential of B16F10 melanoma cells without affecting primary tumor growth. A, tumor growth. Effect of CAV1 expression on B16F10 tumor growth *in vivo* ($n \geq 7$ per group). B and C, experimental lung metastasis assay. Effect of CAV1 expression 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).

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

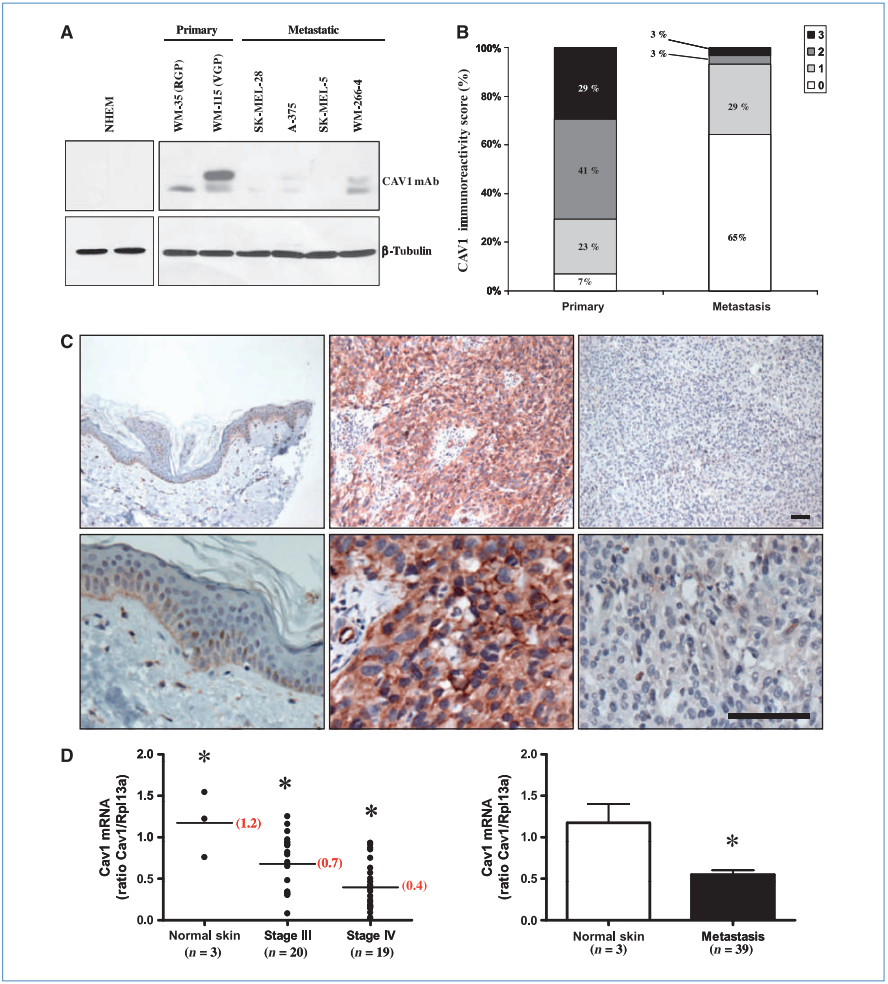

Figure 5. CAV1 expression is reduced in human metastatic melanoma cell lines and human tissue samples derived from metastatic lesions. A, immunoblot analysis showing CAV1 expression in primary human melanoma cell lines (WM35, WM115) compared with a reduced or absent CAV1 expression in primary melanocytes (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).

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

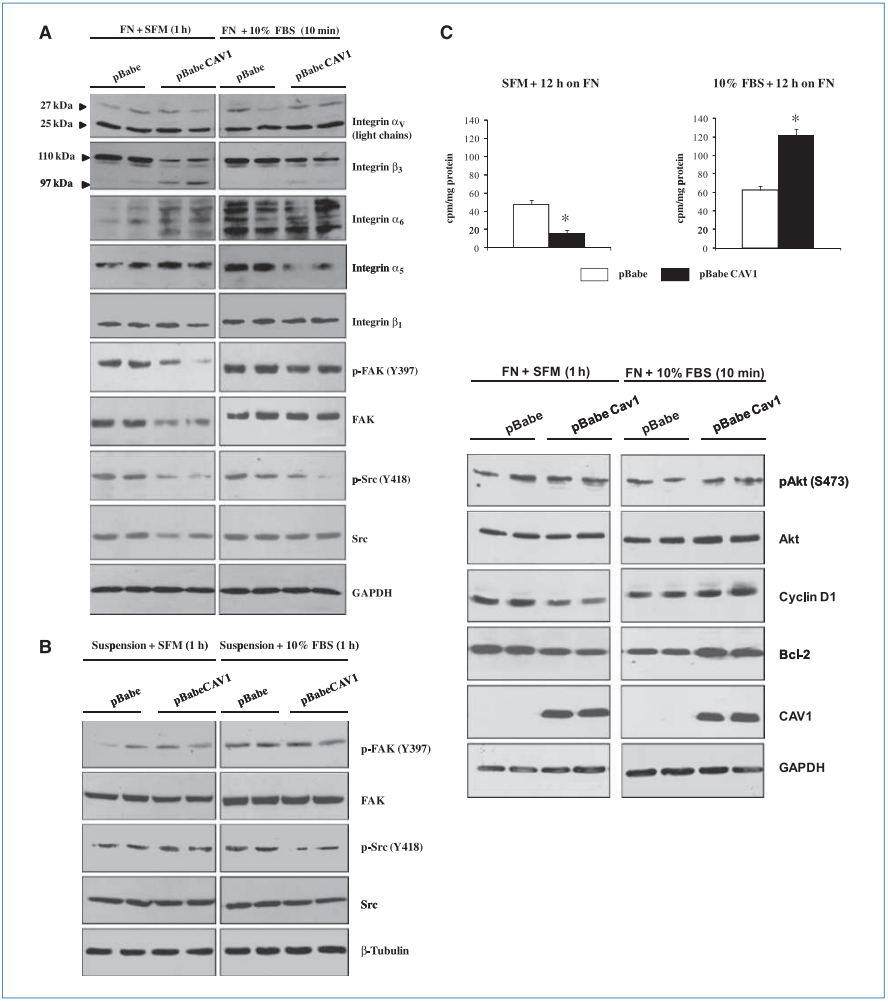

Figure 6. CAV1 suppresses the integrin/Src/FAK pathway following integrin engagement in B16F10 melanoma cells. A, pBabe and pBabeCAV1-B16F10 melanoma cells were serum starved for 18 h, replated on FN-coated plates for 1 h in SFM, and either lysed (left) or pulsed for 10 min with 10% FBS-containing medium 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.

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

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

Published OnlineFirst August 13, 2010; DOI:10.1158/0008-5472.CAN-10-0900

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.

Acknowledgments

We thank Dr. Jason B. Lee of Thomas Jefferson University for help in evaluating melanoma tissue sections.

Grant Support

American Heart Association (Beginning Grant in Aid; F. Capozza); NIH/National Cancer Institute (grants R01-CA-080250, R01-CA-098779, R01-CA-120876, and R01-AR-055660), Susan G. Komen Breast Cancer Foundation, Margaret Q. Landenberger Research Foundation, and Pennsylvania Department of Health (M.P. Lisanti); W.W. Smith Charitable Trust, Breast Cancer Alliance, and Research Scholar Grant from the American Cancer Society (F. Sotgia); and NIH Graduate Training Program Grant T32-CA09678 (C. Trimmer).

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Received 03/18/2010; revised 07/20/2010; accepted 08/02/2010; published OnlineFirst 08/13/2010.

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SUPPLEMENTAL MATERIALS AND METHODS

Immunohistochemistry of Tissue Sections. It was essentially performed as previously described by us (9). A Tissue microarray (TMA) of paraffin embedded human melanoma tissue samples was obtained from US Biomax (Mel207; 69 cases/207 cores). Slides were de-paraffinized in xylene, dehydrated through a graded series of ethanol washes, and placed in PBS. After performing antigen retrieval by heating the sections in 10 mM Citrate Buffer (pH 6.0) in a pressure cooker, tissue sections were incubated for 15 min in 3% H_2O_2 to quench endogenous peroxidase. After rinsing samples with PBS, sections were blocked with 10% goat serum in PBS for 30 minutes at RT. Samples were then incubated with primary antibodies diluted in 1% BSA in PBS overnight at 4°C. Primary antibodies were used at the following dilutions: anti-Cav1 pAb (1:500; N-20 Santa Cruz). Binding of primary antibody was visualized using an IHC detection kit from Vector Labs. A Nova Red Kit (Vector Labs) was used as the HRP substrate. Images were acquired using an Olympus (BX 51) inverted microscope.

Semi-quantitative Analysis of Immunoreactivity. After performing CAV1 immunostaining (see above), individual tissue melanoma cores were scored for intensity of CAV1 immunoreactivity. An expert dermatopathologist (JBL) blindly scored all the samples giving a score of 0 to absence of CAV1 staining, 1 for weak CAV1 staining, 2 for moderate CAV1 staining, and 3 for strong CAV1 staining. CAV1 immunoeactivity scores for primary melanoma and metastatic melanoma lesions were then grouped for analysis. Statistical significance between categories (immunoreactivity scores) was determined by χ^2 test for independence.

Orthotopic Injections in Mice. Injctions were performed essentially as previously described (16). 10^6 B16F10 melanoma cells were suspended in 100 µl of PBS and intradermally injected in 3-4 mo old C57Bl/6J female mice. Tumor growth was monitored weekly by measuring the length

(*L*) and width (W) of the tumor using a caliper. Tumor volume was determined by the following formula: V= 0.52 LW^2. Body weight was also recorded at the beginning and at the end of the experiment. 18 days after cell injections, tumors were harvested, weighted, and processed for IHC.

Experimental Metastasis Assay. Metastatic ability was assessed essentially as previously described (17). 10^5 B16F10 melanoma cells were suspended in 100μl of PBS and intravenously injected in 3-4 month old C57Bl/6 female mice. 18 days after injections, lungs were removed, insufflated with 10% buffered formalin and the number of visible pulmonary metastases on the surface of the lungs was counted. Lungs were imaged using a low power stereomicroscope (Nikon SMZ-1500).

Table 1. Cell Cycle Analysis of B16F10 pBabe and B16F10 pBabeCAV1

	G_0/G_1	S	G_2/M
pBabe	57.64 ± 0.72	26.66 ± 0.77	15.71 ± 0.37
pBabe- Cav-1	51.15 ± 0.61 *	29.49 ± 0.23 *	19.36 ± 0.58 *

Values are means ± SEM; n = 4, *per* each group; * $P < 0.05$

Table 2. Experimental Lung Metastasis

Cell Line	Mice w/ mets	Mice w/o mets	Total mice injected
B16F10 pBabe	17 (94%)	1 (6%)	18
B16F10 pBabe-Cav-1 **	8 (42%)	11 (58%)	19

****P* = 0.0014** as determined by Two Tailed Fisher's Exact Test

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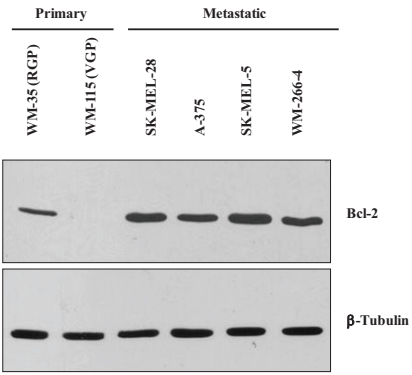
Fig S1



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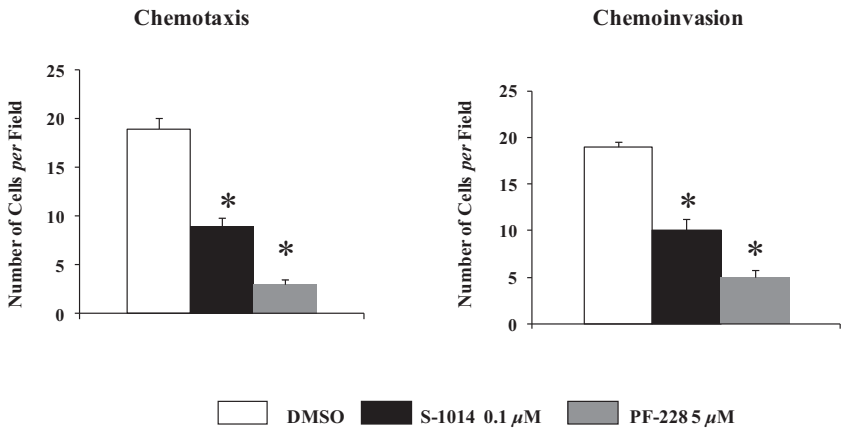

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Increased Bcl-2 expression levels in metastatic melanoma cell lines. Western blot analysis of Radial Growth Phase derived melanocytes (WM-35), Vertical Growth Phase derived melanocytes (WM-115), and metastatic melanoma cell lines (SK-MEL-28, A-375, SK-MEL-5, WM-266-4), for Bcl-2. β-Tubulin was used as loading control. Note that Bcl-2 expression is low or undetectable in primary melanoma derived cell lines (WM35, WM115), while metastatic melanoma cell lines display increased expression levels of Bcl-2 protein.

Figure S2. Src and FAK inhibitors (SKI-606 and PF-574,228 respectively) effectively block migration and invasion of B16F10 melanoma cells. 5 x 10^4 B16F10 cells were seeded in the upper wells of matrigel coated (for chemoinvasion) and 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 chemo-attractant in the lower wells. DMSO (vehicle), 0.1 μM Bosutinib (SKI-606), and 5 μM PF-574,228 were added to both the upper and lower wells of the transwell chambers. After 6h, transwells were washed with PBS, wiped with cotton swabs and stained with crystal violet to determine migrated cells. Data represent the average of three independent experiments. Five fields *per* sample were counted. Results are reported as means ± SEM (* $P < 0.05$ for SKI 606 and PF 574,228 vs DMSO; as determined by Dunnett Multiple Comparisons Test).

Chapter 2

Genetic Ablation of Cav1 Differentially Affects Melanoma Tumor Growth and Metastasis in Mice: Role of Cav1 in Shh Heterotypic Signaling and Transendothelial Migration

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

Molecular and Cellular Pathobiology

Cancer Research

Genetic Ablation of Cav1 Differentially Affects Melanoma Tumor Growth and Metastasis in Mice: Role of Cav1 in Shh Heterotypic Signaling and Transendothelial Migration

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Abstract

Both cell-autonomous and non–cell-autonomous factors contribute to tumor growth and metastasis of melanoma. The function of caveolin-1 (Cav1), a multifunctional scaffold protein known to modulate several biologic processes in both normal tissue and cancer, has been recently investigated in melanoma cancer cells, but its role in the melanoma microenvironment remains largely unexplored. Here, we show that orthotopic implantation of B16F10 melanoma cells in the skin of Cav1KO mice increases tumor growth, and co-injection of Cav1-deficient dermal fibroblasts with melanoma cells is sufficient to recapitulate the tumor phenotype observed in Cav1KO mice. Using indirect coculture experiments with fibroblasts and melanoma cells combined with cytokine analysis, we found that Cav1-deficient fibroblasts promoted the growth of melanoma cells via enhanced paracrine cytokine signaling. Specifically, Cav1-deficient fibroblasts displayed increased ShhN expression, which heterotypically enhanced the Shh signaling pathway in melanoma cells. In contrast to primary tumor growth, the ability of B16F10 melanoma cells to form lung metastases was significantly reduced in Cav1KO mice. This phenotype was associated mechanistically with the inability of melanoma cells to adhere to and to transmigrate through a monolayer of endothelial cells lacking Cav1. Together, our findings show that Cav1 may regulate different mechanisms during primary melanoma tumor growth and metastatic dissemination. *Cancer Res; 72(9); 2262–74. ©2012 AACR.*

Introduction

Tumors are heterogeneous microenvironments that consist of both neoplastic and nonneoplastic cells (tumor–stroma). Tumor growth and the consequent metastatic dissemination of tumor cells result from continuous reciprocal interactions between cancer cells and their surrounding stroma (1, 2). Cutaneous melanoma remains the most aggressive type of skin cancer and both cell-autonomous and non–cell-autonomous mechanisms are necessary for melanoma growth and metastasis (3). Recent research, in fact, has shown that stromal cells (fibroblasts and endothelial cells) support the growth and dissemination of melanoma cells by modulating angiogenesis, secreting growth factors and cytokines, and contributing to extracellular matrix deposition and degradation (4). Thus, identifying novel mechanisms critically regulating tumor–stroma interactions may be therapeutically relevant in this type of cancer.

Caveolae are specialized microdomains of the plasma membrane enriched in the scaffold protein caveolin-1 (Cav1; refs. 5, 6). Because of the multitude of interacting proteins described, Cav1 has been implicated in the modulation of many biologic processes in both normal tissues and cancer (7, 8). Although much research has primarily focused on determining the function of Cav1 in cancer cells, recent studies have started to investigate the function of Cav1 protein in the tumor microenvironment (9, 10). Indeed, Cav1 is highly expressed in endothelial cells and fibroblasts, two of the cell types that are normally involved in stromal remodeling during melanoma progression (3). In addition, the angiogenesis defects (11) and impaired skin wound healing (12) displayed by Cav1KO mice suggest that loss of Cav1 in the stromal compartment may functionally affect tumor–stroma interactions in melanomagenesis.

To examine this issue, we used Cav1KO mice to determine whether stromal Cav1 may affect the growth and metastatic ability of B16F10 melanoma cells. We show that absence of Cav1 promotes the growth of intradermally implanted B16F10 melanoma cells in mice. Indirect coculture experiments and

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-11-2593

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

co-injections of fibroblasts and melanoma cells show that lack of Cav1 in dermal fibroblasts promotes the growth of melanoma cells *in vitro* and *in vivo* via paracrine cytokine signaling. In contrast, the ability of B16F10 cells to form lung metastases in Cav1KO mice was significantly impaired. These results were consistent with the inability of B16F10 cells to transmigrate through a monolayer of human umbilical vein endothelial cells (HUVEC) lacking Cav1. Collectively, our data suggest functionally distinct roles for stromal Cav1 in melanoma primary tumor growth and metastasis.

Materials and Methods

Materials

Antibodies and their sources were as follows: Cav1 (N-20), PECAM-1 (M20), eGFP (sc-8334), and Shh (N-19) were from Santa Cruz. Keratin-14 (K14) was from Covance. Rat anti-mouse VCAM-1 and ICAM-1 were from R&D. Gli-1 was from Cell Signaling. β-Tubulin was from Sigma and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was from Fitzgerald.

Animal studies

Three- to four-month-old Cav1WT (WT), Cav1KO (13), and Cav2KO (14) C57Bl/6 female mice were used for orthotopic and i.v. injections of B16F10 cells. For co-injection experiments, 3- to 4-month-old athymic female mice (NCr-Nu; Taconic) were used (15, 16). All *in vivo* studies were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University (Philadelphia, PA).

Cell lines

B16F10 and A-375 were from American Type Culture Collection, whereas human immortalized dermal foreskin fibroblasts (hTBJ1) were originally from Clontech. HUVECs were purchased from ALLCELLS. Only early passages of these cell lines were used in experiments.

Neonatal dermal fibroblasts

Dermal fibroblasts and primary mouse keratinocytes were isolated from the skin of newborn mice (1- to 3-day-old) as described in details by others (17). Fibroblasts were suspended in 10% FBS-Dulbecco's Modified Eagle's Media (DMEM; Invitrogen) and initially plated at a density of 5,000 cells per cm^2.

Co-injection experiments

Wild-type (WT), Cav1KO, and Cav2KO neonatal fibroblasts (see above) were intradermally co-injected with 10^5 B16F10 cells in nude mice at 5:1 ratios. Similarly, hTBJ1 fibroblasts were co-injected with human A-375 melanoma cells (18, 19).

Lentiviral vectors

For lentivirus-mediated silencing of the *Cav1* gene, predesigned control shRNAmiR (shCtrl-miR) and shRNAmiRs (shCAV1-miRs) targeting the human CAV1 mRNA (NM_001753.3) were purchased from Invitrogen and subcloned into the pRRLsin.cPPT.hCMV.eGFP.WPRE (LV-eGFP) lentiviral vector (20). The resulting constructs (LV-shCtrl-miR-eGFP and LV-shCAV1-miRs-eGFP) were packaged according to standard protocols (20). Effective CAV1 knockdown in target cells was determined by Western blot analysis of fluorescence-activated cell-sorting (FACS)-sorted eGFP-positive cells. A lentiviral vector (Lv105-Puro) encoding mCherry cDNA was from GeneCopoeia whereas ready to use shCtrl-Puro and shGli-1-Puro (gene ID: 14632) lentiviral particles were from Santa Cruz.

Cocultures of fibroblasts and melanoma cells

Direct cocultures (direct cell–cell contact) were established by seeding 10^4 cells per cm^2 mCherry-labeled melanoma cells on eGFP-labeled (shCtrl/shCAV1 cells) or on unlabeled fibroblasts (1:5 ratios) that had previously been serum-activated as described later for the conditioned medium (CM) experiments. Forty-eight or 72 hours after the initial plating (21), cocultures were harvested and their relative growth determined by flow cytometric analysis (FACS). Transwell cocultures of fibroblasts and melanoma cells (no cell–cell contact) were established as described earlier by using 0.4-μm pore size transwell membrane inserts (22).

Conditioned medium experiments

Conditioned medium was collected from dermal fibroblasts that were maintained in serum-free medium for 48 hours. Fibroblasts were then serum-activated (SA) by maintaining them for 12 hours in 10%FBS-DMEM and 24 hours in 1%FBS-DMEM (23). Conditioned medium from these cells (SA-CM) was then incubated with melanoma cells for 48 hours. For Shh-pathway inhibition studies, cyclopamine (Selleck) or dimethyl sulfoxide (DMSO) were added to conditioned medium for 48 hours. Recombinant Shh protein (PeproTech) was used for hedgehog signaling activation in melanoma cells.

Cytokine array and ELISA

Cytokine array and ELISA for ShhN were carried out on FB-CM using commercially available kits from RayBiotech and R&D.

Immunohistochemistry

S100 (RAb; Dako) immunohistochemical staining of 5-μm paraffin-embedded tumor sections was carried out as previously described by us (15).

Tumor angiogenesis

Microvessel density (MVD) was determined by CD31 (1:500; sc-1506) immunohistochemical staining of 5-μm paraffin-embedded tumor sections (24). CD31-positive vessels were counted in 5 to 6 specimens per group (5 fields/sample) using a 20× objective and an ocular grid (0.25 mm^2 per field). CD31 immunoblotting of whole-tumor lysates was also carried out (25, 26).

