Caveolin-1 Gene Disruption Promotes Mammary Tumorigenesis and Dramatically Enhances Lung Metastasis in Vivo

ROLE OF CAV-1 IN CELL INVASIVENESS AND MATRIX METALLOPROTEINASE (MMP-2/9) SECRETION*

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Caveolin-1 (Cav-1) is the principal structural component of caveolae membrane domains in non-muscle cells, including mammary epithelia. There is now clear evidence that caveolin-1 influences the development of human cancers. For example, a dominant-negative mutation (P132L) in the Cav-1 gene has been detected in up to 16% of human breast cancer samples. However, the exact functional role of caveolin-1 remains controversial. Mechanistically, in cultured cell models, Cav-1 is known to function as a negative regulator of the Ras p42/44 MAP kinase cascade and as a transcriptional repressor of cyclin D1 gene expression, possibly explaining its in vitro transformation suppressor activity. Genetic validation of this hypothesis at the in vivo whole organismal level has been prevented by the lack of a Cav-1 (-/-)-null mouse model. Here, we examined the role of caveolin-1 in mammary tumorigenesis and lung metastasis using a molecular genetic approach. We interbred a well characterized transgenic mouse model of breast cancer, MMTV-PyMT (mouse mammary tumor virus-polyoma middle T antigen), with Cav-1 (-/-) mice. Then, we followed the onset and progression of mammary tumors and lung metastases in female mice over a 14-week period. Interestingly, PyMT/Cav-1 (-/-) mice showed an accelerated onset of mammary tumors, with increased multiplicity and tumor burden (2-fold). No significant differences were detected between PyMT/Cav-1 (+/+) and PyMT/Cav-1 (-/-) mice, indicating that complete loss of caveolin-1 is required to accelerate both tumorigenesis and metastasis. Molecularly, mammary tumor samples derived from PyMT/Cav-1 (-/-) mice showed ERK-1/2 hyperactivation, cyclin D1 up-regulation, and Rb hyperphosphorylation, consistent with dysregulated cell proliferation. PyMT/Cav-1 (-/-) mice also developed markedly advanced metastatic lung disease. Conversely, recombinant expression of Cav-1 in a highly metastatic PyMT mammary carcinoma-derived cell line, namely Met-1 cells, suppressed lung metastasis by ~4.5-fold. In vitro, these Cav-1-expressing Met-1 cells (Met-1/Cav-1) demonstrated a ~4.8-fold reduction in invasion through Matrigel-coated membranes. Interestingly, delivery of a cell permeable peptide encoding the caveolin-1 scaffolding domain (residues 82–101) into Met-1 cells was sufficient to inhibit invasion. Coincident with this decreased invasive index, Met-1/Cav-1 cells exhibited marked reductions in MMP-9 and MMP-2 secretion and associated gelatinolytic activity, as well as diminished ERK-1/2 signaling in response to growth factor stimulation. These results demonstrate, for the first time, that caveolin-1 is a potent suppressor of mammary tumor growth and metastasis using novel in vivo animal model approaches.

Caveolin-1 (Cav-1) was first discovered as a tyrosine-phosphorylated target in Rous sarcoma virus (RSV)-transformed avian fibroblasts, suggesting a possible role for this protein in cellular transformation (1). Subsequent studies identified caveolin-1 as a component of plasma membrane caveolae, small 50–100-nm omega-shaped invaginations involved in vesicular trafficking and cholesterol homeostasis (2, 3). Analysis of its protein expression pattern revealed that Cav-1 is found in a diverse range of cell types, including adipocytes, fibroblasts, endothelial cells, smooth muscle cells, and mammary epithelial cells (4–7). It is now clear that the majority of caveolae require caveolin-1 for proper formation, indicating that Cav-1 is a requisite caveolar structural protein. For example, Cav-1

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hypothesis is that loss of Cav-1 expression in vivo may lead to hyperactivation of p42/44 MAP kinase signaling. Potentially, this dysregulation could drive increases in tumorigenesis and/or metastasis in Cav-1 (-/-)-deficient mice, if they are provided with an oncogenic stimulus. Here, we test this hypothesis experimentally, using the mouse mammary gland as a model system.

Elucidation of a role for Cav-1 in cellular transformation began with the observation that Cav-1 is down-regulated in oncogenically transformed cells (20). Additional research demonstrated that the Cav-1 protein is down-regulated in human cancers, tumors derived from transgenic mice, and in human or rodent cancer cell lines, including breast tumors or breast cancer cell lines. Furthermore, Lee et al. (7) demonstrated that Cav-1 overexpression in human mammary cancer cells (T47D) results in tumor cell growth inhibition. Genetically, the Cav-1 gene is localized to a region on human chromosome 7q31.1 near microsatellite marker D7S522, a known fragile site (FRA7G) that is commonly deleted in a number of human cancers, including squamous cell carcinomas, ovarian carcinomas, colon carcinomas, and breast carcinomas (reviewed in Ref. 21).

In terms of mammalian breast cancer, one particular study demonstrated that up to 16% of patients with primary mammary tumors harbor a sporadic mutation at codon 132 (P132L) in Cav-1 (22). Interestingly, the Cav-1 (P132L) mutation behaves in a dominant-negative fashion, causing the intracellular retention of wild-type Cav-1 at the level of the Golgi complex (27). Recently, Han et al. (23) have also identified novel Cav-1 mutations in human oral squamous cell carcinomas.

Mechanistically, in cultured cell models, Cav-1 is known to function as a negative regulator of the Ras-p42/44 MAP kinase cascade and as a transcriptional repressor of cyclin D1 gene expression, possibly explaining its in vivo transformation suppressor activity. However, genetic validation of this hypothesis at the in vivo and whole organismal level has been prevented by the lack of a Cav-1 (-/-)-null mouse model.

In this report, we have employed MMTV-PyMT transgenic mice to directly explore the role of Cav-1 in tumor development and metastasis, using an autochthonous animal model. PyMT transgenic mice express high levels of this transforming oncogene under the control of the MMTV-LTR promoter, which specifically directs expression to the mammary epithelium (24). All female virgin PyMT transgenic mice rapidly develop multifocal mammary adenocarcinomas that are palpable, as early as 6–7 weeks of age. The benefit of this mouse tumor model over others is that female PyMT mice also develop pulmonary metastases by 3–4 months of age, with an extremely high penetrance (~90–100%). Importantly, this mouse tumor model has been shown to recapitulate human breast cancer progression, from early hyperplasia to malignant breast carcinoma, including the up-regulation of ErbB2/Neu and cyclin D1 expression (25, 26).