[^{3}H]Thymidine incorporation assay and growth curves

DNA synthesis was determined by incubating asynchronously growing cells with 0.5 μCi/mL of [^{3}H]thymidine (Perkin-Elmer) for 18 hours (27). Growth of melanoma cells was

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

assessed by MTT assay at 0, 24, 48, 72, and 96 hours after plating (28).

Western blotting

Homogenized tissue samples and cells were sonicated and lysed in a modified radioimmunoprecipitation assay buffer and processed for immunoblot analysis as previously described (26).

Tumor–cell transendothelial migration assay

HUVECs (5×10^4) were grown to confluence (72 hours) on top of an 8-μm pore size gelatin-coated membrane (Transwells; BD Biosciences). A total of 1×10^5 [^{3}H]thymidine- or mCherry-labeled B16F10 cells in 500 μL of 0.1% bovine serum albumin (BSA)-DMEM were added to the Transwell inserts. Serum-free NIH3T3 conditioned medium (48 hours) was used as a chemoattractant. After 6 hours, Transwells were washed with PBS and wiped with cotton swabs. Membranes were removed and the amount of radioactivity determined by liquid scintillation counting (LSC; ref. 29). Alternatively, inserts were fixed with 4% paraformaldehyde, wiped with cotton swabs, and mounted onto glass slides. Migrated mCherry-B16F10 cells were imaged by confocal microscopy (LSM510.META.Confocal; Zeiss).

Tumor–endothelial cell adhesion assay

[^{3}H]Thymidine-labeled B16F10 cells (1×10^5) were incubated on top of a monolayer of HUVECs in 0.1% BSA-DMEM at 37°C for 30 minutes. After being washed with PBS, cells were solubilized in 0.5N NaOH/0.1% SDS and the amount of radioactivity determined by LSC (29). For neutralizing antibody experiments, HUVEC monolayers were pretreated for 30 minutes with VCAM-1 (sc-20070), ICAM-1 (sc-59787), or IgG isotype control (10 μg/mL each; ref. 30).

TNFα-induced ICAM-1 and VCAM-1 expression in mice

Three- to four-month-old WT and Cav1KO female mice were intraperitoneally injected with 25 μg/kg of mouse recombinant TNFα (R&D). After 5 hours, mice were sacrificed and lungs were cleared of blood by infusing cold PBS through the right ventricle (31). The left lung lobe was then collected and processed for Western blot analysis (26).

Statistical analysis

Results are represented as means ± SEM. Statistical analysis was conducted using GraphPad Software.

Results

Cav1 ablation in mice promotes the growth of B16F10 melanoma cells independently of Cav2

To determine whether absence of Cav1 in the skin affects B16F10 cell growth, we examined the growth of B16F10 cells orthotopically (intradermally) implanted in the skin of WT and Cav1KO C57Bl/6 female mice. After 18 days, analysis of tumor size revealed that their growth was enhanced (~2-fold) in Cav1KO mice (Fig. 1A). Caveolin-2 (Cav2) is normally coexpressed and hetero-oligomerizes with Cav1. Previous studies have reported reduced Cav2 levels in Cav1KO mice (13). Thus, the Cav1KO tumor phenotype may be confounded by Cav2 loss or reduced function. Relative to WT and Cav1KO animals, B16F10 cells implanted in the skin of Cav2KO mice grew more slowly (~1.5-fold), indicating that the Cav1KO tumor phenotype was Cav2-independent (Fig. 1B). Similar reductions in tumor growth were observed in subcutaneously injected Cav2KO mice (Supplementary Fig. S1), indicating a growth-promoting role for Cav2 in melanoma. CD31 immunohistochemical staining of Cav1KO tumor sections revealed increased MVD relative to WT and Cav2KO tumors, respectively. These results were corroborated by CD31 immunoblots of whole-tumors lysates (Fig. 1C). Taken together, these findings suggest that growth of B16F10 in Cav1KO mice correlates with their MVD and this effect is independent of Cav2.

Absence of Cav1 but not Cav2 in dermal fibroblasts enhances the growth of melanoma cells in co-injection experiments

Dermal fibroblasts are the main cell components of the skin, which are important in maintaining the normal physiologic functions of this organ. To determine whether loss of Cav1 in dermal fibroblasts was sufficient to recapitulate the tumor phenotype observed in Cav1KO mice, dermal xenografts were established in nude mice by co-injecting WT and Cav1KO neonatal dermal fibroblasts with B16F10 melanoma cells at 5:1 ratios. After 14 days, analysis of tumor size showed that Cav1KO fibroblasts promoted the growth of B16F10 cells. S100b immunohistochemical staining (melanoma cell marker) and trichrome staining of tumor sections showed that collagen deposition and/or stromal cell proliferation was unchanged in B16F10/WT and B16F10/Cav1KO tumors (Fig. 2A). In contrast, the growth of tumors resulting from co-injecting B16F10/Cav2KO fibroblasts was comparable with the growth of their controls. Interestingly, immunoblot analysis revealed that Cav1 expression was maintained in Cav2KO fibroblasts, whereas Cav2 levels were slightly diminished in Cav1KO fibroblasts (Fig. 2B). The tumor-promoting role of Cav1-deficient fibroblasts was further shown by co-injecting human A-375 melanoma cells with hTBJ1-shCtrl or hTBJ1-shCAV1 fibroblasts in nude mice (Fig. 2C). Together, these results show that Cav1-deficient fibroblasts, but not Cav2KO cells, are sufficient to recapitulate the tumor phenotype of Cav1KO mice.

Fibroblasts lacking Cav1 promote the growth of melanoma cells in noncontact cocultures but not in direct contact cocultures

To investigate possible mechanisms whereby Cav1-deficient fibroblasts may promote B16F10 tumor growth, we cocultured (under cell–cell contact conditions) mCherry-B16F10 cells with serum-activated WT or Cav1KO dermal fibroblasts or mCherry-A-375 cells with serum-activated eGFP-labeled hTBJ1-shCtrl or hTBJ1-shCAV1 at 1:5 ratios in low-serum medium (1% FBS). After 48 and 72 hours, FACS analysis of mCherry-B16F10 or mCherry-A-375 cells revealed that Cav1-deficient fibroblasts were unable to promote the growth of melanoma cells under these conditions. Immunoblot analysis of primary Cav1KO fibroblasts or shCAV1 fibroblasts confirms

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

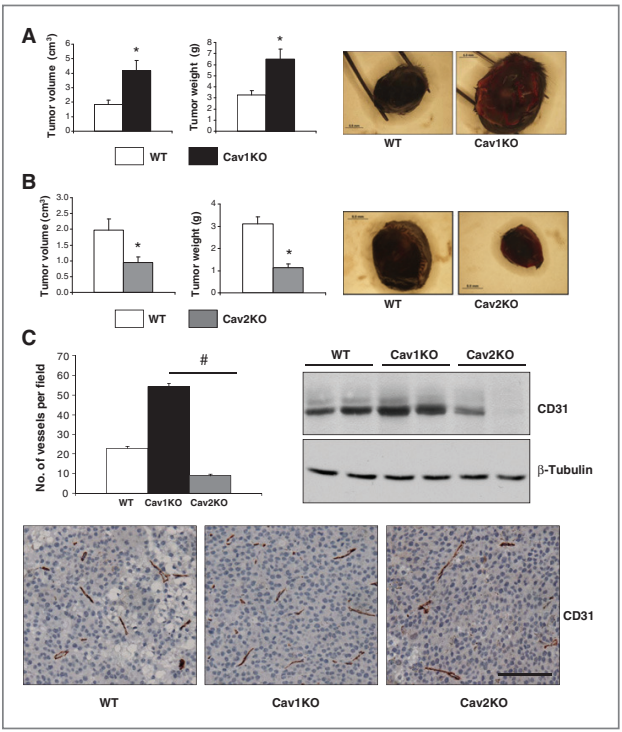

Figure 1. Cav1 ablation in mice promotes the growth of B16F10 melanoma cells independently of Cav2. A total of 10^6 B16F10 melanoma cells were orthotopically (intradermally) implanted in the skin of 3- to 4-month-old WT, Cav1KO (A) and Cav2KO (B) C57Bl/6 female mice ($n \geq 8$ per group). After 18 days, tumors were excised and their size determined. Representative images of tumors are displayed on the right. C, CD31 immunohistochemistry of tumor sections showing that microvascular density correlates with tumor size in Cav1KO and Cav2KO mice ($n = 5$ per group). CD31 immunoblotting of whole-tumor lysates is shown. Results are means ± SEM shown (*, #, $P < 0.05$, by 2-tailed Mann–Whitney and by Dunnett's multiple comparisons test; scale bar, 100 μm).

absence/knockdown of Cav1 protein relative to WT and shCtrl fibroblasts, respectively. Furthermore, primary cultures were negative for the keratinocyte cell marker K14, confirming the purity of these cell populations (Fig. 3A and B). In contrast, a [^{3}H]thymidine incorporation assay shows that proliferation of melanoma cells was significantly increased when cocultured (72 hours) with fibroblasts lacking Cav1 under noncontact conditions (Fig. 3C). These findings suggest that soluble secreted factors may be mediating the proproliferative effects of Cav1-deficient fibroblasts on melanoma cells.

Serum-activated Cav1KO dermal fibroblasts display increased amounts of protumorigenic cytokines

To determine whether Cav1 expression may regulate secreted soluble factors in fibroblasts, a cytokine array was done on conditioned medium from serum-activated WT and Cav1KO dermal fibroblasts. Conditioned medium from serum-activated Cav1KO fibroblasts displays increased amount of ShhN, basic fibroblast growth factor (bFGF), and matrix metalloproteinase (MMP)2/3, cytokines known to promote proliferation, invasion, and angiogenesis during melanomagenesis. Decoy receptors [hepatocyte growth factor receptor (HGFR), VEGF receptor (VEGFR)2] and an inhibitor of MMPs (TIMP1) were reduced in Cav1KO-CM (Fig. 4A). Increased expression of ShhN was also confirmed by ELISA assay and by immunoblot analysis of conditioned medium and cell lysates from Cav1-deficient fibroblasts (Fig. 4A). In addition, B16F10 cells incubated with conditioned medium from serum-activated Cav1KO fibroblasts (48 hours) display increased cell proliferation/growth and hyperactivation of the Shh signaling pathway as evidenced by increased cyclin D1/A and Gli-1 expression (an Shh target gene) and by increased [^{3}H]thymidine incorporation and MTT assay (Fig. 4B). Similar outcomes were obtained when A-375 cells were incubated with conditioned medium from serum-activated hTBJ1-shCAV1 cells (Fig. 4A and C). Thus, our results indicate that fibroblasts lacking Cav1 secrete factors that promote proliferation, invasion, and angiogenesis.

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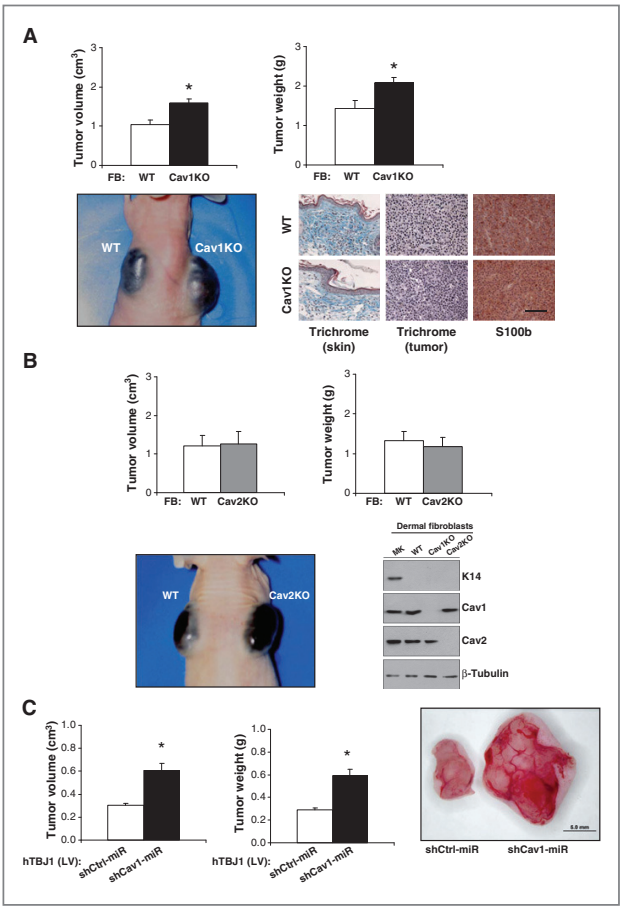

Figure 2. Absence of Cav1 but not Cav2 in dermal fibroblasts enhances the growth of melanoma cells in co-injection experiments. A, B16F10 melanoma cells (1×10^5) were intradermally co-injected with Cav1KO neonatal dermal fibroblasts at 1:5 ratios in 3- to 4-month-old nude female mice. After 14 days, tumors were dissected and their size determined. Masson's trichrome staining and S100b (melanoma cell marker) immunohistochemistry of tumor sections revealed similar intratumoral collagen deposition between WT-FB/B16F10 and Cav1KO-FB/B16F10 xenografts ($n = 5$ per group). B, tumor size of B16F10 cells co-injected with WT and Cav2KO dermal fibroblasts as in A. K14 (keratinocyte marker), Cav1 and Cav2 immunoblots of freshly isolated dermal fibroblast, and mouse keratinocytes (MK) are also shown. β-Tubulin immunoblot is shown as loading control. C, lentivirus-mediated CAV1 silencing (Lv-shCAV1-miR) in hTBJ cells promotes the growth of A-375 cells as determined by co-injection experiments. Results are means ± SEM ($n \geq 5$ per group; *, $P < 0.05$, by 2-tailed Mann–Whitney test; scale bar, 100 μm).

Inhibition of Shh signaling pathway in melanoma cells reverses the proproliferative/protumorigenic effects of fibroblasts lacking Cav1

Recent studies have shown aberrant activation of Shh signaling in several cancer types including melanoma (32). To determine whether pharmacologic inhibition of the Shh pathway in B16F10 cells can reverse the proproliferative effect of Cav1KO-CM, we conducted a [^{3}H]thymidine incorporation assay on melanoma cells incubated for 48 hours with Cav1KO-CM containing cyclopamine, a specific inhibitor of the Shh pathway. Our results show that low concentrations of cyclopamine (5–10 μmol/L) were effective in blocking the proproliferative effects of Cav1KO-CM on B16F10 melanoma cells. Interestingly, the proliferation of B16F10 cells incubated with WT-CM containing cyclopamine remains unchanged (Fig. 5A). Similarly, cyclopamine prevented the proproliferative effects of conditioned medium from serum-activated hTBJ1-shCAV1 cells on A-375 human melanoma cells (Fig. 5B). To examine whether inhibition of the Shh signaling pathway in B16F10 melanoma cells abolishes the protumorigenic

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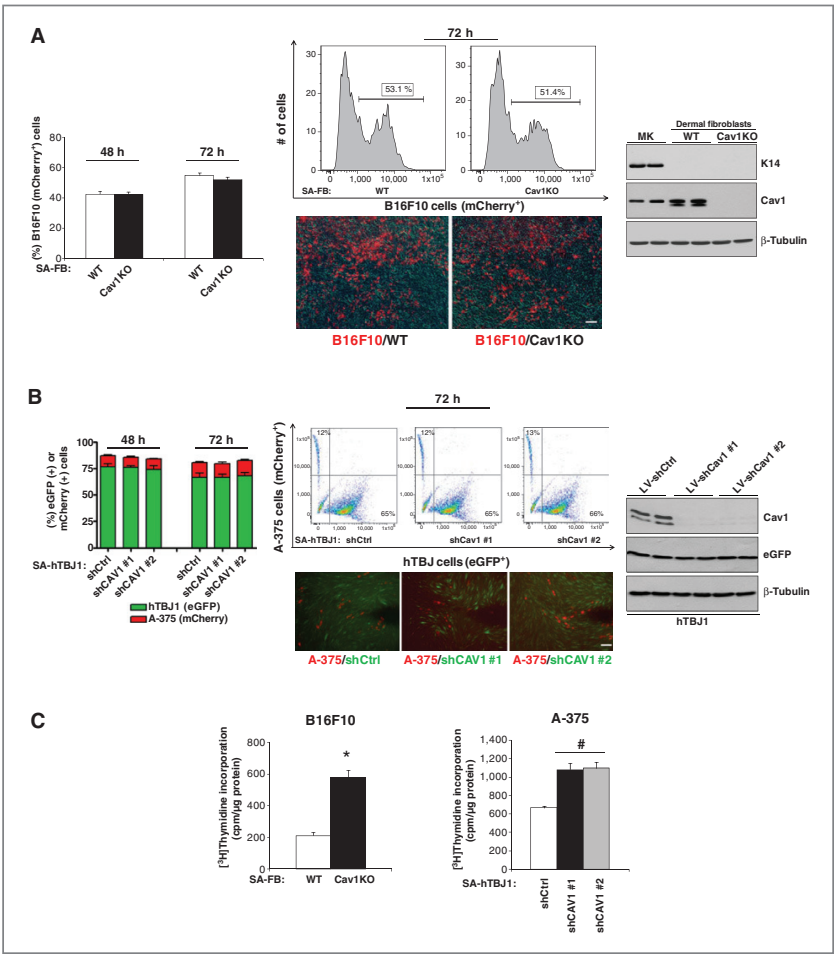

Figure 3. Fibroblasts lacking Cav1 promote the growth of melanoma cells in noncontact cocultures but not in direct-contact cocultures. A, representative flow cytometric plots, photomicrographs, and quantification of B16F10-mCherry cells cocultured for 48 to 72 hours with SA-WT and SA-Cav1KO neonatal dermal fibroblasts in 1%FBS-DMEM ($n = 5$ per group). Cav1 and K14 (keratinocyte marker) immunoblots of freshly isolated dermal fibroblasts and keratinocytes are shown (right). B, representative flow cytometric plots, photomicrographs, and quantification of mCherry-A-375 cells growth, cocultured for 48 to 72 hours with SA-eGFP–labeled hTBJ1-shCtrl-miR or SA-hTBJ1-shCAV1-miR dermal fibroblasts in 1%FBS-DMEM ($n = 5$ per group). Cav1 and eGFP immunoblots of SA-hTBJ1-shCtrl-miR or SA-hTBJ1-shCAV1-miR are shown (right). β-Tubulin immunoblots are shown as loading controls. C, increased proliferation ([^{3}H]thymidine incorporation assay) of B16F10 and A-375 cells cocultured in absence of cell–cell contact (Transwell; 48 hours) with SA-FBs lacking Cav1 ($n = 5$ per group). Results are means ± SEM (*,#, $P < 0.05$, by 2-tailed Student t test and by Dunnett's multiple comparisons test; scale bar, 100 μm).

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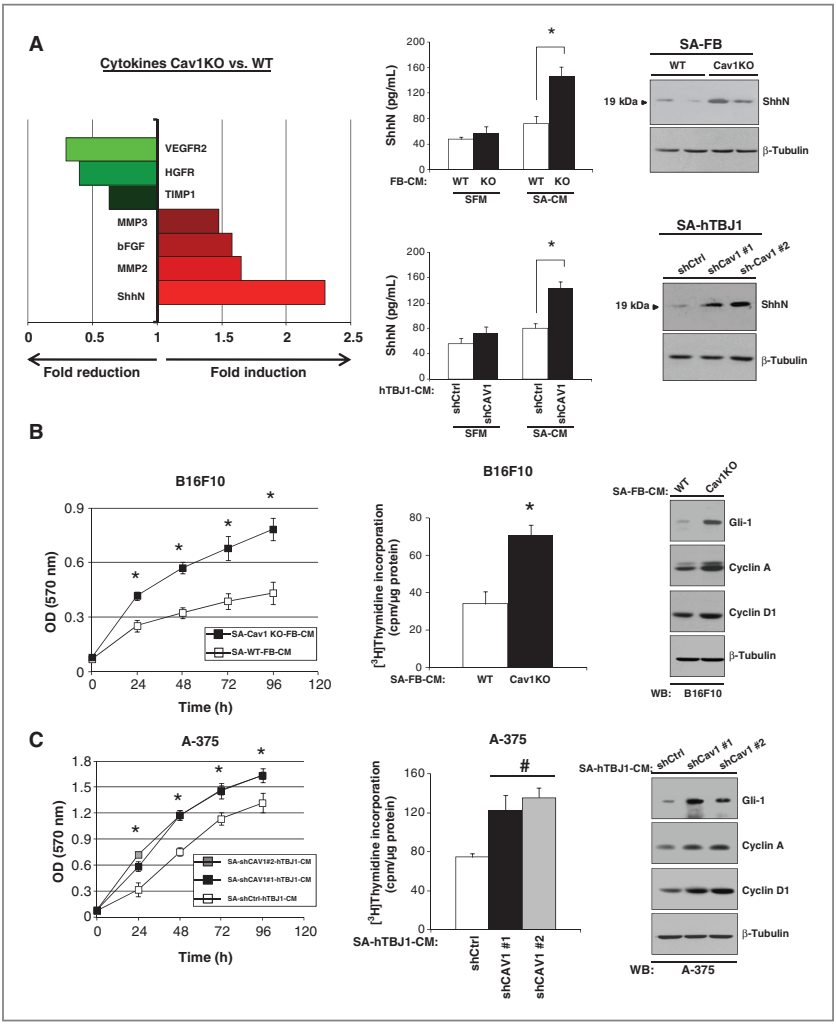

Figure 4. SA-Cav1KO dermal fibroblasts display increased amounts of protumorigenic cytokines. A, left, cytokines differentially regulated in conditioned medium from SA-WT and SA-Cav1KO neonatal dermal fibroblasts; middle, ELISA showing increased ShhN levels in the conditioned medium of serum-activated dermal fibroblasts (SA-CM) lacking Cav1 ($n = 4$ per group). ShhN immunoblots of serum-activated dermal fibroblasts are displayed (right). B, MTT assay and [^{3}H]thymidine incorporation assay (48 hours) of B16F10 melanoma cells treated with SA-CM from WT and Cav1KO fibroblasts. Immunoblot analysis showing increased expression of Gli-1, cyclin D1, and cyclin A in B16F10 melanoma cells incubated (48 hours) with SA-CM from Cav1KO dermal fibroblasts ($n = 8$ per group). Similar results are shown (C) for human A-375 melanoma cells treated with SA-CM from hTBJ1-shCtrl and hTBJ1-shCAV1 cells. Results are means ± SEM ($n \geq 6$ per group; *,$^\#$, $P < 0.05$, by 2-tailed Student t test and by Dunnett's multiple comparisons test).

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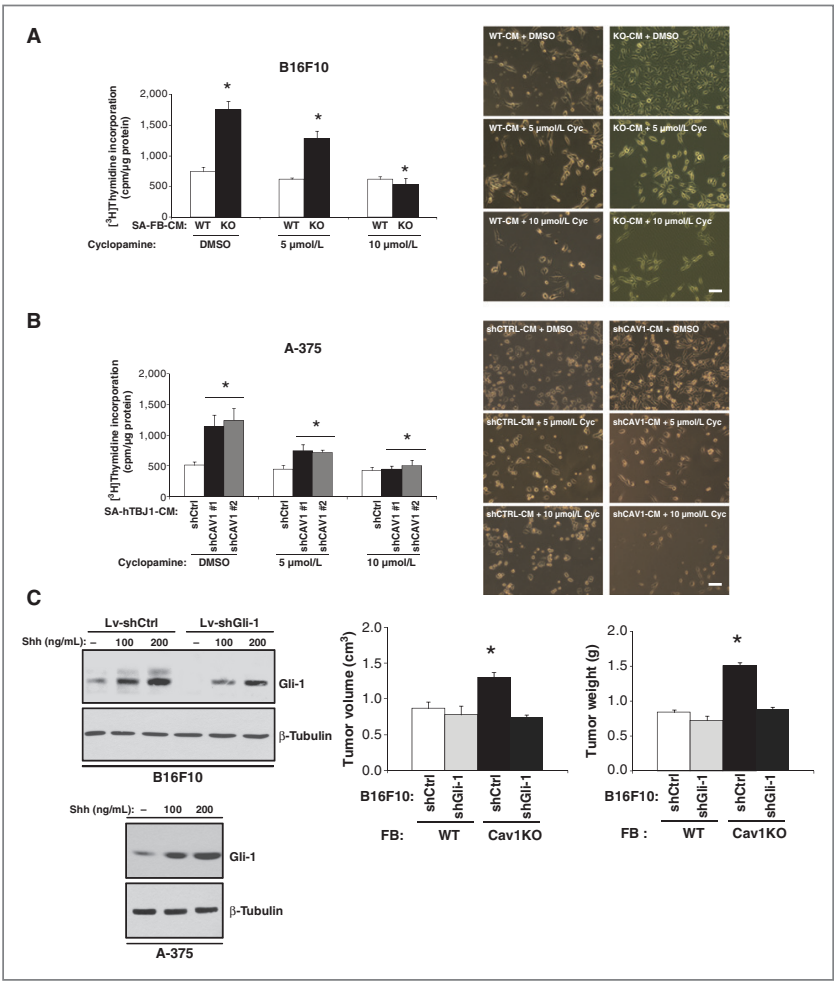

Figure 5. Inhibition of Shh signaling pathway in melanoma cells reverses the proproliferative/protumorigenic effects of fibroblasts lacking Cav1. A, [^{3}H]thymidine incorporation assay of B16F10 melanoma cells treated with DMSO or cyclopamine after incubation with SA-CM from WT and Cav1KO dermal fibroblasts. Representative phase-contrast images of B16F10 cells treated with SA-CM from WT and Cav1KO fibroblasts with cyclopamine are shown on the right. Similar experiments (B) were done with A-375 cells incubated with SA-CM from hTBJ1-shCtrl and hTBJ1-shCAV1 cells. C, Gli-1 and β-tubulin immunoblots of B16F10-shCtrl, B16F10-shGli-1, and A-375 cells before and after being treated with Shh (left). Gli-1 knockdown in B16F10 cells reverses the tumor-promoting effects of Cav1KO fibroblasts as (right) determined by co-injection experiments. Results are means ± SEM ($n \geq 4$ per group; *, $P < 0.05$, by Tukey's multiple comparisons test; scale bar, 200 μm).

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

properties of Cav1-deficient fibroblasts *in vivo*, we stably silenced the Gli-1 gene by lentiviral shRNA technology. Complete Gli-1 knockdown and reduced Gli-1 expression levels were achieved in absence and presence of Shh, respectively. Similar Gli-1 expression levels were also observed in A-375 cells treated with Shh, suggesting a fully functional Shh signaling pathway in both melanoma cell types (Fig. 5C, left). Co-injection experiments conducted as in Fig. 2A showed that Gli-1 knockdown in B16F10 cells was sufficient to reverse the tumor-promoting effects of Cav1-deficient fibroblasts (Fig. 5C, right). In summary, these data show that Shh heterotypic signaling is critical for B16F10 melanoma cell proliferation and melanoma tumor growth when Cav1 is absent in dermal fibroblasts.

Cav1 deficiency inhibits lung colonization and transendothelial migration of B16F10 melanoma cells

Given the absence of spontaneous metastasis formation in B16F10 orthotopic tumor–bearing WT and Cav1KO mice (data not shown), we i.v. injected 10^5 B16F10 cells to determine their ability to colonize the lungs of WT and Cav1KO mice (experimental metastasis assay). Interestingly, the ability of B16F10 cells to colonize the lungs of Cav1KO mice was significantly impaired (Fig. 6A). To identify possible mechanisms accounting for these findings, we determined the ability of mCherry-labeled or [^{3}H]thymidine-labeled B16F10 cells to transmigrate through a monolayer of lentivirally transduced shCtrl and shCAV1-HUVEC cells. Consistent with our *in vivo* data, the ability of B16F10 cells to adhere to and to transmigrate through a HUVEC monolayer was significantly reduced in CAV1 knockdown cells (Fig. 6B and C). Interestingly, incubation of HUVEC with ICAM-1 and VCAM-1 antibodies reduced the adhesion of B16F10 cells to levels similar to those observed with the HUVEC-shCAV1, suggesting a critical role for Cav1 in regulating the process of metastatic extravasation (Fig. 6C, left). TNFα-induced VCAM-1 and ICAM-1 expression has been described as being critical in cancer cells–endothelium interactions (33). Interestingly, our *in vitro* results were corroborated by reduced VCAM-1 and ICAM-1 expression levels in lungs of 5h-TNFα–treated Cav1KO mice (Fig. 6C, right). Collectively, these results show that CAV1 has a key role in the endothelium and regulates processes such as adhesion and transmigration that are ultimately relevant for the establishment of lung metastases *in vivo*.

Discussion

In this study, we show that Cav1 gene disruption promotes the growth of B16F10 melanoma cells in the skin of mice, whereas it inhibits the formation of lung metastases. Our data indicate that lack of Cav1 in dermal fibroblasts contributes to primary melanoma tumor growth by increased paracrine cytokine signaling, whereas the inability of B16F10 cells to form lung metastases is attributed to defects in VCAM-1- and ICAM-1–mediated adhesion to endothelial cells.

Although the function of Cav1 has been recently examined in melanoma cancer cells (8, 15), the role of stromal Cav1 in melanoma tumor growth and metastasis remains less well studied. Here, we show that the difference in tumor growth seen in Cav1KO and Cav2KO mice appears to correlate well with differences in their microvascular density. However, in contrast to our results, previous studies have shown reduced tumor growth and reduced MVD in Cav1KO mice subcutaneously injected with B16F10 cells (34, 35), indicating that the injection site (intradermal vs. subcutaneous) and consequently the different tumor microenvironments may significantly affect melanoma tumor growth (36, 37). Although our findings are consistent with other studies showing a direct positive relationship between lack of Cav1 and higher microvascular density *in vivo* (11, 38), we cannot exclude the possibility that other stromal factors other than the endothelial cells are responsible for the Cav1KO and Cav2KO tumor phenotypes. Dermal fibroblasts, in fact, are abundant cellular components of the skin and they exert important biologic functions to maintain normal skin homeostasis (39). Our co-injection experiments show that absence of Cav1 in dermal fibroblasts is sufficient to recapitulate the tumor phenotype of Cav1KO mice. Interestingly, Cav2-deficient fibroblasts that express Cav1 (14) fail to replicate the tumor phenotype of Cav2KO (and Cav1KO) mice, indicating that the tumor-promoting effects of Cav1-deficient fibroblasts were Cav2-independent.

On the basis of these findings, we postulated that Cav1-deficient fibroblasts promote the growth of melanoma cells by either direct cell–cell contact or paracrine signaling. To test this hypothesis, we did direct and indirect cocultures of fibroblasts and melanoma cells. Interestingly, our results from coculture experiments suggest that the growth-promoting features of Cav1-deficient fibroblasts may be attributed to enhanced paracrine signaling that does not require direct cell–cell contact. However, the inability of Cav1-deficient fibroblasts to promote the growth of melanoma cells in direct cell–cell contact cocultures can most likely be attributed to direct cell–cell contact inhibitory mechanisms exerted by normal fibroblasts (primary and/or immortalized cells) that are able to overcome the proproliferative effects of secreted soluble factors. To identify possible secreted factors, we conducted a cytokine array on conditioned medium from serum-activated dermal fibroblasts. The increased secretion of cytokines, such as ShhN, MMP2/3, and bFGF, and the reduced expression of VEGFR2, HGF-R (decoy receptors; ref. 40) and TIMP1 (MMPs inhibitor) observed in Cav1KO dermal fibroblasts further confirms their protumorigenic phenotype, and this cytokine signature correlates well with the tumor phenotype of Cav1KO mice. In addition, these results are in agreement with many published studies that identified similar factors associated with the stromal remodeling of tumors (3, 41). A key finding of our study is the increased amount of the soluble form of the Shh protein (ShhN) observed in the conditioned medium of serum-activated Cav1KO dermal fibroblasts. Aside from having an essential role in embryonic development, Shh modulates many aspects of skin biology including wound healing (42), proliferation, and transformation (43). Furthermore, although Shh has been described to mainly function in an

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

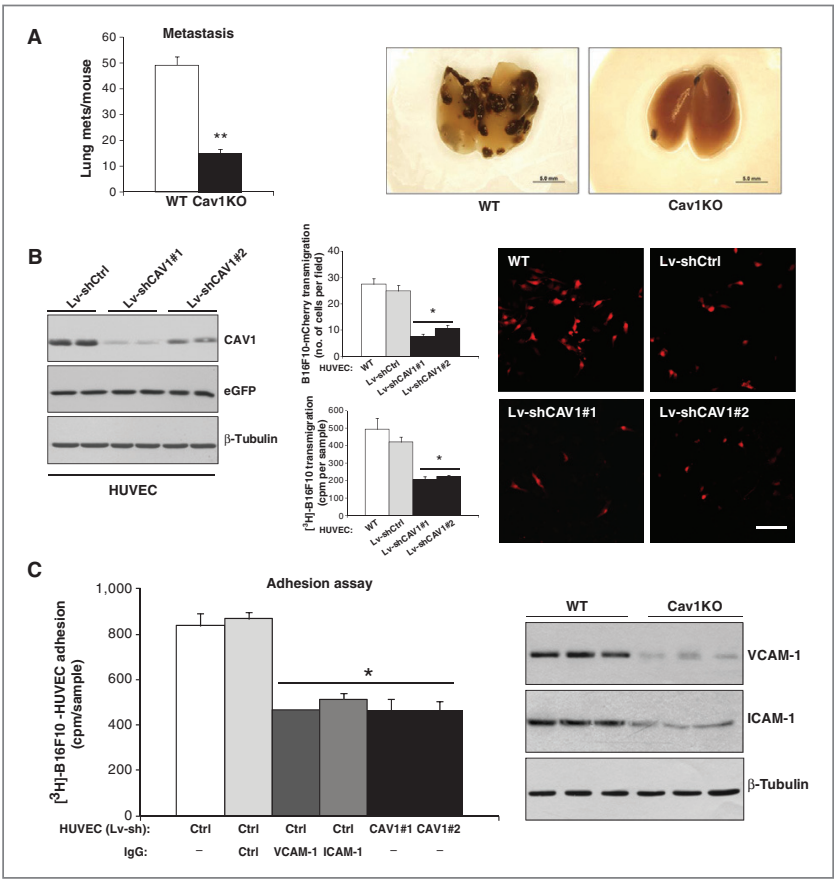

Figure 6. Cav1 deficiency inhibits lung colonization and transendothelial migration of B16F10 melanoma cells. A, left, experimental lung metastasis (mets) assay showing that the ability of B16F10 cells to colonize the lungs of Cav1KO mice is markedly reduced in ($n = 9$ per group). Representative images of WT and Cav1KO lungs dissected 18 days after i.v. injections of 10^5 B16F10 cells are shown (right). B, transmigration (6 hours) of mCherry- and/or [^{3}H]thymidine-labeled B16F10 cells across a confluent monolayer of HUVEC-WT, HUVEC-Lv-shCtrl, and HUVEC-Lv-shCAV1-#1/#2 ($n = 4$ per group). Representative images of transmigrated B16F10-mCherry cells are shown (right). C, adhesion assay of [^{3}H]thymidine-labeled B16F10 cells on confluent monolayers of HUVEC-shCtrl and HUVEC-shCAV1 untreated or treated with IgG1-isotype, ICAM-1, and VCAM-1 antibodies ($n = 4$ per group). VCAM-1, ICAM-1 immunoblots of lungs from TNFα-treated WT and Cav1KO mice (right). Results are means ± SEM (*, *, $P < 0.05$, by 2-tailed Mann–Whitney test and by Dunnett's Multiple Comparisons test; scale bar, 100 μm).

autocrine manner in melanomagenesis (32), it is now becoming increasingly evident that Shh may contribute to tumor growth in a paracrine manner (44). Our results, showing increased DNA synthesis and increased Gli-1 expression in melanoma cells incubated with conditioned medium from Cav1-deficient fibroblasts, provide evidence that absence of Cav1 enhances Shh heterotypic signaling. Consequently, the proproliferative and protumorigenic

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

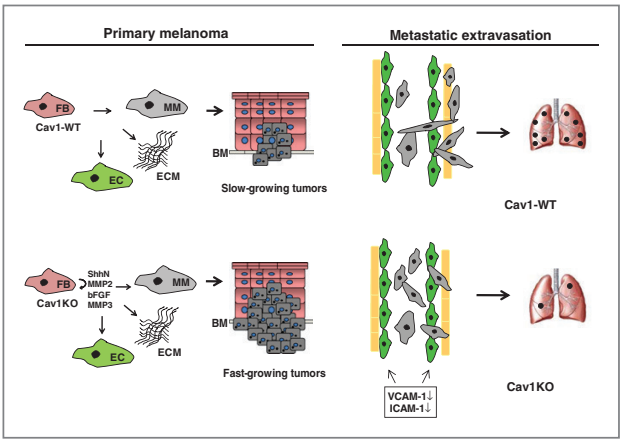

Figure 7. Schematic representation of Cav1-mediated mechanisms regulating primary melanoma tumor growth and metastasis in mice. In primary melanoma, absence of Cav1 in dermal fibroblasts promotes the growth of B16F10 cells by enhanced expression of protumorigenic cytokines (left). In contrast, absence of Cav1 results in reduced VCAM-1/ICAM-1 expression levels and in inhibition of transendothelial migration and lung colonization of B16F10 cells (right). BM, basement membrane; EC, endothelial cells; ECM, extracellular matrix; MM, malignant melanoma.

effects of Cav1-deficient fibroblasts are reversed by inhibiting the Shh pathway with cyclopamine and by silencing Gli-1 in B16F10 cells.

Another important key finding of our study is the inability of B16F10 cells to form lung metastases in Cav1KO mice. The dissemination of cancer cells to metastatic sites is a stepwise process that begins with the invasion of the dermis surrounding the primary tumor and ends with metastatic extravasation and colonization of ectopic sites (36, 45). Metastatic extravasation from the bloodstream is a critical last step of the metastatic cascade that similarly to leukocyte transmigration requires the firm binding of cancer cells to the endothelial adhesion molecules VCAM-1 and ICAM-1. Blockade of VCAM-1- and ICAM-1–mediated interactions has been shown to effectively prevent the development of metastasis in a preclinical setting (30, 46, 47). Given these considerations, our adhesion assay results and our data showing reduced ICAM-1 and VCAM-1 expression in the lungs of TNFα-treated Cav1KO mice suggest that the inability of B16F10 cells to form metastases and to extravasate may be attributed to defects in VCAM-1- and ICAM-1–mediated adhesion to endothelial cells. Given our primary tumor results, the metastasis phenotype of Cav1KO mice appears quite paradoxical. However, recent work reveals that Cav1KO mice display several non-cancer–related phenotypes that support our observations. For instance, previous studies have shown that the resistance of Cav1KO mice to atherosclerosis development may be attributed to impaired endothelial VCAM-1 and ICAM-1 functions that ultimately result in reduced inflammation and impaired macrophage migration throughout the endothelium (48–50). Thus, it appears that similar endothelial defects may cause resistance to atherosclerosis and reduce melanoma metastasis in Cav1KO mice.

In summary, we show that loss of Cav1 promotes the growth of B16F10 tumors in the skin, whereas it suppresses B16F10 lung metastasis. Mechanistically, this phenotype is associated with enhanced paracrine cytokine signaling in Cav1KO dermal fibroblasts and with defects in endothelial cell–mediated transmigration of melanoma cells (Fig. 7). Thus, these findings support the notion that effective anticancer therapies will have to take into account the complex interactions between cancer cells and their microenvironment in both primary tumors and metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F. Capozza, S. Katiyar, F. Sotgia, M.P. Lisanti
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Acquisition of data: F. Capozza, C. Trimmer, M. Crosariol, G. Llaverias, R.G. Pestell
Analysis and interpretation of data: F. Capozza, R. Castello-Cros, F. Sotgia
Writing, review, and/or revision of the manuscript: F. Capozza, C. Trimmer, A. Follenzi, R.G. Pestell, M.P. Lisanti
Administrative, technical, or material support: F. Capozza, R. Castello-Cros, D. Whitaker-Menezes, A. Follenzi, R.G. Pestell, M.P. Lisanti
Study supervision: F. Capozza, M.P. Lisanti
Carried out experiments: C. Trimmer, D. Whitaker-Menezes, R. Castello-Cros, S. Katiyar
Making of the LV constructs and stable cell lines: F. Capozza, A. Follenzi
Isolating primary fibroblasts**and conducting co-injections in mice:** F. Capozza, C. Trimmer
Sharing reagents: G. Llaverias, M. Crosariol, F. Sotgia, R.G. Pestell, M.P. Lisanti

Acknowledgments

The authors thank Dr. Philippe Frank for kindly providing WT/Cav2KO C57Bl/6 mice, Matthew Farabaugh (Thomas Jefferson University) for excellent technical assistance with FACS analysis, Dr. Kyung-Min Noh (Rockefeller University, New York, NY), and Dr. Gino Cingolani (Thomas Jefferson University) for the critical reading of the manuscript and insightful discussions.

Published OnlineFirst March 6, 2012; DOI:10.1158/0008-5472.CAN-11-2593

Grant Support

F. Capozza was supported by a grant from the American Heart Association (BGIA). M.P. Lisanti was supported by NIH/NCI grants (R01CA120876; R01CA098779), the Susan G. Komen Breast Cancer Foundation, the Margaret Q. Landenberger Research Foundation, and in part by the Pennsylvania Department of Health. C. Trimmer was supported by NIH Graduate Training Program Grant T32-CA09678.

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Received August 3, 2011; revised February 23, 2012; accepted February 25, 2012; published OnlineFirst March 6, 2012.

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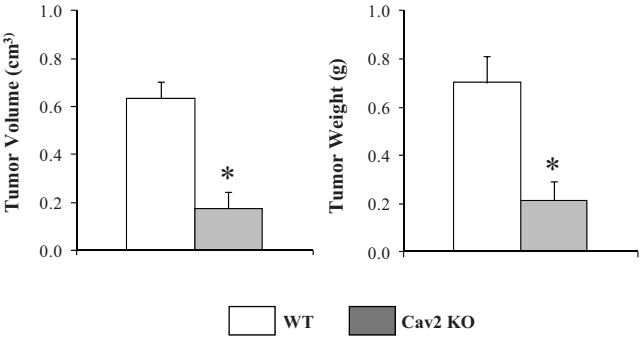
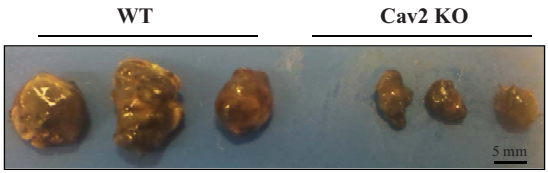
Fig S1





SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Tumor growth of subcutaneously implanted B1610 cells is significantly reduced in Cav2 KO mice. B16F10 melanoma cells ($1x10^6$) were subcutaneously (SC) injected in the skin of 3-4-month-old WT, and Cav2 KO C57Bl/6 female mice ($n = 6$, *per* group). After 18 days, tumors were excised and their volume and weight determined. Representative images of tumors dissected from WT and Cav2 mice are also shown (*, $P < 0.05$, by two tailed Mann-Whitney Test). Results are means ± SEM.

Chapter 3

Cav1 inhibits benign skin tumor development in a two-stage carcinogenesis model by suppressing epidermal proliferation

Am J Transl Res 2013;5(1):80-91
www.ajtr.org /ISSN:1943-8141/AJTR1211010

Original Article

Cav1 inhibits benign skin tumor development in a two-stage carcinogenesis model by suppressing epidermal proliferation

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Received November 28, 2012; Accepted December 30, 2012; Epub January 21, 2013; Published January 30, 2013

Abstract: Caveolin-1 (Cav1) is the main protein component of the membrane lipid rafts caveolae. Cav1 serves as a scaffolding protein that compartmentalizes a multitude of signaling molecules and sequesters them in their inactive state. Due to its function in the negative regulation of signal transduction, loss of Cav1 has been implicated in the pathogenesis of many cancers, but its role in cutaneous squamous cell carcinoma (cSCC) is largely unexplored. cSCC is a multi-stage disease characterized by the development of benign, premalignant lesions and their progression into malignant cancer. Here, we use a two-stage carcinogenesis protocol to elucidate the function of Cav1 in the different stages of benign papilloma development: initiation and promotion. First, we demonstrate that Cav1 knock-out (KO) mice are more susceptible to benign papilloma development after being subjected to a DMBA/TPA initiation/promotion protocol. Treatment of wild-type (WT) and Cav1 KO mice with DMBA alone shows that both groups have similar rates of apoptosis. In contrast, treatment of these groups with TPA alone indicates that Cav1 KO mice are more susceptible to promoter treatment as evidenced by increased epidermal proliferation. Furthermore, primary keratinocytes isolated from Cav1 KO mice have a proliferative advantage over WT keratinocytes in both low- and high-calcium medium, conditions that promote proliferation and induce differentiation, respectively. Collectively, these data indicate that Cav1 functions to suppress proliferation in the epidermis, and loss of this function promotes the development of benign skin tumors.