We interbred PyMT transgenic mice with Cav-1 null (−/−) mice to generate three genetically distinct cohorts of female mice that are all hemizygous for the PyMT transgene: 1) PyMT/Cav-1 (+/+); 2) PyMT/Cav-1 (−/−); and 3) PyMT/Cav-1 (−/−) mice. During a 14-week observation period, mice were evaluated for tumor onset, incidence, burden, and metastasis. We now demonstrate that genetic disruption of the caveolin-1 gene markedly increases tumorigenesis by reducing tumor latency, and increasing tumor multiplicity/burden, without altering histopathological progression. Mammary tumor samples derived from PyMT/Cav-1 (−/−) mice showed ERK-1/2 hyperactivation, cyclin D1 up-regulation, and Rb hyperphosphorylation, consistent with dysregulated cell proliferation. However, (i) no differences in PyMT expression or kinase-associated activity were detected in the absence of Cav-1; (ii) no alterations in the expression levels or phosphoactivation states among PyMT-associated proteins (Src, Shc, PI 3-kinase) were observed; and (iii) the genetic disruption of Cav-1 had no effect on the estrogen receptor or progesterone receptor status of these tumors. In addition, we find that an absence of caveolin-1 results in striking increases in the frequency of distant lung metastases from the primary site of origin. Finally, metastatic mammary tumor cells engineered to recombinantly express Cav-1 show significant reductions in Matrigel invasion and dramatically reduced MMP-9/MMP-2 activities. As such, this is the first demonstration that loss of caveolin-1 promotes mammary tumorigenesis and lung metastasis in an in vivo animal model.

**EXPERIMENTAL PROCEDURES**

**Materials and Expression Vectors—**Mouse monoclonal antibodies to caveolin-1 (clones 2297 and 2234) were the generous gift of Dr. Roberto Camps-Gonzalez (BD Pharmingen, Inc.). Rabbit polyclonal antibodies directed against total ERK-1/2, activated phospho-ERK-1/2, phospho-Rb (Ser-780), and phospho-Src (Tyr-416) were obtained from Cell Signaling, Inc. The anti-Rb rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology, Inc., as well as rabbit polyclonal antibodies to ER, PR, c-Src, and MMP-2. Monoclonal antibodies directed against phospho-Shc, phospho-Akt (Ser-473), and PI 3-kinase were obtained from BD Pharmingen, Inc. A rabbit polyclonal antibody specific for mouse MMP-9 was obtained from Tripl Tone Biologics, Inc. (Forest Grove, OR). Additional antibodies and their sources include anti-cyclin D1 rabbit pAb (NeoMarkers, Fremont, CA), anti-β-actin mAb AC-15 (Sigma), anti-β-tubulin mAb (Sigma), and anti-MT1-MMP mAb (Chemicon). Dr. William J. Muller generously provided a mouse monoclonal antibody (PAB 762) directed against PyMT. We have previously reported on the use of the retroviral vector pBABE for the delivery of cloned cDNAs (27). A construct bearing the PyMT cDNA was generously provided by Dr. Robert Freund. Murine EGF and FGF-2 were purchased from PeproTech, Inc. (Rocky Hill, NJ). Heparin, 4-aminophenylmercuric acetate (APMA), gelatin, and crystal violet were obtained from Sigma. GM6001 and N-sulfonylamino acid MMP-2/MMP-9-specific inhibitors I and II were all obtained from Calbiochem/EMD Biosciences, Inc. (San Diego, CA).

**Cell Culture—**All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Animal Studies—**All animals were housed and maintained in a barrier facility at the Institute for Animal Studies, Albert Einstein College of Medicine (AECOM). Mice were kept on a 12-hour light/dark cycle with ad libitum access to chow (Picolab 20, PMI Nutrition International) and water. Animal protocols used for this study were approved by the AECOM Institute for Animal Studies. Cav-1-null mice and MMTV-PyMT transgenic mice (strain 634) were generated as previously described (8, 28). All the mice used in this study were in the FVB/N background. Matings were performed with PyMT male hemizygous.
gous mice. PyMT/Cav-1 (+/−) and PyMT/Cav-1 (+/+) male mice were interbred with Cav-1 (+/+) or Cav-1 (−/−) female mice to generate a cohort of Cav-1 (+/−), Cav-1 (+/−), or Cav-1 (−/−) female mice, all hemizygous for the PyMT transgene. None of the PyMT transgenic, negative control mice developed tumors (n = 40). For the female tumor study, all the mice analyzed were virgin.

**Tumor Palpation and Excision**—Female mice were palpated twice weekly beginning at 6 weeks of age for the development of tumors in their mammary glands. Mice were examined in a genotype-blinded fashion and palpated in each of the ten mammary glands up until 12 weeks of age. Beginning at 12 weeks, female mice were sacrificed, and all mammary tumors were carefully excised and weighed. Portions of the tumors were also frozen in liquid nitrogen or stored in formalin for fixation purposes. Identical procedures were performed on mice sacrificed at 13 and 14 weeks of age. For the male tumor study, mice were sacrificed at 23 weeks of age, and all tumors were excised, weighed, and fixed in formalin.

**Histological Analysis of Mammary Tumors**—Mammary tumors were excised, cut into smaller portions, and fixed with 10% neutral buffered formalin for over 24 h before embedding in paraffin. Sections were cut at 5 microns, stained with hematoxylin and eosin, and evaluated by an experienced histopathologist (Dr. Neeru G. Chopra). Analyses, and descriptions were performed in accordance with the guidelines put forth by the mouse mammary gland pathology consensus meeting in Annapolis (29).

**Lung Metastasis Analysis**—Female PyMT mice at 13 and 14 weeks of age were sacrificed and the lungs exposed by thoracic and tracheal dissection. Before removal, lungs were injected with 2 ml of 10% neutral buffered formalin by tracheal cannulation in order to fix the inner airspaces and inflate the lung lobes. Lungs were then excised and placed into formalin for 48 h. Lung lobes were separated in order to visualize all surfaces of each lobe. During counting, an equal number of lobes were matched for size and location in the thorax. Representative lungs were also paraffin-embedded and processed for histological analysis. For the Met-1 cell metastasis study, 1 × 10^6 cells suspended in 0.1 ml of PBS were injected through the tail vein of female athymic nude mice for each cell line. After 3 weeks, the lungs were removed and inflated with formalin for 48 h. Lung lobes were separated in order to visualize all surfaces of each lobe. During counting, an equal number of lobes were matched for size and location in the thorax. Representative lungs were also paraffin-embedded and processed for histological analysis.