Keywords: Cav1, caveolin, skin cancer, skin, two stage carcinogenesis

Introduction

Caveolae were originally identified by electron microscopy in the 1950's as flask-shaped cavities (literally "little caves") in the cell membrane [1]. These specialized lipid rafts function in various cellular processes including signal transduction events [2, 3]. The key structural components of these membrane organelles are the three Caveolins (Cav1, 2, and 3), which vary in their tissue specificity [4-7]. The most ubiquitously-expressed and the best-characterized is Caveolin-1 (Cav1). Cav1 contains a scaffolding domain that is able to compartmentalize and negatively regulate the function of many signaling molecules, including MAPK and AKT pathway members [3]. Accordingly, Cav1 expression has been shown to be decreased or lost in several cancer types.

The human *CAV1* gene maps to a known fragile site on chromosome 7 that is frequently lost in human cancers, including head and neck squamous cell carcinomas [8-10]. Furthermore, methylation of CpG islands in the promoter region of the *CAV1* gene has been demonstrated in ovarian and breast cancer, and a dominant negative Cav1 mutation—P132L—is found in roughly 16% of ER-positive breast cancers [11-15]. In addition to evidence from human cancers, the increased susceptibility of the Cav1 knock-out (Cav1 KO) mouse to mammary epithelial cell hyperplasia [15] and oncogene- and carcinogen-induced breast and skin can-

cers [16, 17] indicates that Cav1 functions as a tumor suppressor gene in several cancer types. However, the role of Cav1 in cancer seems to be tissue-specific, as this protein is able to act as a growth promoter in some malignancies, such as prostate cancer [18, 19]. Although little research has examined the function of Cav1 in cutaneous squamous cell carcinoma (cSCC), recent work indicates a tumor suppressive function for Cav1 in this type of cancer [17, 20].

With roughly 700,000 new cases diagnosed annually in the United States, cSCC is the second most commonly diagnosed malignancy among white populations, with an incidence that is increasing worldwide [21-23]. cSCC development is a multi-stage process which requires the accumulation of genetic alterations [24]. Tumorigenesis begins with the initiation of a single epidermal cell and proceeds through the promotion of benign tumor growth and finally the progression of the benign tumor into a malignant and potentially metastatic lesion [24, 25]. As with many cancers, the ultimate cause of death for cSCC is metastasis to the lymph nodes or distal sites [26, 27]; however, both pre-malignant lesions, such as actinic keratoses, and malignant cSCCs have significant consequences for human health due to their potential for local disfigurement and invasion [28]. Therefore, identifying proteins involved in the promotion of benign tumor growth and the progression of malignant lesions could provide biomarkers for predicting tumor outcome and targets for better therapeutic intervention [29]. Interestingly, previous work has demonstrated that loss of Cav1 may contribute to the pathogenesis of psoriasis, a benign proliferative disorder of the epidermis, and increases susceptibility to benign tumor development in mice [17, 30, 31]. However, the specific contribution of Cav1 loss to the different stages of skin tumor development, i.e. initiation, promotion, and progression, has not been explored.

In an effort to better elucidate the role of Caveolin-1 in skin cancer, we decided to examine the sensitivity of the Cav1 KO mouse model to a classic two-stage carcinogenesis protocol. In accordance with previous work using chronic carcinogen treatment [17], Cav1 KO mice subjected to a two-stage carcinogenesis protocol are more susceptible to the development of benign papillomas. Specifically, Cav1 KO mice display an increase in tumor incidence and size following 6 weeks of treatment. In addition, Cav1 ablation results in an increase in tumor multiplicity throughout the entire course of the study, and after 6 months of treatment, Cav1 KO mice have a two-fold higher tumor burden than WT mice. Separate treatment with the initiator, DMBA, and the promoter, TPA, revealed that loss of Cav1 does not affect epidermal apoptosis following DMBA treatment but does significantly increase epidermal proliferation following TPA treatment. In support of these results, keratinocytes isolated from Cav1 KO mice have an increased proliferative capacity and are resistant to calcium-induced differentiation. In summary, our *in vivo* and *in vitro* results demonstrate a role for Cav1 in suppressing epidermal proliferation in the promotion stage of skin tumor development.

Materials and methods

Materials

DMBA (7,12-Dimethylbenz(a)anthracene) and BrdU were from Sigma Aldrich (St. Louis, MO). TPA (12-O-tetradecanoylphorbol-13-acetate) was from LC Laboratories (Woburn, MA). Collagen I and fibronectin were from BD Biosciences (Franklin Lakes, NJ). Antibodies and their sources were as follows: Cav1 was from Santa Cruz (Santa Cruz, CA) and Cav2 was from BD Biosciences (Franklin Lakes, NJ). K14 and Loricrin were from Covance (Princeton, NJ) while GAPDH was from Sigma Aldrich (St. Louis, MO).

Two-stage carcinogenesis

Age-matched FVB/N WT and Cav1 KO mice were subjected to a two-stage carcinogenesis protocol [25]. Mice were shaved two days before initiation. Mice were topically treated with 0.2 mL acetone vehicle or initiated with 200 nmols of DMBA in 0.2 mL acetone. One week later, mice were treated with twice-weekly applications of 0.2 mL acetone vehicle or 10 nmols TPA in 0.2 mL acetone. Tumor incidence and multiplicity were monitored weekly. Tumor volume was calculated at various timepoints using the equation (width^2 x length)/2. To examine the effect of DMBA treatment alone, age-matched WT and KO mice were treated with a single dose of 200 nmols DMBA and sacrificed 24 hours later. Similarly, age-matched WT and KO mice were shaved and treated with 3 appli-

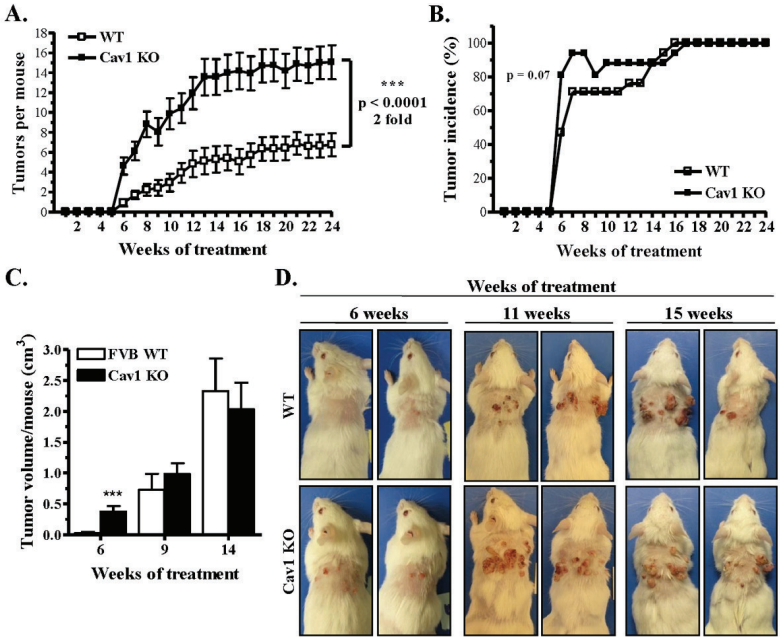

Figure 1. Cav1 KO mice subjected to a two-stage carcinogenesis protocol show increased susceptibility to benign tumor development. A. WT and Cav1 KO mice were initiated with 200 nmols DMBA followed by twice weekly applications of TPA. Tumor multiplicity is significantly higher in Cav1 KO mice throughout the entire course of the study (n = at least 16 mice per group). Results are reported as mean ± SEM ($p < 0.0001$ via a Mann-Whitney non-parametric test). B. Tumor incidence is similar between WT and Cav1 KO mice, although KO mice display a non-significant trend toward greater tumor incidence between 6 and 12 weeks of treatment (via a Fisher's Exact Test). C. The average tumor volume per mouse is greater in Cav1 KO mice after 6 weeks of treatment, but this difference is not observed at 9 and 14 weeks of treatment. Results are reported as mean ± SEM ($p < 0.05$ by unpaired t-test). D. Pictures taken at different timepoints over the course of the study illustrate the significant increase in tumor volume in Cav1 KO mice at 6 weeks and the increase in tumor multiplicity displayed by Cav1 KO throughout the course of the study.

cations (Days 1, 4, and 8) of 10 nmol TPA in acetone. 24 hours following the final treatment, mice were injected with 200 mg/kg BrdU, sacrificed 30 minutes later, and their skin collected for histology. Mice were maintained in a barrier facility with a 12 hour light/dark cycle and *ad libitium* access to chow. All experiments were conducted in accordance with IACUC approval.

Primary keratinocytes isolation

Primary keratinocytes were isolated from WT and Cav1 KO mice as previously described [32, 33]. Briefly, the skin was removed from 1-2 day old pups and placed in 0.25% trypsin overnight at 4°C. The following day, the epidermis was separated from the dermis, minced and placed in 50-mL conical in low-calcium medium for 30 minutes at 37°C. After straining, cells were counted and plated at a density of $5x10^5$ cells/well of a 6-well plate coated with 20 µg/mL collagen I and 10 µg/mL fibronectin. Low calcium medium was Keratinocyte Growth Medium-2 (KGM-2) from Lonza (Walkersville, MD) with 8% chelated-FBS, penicillin/streptomycin, and 0.05 mM calcium chloride. High calcium medium was the same formulation with 1.2 mM calcium chloride.

TUNEL staining

Paraffin-embedded skin sections were stained for terminal deoxynucleotidyltransferase-mediated dUTP nick-end label (TUNEL) positive cells. Briefly, samples were deparaffanized and rehydrated, and tissue was permeabilized with 20 μg/mL Proteinase K (Roche Diagnostics, Indianopolis, IN). Slides were washed, endogenous peroxidase activity was blocked for 10 minutes with 3% H_2O_2, and slides were washed again. TdT enzyme (30% enzyme, 70% reaction buffer; Millipore, Billerica, MA) was added for 30 minutes followed by anti-digoxigenin HRP antibody (Roche Diagnostics). Slides were developed with DAB substrate and counterstained with hematoxylin before mounting.

BrdU incorporation

Paraffin-embedded skin sections were stained for BrdU incorporation using a BrdU immunohistochemistry kit as per manufacturer's instructions (EMD Chemicals, Gibbstown, NJ).

Western blot analysis

Cells were lysed in a modified radioimmunoprecipitation assay (RIPA) buffer and subjected to immunoblot analysis as previously described [34]. Briefly, protein samples were separated via an SDS-PAGE gel and transferred to a nitrocellulose membrane. Following blocking in 5% BSA (Sigma Aldrich) in wash buffer (TBS with 0.1% Tween), primary antibody was added for one hour at room temperature or overnight at 4°C. Membranes were washed, HRP-conjugated secondary antibody was added for one hour, washed again, and developed using Peirce Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL).

Confocal microscopy

Cells were subjected to staining for Cav1 and Cav2 as previously described [35].

Proliferation assay

[^{3}H]Thymidine incorporation was performed as previously described [36]. Briefly, primary keratinocytes were plated at the same density in collagen/fibronectin-coated 6 well plates. Complete medium (either low- or high-calcium) with 0.1 μCi/mL [^{3}H]Thymidine was added for 4 hours. Cells were fixed in 10% trichloroacetic acid and lysed in SDS-NaOH solublization buffer. [^{3}H]Thymidine levels were quantified by liquid scintillation counting and normalized for sample protein concentration.

Statistical analysis

All results are represented as mean ± SEM. Statistical analyses were performed using Prism 4.0 (GraphPad Software, Inc. San Diego, CA).

Results

Cav1 KO mice subjected to a two-stage carcinogenesis protocol show increased susceptibility to benign tumor development

Previous work conducted by our laboratory demonstrated that C57BL/6J Cav1 KO mice chronically treated with a carcinogenic compound display an increase in benign tumor incidence, multiplicity, and size [17]. In this protocol, DMBA functions as a complete carcinogen; therefore, it does not allow for the separation of the different stages of benign tumor development in these mice (i.e. initation and promotion). In addition, C57BL/6 mice are resistant to malignant progression, while FVB/N mice subjected to a two-stage carcinogenesis protocol are more sensitive to the development of SCC [37, 38]. In an effort to corroborate the results reported by Capozza and colleagues [17] and examine the different stages of skin tumor development, a two-stage carcinogenesis protocol was undertaken using FVB/N WT and Cav1 KO mice.

Age-matched FVB/N WT and Cav1 KO mice were initiated with a dose of 200 nmols DMBA and promoted with twice-weekly applications of 10 nmols TPA. Mice were then monitored weekly for tumor development. Similarly to the study conducted by Capozza and colleagues [17], Cav1 KO mice display a significant increase in tumor multiplicity throughout the course of the study (**Figure 1A**). Indeed, after 24 weeks of promotion, Cav1 KO mice show an almost 2-fold increase in tumor number in comparison to WT mice. Starting at 6 weeks of treatment, Cav1 KO mice also display a non-significant increase in tumor incidence that continues until 12 weeks of treatment (**Figure 1B**). In addition, average papilloma volume per mouse is significantly increased in Cav1 KO mice following 6 weeks of treatment, but this size difference dis-

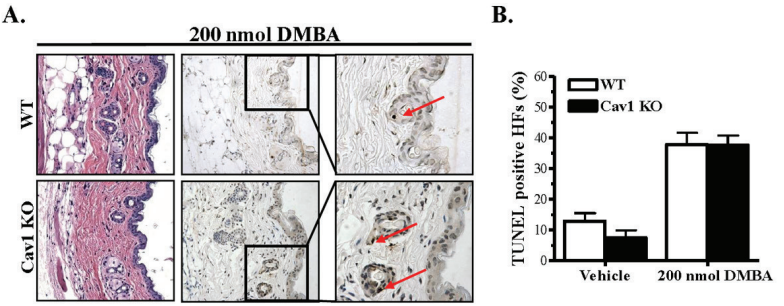

Figure 2. WT and Cav1 KO mice display similar rates of apoptosis following DMBA treatment. A. WT and Cav1 KO mice were treated with a single dose of 200 nmols DMBA and sacrificed 24 hours later. H&E staining demonstrates that there is no morphological difference between WT and Cav1 KO epidermis following treatment. Note that TUNEL staining mainly occurs in the hair follicles (HFs) as opposed to the interfollicular epidermis. No difference in the TUNEL-positive hair follicles was noted between WT and Cav1 KO mice. Arrows indicate TUNEL-positive cells. Pictures were taken at 400x magnification. B. Quantification of the percentage of TUNEL positive hair follicles out of approximately 100 hair follicles/mouse, 5 mice per group. Note the similar percentage of apoptotic hair follicles in WT and Cav1 KO mice. Results are reported as mean ± SEM (compared via an unpaired t-test).

appears by 9 and 14 weeks (**Figure 1C**). Pictures taken throughout the course of the study illustrate the increase in tumor multiplicity observed in Cav1 KO mice (**Figure 1D**). These results corroborate work previously conducted in C57BL/6 mice, as FVB Cav1 KO mice display an increase in benign tumor multiplicity in both carcinogenesis studies [17].

WT and Cav1 KO mice display similar rates of apoptosis following DMBA treatment

The use of a two-stage carcinogenesis protocol allows for the dissection of the initiation and promotion stages of tumor development. This task is accomplished through treating the skin with each chemical separately and evaluating the relative effect on the epidermis. DMBA initiates tumorigenesis by inducing mutation of the H-Ras oncogene in the epidermal stem cells of the hair follicle or the basal layer; these cells subsequently undergo apoptosis or survive to become a target for promoter treatment [25, 39]. Treatment of WT and Cav1 KO mice with a single dose of 200 nmols DMBA results in apoptosis mainly in the stem cell compartment of the hair follicle, as previously noted by other groups (**Figure 2A**) [39]. Examination of the percentage of hair follicles positive for TUNEL staining shows that WT and Cav1 KO mice have similar rates of apoptosis following DMBA treatment (**Figure 2A** and **B**). These results indicate that loss of Cav1 does not affect the initiation stage of tumor development.

Cav1 KO mice display increased sensitivity to TPA treatment

In an effort to determine if Cav1 ablation in these mice affects the promotion stage of the carcinogenesis protocol, we examined the sensitivity of Cav1 WT and KO mice to TPA treatment. Age-matched Cav1 WT and KO were treated with three applications of acetone or 10 nmol TPA and sacrificed 24 hours later. Treatment with TPA alone results in epidermal hyperplasia in both Cav1 WT and KO mice, with no observable difference in epidermal thickness between the two groups (**Figure 3A**). However, examination of BrdU incorporation indicates that Cav1 KO mice display a significant increase in epidermal proliferation following TPA treatment (**Figure 3B**). Quantification of the labeling index indicates Cav1 KO mice show an almost 2-fold increase in BrdU staining in comparison to WT mice. These results indicate that Cav1 KO mice are more sensitive than WT mice to the promotion stage of the two-stage carcinogenesis protocol.

Cav1 ablation increases the proliferative ability of primary murine keratinocytes

Given the increased sensitivity of Cav1 KO mice to a two-stage carcinogenesis protocol and TPA

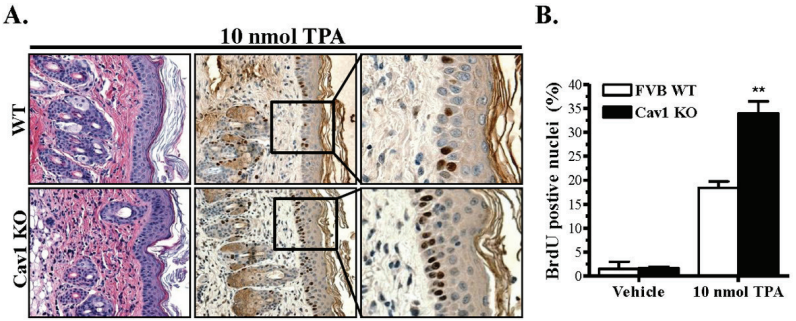

Figure 3. Cav1 KO mice display increased sensitivity to TPA treatment. A. WT and Cav1 KO mice were treated with three doses of vehicle or 10 nmols TPA (Days 1, 4 and 8) and sacrificed 24 hours following the final dose. H&E staining shows there is no difference in epidermal hyperplasia between the two groups treated with TPA. Immunohistochemical staining for BrdU shows that Cav1 KO animals show an increase in BrdU incorporation in the basal layer of the epidermis. Pictures were taken at 400x magnification. B. Quantification of the percentage of BrdU positive nuclei out of total nuclei in 5-6 fields/mouse, 4 mice per group. WT and Cav1 KO mice show no difference in BrdU incorporation in vehicle-treated skin. Note that Cav1 KO mice show a significant increase in BrdU incorporation in TPA-treated skin. Results are reported as mean ± SEM ($p < 0.01$ via an unpaired t-test).

treatment, we next examined the *in vitro* growth characteristics of keratinocytes isolated from WT and KO mice. Keratinocytes isolated from Cav1 KO mice show complete absence of Cav1 protein, as demonstrated by both Western blot and confocal microscopy (**Figure 4A** and **B**). Previous work has shown that Cav1 and Cav2 co-localize at the plasma membrane and that Cav1 is necessary for the stabilization and proper localization of Cav2 [5, 6, 40, 41]. Accordingly, Cav2 levels are decreased (**Figure 4A**) and Cav2 is mislocalized around the nucleus (**Figure 4B**) in Cav1 KO keratinocytes. In addition, the levels of keratin 14 (K14), a marker of proliferating keratinocytes, and Loricrin , a marker of differentiating keratinocytes, are similar between WT and Cav1 KO cells.

In an effort to examine the effect of Cav1 loss on proliferative capacity, [^{3}H]Thymidine incorporation was examined under low-calcium and high calcium growing conditions. In low-calcium (0.05 mM) medium, primary keratinocytes proliferate exponentially, while high-calcium (1.2 mM) medium induces the cells to stop proliferating and undergo terminal differentiation [32, 33]. Cav1 ablation increases proliferation in keratinocytes grown under low-calcium conditions when cells are plated at two different densities (**Figure 4C**). This finding is further illustrated by phase contrast microscopy showing increased cell density of Cav1 KO keratinocytes 48 and 72 hours after plating (**Figure 4D**). In addition, when the calcium concentration in the medium is increased, proliferation in both WT and KO keratinocytes decreases, but [^{3}H]thymidine incorporation is significantly higher in Cav1 KO keratinocytes versus WT cells (**Figure 4C**). Collectively, these results indicate that Cav1 KO keratinocytes have a proliferative advantage over WT cells in conditions that promote proliferation and induce differentiation. In addition, these data correspond to our results indicating a role for Cav1 in suppressing proliferation following promoter treatment in the epidermis.

Discussion

In the present study, we examined the function of Caveolin-1 in the initiation and promotion of benign tumor development. Using a two-stage carcinogenesis protocol, we provide evidence that the increase in benign tumor development observed in Cav1 KO mice is due to increased epidermal proliferation following promoter treatment and is not a function of increased initiated cell survival. We first show that Cav1 KO mice subjected to a classic two-stage carcinogenesis protocol are more susceptible to

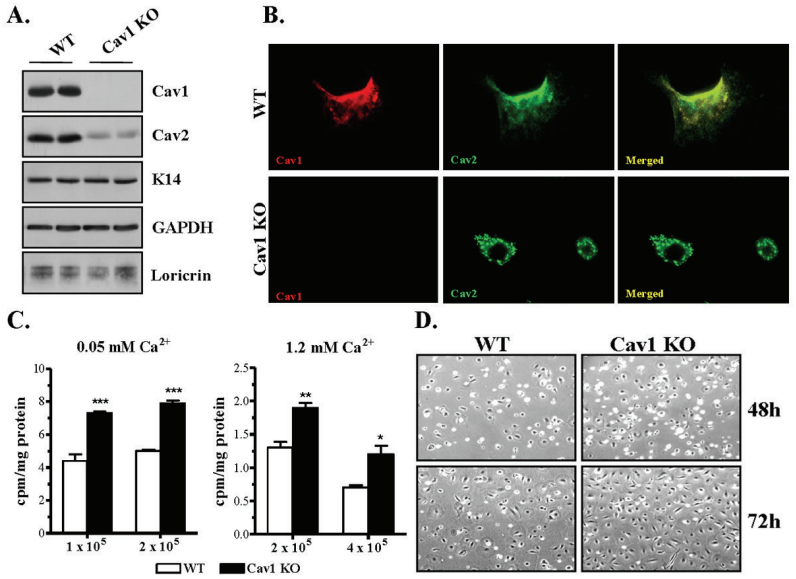

Figure 4. Cav1 ablation increases the proliferative ability of primary murine keratinocytes. A. Primary keratinocytes isolated from Cav1 KO mice show a complete absence of Cav1 protein and a concurrent decrease in Cav2 protein. K14 and Loricrin, markers for keratinocyte proliferation and differentiation, respectively, are unaffected by loss of Cav1. B. Confocal microscopy shows that Cav1 and Cav2 co-localize in WT keratinocytes, but with loss of Cav1 in Cav1 KO keratinocytes, Cav2 remains sequestered around the nucleus. Pictures were taken at 600x magnification. C. [^{3}H]thymidine incorporation assays show that in both pro-proliferative (0.05 mM calcium) or pro-differentiative (1.2 mM calcium) medium conditions, Cav1 ablation in keratinocytes increases proliferation at different plating densities. Results are reported as mean ± SEM ($n \geq 3$ *per* group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ via an unpaired t-test). D. Phase-contrast microscopy of WT and Cav1 KO keratinocytes plated at the same density. Cav1 KO keratinocytes grow to a higher cell density 48 and 72 hours after plating, indicating these cells have a proliferative advantage over WT cells. Pictures were taken at 100x magnification.

benign tumor development than their wild-type counterparts. Specifically, Cav1 KO mice display an increase in tumor multiplicity throughout the entire course of the study. After 6 months of treatment, the tumor burden of Cav1 KO mice is two-fold higher than that of WT mice. In addition, tumor volume is increased in Cav1 KO mice following 6 weeks of promotion, and Cav1 KO display a non-significant trend toward increased tumor incidence between 6 and 12 weeks of treatment. We attribute the increase in tumor multiplicity in Cav1 KO mice to their increased sensitivity to promoter treatment, as evidenced by more proliferation following TPA treatment. Finally, keratinocytes isolated from Cav1 KO mice display an increased proliferative ability. Collectively, these results indicate a role for Cav1 in the suppression of proliferation during benign skin tumor development.

The results discussed herein are in accordance with previous work conducted by our laboratory demonstrating that Cav1 KO mice are more susceptible to benign tumor development following chronic treatment with 7,12-Dimethylbenz [a] anthracene (DMBA) [17]. Two-stage carcinogenesis is a different model in which mice are subjected to an initiating dose of a carcinogen, most commonly DMBA, followed by twice-weekly applications of a growth

promoter, commonly 12-O-tetradecanoyl -phorbol-13-acetate (TPA). Tumorigenesis is dependent upon the survival of initiated cells, their promotion into benign tumors, and the progression of these benign tumors into a squamous cell carcinomas [25]. The advantage of using a two-stage carcinogenesis protocol is that it allows for the dissection of the contribution of protein gain- or loss-of function to the different stages of tumorigenesis. The skin can be treated separately with each chemical, initiator or promoter, and monitored for the relative amount of apoptosis or proliferation, respectively [25, 39]. In the study conducted by Capozza and colleagues [17], DMBA was used as a complete carcinogen, and therefore the contribution of loss of Cav1 to the different stages of tumor development could not be determined.

Following the observation that Cav1 KO mice subjected to a two-stage carcinogenesis protocol are more susceptible to benign tumorigenesis, these mice were treated separately with DMBA and TPA to determine which stage of tumor development is more sensitive to loss of Cav1. Interestingly, both WT and Cav1 KO mice display similar rates of apoptosis following treatment with DMBA, indicating that Cav1 loss does not affect the survival of cells in the treated epidermis and consequently the initiation phase. In contrast, Cav1 KO mice are more sensitive to TPA treatment, and display increased BrdU incorporation in comparison to WT mice. These results indicate that the increased susceptibility to benign tumor development observed in Cav1 KO mice is a product of augmented proliferation in the promotion of benign skin tumors versus increased initiated cell survival. In further support of these results, keratinocytes isolated from Cav1 KO mice have a proliferative advantage over WT keratinocytes in both low- and high-calcium medium. These results are especially relevant in light of other research demonstrating resistance to calcium-induced cell cycle inhibition in keratinocytes isolated from mouse models with increased susceptibility to skin carcinogenesis [42]. The two-stage carcinogenesis model is also useful for analyzing the progression of benign tumors to malignant lesions [25]. It should be noted that no difference was observed in the rate of conversion to malignancy between WT and Cav1 KO mice, due in part to unexpected mouse death and the necessity of mouse sacrifice due to large tumor size (data not shown). Collectively, these results indicate that Cav1 functions to suppress keratinocyte proliferation, both *in vitro* and *in vivo*.

Caveolin-1 is a major regulator of signaling transduction events in the cell, accomplished through its scaffolding domain that functions to compartmentalize many signaling molecules and inhibit their activity [3, 43]. One of the major targets of Cav1 signaling suppression is the Ras/Erk1/2 mitogen-activated protein kinase (MAPK) cascade, and several components of this signaling pathway, including EGFR, Ras, Mek1/2, and Erk1/2 have been demonstrated to localize with caveolae and interact with Cav1 [44-50]. Furthermore, Cav1 depletion has been shown to hyperactivate Erk1/2 signaling both *in vitro* and *in vivo* [46-48, 51, 52]. The Erk1/2 signaling cascade is a major regulator of proliferation, in part through the promotion of cyclin D1 expression [53, 54]. Interestingly, Capozza and colleagues demonstrate that the hyperplastic epidermis of Cav1 KO mice treated with DMBA have increased expression of both activated Erk1/2 and cyclin D1 [17]. Although the expression of these proteins was not investigated in the current study, it is interesting to speculate that the increased proliferation exhibited by Cav1 KO keratinocytes both *in vitro* and *in vivo* is due at least in part to hyperactivation of the Erk1/2 MAPK cascade. The results presented herein are also in accordance with other work that has demonstrated a role for Cav1 in suppressing experimentally-induced hyperplasia and epidermal proliferation following tape stripping [30]. Taken together, Cav1 functions to suppress epidermal proliferation under a variety of conditions, including chemical treatment and disruption of the epidermal barrier.

The function of Cav1 in inhibiting the promotion of benign growth has important ramifications for human disease. Interestingly, Cav1 loss has been implicated in the pathogenesis of two benign hyperproliferative disorders of the epidermis, psoriasis and Netherton syndrome [30, 31, 55]. Specifically, Campbell and colleagues demonstrate decreased expression of Cav1 in a significant portion of human psoriasis plaques [31]. These results correspond to the work discussed herein in which loss of Cav1 increases

benign proliferation following TPA treatment. In our model, this increase in proliferation corresponds to an increased susceptibility to benign tumor development. Human skin tumor development is a multi-stage process characterized by the development of pre-malignant lesions such as actinic keratoses and keratoacanthomas [24]. Although Cav1 expression has been shown to be significantly decreased in a subset of human squamous cell carcinoma tumors, the expression of Cav1 in human pre-malignant skin tumors has never been examined. The results presented herein provide evidence that Cav1 functions to suppress promotion of benign tumor growth in mice, and it therefore may have a similar function in human tumors. Furthermore, the promotion and progression stages of human tumor development have the most significant consequences for human health due to the potential for local disfigurement, invasion, and metastasis [26, 28]; therefore, proteins involved in these stages of tumor development are important potential targets for future therapeutics [29]. As a modulator of epidermal proliferation and benign tumorigenesis, Cav1 may be an excellent target for therapeutic intervention.

In summary, our results demonstrate that the increased susceptibility of Cav1 KO mice to benign tumor development is due to enhanced epidermal proliferation following promoter treatment, and is not a function of increased initiated cell survival. Indeed, Cav1 ablation confers a proliferative advantage to both primary keratinocytes *in vitro* and treated epidermis *in vivo*. Further work should assess the relevance of these findings to human premalignant tumors.

Abbreviations

Cav1, caveolin-1; Cav2, caveolin-2; cSCC, cutaneous squamous cell carcinoma; K14, keratin 14; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Erk, extracellular-signaling related kinase; MAPK, mitogen-activated protein kinase; BrdU, 5-bromodeoxyuridine; H&E, hematoxylin and eosin; FBS, fetal bovine serum.

Acknowledgement

F.C. was supported by a grant from the American Heart Association (BGIA). C.T. was supported by NIH Graduate Training Program Grant T32-CA09678.

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Chapter 4

Cav1 suppresses tumor growth and metastasis in a murine model of Cutaneous SCC through modulation of MAPK/AP-1 activation

The American Journal of Pathology, Vol. 182, No. 3, March 2013

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The American Journal of PATHOLOGY

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Cav1 Suppresses Tumor Growth and Metastasis in a Murine Model of Cutaneous SCC through Modulation of MAPK/AP-1 Activation

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Caveolin-1 (Cav1) is a scaffolding protein that serves to regulate the activity of several signaling molecules. Its loss has been implicated in the pathogenesis of several types of cancer, but its role in the development and progression of cutaneous squamous cell carcinoma (cSCC) remains largely unexplored. Herein, we use the keratinocyte cell line PAM212, a murine model of cSCC, to determine the function of Cav1 in skin tumor biology. We first show that *Cav1* overexpression decreases cell and tumor growth, whereas *Cav1* knockdown increases these attributes in PAM212 cells. In addition, *Cav1* knockdown increases the invasive ability and incidence of spontaneous lymph node metastasis. Finally, we demonstrate that *Cav1* knockdown increases extracellular signaling—related kinase 1/2 mitogen-activated protein kinase/activator protein-1 pathway activation. We attribute the growth and invasive advantage conferred by *Cav1* knockdown to increased expression of activator protein-1 transcriptional targets, including cyclin D1 and keratin 18, which show inverse expression in PAM212 based on the expression level of Cav1. In summary, we demonstrate that loss of Cav1 affects several characteristics associated with aggressive human skin tumors and that this protein may be an important modulator of tumor growth and invasion in cSCC. *(Am J Pathol 2013, 182: 1—13; http://dx.doi.org/10.1016/j.ajpath.2012.11.008)*

Nonmelanoma skin cancer, comprising both basal and squamous cell carcinomas, is the most prevalently diagnosed malignancy among white populations, and its incidence is increasing worldwide.[1—3] Unlike basal cell carcinomas, cutaneous squamous cell carcinomas (cSCCs) can be aggressive cancers that carry a significant risk of metastasis. Of the 700,000 cSCCs diagnosed yearly in the United States,[3] approximately 5% will metastasize to lymph nodes and distant organs.[4,5] Various markers are used to assess the risk for metastatic progression in these lesions: tumor size and depth, degree of differentiation, and involvement of vascular or lymphatic vessels, among others.[6,7] Thus, given the prevalence of SCCs and their potential for developing into life-threatening malignancies, the identification of novel mechanisms contributing to tumor development and progression into invasive lesions could provide better prognostic markers to predict disease outcome and improve therapeutic treatments. Q9

Caveolae are a specialized form of membrane lipid raft characterized as flask-shaped cavities in the cell membrane.[8,9] One of their main biological functions is signal transduction, accomplished through the proteins that preferentially localize

Supported by an American Heart Association grant (Beginning Grant-in-Q3 Aid to F.C.), NIH/National Cancer Institute grants (R01CA120876 and R01-CA-098779 to M.P.L.), the Susan G. Komen Breast Cancer Foundation (M.P.L.), the Margaret Q. Landenberger Research Foundation (M.P.L.), in part by the Pennsylvania Department of Health (M.P.L.), and an NIH Graduate Training Program grant (T32-CA09678 to C.T.).

A guest editor acted as editor-in-chief for this manuscript. No person at Thomas, Jefferson University was involved in the peer review process or final disposition of this article.

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http://dx.doi.org/10.1016/j.ajpath.2012.11.008

to these organelles.[10] The essential protein components of these cellular structures are the caveolins, encoded by three different genes (*CAV1*, *CAV2*, and *CAV3*) that vary in their tissue specificity.[9,11,12] Caveolin-1 (Cav1) contains a scaffolding domain that compartmentalizes a multitude of signaling molecules within caveolae, modulating their activity and preferentially binding them in their inactive state.[9] Cav1, therefore, functions as a negative regulator of numerous signaling molecules, and its misregulation, deletion, or mutation has been implicated in the pathological characteristics of a variety of human diseases, including cancer.

Despite the substantial amount of research on the function of Cav1 in various cancer types, its role in the pathogenesis of nonmelanoma skin cancer remains largely unexplored. Previous research has shown that Cav1 ablation in murine skin increases basal layer proliferation[13] and benign papilloma incidence, multiplicity, and size after carcinogenic treatment.[14] In addition, in human skin, Cav1 is lost during the progression of psoriasis[15,16] and significantly decreased in cutaneous squamous cell carcinoma.[17] To further examine the role of Cav1 in squamous cell carcinoma development, we used a murine keratinocyte cell line, PAM212, that is able to form squamous cell carcinomas *in vivo*.[18] By using this system, we were able to investigate how altered Cav1 expression affects aspects of cancer development and progression.

In the current study, we show that overexpression of *Cav1* in PAM212 cells results in decreased cell proliferation *in vitro*, which corresponds to a dramatic decrease in tumor incidence and size *in vivo*. In contrast, the knockdown of *Cav1* in these cells increases tumor growth and enhances their invasive ability both *in vitro* and *in vivo*. Mechanistically, *Cav1* knockdown is associated with hyperactivation of the extracellular signaling−related kinase (Erk) 1/2 mitogen-activated protein kinase (MAPK) signaling pathway and increased activator protein (AP)-1 transcription factor activation in response to two different growth stimuli. We implicate several AP-1−responsive genes as mediators of the proproliferative and invasive phenotype in *Cav1* knockdown PAM212 cells, as determined by quantitative RT-PCR (RT-qPCR). Finally, we show that *Cav1* overexpression in the human cSCC cell line SCC13 decreases *in vitro* proliferation, migration, and invasion, indicating that our results are able to translate to human skin cancer. In summary, these results demonstrate that loss of Cav1 negatively affects several markers for metastatic potential in human skin tumors, including tumor size and invasiveness, and this is mechanistically associated with MAPK/AP-1 hyperactivation.

Materials and Methods

Materials

Antibodies and their sources were as follows: Cav1 (N-20), cyclin D1, cyclin A, and matrix metalloproteinase 2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Cav2 and epidermal growth factor receptor (EGFR) were from BD Biosciences (Franklin Lakes, NJ). Keratin 14 (K14) and keratin 10 (K10) were from Covance (Princeton, NJ). Keratin 18 (K18) and CD31 were from Abcam (Cambridge, MA). Keratin 8 (K8) was from Epitomics (Burlingame, CA). *p*-Histone H3 (S10) was from Upstate (Billerica, MA). *p*-Erk (T202/Y204), Erk, *p*-EGFR (Y1173), c-Fos, *p*-c-Jun (S73), c-Jun, and *p*-c-Jun N-terminal kinase (JNK; T183/Y185) were from Cell Signaling Technology (Beverly, MA). Glyceraldehyde-3-phosphate dehydrogenase was from Fitzgerald (Acton, MA), and β-actin and β-tubulin were from Sigma-Aldrich (St. Louis, MO). U0126 and SP600125 were from Cell Signaling Technology and LC Laboratories (Woburn, MA), respectively.

Cell Culture

PAM212 cells were a generous gift from Dr. Ulrich Rodeck (Thomas Jefferson University, Philadelphia, PA). They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). HaCaT human immortalized keratinocytes were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. SCC13 cells were obtained from The Harvard Skin Disease Research Center (Boston, MA).[19,20] They were maintained in a 1:1 mix of Keratinocyte Serum Free Medium (Life Technologies, Grand Island, NY) and DF-K medium (a 1:1 mix of calcium-free Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient Mixture; Life Technologies) supplemented with 25 μg/mL bovine pituitary extract, 0.2 ng/mL EGF, and 0.3 mmol/L calcium chloride. All cells were kept in an incubator at 37°C with 5% CO_2.

Stable Cell Lines

PAM212 and SCC13 cells were stably transduced to express either pBabe or pBabe-Cav1.[21] To stably knock down *Cav1*, shRNA scramble control and predesigned shRNAs targeting nucleotides 185 to 205 and 482 to 502 of Cav1 mRNA (NM_007616.4) were obtained from Invitrogen (Carlsbad, CA) and subcloned into the pQCXIP-GFP retroviral vector (Clontech, Mountain View, CA). PAM212 cells were infected, and a stable cell population was selected, as previously described.[22] Successful overexpression and knockdown of *Cav1* were verified by using Western blot analysis. For Cav1 re-expression in *Cav1* knockdown cells, short hairpin RNAs (shRNAs) specific for *Cav1* (sh-Cav1) PAM212 cells were stably transduced to express pBabe-Cav1 as above. The Cav1 expressed by this vector is resistant to the sh-Cav1 constructs already being expressed in these cells because of nucleotide mismatch.

Western Blot Analyses

Cells were lysed in a modified radioimmunoprecipitation assay buffer and analyzed as previously described.[23] Briefly,

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protein was separated via an SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% bovine serum albumin (BSA; Sigma-Aldrich) in Tris-buffered saline with 0.1% Tween. Primary antibody diluted in blocking buffer was added for either 1 hour at room temperature or overnight at 4°C. Membranes were washed three times in wash buffer, and horseradish peroxidase–conjugated secondary antibody was added for 1 hour at room temperature. After washing, signal was developed using Pierce Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL).

Confocal Microscopy

Cells were double immunostained for Cav1 and Cav2, as previously described.[21] Pro-Long Gold Antifade reagent (Molecular Probes, Eugene, OR) was used to mount the coverslips, which were subsequently imaged by confocal microscopy (LSM 510.META.Confocal; Carl Zeiss Inc., Jena, Germany).

Growth Curves and Proliferation Assay

Proliferation was measured using a 5-bromodeoxyuridine (BrdU) incorporation enzyme-linked immunosorbent assay (Roche Diagnostics, Indianapolis, IN). Cells were labeled with BrdU in RPMI 1640 medium with 10% FBS for 4 hours. Growth curves of PAM212 and SCC13 cells were generated by plating 10.5 cells/cm^2 and counting cell number daily for 4 days. Alternatively, growth curves were generated by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays (Promega, Madison, WI) and performed according to the manufacturer's instructions.

Tumor/Metastasis Studies

PAM212 cells (5×10^6) were intradermally injected into the back skin of 5- to 6-week-old BALB/c nude mice, as previously described.[24] Overexpression and knockdown cells were pair injected with their respective controls. Tumor volume was calculated weekly using the following equation: (width^2 × length)/2. For spontaneous metastasis studies, one cell type per mouse was injected as previously described. Tumors were surgically excised after 5 weeks, and mice were sacrificed 4 weeks after excision and examined for visible (macroscopic) lymph node metastasis.[25] Mice were maintained in an animal barrier facility with a 12-hour light/dark cycle with ad libitum access to chow. All mouse work was conducted in accordance with Institutional Animal Care and Use Committee approval.

RT-qPCR Data

RT-qPCR was performed using ready-to-use primers (Real Time Primers, LLC, Elkins Park, PA) and SYBR master mix (Applied Biosystems by Life Technologies). Quantitative expression data were acquired using the ABI-Prism 7900HT Sequence Detection System (Applied Biosystems), and results were analyzed by the $\Delta\Delta C_T$ method.[26]

IHC Analysis

Tumor sections were stained for K18 expression using standard immunohistochemical (IHC) techniques.[14] Briefly, paraffin-embedded sections were rehydrated and antigen retrieval was performed using citrate buffer. Endogenous peroxidase activity was blocked with 3% H_2O_2, and sections were blocked in 10% goat serum and incubated with primary antibody overnight. After washing, sections were incubated with biotinylated secondary antibody for 30 minutes, washed again, and incubated with streptavidin for 30 minutes. After signal development with 3,3′-diaminobenzidene, slides were counterstained with hematoxylin, dehydrated, and mounted.

Growth Factor Stimulation

PAM212 cells were plated in normal medium, serum starved for 18 hours in serum-free medium (SFM) with 0.1% BSA, and treated with 50 ng/mL EGF (Peprotech, Rocky Hill, NJ) in SFM with 0.1% BSA. After treatment, cells were lysed and used in Western blot analysis, as previously described.

Inhibitor Treatments

PAM212 cells were pre-incubated with dimethyl sulfoxide or 40 μmol/L inhibitor in SFM for 2 hours, and then stimulated with 10% FBS with dimethyl sulfoxide or inhibitor for 1 hour. After treatment, cells were lysed and used in Western blot analysis, as previously described.

Migration and Invasion Assays

For PAM212 transwell assays, 10^5 cells (migration) or 1.5×10^5 cells (invasion) were suspended in 0.5 mL SFM with 0.1% BSA and added to 8-μm transwell chambers (BD Biosciences) without (migration) or with (invasion) Matrigel. Cells were allowed to migrate for 6 hours or invade for 18 hours, after which cells that failed to migrate or invade were removed with a cotton swab. Cells were fixed and stained with 0.5% crystal violet in methanol. For assays using EGF, 50 ng/mL EGF in SFM with 0.1% BSA was used as the chemoattractant in the bottom well, and cells were allowed to migrate for 24 hours or invade for 48 hours. For inhibitor assays, dimethyl sulfoxide or inhibitor was added to both the top chamber and the bottom well, and cells were allowed to migrate for 24 hours or invade for 48 hours. For SCC13 assays, 7.5×10^4 cells (migration) or 10^5 cells (invasion) were plated using 10% FBS as the chemoattractant and allowed to migrate or invade for 24 hours.

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Statistical Analysis

All results are represented as mean ± SEM. Statistical analyses were performed using Prism software version 4.0 (Graph Pad Software, Inc., San Diego, CA).

Results

Cav1 Overexpression Decreases *in Vitro* Proliferation and *in Vivo* Tumor Incidence and Growth in PAM212 Keratinocytes

PAM212 murine keratinocytes were stably transduced to express either pBabe empty vector or pBabe-Cav1 overexpression vector. PAM212-Cav1 cells showed an approximately twofold increase in Cav1 expression, whereas Cav2, which colocalized with Cav1, remained unaffected. In addition, the expression of K14, a marker of proliferating keratinocytes, and K10, a marker of differentiating keratinocytes, were unchanged by Cav1 overexpression (Figure 1A). Furthermore, Cav1 overexpression diminished the proliferative ability of PAM212 cells, as evidenced by decreased BrdU incorporation and expression of cyclin D1 and *p*-histone H3 (Figure 1B).

To examine the *in vivo* impact of Cav1 overexpression, pBabe and Cav1 cells were intradermally pair injected into the back skin of Nude mice. Beginning at 2 weeks after injection, analysis of tumor growth revealed that Cav1 overexpression significantly decreased the tumorigenicity of PAM212 cells (tumor incidence) from 100% to 50% (Figure 1C). In addition, overexpression of Cav1 reduced tumor volume by approximately 27-fold (Figure 1D) and tumor weight by 30-fold (data not shown) by 5 weeks after injection. These data indicated that Cav1 functions as a negative regulator of cell and tumor growth in PAM212 cells.