**Immunoblot Analysis**—150 to 200 mg portions of tumor tissue frozen in liquid nitrogen were homogenized in 2 ml of boiling lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 60 mM octyl glucoside), containing 15% India Ink. Lung homogenates on ice were homogenized by glass tissue grinders. Tissue lysates were then centrifuged at 12,000 × g for 10 min to remove insoluble debris. For analyzing PyMT expression, tissue lysates were also pre cleared with protein G following centrifugation. Protein concentrations were analyzed using the BCA reagent (Pierce) and the volume required for 20–100 µg of protein was determined. Tissue or cell lysates were separated by SDS-PAGE (8–12% acrylamide) and transferred to nitrocellulose. The nitrocellulose membranes were stained with Ponceau S (to visualize protein bands), followed by immunoblot analysis. Subsequent wash buffers contained 10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBS-Tween), which was supplemented with 1% bovine serum albumin (BSA) and 4% nonfat dry milk (Carnation) for the blocking solution and 1% BSA for the antibody diluent. For phosphoantibody analysis, the blocking solution contained only 5% BSA in TBS-Tween (without nonfat milk). Primary antibodies were used at a 1:500 dilution. Horseradish peroxidase-conjugated secondary antibodies (anti-mouse 1:5,000, Pierce) or anti-rabbit 1:5,000 (BD Pharmingen) were used to visualize bound antibodies, with the Supersignal chemiluminescence substrate (Pierce).

**Retroviral Transduction**—The retroviral pBABE constructs were transduced into Phoenix cells by a calcium phosphate precipitation method. About 6–9 h post-transfection, cells were washed once with PBS, and the media was changed to complete growth medium (DMEM, 10% FBS, glutamine, penicillin/streptomycin). Approximately 48 h post-transfection, the supernatant from transduced Phoenix cells was harvested, passed through a 0.45-µm filter to remove cellular debris, and mixed with polybrene (5 µg/ml). 2 × 10^5 cells of each cell line were infected with a 1:1 mixture of viral-containing supernatant and complete growth medium. Aspiration and infection was repeated every 12 h for a total of 3 times. To generate stable pools of pBABE-transfected cell lines, the cells were placed in complete growth media supplemented with puromycin (2.5 µg/ml) for 1 week, thus avoiding clonal variability.

**Cell Migration and Invasion Assays**—The invasive potential of the tumor-derived cell lines was measured by an in vitro Boyden chamber assay (30). Briefly, 10^5 cells in 0.5 ml of serum-free DMEM were added to the wells of 8 µm pore membrane Boyden chambers, either coated with (for invasion assays) or without (for migration assays) Matrigel (BD Biosciences). The lower chambers contained either 0.5 or 10% FBS in DMEM to serve as a chemoattractant. Cells were allowed to migrate or invade over the course of 24 h. Cells that had not penetrated the filters were removed by scrubbing with cotton swabs. Chambers were fixed in 100% methanol for 2 min, stained in 0.5% crystal violet for 2 min, rinsed in water, and examined under a bright-field microscope. Values for invasion and migration were obtained by counting 5 fields per membrane (×20 objective) and represent the average of three independent experiments performed over multiple days. For penetratin experiments, Met-1 cells were incubated with penetratin alone or in combination with Cav-1 (32). Penetratin concentration of 5 µM during the invasion assay. Penetratin peptides are the biotinylated 3-proline forms of penetratin that are excluded from the nucleus but are retained in the cytoplasm (31). For MMP inhibitor experiments, inhibitors were placed in both the lower and upper chambers (10 µM), and cells were allowed to invade over the course of 24 h. The K_i and I_{50} values for these inhibitors are as follows: GM6001, K_i = 500 µM for MMP-2 and K_i = 200 µM for MMP-9; MMP-2/MMP-9 Inhibite.
and were incubated in collagenase buffer (100 mM Tris-HCl, pH 8.0, 5 M CaCl$_2$, 0.02% NaN$_3$, and 30 mM DMEM) at 37°C for 30 min. Lytic bands were further sonicated for 5 s, and the supernatants were collected by centrifugation at 14,000 rpm for 30 min.  

MT-MMP Activity Assay—Equal numbers of cells were plated onto 15-cm plastic dishes and grown to confluence over 1–2 days. Plasma membrane preparations were obtained by washing the cells twice in PBS, collecting the cells with a cell scraper, and resuspending in 1 ml of ice-cold PBS containing protease inhibitors. Cells were washed twice with PBS then subjected to three rounds of freeze-thaw in dry ice-ethanol/37°C bath. Lysates were further sonicated for 5 s, and the membranes pelleted by centrifugation for 30 min at 4°C (14,000 rpm). Pelleted membranes were washed once and resuspended in 0.5 ml complete PBS. Protein concentrations were determined by the BCA reagent, and samples were diluted with complete PBS to obtain starting concentrations of 0.05 mg/ml. E. coli bioassay screening for MT-MMP activity was performed according to the manufacturer’s protocol (Chemicon, Temecula, CA). Briefly, varying dilutions of the starting membrane preparations were incubated in zymography gel media containing the MT-MMP substrate (MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$) before reading on a fluorometer plate reader using 320 nm excitation and 390 nm emission filters. Standard curves with the control peptide MT-MMP standard were generated to determine the appropriate sample dilution at which the values fell in the middle of the standard curves. For Met-1 cells, this was determined to be about 1–5 μg of the starting plasma membrane lysates.

Substrate Gel Electrophoresis (Zymography)—Secreted metalloproteinases and their gelatinolytic activity were measured by zymography (32). Equal numbers of cells were plated on 6-well culture dishes and allowed to reach confluence over 24 h. Cultures were washed in PBS and then incubated for 20 h in 1 ml serum-free DMEM in the absence or presence of EGF (100 ng/ml) or FGF-2 (10 ng/ml) with heparin (0.1 mg/ml). Conditioned media samples harvested from four duplicate wells were pooled (4 ml) and concentrated to 200 μl using Amicon Ultra-4 centrifugal filter devices with a 30 kDa exclusion limit (Millipore). Concentrated conditioned media samples (10 μl) were then loaded onto 8% SDS-PAGE gels that had been co-polymerized with 1 mg/ml gelatin. Electrophoresis was performed under non-reducing conditions at 100 volts for 2 h at 4°C. Gels were washed in 2.5% Triton X-100 for 30 min. and then incubated in collagenase buffer (100 mM Tris-HCl, pH 8.0, 5 mM CaCl$_2$, 0.02% NaN$_3$) for 40 h at 37°C. Gels were stained for 30 min. with 0.5% Coomassie Blue in destain solution (30% methanol, 10% acetic acid) and then destained three times for 15 min. The presence of gelatinolytic activity due to secreted metalloproteinases was indicated by an unstained proteolytic region in the gel (MMP-9 corresponds to ~92 kDa (gelatinase B); MMP-2 corresponds to ~72 kDa (gelatinase A) and were confirmed with positive controls from N-cadherin overexpression filters. Standard curves with the control peptide and MT1-MMP-positive control were generated to determine the appropriate sample dilution at which the values fell in the middle of the standard curves. For Met-1 cells, this was determined to be about 1–5 μg of the starting plasma membrane lysates.

RESULTS

Loss of Cav-1 Accelerates Mammary Tumor Onset and Multiplicity in PyMT Transgenic Mice—In order to assess the effect of loss of caveolin-1 on mammary tumor development, we interbred Cav-1 (−/−) null mice with MMTV-PyMT transgenic mice. Female offspring were subdivided into three groups that were all hemizygous for the PyMT transgene: (i) PyMT/Cav-1 (−/+), (ii) PyMT/Cav-1 (−/−), and (iii) PyMT/Cav-1 (−/−). Beginning at 6 weeks of age, mice in the study were palpated twice a week in all ten mammary glands for the development of tumors in a genotype-blinded fashion.

As depicted in Table I, PyMT/Cav-1 (−/−) mice showed a dramatically accelerated onset of a palpable tumor mass, as compared with PyMT/Cav-1 (−/+). No significant differences in palpable tumor onset were observed between the PyMT/Cav-1 (−/+) and PyMT/Cav-1 (−/−) groups. As a result, PyMT/Cav-1 (−/+) and PyMT/Cav-1 (−/−) tumor groups are shown grouped as one cohort. On average, PyMT/Cav-1 (−/−) mice possessed a palpable tumor in one of ten mammary glands by 56.6 days (~8 weeks) of age, 7 days earlier than their Cav-1 (+/+) and Cav-1 (−/+). 

| PyMT/Cav-1 | Mean age (days) | S.E. |
|------------|----------------|------|
| +/+, −/−   | 63.8           | 1.37 |
| +/−, −/−   | 56.6°          | 1.26 |

*p value < 0.0003. S.E., standard error of the mean.

| PyMT/Cav-1 | Mean age (days) | S.E. |
|------------|----------------|------|
| +/+, −/−   | 68.4           | 1.55 |
| +/−, −/−   | 59.5°          | 1.19 |

*p value < 0.00004.

Mice were continually monitored after the appearance of the first palpable tumor in order to determine when at least 5 of 10 mammary glands (50%) were positive for palpable tumors. As demonstrated in Table II, PyMT/Cav-1 (−/−) mice had palpable masses in at least 50% of their mammary glands ~9 days earlier than PyMT/Cav-1 (+/+, +/−) mice (p value < 0.00004). This observation suggests that loss of caveolin-1 not only affects the emergence of the first tumor, but also the frequency of tumors. Taken together, complete ablation of Cav-1 results in both decreased tumor latency and increased tumor multiplicity.

A Complete Absence of Cav-1 Increases Mammary Tumor Burden in PyMT Transgenic Mice—While the emergence of palpable tumors in the mammary gland is clearly affected by the absence of Cav-1, the growth of these small masses to large, bulky tumors may be unaffected. In order to assess this possibility, we sacrificed at least 10 mice per cohort at 12, 13, and 14 weeks of age, excised the tumors, and weighed them. By these ages, 100% of mammary glands in PyMT mice contained histologically identifiable tumors.

Taking the single largest tumor per mouse, PyMT/Cav-1 (−/−) mice show substantially increased tumor wet weight as compared with the PyMT/Cav-1 (+/+, +/−) cohort at 12, 13, and 14 weeks (Fig. 1A). This difference is ~1.7-fold at 12 weeks and climbs to 1.9- and 2.0-fold for 13 and 14 weeks, respectively.

As a reflection of total tumor burden, we also determined the total wet weight of the three largest tumors per mouse. The results, shown in Fig. 1B, demonstrate that the absence of Cav-1 results in increased total tumor burden, ranging from 1.5-fold at 12 weeks to 1.8–2.0-fold at 13 and 14 weeks. These results were extremely statistically significant at 13 and 14 weeks of age (p value < 0.0008) and nearly significant at 12 weeks of age (p value = 0.09). No significant differences in tumor weights were observed between PyMT/Cav-1 (+/+) and PyMT/Cav-1 (+/−) mice, indicating that partial loss of Cav-1 does not elicit the accelerated tumor development phenotype observed in those mice completely lacking Cav-1 (−/−).

In general, both the mean age when 50% of mammary glands have palpable masses and the mean age when 50% of mammary glands have palpable masses are all significantly affected by the loss of caveolin-1 on mammary tumor development, resulting in advanced tumor onset (decreased tumor latency), greater tumor multiplicity, and substantially increased tumor burden. These data indicate that the presence of Cav-1 in the mammary gland normally serves to suppress tumor development.

Gross and Histopathological Appearance of PyMT Mammary Tumors—Representative images of PyMT mice from both cohorts at 13 weeks are depicted in Fig. 2A. In general, both the size and frequency of tumors in PyMT/Cav-1 (−/−) mice were dramatically increased, as compared with mice from the PyMT/Cav-1 (+/+, +/−) cohort. Excision of the tumors from the two cohorts revealed that they generally appeared as pale firm masses, consistent with adenocarcinoma (Fig. 2B). However,
Note that PyMT/Cav-1 (−/−) mice demonstrate a substantial increase in the single largest tumor isolated from each mouse. The differences are 1.7-fold at 12 weeks, 1.9-fold at 13 weeks, and 2.0-fold at 14 weeks of age. No statistical differences were detected between PyMT/Cav-1 (+/+) and PyMT/Cav-1 (+/−) mice. B, bar graph representing the sum of the three largest tumors (a reflection of total tumor burden) excised from each mouse at 12, 13, and 14 weeks of age. Note that the absence of Cav-1 dramatically affects total tumor burden, resulting in increases of 1.5-fold at 12 weeks, 1.7-fold at 12 weeks, 1.9-fold at 13 weeks, and 2.0-fold at 14 weeks of age. No statistical differences were detected between PyMT/Cav-1 (−/−) mice.