Cav1 Knockdown Increases *in Vitro* Proliferation and *in Vivo* Tumor Growth in PAM212 Keratinocytes

PAM212 cells that were stably transduced to express sh-Cav1 displayed complete absence of Cav1 protein with a concurrent decrease in Cav2 expression levels. Immunoblot analysis showed that K14 and K10 levels were unaffected by *Cav1* knockdown (Figure 2A). As previously described in cells and the *Cav1* knockout mouse, Cav1 and Cav2 colocalize,[27] and Cav1 is required to stabilize and transport Cav2 to the plasma cell membrane.[12] Accordingly, confocal microscopy analysis revealed that Cav1 and Cav2 colocalized as normal in short hairpin control (shCtl) cells, whereas in sh-Cav1 cells, Cav2 was mainly localized around the nucleus (Figure 2B). These results indicated that *Cav1* knockdown in these cells closely mimicked the phenotype observed in *Cav1* knockout mice in that Cav2 was reduced and failed to localize to the membrane.

In addition, *Cav1* knockdown significantly enhanced the proliferation of PAM212 cells *in vitro*, as demonstrated by increased BrdU incorporation and expression of cyclins D1 and A (Figure 2C). To examine *in vivo* growth, *Cav1* knockdown and control cells were pair injected into the back skin of Nude mice. Analysis of tumor volume revealed that *Cav1* knockdown PAM212 cells formed tumors that were significantly larger compared with shCtl cells throughout the entire study (Figure 2D). By 5 weeks after injection, shCav1 tumors were approximately fivefold larger in volume and fourfold larger in mass (Figure 2D). In addition, the effect of *Cav1* knockdown on *in vitro* proliferation and *in vivo* tumor growth was validated

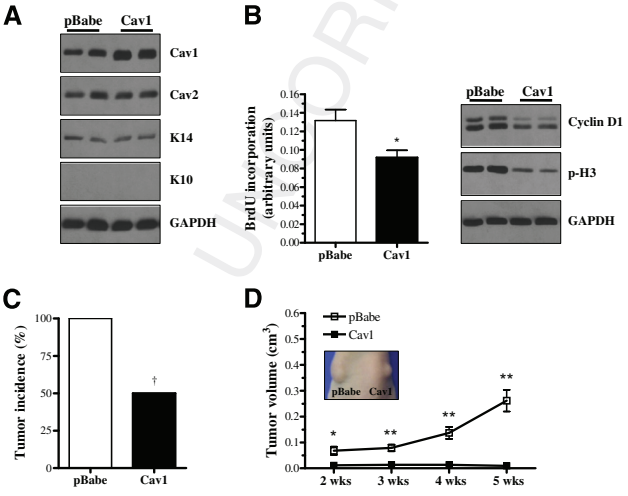

Figure 1 Cav1 overexpression decreases *in vitro* proliferation and *in vivo* tumor incidence and growth in PAM212 keratinocytes. **A:** Western blot analysis of PAM212 cells retrovirally transduced with either empty pBabe plasmid (pBabe) or pBabe-Cav1 overexpression plasmid (Cav1) shows increased Cav1 expression, but no change in the expression of Cav2; K14, a marker of proliferating keratinocytes; or K10, a marker of differentiating keratinocytes. **B:** Cav1 PAM212 cells display reduced proliferation, as determined by a BrdU incorporation assay (4 hours; $n = 16$ per group) and decreased expression of cyclin D1 and *p*-histone H3 proteins. Cav1 overexpression dramatically reduces tumor incidence (**C**) and growth (**D**) in PAM212 cells intradermally injected in the back skin of mice ($n = 10$ per group). $^{\dagger}P < 0.05$ (by Fisher's exact test). **Inset:** Tumor growth 4 weeks after injection. Results are reported as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ by the unpaired *t*-test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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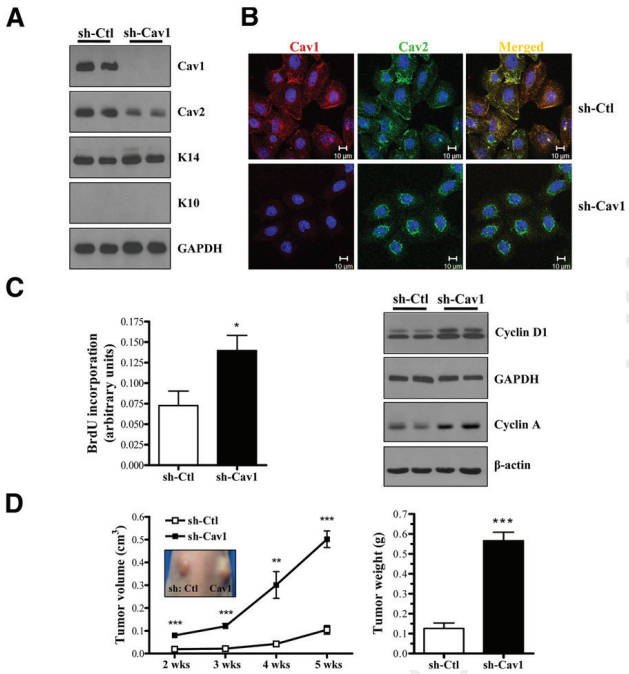

Figure 2 *Cav1* knockdown increases *in vitro* proliferation and *in vivo* tumor growth in PAM212 cells. **A:** Western blot analysis of PAM212 cells stably transduced via retroviral infection with either scramble shCtl or shCav1. *Cav1* knockdown results in a decrease in Cav2 expression, whereas K14 and K10 expression is unaffected. **B:** Confocal microscopy of shCtl and shCav1 PAM212 cells immunostained with antibodies against Cav1 and Cav2. Hoechst 33342 is shown as a nuclear marker. In the absence of Cav1, Cav2 remains sequestered around the nucleus. **C:** *Cav1* knockdown increases the proliferative ability of PAM212 cells, as determined by a BrdU incorporation assay (4 hours; $n = 4$ per group) and increased expression of cyclins A and D1. **D:** *In vivo* tumor growth was assessed as in Figure 1D. *Cav1* knockdown results in a dramatic increase in tumor volume and weight. **Inset:** Tumor growth 4 weeks after injection. Results are reported as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by the unpaired *t*-test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Q30

using a second shRNA construct (Supplemental Figure S1). Cav1 ablation in these cells increased BrdU incorporation by 1.9-fold, tumor volume by 3.6-fold, and tumor weight by 2.3-fold. Finally, we showed that re-expressing an shRNA-resistant Cav1 in both shCav1 cell lines rescued the increase in cell growth caused by *Cav1* knockdown in these cells (Supplemental Figure S2). Collectively, these data illustrated the growth-inhibitory function of Cav1 in PAM212 cells.

Cav1 Knockdown Increases the Invasive and Metastatic Ability of PAM212 Cells

To determine whether *Cav1* knockdown affected the metastatic ability of these cells, we undertook a spontaneous metastasis study. Tumors were allowed to grow for 5 weeks and were excised, and animals were examined for evidence of visible lymph node metastasis 4 weeks after excision. Interestingly, down-regulation of *Cav1* significantly increased the incidence of visible lymph node metastasis from approximately 50% to approximately 100%. In addition, lymph nodes dissected from animals with metastasis were significantly larger (approximately threefold) in mice injected with shCav1 cells versus shCtl-injected animals (Figure 3A). [F3]

To determine why shCav1 tumors colonized a secondary site with greater frequency, we examined the histological characteristics of the primary tumors. Accordingly, histological analysis revealed abundant keratin pearls (pink deposits) in shCtl tumors, an indication of a well-differentiated lesion.[28] shCav1 primary tumors also showed a dramatic increase in the expression of K18, a marker for less-differentiated and more invasive SCCs[29–33] (Figure 3B). In addition, CD31 staining suggested that shCav1 tumors showed a trend toward greater vessel density (Figure 3C), a requirement for tumor growth and metastasis.[34,35]

Finally, we examined the *in vitro* migratory and invasive ability of these cells using transwell assays. *Cav1* knockdown in PAM212 conferred an increased ability to migrate through a transwell chamber by approximately fourfold and to invade through Matrigel by 11-fold (Figure 3D). This enhanced invasion was associated with increased expression of matrix metalloproteinase 2, a protein involved in extracellular matrix breakdown during tumor cell invasion and metastasis[36,37];

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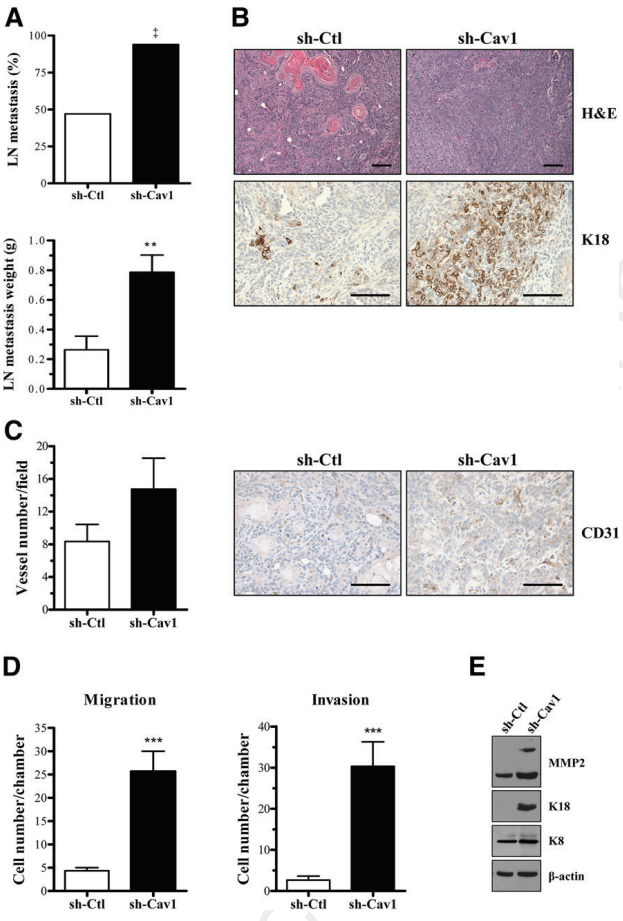

Figure 3 *Cav1* knockdown increases the invasive and metastatic ability of PAM212. **A:** Spontaneous metastasis formation was examined 9 weeks after intradermal injection of shCtl and shCav1 cells. *Cav1* knockdown results in an increase in the incidence of visible lymph node metastasis (approximately twofold) and weight ($n \geq 15$ per group). $^{\ddagger}P < 0.01$ by Fisher's exact test. **B:** Representative H&E staining of paired shCtl and shCav1 primary tumors demonstrate that both cell types form squamous cell carcinomas. There is increased expression of K18 in shCav1 tumors, indicative of a less-differentiated tumor. **C:** CD31 IHC staining reveals a trend toward increased vessel formation in *Cav1* knockdown cells ($n \geq 4$ per group). **D:** By using 10% serum as a chemoattractant, transwell migration and invasion assays show that *Cav1* knockdown dramatically increases the ability of PAM212 to migrate through a transwell chamber and invade through Matrigel ($n = 6$ per group). **E:** Western blot analysis of invasive markers shows that *Cav1* knockdown increases the expression of matrix metalloproteinase (MMP) 2, K18, and its binding partner, K8. Results are reported as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired *t*-test. Scale bar = 10 μm.

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K18; and its binding partner K8, which is also associated with invasion[29–33] (Figure 3E and Supplemental Figure S1). In addition, overexpression of Cav1 had the opposite effect on migration, invasion, and K8 and K18 expression (Figure 4, A and B). *Cav1* overexpression decreased K8 and K18 expression and migratory ability by threefold and invasive ability by 1.9-fold. Collectively, these data suggested that *Cav1* knockdown positively affected both the *in vitro* and *in vivo* invasive capacity of these cells.

Cav1 Knockdown Increases MAPK Pathway Activation in Response to Serum or Stimulation with EGF

To determine a mechanism by which *Cav1* knockdown enhanced tumor growth and invasion in PAM212 cells, we examined signaling pathway activation in response to various growth stimuli. When grown in medium containing FBS, shCav1 PAM212 cells showed increased Erk1/2 activation, whereas *p*-EGFR expression levels were unchanged. Serum-starved shCav1 PAM212 cells treated with EGF displayed enhanced *p*-EGFR at 1 minute after treatment, and *p*-Erk1/2 expression levels increased after 1 and 10 minutes (Figure 5A). Notably, *Cav1* knockdown increased the ability of PAM212 cells to migrate through a transwell chamber (approximately ninefold) and invade through Matrigel (approximately twofold) when EGF was used as a chemoattractant (Figure 5B).

An examination of signaling molecules downstream of Erk1/2 activation revealed that shCav1 PAM212 cells grown in medium containing FBS showed increased total

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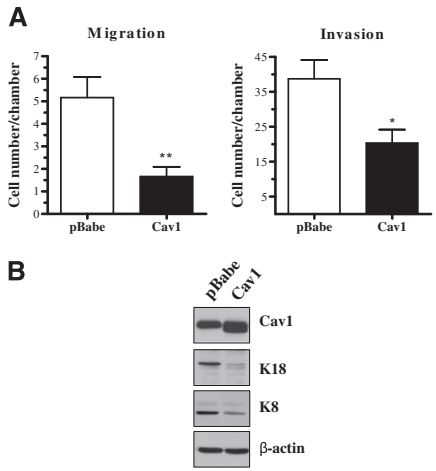

Figure 4 Cav1 overexpression decreases migration, invasion, and expression of K8 and K18 in PAM212 cells. **A**: By using 10% serum as a chemoattractant, transwell assays show that *Cav1* overexpression decreases the ability of PAM212 to migrate and invade ($n \geq 3$ per group). **B**: Western blot analysis shows that Cav1 overexpression decreases the expression of both K8 and K18. Results are reported as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ by the unpaired *t*-test.

c-Fos and *p*-c-Jun levels. Similarly, serum-starved cells treated with EGF showed increased total c-Fos and *p*-c-Jun levels by 60 minutes after treatment, indicating that loss of Cav1 enhanced MAPK and AP-1 transcription factor activation (Figure 5C). Finally, shCav1 cells stimulated by either serum or EGF showed an increase in the expression of K18, an AP-1–responsive gene,[32,38–41] and its binding partner, K8. This phenotype was reversed by Cav1 overexpression (Figure 4B). These findings were consistent with our *in vivo* results (Figure 3B) and with previous studies that have shown that K8 and K18 expression positively correlated with invasive potential in several cell types,[42–45] including PAM212,[33] and in mouse and human skin tumors.[29,33] In addition, RT-qPCR analysis of AP-1 target genes in *Cav1* knockdown cells revealed increased expression of genes associated with tumor growth and invasion (Figure 5D).

Collectively, these data indicated that the MAPK pathway was hyperactivated in *Cav1* knockdown cells in response to two different growth stimuli, which, in turn, increased AP-1 activation and increased transcription of AP-1 target genes.

Dual Inhibition of Erk1/2 and JNK Decreases Expression of K18 and Eliminated the Invasive Advantage Conferred by *Cav1* Knockdown in PAM212 Cells

To assess the functional significance of MAPK activation in shCav1 PAM212 cells, we treated cells with inhibitors for both the Erk1/2 and JNK pathways. JNK is another activator of AP-1 signaling through its ability to phosphorylate c-Jun. U0126 is an MAPK inhibitor, a molecule that activates Erk1/2, and SP600125 is a JNK inhibitor. We confirmed that 40 μmol/L of each inhibitor was sufficient to decrease its respective signaling pathways (Figure 6A). In addition,

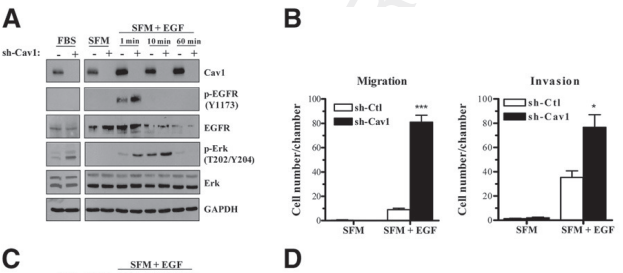

Figure 5 *Cav1* knockdown increases MAPK pathway activation in response to serum or stimulation with EGF. **A**: Western blot analysis of shCtl and shCav1 PAM212 cells grown in complete media with serum (**left panel**) or serum starved for 18 hours and treated with 50 ng/mL EGF (**right panel**). *Cav1* knockdown cells treated with EGF show an increase in EGFR activation. When grown in either complete or EGF-supplemented media, *Cav1* knockdown cells show an increase in activated Erk1/2. **B**: *Cav1* knockdown increases the ability of PAM212 to migrate and invade through transwell chambers when EGF is used as the chemoattractant in serum-free conditions ($n \geq 3$ per group). Results are reported as mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$ by the unpaired *t*-test. **C**: Examination of signaling molecules downstream of MAPK activation reveals that shCav1 cells grown in serum or EGF show an increase in total c-Fos protein and activated c-Jun, an indication that these cells have increased activity of the AP-1 transcription factor. In addition, *Cav1* knockdown increases expression of K18, an AP-1–responsive gene, and its binding partner, K8. **D**: RT-qPCR analysis shows increased expression of AP-1 target genes in *Cav1* knockdown PAM212 cells ($n = 3$ per group). Results are reported as mean ± SEM. $^*P < 0.05$ by Dunnet's multiple comparisons test.

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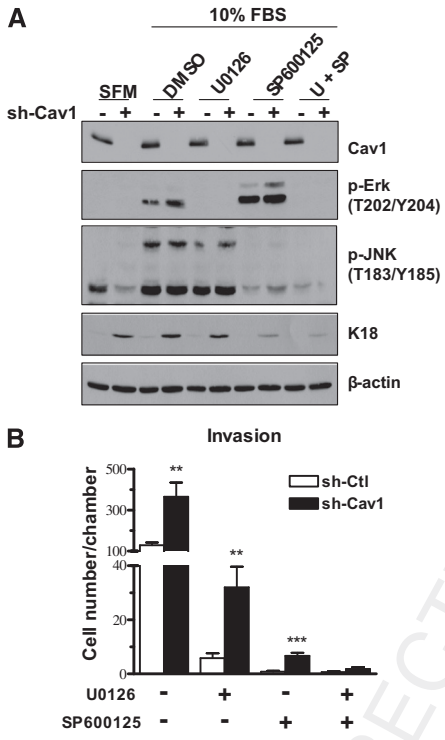

Figure 6 Dual inhibition of Erk1/2 and JNK signaling decreases expression of K18 and abolishes the invasive difference conferred by *Cav1* knockdown in PAM212. **A:** Western blot analysis of PAM212 cells treated with 40 μmol/L of U0126, an MAPK inhibitor, or SP600125, a JNK inhibitor, either singly or in combination. Dual inhibition results in the greatest decrease in K18. **B:** A Matrigel invasion assay shows that inhibition of either Erk1/2 or JNK decreases the overall invasive ability of both shCtl and shCav1 PAM212 cells, but does not eliminate the difference between the two groups. When both inhibitors are used in combination, the significant difference in invasive ability conferred by *Cav1* knockdown is abolished ($n = 3$ per group). Results are reported as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ by the unpaired *t*-test. DMSO, dimethyl sulfoxide.

we verified that inhibiting these pathways decreased the expression of K18. Maximum inhibition of K18 expression occurred when both pathways were inhibited, indicating both pathways were contributing to the expression of this protein. Finally, an invasion assay performed with these inhibitors showed that both Erk1/2 and JNK inhibition dramatically decreased the invasive ability of both shCtl and shCav1 PAM212 cells; however, dual inhibition was necessary to abolish the significant increase in invasion observed in shCav1 cells (Figure 6B). These results indicated that the invasive advantage conferred by *Cav1* knockdown was mediated through two different MAPK pathways: Erk1/2 and JNK. In addition, K18 could be a downstream mediator of the invasive phenotype, because this was one protein that was affected by inhibition of these two pathways.

Cav1 Expression Is Decreased in the Human cSCC Cell Line, SCC13, and Cav1 Overexpression Decreases *in Vitro* Proliferation, Migration, and Invasion

To assess whether our results in PAM212 translated to human cells, we used the human cSCC cell line, SCC13. We first compared Cav1 expression in these cells with that of the human immortalized (ie, non-transformed) keratinocyte cell line, HaCaT (Figure 7A). SCC13 carcinoma cells showed [F7] a dramatic decrease in Cav1 and Cav2 expression compared with noncancerous cells. Next, we successfully expressed either pBabe empty vector or Cav1 overexpression vector in these cells without altering Cav2 expression (Figure 7B). Cav1 overexpression resulted in a significant decrease in cell growth, as evidenced by a growth curve (Figure 7C), and in cell migration and invasion (Figure 7D), as evidenced by transwell chamber assays. Surprisingly, despite the similar phenotype conferred by Cav1 overexpression in PAM212 and SCC13, Erk activation and K18 expression levels were largely unchanged by Cav1 overexpression in SCC13 cells (Figure 7E). These results indicated that the inhibitory role of Cav1 in growth and invasion acted through a different mechanism than that observed in PAM212. However, these data indicated that the phenotype conferred by Cav1 in murine PAM212 cells could be recapitulated in the human cell line, SCC13.

Discussion

In the present study, we have examined the function of Cav1 in the development and progression of skin cancer. By using a murine model of cutaneous squamous cell carcinoma, the PAM212 cell line, we provide evidence that loss of Cav1 functions in primary tumor growth and also in the progression of primary tumors into less-differentiated and metastatic lesions. PAM212 cells are spontaneously transformed BALB/c keratinocytes.[18] They are commonly used in skin research to study *in vivo* tumor growth,[18] gene and protein expression changes during tumor progression,[46–51] and response to cytokines and chemicals.[50–53] PAM212 cells form tumors with 100% incidence but display a lower incidence of metastatic dissemination,[48,54] making them an ideal model to study changes in both tumor growth and progression. By altering Cav1 expression in these cells, we assessed the effect of this protein on both primary and secondary tumor formation.