In conclusion, we detected no differences in the pathological progression of the tumors, we characterized the tumors histopathologically. Our histopathologist examined at least ten tumors per cohort at both 12 and 14 weeks in a blinded fashion in accordance with the guidelines and recommendations set forth by the Annapolis Meeting on the mammary pathology of genetically engineered mice (29). Morphologically, tumors derived from PyMT/Cav-1 (+/+, +/−) and PyMT/Cav-1 (−/−) mice did not show any significant differences. All the tumors are clearly malignant and are morphologically carcinomas (Fig. 2C). Histologically, the grade of these carcinomas ranges from low to medium.

In conclusion, we detected no differences in the pathological grade of the tumors between both cohorts at the ages examined, suggesting that Cav-1 does not have an effect on the final stages of tumor histopathological progression to invasive carcinomas.

**Male PyMT/Cav-1 (−/−) Mice Develop Mammary Tumors with a Dramatically Increased Incidence**—Male mice harboring the PyMT transgene also develop mammary adenocarcinomas with 100% penetrance, albeit with significantly reduced tumor onset and frequency, presumably due to the lower hormonal inducibility of the MMTV promoter and the rudimentary development of the epithelial ductal system in the male mammary gland (24). These tumors are adenocarcinomas that are histologically identical to tumors in PyMT female mice. The benefit of studying tumor development in a male PyMT cohort is that potential differences in tumorigenesis-conferred by a modifier gene such as Cav-1—are likely to be accentuated due to the longer intervals of time needed for tumors to develop.

Therefore, we also generated cohorts of male PyMT/Cav-1 (+/+, +/−) mice (n = 20 mice) and male PyMT/Cav-1 (−/−) (n = 24 mice). At 23 weeks of age, 96% of PyMT/Cav-1 (−/−) mice had at least one tumor evident upon dissection, compared with only 70% of PyMT/Cav-1 (+/+, +/−) mice. In addition, tumor multiplicity was markedly increased in PyMT/Cav-1 (−/−) male mice, with an average of 4.0 tumors per mouse as opposed to only 1.2 tumors per mouse in the PyMT/Cav-1 (+/+, +/−) cohort (Fig. 3A). Additionally, there was a striking increase in tumor size and weight per mouse. As demonstrated in Fig. 3B, PyMT/Cav-1 (−/−) male mice had an 8.4-fold increase in average weight from the single largest tumor isolated per mouse (4.25 grams in PyMT/Cav-1 (+/+, +/−) mice; 0.51 grams in PyMT/Cav-1 (+/+, +/−) mice). Once again, there were no significant differences observed between PyMT/Cav-1 (+/+) and PyMT/Cav-1 (−/−) mice.

Tumors derived from both male cohorts were also processed for histological characterization. The tumor tissues were clearly adenocarcinomas and possessed a pathological grade ranging from low-to-medium, as in the female study. Likewise,
no morphological differences were observed between the tumors derived from both cohorts (data not shown). These results from the male study mirror the findings from the female cohorts by demonstrating that loss of Cav-1 results in accelerated tumor onset, as well as increased tumor incidence, multiplicity, and burden.

Caveolin-1 Acts as a Tumor Suppressor in PyMT-transformed Mouse Embryonic Fibroblasts and Mammary Adenocarcinoma Cells—Our observations that tumorigenesis is markedly accelerated in both PyMT/Cav-1 (−/−) female and male mice prompted us to study the effects of the loss of Cav-1 on cellular transformation. A disadvantage of studying tumorigenesis in PyMT/Cav-1 (−/−) mice is that while tumors arise from transformed mamma epithelial cells lacking Cav-1, they also develop in a setting where Cav-1 is genetically ablated in all host cells and tissues. The complexity caused by the numerous cell types present in the mammary gland necessitated a study of the effect of Cav-1 on cellular transformation independent of other cell types and tissues.

Therefore, we elected to transform previously generated immortalized fibroblastic cell lines (34, 35) with the PyMT oncogene. These immortalized MEFs were generated from wild-type and Cav-1 (−/−)-null mice and, therefore, either endogenously express or completely lack caveolin-1. Using immortalized cell lines (i) facilitates the maintenance of cells in culture, and (ii) facilitates tumorigenesis, since cells require the properties of both immortalization and transformation in order to efficiently form tumors in mice.

We retrovirally infected and transformed Cav-1 (+/+)- and Cav-1 (−/−) MEFs with a pBABE-PyMT construct. After selection with puromycin for 1 week, stable cells were examined by immunoblotting for expression of PyMT and Cav-1. As demonstrated in Fig. 4A, PyMT/Cav-1 (+/+) and PyMT/Cav-1 (−/−) immortalized MEFs express equivalent levels of PyMT, with the former cells expressing significant amounts of Cav-1. Since the PyMT oncogene is sufficient to induce a transformed/oncogenic state in established cell lines (36), we have therefore generated PyMT-transformed immortalized cell lines either expressing or lacking Cav-1.

Next, we injected 10⁶ cells of either PyMT/Cav-1 (+/+)- or PyMT/Cav-1 (−/−) MEFs into the flanks of athymic (nude) mice. After 6 weeks, the tumors were excised and weighed. We found that there was a dramatic increase in the size and weight (−8.6-fold) of tumors derived from PyMT/Cav-1 (−/−) MEFs, as compared with PyMT/Cav-1 (+/+)- MEFs (Fig. 4B and inset). Importantly, both non-PyMT-transformed Cav-1 (+/+)- and Cav-1 (−/−)- MEFs, that were retrovirally infected with an empty pBABE construct, formed no tumors during the same period of evaluation. Once again, histological analysis of the tumors derived from Cav-1 expressing or Cav-1 lacking PyMT-transformed immortalized MEFs showed no morphologic differences (data not shown).

In order to demonstrate that Cav-1 has transformation suppressor activity not only in transformed mesenchymal cells, but also in transformed epithelial cells, we re-expressed Cav-1 in an MMTV-PyMT tumor-derived cell line, Db7. Utilizing a pBABE-Cav-1 construct, we similarly retrovirally infected B7 parental cells, which normally express no detectable Cav-1, to generate Cav-1 expressing Db7 stable cell lines after puromycin selection (Fig. 4C). Next, we injected 10⁶ cells of either Db7/pBABE or Db7/Cav-1 cells into the flanks of nude mice and isolated the tumors after 3 weeks. We found that the re-expression of Cav-1 in these mammary adenocarcinoma-derived cells resulted in significantly reduced tumor burden (−44%) in these mice (Fig. 4D).

In summary, the presence of Cav-1 in either PyMT-transformed MEFs or a mammary adenocarcinoma-derived cell line results in significant reductions in tumor size. These results indicate that Cav-1 possesses transformation suppressor activ-
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Loss of Cav-1 in male PyMT transgenic mice results in even greater increases in tumor multiplicity and tumor burden. A, PyMT male mice at 23 weeks of age were sacrificed, and all tumors were excised and weighed. Tumor multiplicity is markedly augmented by the absence of Cav-1 (Fig. 4A). From the epidermal growth factor receptor, eNOS, Src family tyrosine kinases, and components of the Ras/MAP kinase pathway (14, 16, 18, 19). The ability of PyMT to transform cells occurs through its membrane localization and subsequent association and activation of components of several signaling pathways, including Src, PI 3-kinase, and Shc (36).

To exclude the possibility that the absence of Cav-1 is affecting the activation state of PyMT, we have utilized the above mentioned PyMT-transformed immortalized MEFs to examine the PyMT-associated kinase activity from these cells by using in vitro kinase assays. PyMT was immunoprecipitated from 1 mg of total cell lysates from both PyMT/Cav-1 (+/+) and PyMT/Cav-1 (-/-) cells. Immunoprecipitated PyMT was resuspended in kinase reaction buffer and incubated with [γ-32P]ATP and an acid-denatured enolase substrate for 10 min at room temperature. As demonstrated in Fig. 5B, there were no differences in levels of phosphorylated enolase, indicating that the absence of Cav-1 does not affect the kinase-associated activity of PyMT derived from PyMT-transformed cells. Since PyMT and Cav-1 are both membrane-associated proteins, we also addressed whether or not Cav-1 associates with PyMT at the membrane level. No association between Cav-1 and PyMT was detected by co-immunoprecipitation, although positive controls for both proteins co-immunoprecipitated (data not shown).

These findings indicate that the dramatic increases in tumorigenesis observed in PyMT/Cav-1 (-/-) mice cannot be accounted for by increased PyMT expression or by increased PyMT-associated kinase activity in the absence of caveolin-1.

Estrogen and Progesterone Receptor Expression as Well as PyMT-associated Protein Expression and Activation State Are Unaffected by the Absence of Cav-1—During human breast cancer progression, loss of ER and PR status is associated with less differentiated tumors and poorer clinical outcome, which may be due to the inability of the tumor to respond to hormonal signals (37). PyMT transgenic mice develop primary adenocarcinomas with significant ER and PR expression through the early stages of tumor development (26). In those carcinomas that progress to very advanced malignant stages, a loss of ER and PR expression has been detected (26).

Here, we have examined whether loss of Cav-1 affects ER or PR expression in moderately advanced (14-week-old) tumors. At least 6 different tumors from each genotype were examined for ER-α, ER-β, PR-A, and PR-B levels (Fig. 6A). No significant differences in expression were observed, indicating that loss of Cav-1 does not impart an advantage for these tumors to progress to an ER- or PR-independent status. This finding provides additional molecular support for our observation that there are no differences in histopathological tumor progression between both cohorts.

The potent transforming ability of PyMT is mediated partly through its recruitment and activation of several intracellular signaling molecules, including c-Src, PI 3-kinase/Akt, and Shc (26). Thus, we analyzed the expression of these proteins from tumors derived from 14-week-old mice to determine whether a lack of Cav-1 affects their expression levels (Fig. 6B). From the 6 tumors per genotype evaluated, no differences were observed in the constitutive expression of Src, PI 3-kinase, Akt, and Shc. Additionally, we immunoblotted for c-Src, Akt, and Shc with phosphospecific antibodies, which recognize their active phosphorylated species and found no differences in their levels (data not shown). Thus, a complete Cav-1 deficiency does not alter the expression or activation state of these PyMT-associated signaling proteins.

Genetic Disruption of Cav-1 Results in Significant Hyperactivation of ERK 1/2, Increased Cyclin D1 Levels, and Substantially Increased Levels of phospho-Rb—In order to identify a molecular mechanism for the advanced tumor onset, increased
tumor frequency, and increased tumor burden observed in
PyMT/Cav-1 (−/−) mice, we performed immunoblotting on
tumor tissue from both PyMT/Cav-1 (+/+ ) and PyMT/Cav-1
(−/−) mice at 14 weeks of age. Interestingly, mammary
tumor samples derived from PyMT/Cav-1 (−/−) cells are markedly increased in size and frequently showed areas of necrosis
and hemorrhage (inset).

C, immunoblot analysis of stable Db7 cell lines retrovirally transduced with pBABE (empty) and pBABE-Cav-1 (murine
cDNA) constructs. Db7 parental (not shown) and Db7/pBABE cells demonstrate no detectable Cav-1 expression, as compared with Db7/Cav-1 cells.

D, 10⁶ of either Db7/pBABE or Db7/Cav-1 cells were injected subcutaneously into the flanks of athymic nude mice. Tumors were allowed to form
over 3 weeks before they were excised. Bars indicate the S.E. An ~44% decrease in tumor weight was observed (p value < 0.05, Student’s t test,
arisk).

Fig. 4. PyMT-transformed MEFs and mammary tumor cells expressing Cav-1 form smaller tumors in vivo. A, immortalized MEFs either endogenously expressing (+/+) or lacking Cav-1 (−/−) were retrovirally transduced with a pBABE-PyMT construct and selected with puromycin for 1 week to generate stable pools of transduced cells. PyMT/Cav-1 (+/+ ) and PyMT/Cav-1 (−/−) immortalized MEFs were grown to confluence, collected into lysis buffer, and subjected to immunoblot analysis for Cav-1 and PyMT. Note that levels of Cav-1 are not significantly altered in the presence of PyMT. β-Tubulin levels are shown as an equal loading control. B, 10⁶ MEFs of either PyMT/Cav-1 (+/+ ) or PyMT/Cav-1
(−/−) cell lines were injected subcutaneously into the flanks of athymic (nude) mice (n = 10 mice per cell line), and tumors were allowed to form. After 6 weeks, tumors were excised and weighed. Note that the absence of Cav-1 results in dramatically larger tumors resulting from PyMT/Cav-1
(−/−) MEFs, as compared with PyMT/Cav-1 (+/+ ) MEFs. An ~ 8.6-fold increase in weight was observed (p value < 0.00001, Student’s t test, asterisk).

Importantly, non-PyMT transduced cells infected with an empty pBABE vector did not form tumors during the observation period. Bars indicate the S.E. Excised tumors arising from the PyMT/Cav-1 (−/−) cells are markedly increased in size and frequently showed areas of necrosis
and hemorrhage (inset).
ing Cav-1 (−/−) expression. These molecular findings are suggestive of increased cellular proliferation and provide a molecular framework for understanding the decreased tumor latency, increased tumor multiplicity, and increased tumor burden associated with PyMT/Cav-1 (−/−) mice.