We first show that *Cav1* overexpression decreases cell and tumor growth, whereas *Cav1* knockdown increases these attributes in PAM212 cells. Interestingly, knockdown of *Cav1* also affects the invasive characteristics of these

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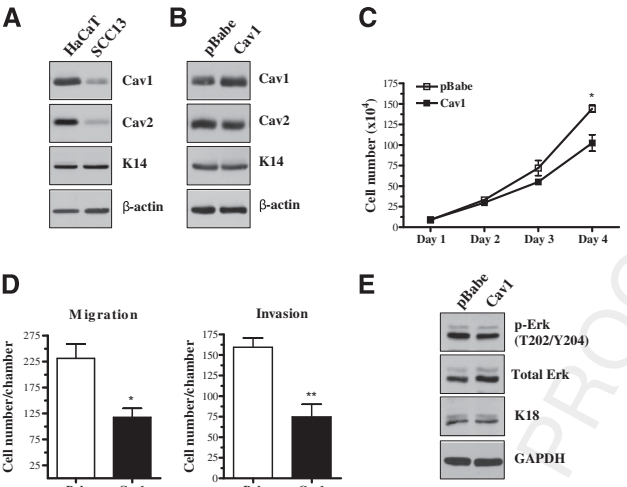

Figure 7 Cav1 expression is decreased in the human cSCC cell line, SCC13, and Cav1 overexpression decreases *in vitro* proliferation, migration, and invasion. **A:** Western blot analysis shows that Cav1 expression is decreased in SCC13 compared with the immortalized human keratinocyte cell line, HaCaT. Cav2 expression is also decreased, whereas K14 expression is unaffected. **B:** Western blot analysis shows successful overexpression of Cav1 in SCC13, whereas Cav2 and K14 expression levels are unaffected. **C:** Cav1 overexpression decreases cell growth in SCC13, as indicated by a growth curve ($n = 3$ per group). **D:** Transwell assays show that overexpression of Cav1 decreases both the migratory and invasive ability of these cells ($n = 4$ per group). **E:** Western blot analysis shows that Cav1 overexpression does not affect Erk activation or the expression of K18. Results are reported as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ by the unpaired *t*-test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells, as evidenced by an increase in the following: i) *in vitro* migratory and invasive ability, ii) vessel density and expression of K18 in primary tumors, and iii) incidence of spontaneous lymph node metastasis. Finally, we show that, in PAM212 cells stimulated with either serum or EGF, *Cav1* knockdown increases Erk1/2 MAPK pathway and AP-1 transcription factor activation. We attribute the increase in invasive ability observed in *Cav1* knockdown cells to downstream mediators of the MAPK and JNK pathways, because dual inhibition of these pathways abolishes the increase in invasion conferred by *Cav1* knockdown. We suggest K18 as one of these mediators. To our knowledge, we provide the first evidence that Cav1 functions in the development and progression of cutaneous squamous cell carcinoma.

Significantly, we corroborated some of our results in a human cSCC cell line, SCC13. SCC13 has substantially lowered levels of both Cav1 and Cav2 compared with a nontransformed human keratinocyte cell line. Overexpression of Cav1 decreased *in vitro* proliferation, migration, and invasion, but it did not affect Erk1/2 activation or K18 expression, as observed in PAM212 cells. One possible explanation is that the level of Cav1 overexpression in SCC13 is lower than that observed in PAM212 cells, potentially making the phenotype less striking. These results indicate that Cav1 functions in the modulation of the malignant phenotype through a different, as yet undetermined, mechanism that is independent of MAPK signaling in these cells.

Our work demonstrates that altered Cav1 expression affects the growth of primary PAM212 tumors and also their level of differentiation and their ability to invade surrounding tissues and metastasize. Given this, we sought to determine a mechanism for the effect of Cav1 on these aspects of tumor biological characteristics. The MAPK pathways are key regulators of cellular proliferation[55,56] and are frequently hyperactivated in cancer.[57–59] One of the downstream mediators of both the Ras-Erk1/2 and the JNK MAPK cascades is the AP-1 transcription factor, composed of both c-Fos and c-Jun proteins.[60–62] Previous work has implicated Cav1 in the negative regulation of the Ras-Erk1/2 MAPK cascade[63,64] and AP-1 transcription factor activation.[65,66] Our current work establishes Cav1 as a key regulator of Erk1/2 MAPK pathway activation in PAM212. By using two different stimuli, we showed increased Erk1/2 activation in *Cav1* knockdown PAM212 cells. In turn, this corresponded to an increase in the expression and activation of AP-1 members, specifically c-Fos and c-Jun. Interestingly, MAPK and AP-1 activation affects the expression of proteins that promote both proliferation and invasion.

The Ras-Erk1/2 MAPK cascade is a critical controller of cell cycle progression in many cell types. Activated Erk1/2 positively regulates cell cycle progression in several ways by contributing to ribosomal RNA and protein synthesis in preparation for translation and inducing positive regulators of cell cycle progression.[56,59] In addition, Erk1/2 activation is essential to G_1-to-S phase progression in many cell types. This is accomplished through increasing the expression and stabilization of AP-1 transcription factor members, which, in turn, promote expression of cyclin D1.[55] Interestingly, we show that overexpression of *Cav1* decreases cyclin D1 expression, whereas knockdown increases expression in PAM212. Our results indicate that *Cav1* knockdown results in hyperactivation of the MAPK pathway, which, in turn, drives proliferation in these cells.

Erk1/2 and AP-1 activation have also been implicated as positive regulators of migration, invasion, and metastasis.

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Specifically, activated Erk1/2 phosphorylates members of focal adhesions promoting motility and, via AP-1, it can induce expression of matrix metalloproteinases that promote extracellular matrix breakdown and invasion.[58,59] In addition, previous work has shown that the *K18* gene has an AP-1–responsive element.[32,38–41] K18 and its binding partner, K8, are intermediate filament molecules normally expressed in non-stratified epithelia.[32] Interestingly, *Cav1* knockdown cells show an increase in both K8 and K18, whereas cells in which *Cav1* is overexpressed show a decrease in these keratins. K8 and K18 have been associated with increased invasion in a variety of cell types.[42–45] Recently, Yamashiro et al[33] have shown that lymph node metastatic derivatives of PAM212 (LY-1 and 2) overexpress both K8 and K18 compared with parental PAM212 cells. Furthermore, the overexpression of K8 and K18 in parental PAM212 confers a more invasive phenotype. Previous work has also shown that both keratins are aberrantly expressed in skin tumors, particularly in less-differentiated areas, and are associated with invasion in human cSCC.[29,33] Given this work, we hypothesized that K8 and K18 are mediators of the phenotype observed in *Cav1* knockdown PAM212 cells. Although previous work has shown that the K8 promoter contains binding sites for the Ras/Erk1/2-responsive Ets transcription factors, relatively little is known about *K8* gene regulation[32,67]; therefore, we sought to inhibit K18 expression by exploiting its known regulation by AP-1. We show that Erk1/2 inhibition modestly decreases K18 expression; to achieve maximum inhibition of K18, it is necessary to dually inhibit both Erk1/2 and JNK, another activator of AP-1 activity. Dual inhibition rescues the increase observed in invasive ability when *Cav1* is knocked down in these cells. This dual contribution of the Erk1/2 and JNK MAPK pathways to cancer development has also been reported in melanoma, in which the two pathways can operate in a feed-forward mechanism, contributing to transcription of downstream target genes, such as cyclin D1.[68] These results indicate that *Cav1* knockdown increases activation of Erk1/2 and AP-1, which, in turn, increase invasive ability; in addition, one of the potential downstream mediators of this phenotype is K18. Our results are interesting given previous studies showing a potential role for Cav1 in both normal skin homeostasis and skin tumor biological characteristics.

Previous work has shown that Cav1 is strongly expressed in the basal layer of both murine and human skin,[14,69] and that expression of Cav1 is lost in a portion of human cSCC tumors.[17] Mechanistically, loss of Cav1 in a subpopulation of human tumors could be an independent event in tumor development or could occur secondarily to other signaling events. As an autonomous event, Cav1 expression or function is lost in various cancers because of the following: i) loss of the chromosomal fragile site where the human *CAV1* gene is located,[70–72] ii) aberrant promoter methylation,[73–76] or iii) protein mislocalization due to mutation.[73,77] Alternatively, loss of Cav1 in skin tumors could be a consequence of other molecular events. For example, previous work has shown that cellular transformation with the H-Ras^{G12V} oncogene decreases *Cav1* promoter activity[78] and results in down-regulation of both caveolin-1 mRNA and protein.[79] Moreover, p53 is a positive regulator of *Cav1* gene transcription.[80] Therefore, p53 loss or Ras activation, two alterations commonly observed in skin cancer,[81–84] could both negatively affect Cav1 expression, providing a potential mechanism by which a decrease in Cav1 protein levels is observed in a subpopulation of cutaneous squamous cell carcinomas. However, the amount of Cav1 expressed in skin tumors with these other molecular alterations could subsequently affect cellular signaling, altering the course of tumor development and progression in this type of cancer. In relation to our current findings, loss of Cav1 after Ras activation could result in a positive feedback loop with even greater Erk1/2 MAPK activation and increased expression of downstream molecules, such as cyclin D1 and K18. Interestingly, we find that our PAM212 cells express constitutively active Ras (Ras^{G12V}) and that the expression of this protein is increased by *Cav1*

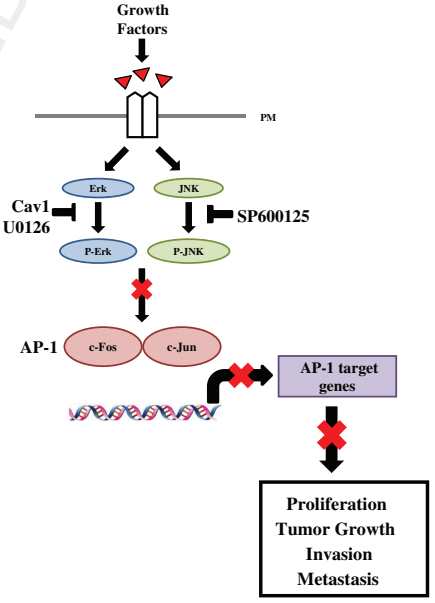

Figure 8 Proposed model for Cav1-mediated inhibition of tumor growth and metastasis in cSCC. Cav1 functions to suppress cell signaling in response to growth factors (eg, serum or EGF) in PAM212 cells, resulting in reduced activation of Erk1/2. This, in turn, decreases expression and activation of c-Fos and c-Jun proteins that combine to form the AP-1 transcription factor, subsequently decreasing transcription of AP-1 target genes. The end result is a decrease in proliferation, tumor growth, invasion, and metastasis. PM, plasma membrane.

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knockdown and decreased by *Cav1* overexpression (Supplemental Figure S3). These results show that, in the context of oncogenic Ras signaling, loss of Cav1, in this case through genetic manipulation, is able to function in a positive feedback loop by augmenting expression of the oncogene. Presumably, this augmentation could exacerbate the malignant phenotype, as demonstrated herein by our work in PAM212. The expression level of Cav1, therefore, could be an important modifier of the skin tumor phenotype.

Our results show that Cav1 affects two major aspects of skin tumor biological characteristics: proliferation and growth of primary tumors and invasion and metastasis to secondary sites. We implicate Cav1 as a modulator of the Ras-Erk1/2 MAPK cascade and AP-1 transcription factor activity. These proteins affect both proliferation and invasion (Figure 8). Our data show that *Cav1* knockdown increases the ability of PAM212 cells to colonize a secondary site. As in many cancers, patient mortality with cSCC is significantly increased in metastatic disease.[5,85] In human tumors, characteristics such as tumor size, degree of differentiation, and involvement of vasculature are used to assess risk of metastasis.[6,7] By using a murine model of cSCC, we show that *Cav1* knockdown increases tumor size, vessel density, and invasiveness. To our knowledge, this is the first study to indicate that Cav1 could be a modulator of invasive and metastatic potential in cutaneous squamous cell carcinoma. In conclusion, we show that Cav1 negatively regulates both the proliferative and invasive capacity of murine cSCC cells and that this is mechanistically associated with inhibition of MAPK and AP-1 activation.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2012.11.008*.

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Supplemental Figure S1 *Cav1* knockdown using a second shRNA construct confirms that loss of *Cav1* in PAM212 increases cell proliferation, migration, and invasion and *in vivo* tumor growth. **A:** Western blot analysis shows successful knockdown of Cav1 protein expression using a second shRNA construct. Similar to the original sh-Cav1 examined (sh-Cav1 1), *Cav1* knockdown using sh-Cav1 2 decreases expression of Cav2 but has no effect on K14 and K10 expression. **B:** Knockdown of *Cav1* using a second shRNA decreases cell proliferation, as shown via BrdU incorporation and a growth curve. The growth curve also shows cell growth for the original sh-Cav1 construct (sh-Cav1 1), and both sh-Cav1 1 and actual hash symbol 2 display similar rates of cell growth. Results for the growth curve are reported as mean $\pm$ SEM. $*P < 0.05$ by Dunnett's multiple comparisons test. **C:** sh-Cav1 2 PAM212 cells show a significant increase in tumor growth beginning at 4 weeks after injection. By 5 weeks after injection, tumor weight is approximately twofold higher in *Cav1* knockdown tumors. **D:** *Cav1* knockdown using sh-Cav1 2 decreases both the migratory and invasive ability of PAM212 cells when serum is used as a chemoattractant. In addition, decreased Cav1 expression increases the expression of K8 and K18 in these cells, similar to what we observe in the original sh-Cav1 cell line. Results are reported as mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ by the unpaired *t*-test. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Supplemental Figure S2 Overexpression of shRNA-resistant *Cav1* in *Cav1* knockdown cells rescues their proliferative advantage. **A:** *Cav1* knockdown using two different shRNA constructs (sh-Cav1 1 and actual hash symbol 2) results in a significant increase in cell growth, as indicated by a cell vitality MTS assay measured over 4 days. **B:** Re-expression of Cav1 in these knockdown cells results in the rescue of this growth advantage, as indicated by an MTS assay measured over 4 days. Re-expression of *Cav1* in shCav 1 cells actually decreases cell vitality to levels significantly lower than that of shCtl cells, whereas re-expression of *Cav1* in shCav1 2 cells decreases vitality to levels similar to that of shCtl cells. Results are reported as mean $\pm$ SEM. $**P < 0.01$ by Dunnett's multiple comparisons test.

Supplemental Figure S3 The expression of constitutively active Ras^{G12V} is regulated by Cav1. Western blot analysis showing that *Cav1* knockdown positively affects the expression of Ras^{G12V} whereas *Cav1* overexpression decreases the expression of Ras^{G12V}. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure S1.
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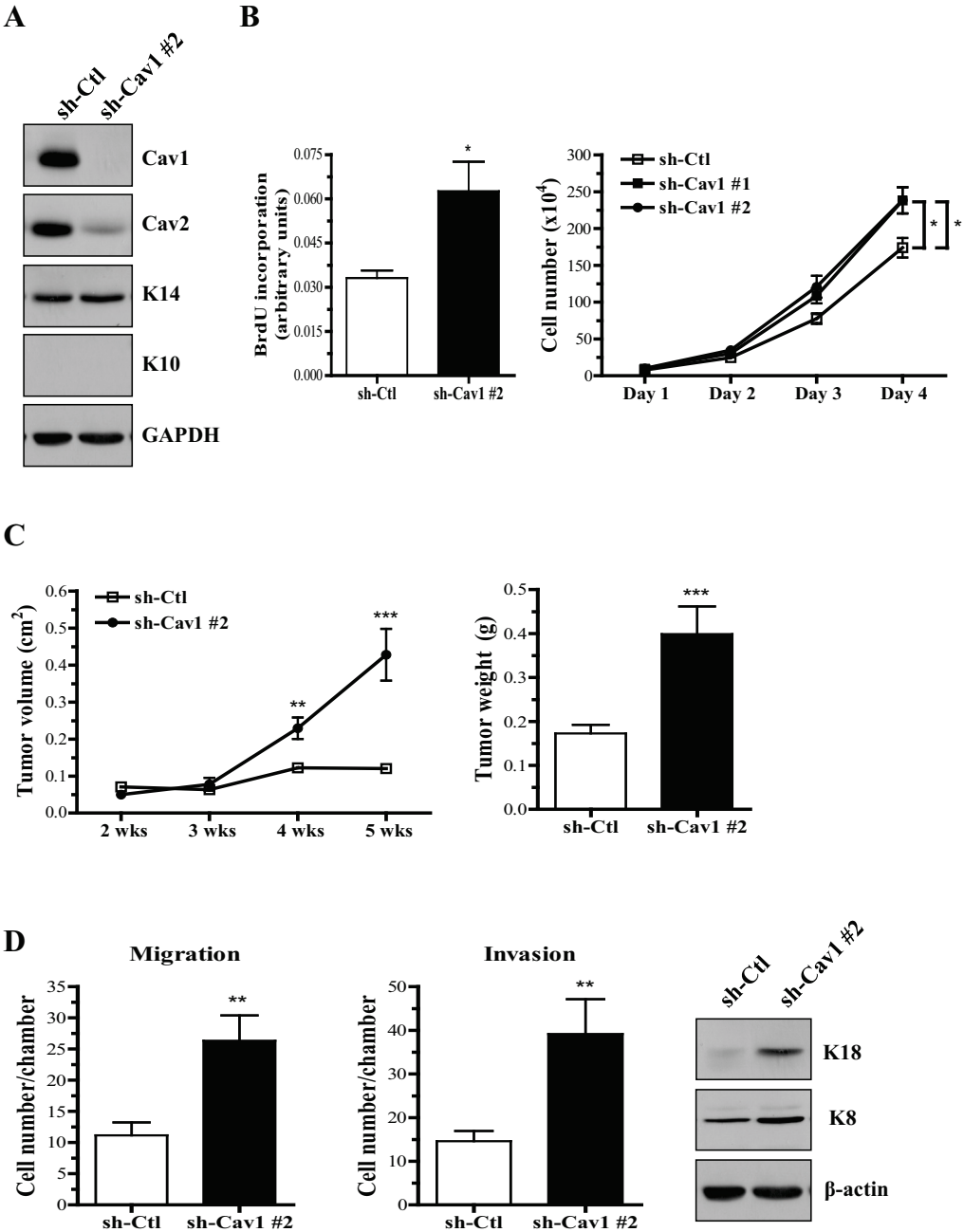


Figure S2.
Trimmer *et al.*

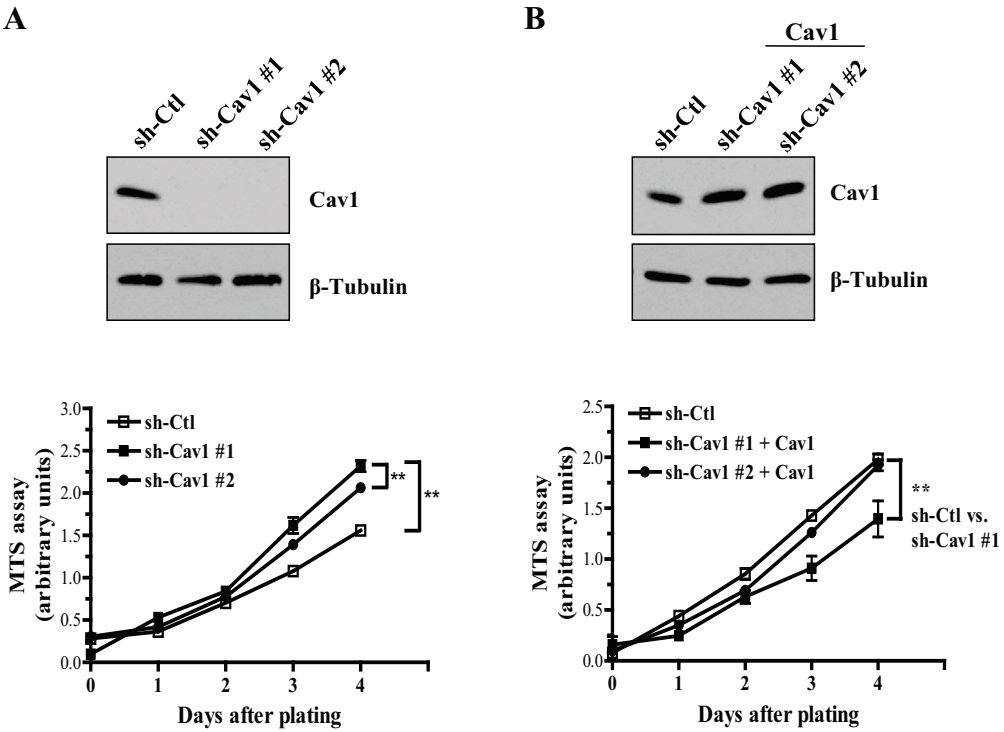
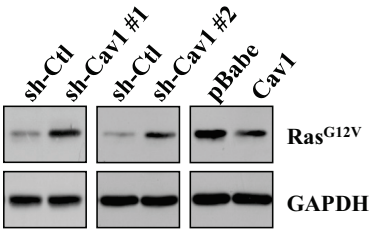


Figure S3.
Trimmer *et al.*



DISCUSSION AND CONCLUSIONS

Modulation of Cav1 expression in cancer cells or their surrounding stroma affects melanoma tumor growth and metastasis in mice.

The work illustrated in **Chapter1** and **Chapter2** of this thesis provides compelling evidence that modulation of Cav1 expression in melanoma cancer cells and/or their surrounding stroma affects the growth and the metastatic dissemination of this type of cancer. Dermal melanocytes derive from a population of cells that originate from the neuronal crest. Melanoma develops starting with a benign nevus, that progress to a hyperplastic lesion. The dysplastic nevus can subsequently progress to a radial growth phase (RGP) lesion that is confined to the epidermis with low invasive potential. In the vertical growth phase (VGP), melanoma cells become able to invade the dermis and are able to metastasize. Besides histological changes, the multistep process of melanomagenesis is characterized by well defined genetic and cellular alterations (signaling pathways, cell cycle, and cell adhesion) that contribute to its development. In melanoma progression, it seems critical the transition from radial melanoma (non invasive) to vertical melanoma (invasive). However, the mechanisms underlying this transition are not completely understood. Several genes have been shown to be upregulated or downregulated during this transition. Markers of angiogenesis and metalloproteases (VEGF, MMPs), are upregulated in vertical growth phase melanoma. Adhesion molecules such as αvβ3 and αvβ5 are observed over expressed in metastatic melanoma while E-Cadherin is decreased in metastatic lesions. Despite the progress in understanding the genetics and biochemistry of malignant melanoma, patients with metastatic disease have a very poor prognosis. Thus, identifying novel mechanisms and factors regulating melanoma progression may be critical for therapeutic intervention in this type of cancer (114). As described above, the function of Cav1 in tumorigenesis seems to be cancer type and/or stage dependent (115) and the specific contribution of Cav1 to primary tumor growth and metastasis in melanoma remains unexplored. Several groups have reported contradictory results for the role of CAV1 in melanoma transformation, migration, and invasion (116) (117) (108, 109). Therefore, to gain a better understanding of the function of Cav1 in melanoma progression, we used the B16F10 melanoma cell line as an experimental system to determine possible Cav1 regulated mechanisms in melanoma tumor growth and metastasis.

The results illustrated in **Chapter 1**, provide for the first time *in vivo* evidence that Cav1 may be functioning as a repressor of metastasis in malignant melanoma. We first showed that introduction of Cav1 by retroviral strategy in B16F10 cells resulted in increased cell proliferation *in vitro* without any affect on 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*. Interestingly, these results correlates with data obtained with human cancer cell lines and melanoma tissue. In fact, 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 expression levels. 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 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. Similar results have been reported by other groups demonstrating analogous reduced expression for Cav1 in melanoma progression using a tissue microarray-based approach (116) (85). 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 ECM-cell interactions being critical to acquire the metastatic phenotype. Integrins are families of surface heterodimeric molecules that link adhesion of different ECM components such as collagen and fibronectin to the actin cytoskeleton of the cell, and these interactions occur at focal adhesions (118). 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 interactions (119). 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 (110) (26). However, it appears 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 (26) (120) (121). Given 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 findings 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* (122) (123). Furthermore, 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 (124) (125) (126). In addition to alterations in Integrin/Src/FAK signaling, integrin engagement in these cells also affects proliferative and apoptotic pathways. When plated on fibronectin (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. These findings suggest that Cav1 promotes proliferative and suppresses apoptotic pathways only in the presence of a proliferative stimulus. In summary, the results provided herein provide evidence that Cav1 is behaving as an antimetastatic gene in melanoma.

Although most of previous cancer research has focused on cancer cells, there is an increasing amount of literature describing the ability of the tumor microenvironment and tumor stroma to support the growth of cancer cells. Tumors are very heterogeneous micro-environments that consist of multiple cell types. Significant amount of research indicates that stromal cells are crucial in contributing to tumor growth and progression. Cancer cells in the tumor are normally surrounded by a scaffold of extracellullar matrix (ECM) and by inflammatory, mesenchymal and endothelial cells (127) (56) (128). Cutaneous melanoma may develop from transformation of normal melanocytes or from preexisting lesions such as congenital nevi or dysplastic nevi. Melanoma cell invasions as well as metastasis formation are multi-factorial processes that require the cooperation of the surrounding stroma. It is becoming increasingly evident that during melanomagenesis melanoma cancer cells establish an active crosstalk with their stroma mainly mediated by direct cell-cell contacts or paracrine cytokine and growth factor signaling. Such crosstalk may activate the tumor stroma at primary or secondary sites and thereby contributing to tumor growth and metastatic dissemination. Cav1 is highly expressed in fibroblasts and endothelial cells two of the main components in the tumor stroma and manipulation of Cav1 expression has been shown to be critical in regulating angiogenesis (129), extracellular matrix deposition (85) and wound-healing (101). Accordingly, previous research has demonstrated that Cav1 KO mice display angiogenesis defects and impaired skin wound healing, suggesting that loss of Cav1 in the stromal compartment may functionally affect tumor growth (101) (130). As described in detail in **Chapter 2**, we decided to further examine this issue by determining whether stromal Cav1 may affect the growth and the metastatic ability of B16F10 melanoma cells. The work presented herein together with previous studies indicates that loss of stromal Cav1 increases 1) melanoma tumor growth, 2) secretion of protumorigenic cytokines by dermal fibroblasts and 3) reduces the transmigration of cancer cells through a monolayer of endothelial cells. Dermal fibroblasts are abundant cellular components of the skin and they exert important biologic functions to maintain normal skin homeostasis (131). Our co-injection experiments show that absence of Cav1 in dermal fibroblasts is sufficient to recapitulate the tumor phenotype of Cav1KO mice. On the basis of these findings, we postulated that Cav1- deficient fibroblasts promote the growth of melanoma cells by either direct cell–cell contact or paracrine signaling. Our results from coculture experiments suggest that the growth-promoting features of Cav1-deficient fibroblasts may be attributed to

enhanced paracrine signaling that does not require direct cell–cell contact. To identify possible secreted factors, we conducted a cytokine array on conditioned medium from serum-activated dermal fibroblasts. The increased secretion of cytokines, such as ShhN, MMP2/3, and bFGF, and the reduced expression of VEGFR2, HGF-R (132) and TIMP1 (MMPs inhibitor) observed in Cav1KO dermal fibroblasts further confirms their protumorigenic phenotype, and this cytokine signature correlates well with the tumor phenotype of Cav1KO mice. In addition, these results are in agreement with many published studies that identified similar factors associated with the stromal remodeling of tumors (133) (134). A key finding of our study is the increased amount of the soluble form of the Shh protein (ShhN) observed in the conditioned medium of serum activated Cav1KO dermal fibroblasts. Shh modulates many aspects of skin biology including wound healing (135), proliferation, and transformation (136). Furthermore, although Shh has been described to mainly function in an autocrine manner in melanomagenesis, it is now becoming increasingly evident that Shh may contribute to tumor growth in a paracrine manner. Interestingly, the proproliferative and protumorigenic effects of Cav1-deficient fibroblasts are reversed by inhibiting the Shh pathway with cyclopamine and by silencing Gli-1 in B16F10 cells. Another important key finding of our study is the inability of B16F10 cells to form lung metastases in Cav1KO mice. The dissemination of cancer cells to metastatic sites is a stepwise process that begins with the invasion of the dermis surrounding the primary tumor and ends with metastatic extravasation and colonization of ectopic sites (137) (138). Metastatic extravasation from the bloodstream is a critical last step of the metastatic cascade that similarly to leukocyte transmigration requires the firm binding of cancer cells to the endothelial adhesion molecules VCAM-1 and ICAM-1. Blockade of VCAM-1- and ICAM-1–mediated interactions has been shown to effectively prevent the development of metastasis in a preclinical setting (139) (140) (141). Given these considerations, our results showing reduced ICAM-1 and VCAM-1 expression in the lungs of TNFa-treated Cav1KO mice suggest that the inability of B16F10 cells to form metastases and to extravasate may be attributed to defects in VCAM-1 and ICAM-1– mediated adhesion to endothelial cells. Accordingly, previous studies have shown that the resistance of Cav1KO mice to atherosclerosis development may be attributed to impaired endothelial VCAM-1 and ICAM-1 functions that ultimately result in reduced inflammation and impaired macrophage migration throughout the endothelium (142, 143). In summary, our findings show that Cav1 is a new key mediator of tumor stromal interactions in melanoma and may function differently in primary tumors and or in the metastatic cascade. These studies support the notion that more effective anticancer therapies have to consider the complex interactions between cancer cells and their microenvironment in both primary tumors and metastases.

Caveolin-1 inhibits the promotion and progression stages of cSCC development
Skin cancer is a multi-stage disease that begins with initiation of a single cell of the epidermis. Next, the single cell clonally expands into a benign tumor, a process called promotion. Finally, the benign tumor progresses into a malignant and potentially metastatic tumor (94). Previous work from us and others has demonstrated that Cav1 KO mice are more susceptible to benign papilloma development following chronic treatment with the carcinogen DMBA and that Cav1 expression is significantly decreased in a significant portion of human skin tumors (107) (78). However, these studies do not examine the specific contribution of Cav1 along the continuum of multi-stage cutaneous squamous cell carcinoma and do not distinguish between the initiation, promotion and progression stages in Cav1 mediated skin carcinogenesis.

Thus, the work undertaken here in **Chapter 3** and **Chapter 4** provides experimental evidence that Caveolin-1 functions in both the promotion and progression stages of this disease. In the first study, a classic two-stage carcinogenesis protocol was undertaken (144). In this protocol, mice are subjected to an initiating dose of a carcinogen, herein DMBA, followed by twice-weekly treatments with a growth promoter, specifically 12-Otetradecanoylphorbol-13-acetate (TPA). Mice develop benign tumors called papillomas that, with continued TPA treatment, eventually progress into malignant squamous cell carcinomas. The molecular alterations that occur in the progression of these tumors are well characterized, and this protocol is a well-established method to determine the effect of protein gain or loss of function on tumor development in mice (144). The development of the tumors in this model is dependent on (1) survival of the initiated cells (i.e. resistance to apoptosis), (2) clonal expansion of the initiated cells into benign tumors (i.e. promotion of proliferation), and (3) progression of the benign tumor into a squamous cell carcinoma. (144). Previous work by Capozza and colleagues demonstrating that C57BL/6 *Cav1* KO mice are more sensitive DMBA induced benign papillomas does not allow for the separation of the different stages of tumor development (78). Given the increased sensitivity of the FVB mouse strain to DMBA/TPA induced skin carcinogenesis (145) (146), the study was performed using FVB/N wild-type (WT) and *Cav1* KO mice.

As detailed in **Chapter 3** and similarly to the study conducted by Capozza and colleagues, FVB *Cav1* KO mice are more susceptible to the development of benign papillomas using a two-stage carcinogenesis protocol. Specifically, *Cav1* KO mice display an increase, albeit non-significant, in incidence following 6 weeks of promotion. More significantly, *Cav1* KO mice have increased tumor multiplicity throughout the entire course of the study. After 6 months of promotion, KO mice have roughly two-fold more tumors than WT mice. In an effort to determine whether this change in multiplicity is a function of increased

initiated cell survival or increased clonal expansion of these cells, *Cav1* KO mice were treated separately with DMBA or TPA. DMBA initiates tumorgenesis by inducing mutation of the H-Ras oncogene in the epidermal stem cells of the hair follicle or the basal layer (144). These initiated cells either undergo apoptosis or survive to become a target for subsequent promoter treatment (144). Treatment of WT and KO mice with DMBA results in apoptosis mainly in the stem cell compartment of the hair follicle, as evidenced by TUNEL staining and as previously noted by other groups (147). Both WT and KO mice display a similar proportion of apoptotic hair follicles, indicating that loss of Cav1 has no effect on initiated cell survival following DMBA treatment. In contrast, the treatment of *Cav1* KO mice with TPA alone results in a significant increase in epidermal BrdU incorporation in comparison to WT mice. These results are in accordance with the study conducted by Capozza and colleagues in which KO mice subjected to chronic carcinogen treatment display increased hyperplasia and expansion of the epidermal proliferative compartment in comparison to WT mice. Furthermore, primary keratinocytes isolated from *Cav1* KO mice display an increased proliferative capacity. Thus, the results presented in **Chapter 3** indicate a role for Cav1 in suppressing the clonal expansion, or promotion, of DMBA initiated cells.

Cell proliferation plays a role not only in the expansion of initiated cells, but also farther along the continuum in the growth of malignant tumors. Further evidence for a role for Cav1 in growth inhibition comes from a second model of cSCC development, the PAM212 cell line. PAM212 cells are spontaneously-transformed murine keratinocytes capable of forming tumors in Balb/c Nude mice (148). As detailed in **Chapter 4**, PAM212 cells were stably transduced to either overexpress or down-regulate Cav1 via retroviral technology. Altered Cav1 expression has significant consequences for both *in vitro* cell growth and *in vivo* tumor growth. Cav1 overexpression decreases BrdU incorporation and the expression of cell cycle markers such as cyclin D1 and phospho-histone H3. Furthermore, Cav1 over-expression decreases tumor incidence from 100% in empty vector expressing cells to 50% in Cav1-expressing cells. Of the tumors that form, those that overexpress Cav1 are very small, roughly 30-fold smaller than empty vector-expressing tumors. In addition, the over-expression of Cav1 in a human squamous cell carcinoma cell line, SCC13 (149), results in a similar decrease in cell growth. In contrast, Cav1 knock-down in PAM212 keratinocytes results in the opposite phenotype: cellular proliferation and expression of cyclin D1 are increased *in vitro* and tumor growth is increased by 5-fold *in vivo*. Collectively, these results indicate that Cav1 contributes to growth suppression, during both benign and malignant tumor development. As previously mentioned, FVB/N mice are particularly susceptible to the development of SCCs when subjected to a two-stage carcinogenesis protocol (144), and this protocol was undertaken in part to observe the effect of Cav1 loss on this stage of carcinogenesis. Unfortunately, a difference

in SCC incidence and multiplicity was not observed using this model, in part because of unexpected mortality in the KO mice and the necessity of early sacrifice due to large tumor size. Despite these results, the PAM212 model allows for the assessment of the effect of modified Cav1 expression on features of tumor progression, including *in vitro* migratory and invasive ability and *in vivo* metastatic ability. Interestingly, Cav1 over-expression in both PAM212 and SCC13 cell lines decreases both migratory and invasive ability. In contrast, these characteristics are increased in PAM212 Cav1 knock-down cells. Furthermore, Cav1 knock-down in PAM212 cells increases their ability to spontaneously metastasize from the skin to the lymph nodes. Examination of the histology of primary tumors revealed that Cav1 knock-down significantly increases the expression of keratin 18, a marker for decreased differentiation and increased invasion in skin tumors (150) (151) (152). Furthermore, Cav1 expression is significantly decreased in the human cell line SCC13 in comparison to the immortalized, non-transformed human keratinocyte cell line HaCaT. These data correspond to work conducted in cell lines indicating that loss of Cav1 could correlate with the progression of human cSCC. Growth and progression of pre-malignant and malignant squamous skin tumors have the most significant consequences for human health due to the possibility of local disfigurement and metastasis. Interestingly, the present work indicates that Caveolin-1 is a *modifier* of several of these characteristics. Specifically, Cav1 functions as a growth suppressor in the promotion of both benign lesions and bona fide SCC tumors, demonstrated in two-stage carcinogenesis and xenograft models. Furthermore, in the PAM212 SCC model, the ability to spontaneously metastasize increases when Cav1 expression is lost. Accordingly, Cav1 knock-down in PAM212 increases *in vitro* invasive ability and *in vivo* expression of K18, a protein expressed in invasive skin tumors (152). These results were corroborated in human cSCC tumors where the expression of Cav1 is decreased in a significant portion of higher-grade (Grade II and III) tumors (*data not shown*).

The work contained herein in combination with previous research indicates that loss of Cav1 increases 1) epidermal hyperplasia in skin chronically treated with a carcinogen, 2) basal layer proliferation in response to a growth promoter, 3) benign tumor development, and 4) growth and progression of malignant cSCC tumors (**Chapters 3, 4**). Cav1 has been demonstrated to inhibit the activity of a multitude of signaling molecules through interaction with its scaffolding domain or protein internalization, and therefore many mechanisms could be contributing to these observed phenotypes. Early research demonstrated that Cav1 is a potent inhibitor of the Erk1/2 signaling cascade and of cyclin D1 expression (153). Given this previous research, aberrant regulation of members of the Ras/Erk1/2 MAPK cascade was a candidate mechanism for the effect of Cav1 loss on growth promotion. The first group to demonstrate a role for Cav1 in the regulation of

epidermal Erk1/2 was Capozza and colleagues; the authors showed that *Cav1* KO mice chronically treated with a carcinogen have increased epidermal expression of activated Erk1/2 in comparison to WT mice (78). In accordance with these results, Cav1 knock-down in PAM212 results in increased activation of Erk1/2 in comparison to control cells in response to two different growth stimuli: serum and epidermal growth factor (EGF) (**Chapter 4**). Furthermore, in the PAM212 model, Cav1 affects the expression of a signaling molecule upstream to Erk1/2, the small GTPase Ras. Ras mediates the transmission of extracellular growth signals to intracellular signaling pathways involved in cell proliferation and survival, including the Raf/Mek/Erk cascade which ultimately results in the phosphorylation and activation of Erk1/2 (154). Interestingly, PAM212 cells express a constitutively active form of Ras, RasG12V, and the expression of this protein varies indirectly with levels of Caveolin-1. Specifically, overexpression of Cav1 in PAM212 decreases expression of RasG12V, while Cav1 knock-down increases its levels (**Chapter 4**). Ras is commonly mutated in many human cancers, including cSCC and Ras mutations have been identified in up to 20% of human cSCCs (154) (155). In human cSCC, Ras mutations arise early in the continuum of tumor development and are observed in actinic keratoses (156). H-Ras is also the primary target of DMBA treatment in epidermal stem cells in the mouse two-stage carcinogenesis model, indicating an important role in tumor development (144). Indeed, expression of constitutively active Ras in mouse skin followed by chemical or genetic promotion is sufficient to cause tumor development in several models. In murine skin, Ras/Erk MAPK hyperactivation results in increased proliferation and defective differentiation, indicating that the activation level of this pathway controls the balance between proliferation and differentiation in the epidermis (157) (154). The role of the Ras/Erk MAPK cascade in normal epidermis and malignant skin tumors is interesting in light of the work presented herein. Ras expression is mainly confined to the basal layer of the epidermis, a pattern that corresponds to that of Cav1 (102). Given the function of Cav1 in the inhibition of multiple members of the Ras/Erk MAPK pathway, this expression pattern may seem counterintuitive in that one would expect Cav1 and Ras expression in different epidermal compartments. However, one hypothesis is that Cav1 is acting as a *brake* to Ras pathway over-activation in the basal layer of the epidermis. In other words, Cav1 is *maintaining* the activation level of this pathway. *Cav1* KO mice do not display epidermal hyperplasia in basal, unstimulated conditions. Presumably, the inhibition conferred by Cav1 is functioning in combination with other inhibitory pathways that are able to compensate under normal growth conditions. However, when the skin is subjected to chemical or physical insult, specifically carcinogens, phorbol esters, or barrier disruption, the critical function of Cav1 in inhibiting proliferation is revealed. *Cav1* KO mice chronically treated with DMBA display epidermal hyperplasia and enhanced Erk1/2 activation (78). In this context loss of the inhibitory signal

provided by Cav1 results in hyperactivation of the pathway. Similarly, treatment with TPA or tape stripping (barrier disruption), increases basal layer proliferation as evidenced by increased BrdU incorporation and PCNA expression, respectively (**Chapter 3**) (100). The inhibitory role of Cav1 on Ras/Erk MAPK cascade activation also has important implications in the development of skin tumors. The increase in tumor multiplicity observed in *Cav1* KO mice when treated with these chemicals indicates that Cav1 loss confers an enhanced sensitivity to the signaling pathways activated following treatment. Additionally, *Cav1* KO mice show enhanced proliferation following TPA treatment, indicating that the *promotion* stage, and not the initiating stage, is most sensitive to loss of Cav1. It is interesting to speculate that this increased sensitivity in the promotion stage is mediated through loss of Erk1/2 MAPK inhibition, ultimately resulting in an increase in benign tumor multiplicity. In the context of malignant tumors, altered Cav1 expression has a significant effect on the Ras/Erk MAPK cascade. Specifically, the expression of RasG12V in PAM212 is inversely related to the level of Cav1 expression. Given how commonly Ras is mutated in human cSCC, the ability of Cav1 to negatively regulate the expression of the oncogene could indicate that Cav1 is capable of *modifying* the tumor phenotype. The AP-1 transcription factor, comprised of both c-Fos and c-Jun, is a downstream mediator of the both the Ras/Erk1/2 and c-Jun N-terminal kinase (JNK) MAPK cascades (158) (159) (160). Erk1/2 functions to stabilize c-Fos, while JNK performs the same function for c-Jun; these two proteins dimerize to bind to DNA and activate the transcription of numerous target genes, many of which are involved in the regulation of proliferation and invasion (161). As described in **Chapter 4**, Cav1 knock-down in PAM212 results in an increase in total c-Fos and activated c-Jun levels in both serum and EGF-treated cells, indicating that AP-1 activation increases with loss of Cav1. Quantitative RT-PCR demonstrates Cav1 knock-down in PAM212 increases mRNA expression of other AP-1 target genes involved in migration and invasion in cancer cells, including matrix metalloproteinases (MMPs) 3 and 9, CD44, cathepsin-L, and keratin 18 (K18) (162, 163) (152). In line with these RT-PCR results, manipulation of Cav1 expression has dramatic effects on migratory and invasive ability in PAM212 and SCC13 cells (**Chapter 4**). Cav1 knock-down in PAM212 increases *in vitro* migration and invasion when either serum or EGF is used as the chemoattractant and *in vivo* lymph node metastasis. Interestingly, studies of mouse and human tissue demonstrate that AP-1 is a significant contributor to cutaneous squamous cell carcinoma development (164). Inhibition of AP-1-regulated signaling dramatically decreases benign tumor multiplicity and growth and halts malignant progression in mouse tumor models induced with activated Ras, chemicals, and ultraviolet radiation, and AP-1 activation may be essential for determining the squamous identity of SCC lesions (165) (98). Furthermore, nuclear localization and expression of c-Fos increases directly with progression to invasive lesions in human cSCCs, and the expression of activated

JNK and c-Jun are found in the majority of these tumors (164) (166) (167). Indeed, Ras activation in conjunction with c-Jun or JNK is sufficient to transform primary human keratinocytes (164) (166) (167). Collectively, this research combined with indicates that aberrant expression of these keratins may be a major mediator of the invasive and metastatic phenotype conferred by Cav1 knock-down.

CONCLUSIONS

Collectively, the work presented here in conjunction with previous studies demonstrate that Cav1 functions to modulate the tumor phenotype in both melanoma and non melanoma skin cancers. What may ultimately determine the contribution of Cav1 loss to epidermal cancers is the context of Cav1 loss, i.e. the presence of other genetic alterations. Loss of Cav1 is insufficient to cause an overt skin phenotype, as illustrated by the normal skin of *Cav1* KO mice; however, in conjunction with other molecular alterations, the loss of Cav1 may be an important *modifier* in skin cancers and other hyperproliferative diseases. In other words, Cav1 loss may increase the sensitivity of epidermal cells to molecular alterations, conferring an increased susceptibility to a variety of epidermal pathologies. In the context of benign skin diseases such as psoriasis, Cav1 loss may contribute to epidermal hyperplasia, a phenotype similar to the increased proliferation observed in Cav1 KO epidermis in experimentally-induced conditions. In models of benign tumor development, loss of Cav1 increases sensitivity to chemical promotion, and future research may demonstrate a similar role in human premalignant tumors. Finally, loss of Cav1 in malignant tumors contributes to tumor progression, conferring a more invasive and metastatic phenotype. Taken together, previous studies and the work herein demonstrate that the function of Cav1 is cancer specific and stage dependent indicating that Cav1 loss may be of prognostic value in predicting tumor recurrence and metastasis in both melanoma and non melanoma skin cancers. A useful future endeavor would be the stratification of human tumors based on Cav1 status followed by an examination of associated molecular alterations and tumor characteristics. Ultimately, careful examination of the genetic context of Cav1 loss will provide a better understanding of the mechanism of Cav1 action in both melanoma and non melanoma skin cancers and will demonstrate its utility as a prognostic and therapeutic target.

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