**Genetic Ablation of Cav-1 Results in Increased Mammary Tumor-derived Metastatic Disease**—The utility of the MMTV-PyMT adenocarcinoma mouse model extends beyond that of studying mammary tumor development. By 16 weeks of age, 100% of the mice develop metastases to the lung that can be observed both microscopically and grossly (24). Since the ability of tumor cells to metastasize closely correlates with the pathological grade of the tumor, our finding that the loss of Cav-1 does not affect tumor histopathological progression provides an exceptional opportunity to study the effect of Cav-1 on metastasis. Here, we have chosen to examine PyMT mice, at 13 and 14 weeks of age, for the appearance of lung metastases.

At 13 weeks of age, 95.8% of the lobes examined from PyMT/Cav-1 (−/−) mice (n = 68 lobes from 17 mice) had visible metastases compared with only 55.8% (n = 45 lobes from 11 mice) from the PyMT/Cav-1 (+/+; +/+), +/+ cohort (Table III). This trend continued at 14 weeks of age, where 100% of PyMT/Cav-1 (−/−) lobes had metastases (n = 17 lobes from 8 mice) compared with 70.8% (n = 17 lobes from 11 mice) of lobes from the PyMT/Cav-1 (+/+; +/+; +/+), +/+ cohort. Importantly, Cav-1 (+/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/+; +/...
8A). In fact, the average number of metastases in PyMT/Cav-1 (−/−) lobes was 39.7 (S.E. = 7.0, n = 17) compared with 9.5 (S.E. = 4.6, n = 17) in the PyMT/Cav-1 (+/+, +/-) group (p value < 0.002; Mann-Whitney test). No significant differences in lung metastasis incidence or frequency were detected between PyMT/Cav-1 (+/+) and PyMT/Cav-1 (+/−) lobes, indicating that it is the complete lack of caveolin-1 expression that is responsible for the observed differences.

In addition to the increased incidence and frequency detected as a result of Cav-1 genetic ablation, the size of the lung metastases generally appeared greater in PyMT/Cav-1 (−/−) lobes (Fig. 8C). Microscopically, there are no morphological differences between lung metastases derived from PyMT/Cav-1 (+/+, +/−), (+/−), or (−/−) tumors (data not shown).

Because Cav-1 (−/−) mice demonstrate an aberrant lung phenotype (8, 10), we next sought to determine whether host-mediated lung defects in Cav-1 (−/−) mice are partially responsible for the increased number of pulmonary metastases observed in PyMT/Cav-1(−/−) mice. Therefore, we injected non-PyMT transgenic Cav-1 (+/+) and Cav-1 (−/−) mice through the tail vein (experimental metastasis) with Met-1 cells, a highly metastatic cell line established from a mammary tumor isolated from an MMTV-PyMT transgenic mouse (42). After 4 weeks, lungs were isolated, stained with India Ink, and the total number of surface metastases were counted under a stereo microscope.

As depicted in Fig. 8D, there were no significant differences in the number of metastases observed in Cav-1 (+/+) mice compared with Cav-1 (−/−) mice. In fact, Cav-1 (−/−) mice even demonstrated an ~43% decrease in the number of total pulmonary metastases compared with wild-type mice. Cav-1 (+/+) had an average number of 6.9 total foci per lung (S.E. = 3.2, n = 6), while Cav-1 (−/−) mice had an average of 3.9 total foci per lung (S.E. = 0.5, n = 5). While these numbers are developing a trend toward suggesting that the lungs of Cav-1 (−/−) mice have a negative impact on the development of metastases, the data did not reach statistical significance. We confirmed these results by repeating experimental metastasis utilizing another highly metastatic cell line, B16-F10 melanoma cells. Once again, Cav-1 (−/−) mice demonstrated reductions (~21%) in the number of pulmonary metastases compared with wild-type mice (data not shown). Taken together the lung tissue defects in Cav-1 (−/−) mice do not account for the observed increases in metastases in PyMT/Cav-1 (−/−) mice. In fact, the above mentioned increases in lung metastases observed in PyMT/Cav-1 (−/−) mice would likely be accentuated if the lung tissue had normal wild-type expression of caveolin-1.

Recombinant Expression of Cav-1 in a Highly Metastatic Mammary Tumor-derived Cell Line Reduces Experimental Metastasis—In order to further establish the importance of the intrinsic levels of caveolin-1 in affecting the metastatic potential of mammary tumor cells, we utilized Met-1 cells to study the effect of Cav-1 expression on the ability of these cells to metastasize to the lung. Initial characterization by immunoblotting revealed that these cells express extremely low levels of Cav-1. Using a retroviral approach, we re-expressed Cav-1 in Met-1 cells and generated two stable cell lines, Met-1/pBABE (empty vector) and Met-1/pBABE-Cav-1 (Fig. 9A). Immunofluorescence demonstrates that recombinant Cav-1 localizes properly to the plasma membrane, showing a characteristic punctuate membrane-staining pattern (Fig. 9B).

Next, we performed experimental metastasis by injecting $10^5$ cells in each mouse through the tail vein and harvesting the lungs after 3 weeks. Lungs were then stained with India Ink, bleached in Fekete’s solution, and scored under a stereo microscope. As demonstrated graphically in Fig. 9C, the number of metastases from Met-1/Cav-1 cells was reduced by ~4.5-fold (p value < 0.05; Mann-Whitney), indicating that overexpression of caveolin-1 reduces the efficiency of these mammary tumor cells to metastasize to the lung. Quantitatively, injections of Met-1/pBABE cells resulted in an average of 437 (S.E. = 79, n = 5) metastases per lung, as compared with 96 (S.E. = 19, n = 5) metastases per lung using Met-1/Cav-1 cells. This experiment was performed multiple times with virtually identical results. As depicted visually in Fig. 9D, recombinant expression of Cav-1 potently inhibits experimental metastasis. These results indicate that differences in Cav-1 expression affect the intrinsic ability of mammary tumor cells to metastasize in vivo.

Taken together, we have definitively demonstrated that loss of Cav-1 predisposes mice to increased incidence, frequency, and size of lung metastases derived from mammary adenocarcinomas. This is the first demonstration that Cav-1 can suppress mammary tumor metastasis in vivo and that loss of Cav-1 expression results in advanced metastatic disease.

Caveolin-1 Expression Potently Inhibits Matrigel Invasion by Metastatic Mammary Tumor Cells—In order to investigate the potential mechanisms for the observed decreases in experimental metastasis, we examined whether the expression of Cav-1 in Met-1 cells altered their invasive potential. Invasion through Matrigel-coated membranes is a widely accepted method for assessing the metastatic potential of virtually all types of tumor cells (30). Therefore, we performed Matrigel invasion assays to measure the invasiveness of Met-1/pBABE versus Met-1/Cav-1 cells in response to chemoattractants (0.5 or 10% FBS in DMEM) over a 24 h incubation period. As shown in Fig. 10, A and B, Met-1/pBABE cells demonstrated a high degree of invasiveness as compared with control NIH-3T3 cells, a non-invasive fibroblast cell line (negative control). In comparison, Met-1/Cav-1 cells showed a ~4.8-fold reduction in Matrigel invasion in response to 10% FBS (Fig. 10B, asterisk). Quantitatively, the average number of Met-1/pBABE cells that invaded per field was 148.4 (S.E. = 14.1, n = 15) as compared with 31.2 (S.E. = 3.9, n = 15) for Met-1/Cav-1 cells (p value < 10⁻⁸). A significant defect in invasion was also observed using 0.5% FBS as a chemoattractant (~3.0-fold; p value < 0.0002).

In parallel, we assessed the migration of Met-1/pBABE and Met-1/Cav-1 cells using the same 8-μm pore membrane chambers used in the invasion assay, albeit not coated with Matrigel. As demonstrated in Fig. 10C, Met-1/Cav-1 cells demonstrated a minor defect (~1.2-fold reduction) in motility as compared with Met-1/pBABE cells in response to 10% FBS as a chemoattractant, with this difference approaching statistical significance (p value = 0.08). Using the migration data in combination with the invasion data, the percent of invading cells for Met-1/pBABE cells was 57.6% compared with 14.6% for Met-1/Cav-1 cells-in response to 10% FBS (invasion index = 3.9).

In order to determine if the caveolin-1 scaffolding domain (CSD, residues 82–101) is sufficient to inhibit matrix invasion, we next treated Met-1 cells with a cell-permeable CSD fused to the C terminus of penetratin, a 16 amino acid peptide derived from the Drosophila antennapedia transcription factor. Fusion of penetratin to small peptides allows their efficient delivery across the plasma membrane into the cytoplasm of living cells.
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Tissue degradation by metalloproteinases

Matrix metalloproteinases (MMPs) are a family of enzymes that degrade extracellular matrix components. These enzymes act not only as extracellular matrix degrading enzymes but also serve to activate other MMPs through cleavage of their propeptide domains, as in the case of MT1-MMP (aka MMP-14) activating pro-MMP-2 (47).

Many studies have shown that MT1-MMP co-localizes with caveolae and caveolin-1 (48–50). Further, it has been demonstrated that caveolae and caveolin-1 are required for proper MT1-MMP localization and activity in migrating endothelial cells (50). Therefore, we tested whether overexpression of Cav-1 in our malignant cells affected MT-MMP-associated activity, with regard to the ability of MT-MMPs to activate other

via a non-endocytic and non-degradative pathway (43, 44). We have previously reported the generation, sequence, and internalization of these penetratin peptides within 2 h in HMEC-1 cells (31).

Therefore, we treated Met-1 parental cells with either penetratin alone or penetratin-CSD during the invasion assay. We found that cytoplasmic delivery of the CSD peptide was sufficient to inhibit invasion > 3.5-fold (Fig. 10D). These results dramatically show that Cav-1 inhibits the invasion of metastatic mammary tumor cells and directly maps this invasion-inhibiting activity to the caveolin-1 scaffolding domain (residues 82–101).

Cav-1 Expression in Metastatic Mammary Tumor Cells Inhibits MMP-2 and MMP-9 Activities—Since the relatively minor defect in motility observed in Cav-1 expressing Met-1 cells cannot sufficiently account for the reduced invasiveness, we sought to determine whether alterations in the expression and activity of matrix metalloproteinases might be responsible for the observed decreases in invasion. Matrix metalloproteinases (MMPs), secreted by tumor cells, function as extracellular matrix-degrading enzymes whose activity enhances tumor invasiveness and metastasis (45). Of the known MMPs, there are currently eight structurally distinct groups, five of which are secreted and three that are membrane-anchored (46). The membrane-tethered groups, known collectively as MT-MMPs, function not only as extracellular matrix degrading enzymes but also serve to activate other MMPs through cleavage of their propeptide domains, as in the case of MT1-MMP (aka MMP-14) activating pro-MMP-2 (47).

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MMPs from their inactive pro-MMP form to their cleaved, active forms. Utilizing an in vitro MT-MMP assay which measures cleavage of a universal MMP peptide substrate, we found that recombinant expression of Cav-1 increases the activity of MT-MMPs 2.0-fold in Met-1/Cav-1 cells (Fig. 11A). Immuno-blotting for MT1-MMP revealed that MT1-MMP expression levels are 1.5–2-fold higher in Met-1/Cav-1 cells (Fig. 11A, top panel). These results are consistent with the findings of Galvez et al., which argue that Cav-1 is required for proper MT1-MMP localization, stabilization, and function. However, these results do not provide a molecular mechanism for the decreased metastasis and invasion observed in Met-1/Cav-1 cells.

Therefore, we next examined the status of MMP-2 (gelatinase A) and MMP-9 (gelatinase B), two well-characterized secreted MMPs which exhibit collagenolytic and gelatinolytic activity. MMP-2 and MMP-9 are clearly linked to breast cancer metastatic potential since MMP-2- and MMP-9-deficient mice are both much less susceptible to experimental metastasis (51, 52). Subsequently, we performed conventional gelatin zymography (32) to assess whether expression of Cav-1 affects the expression and activity of MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Conditioned medium from equal numbers of Met-1/pBABE or Met-1/Cav-1 cells incubated overnight in serum-free medium was subjected to gelatin zymography. This technique measures total MMP gelatinolytic activity (i.e. both active and pro-MMP forms) since pro-MMPs become activated during zymography. Remarkably, Met-1/Cav-1 cells display a dramatic reduction in MMP-9 and MMP-2 associated gelatinolytic activity as compared with Met-1/pBABE cells (Fig. 11B).

Immunoblotting for MMP-9 in whole cell lysates from Met-1/pBABE and Met-1/Cav-1 cells revealed that endogenous expression of MMP-9 is unaltered (Fig. 11C, top panel). However, immunoblotting of conditioned medium from Met-1/pBABE and Met-1/Cav-1 cells demonstrated no detectable secretion of MMP-9 into the medium by Cav-1 re-expressing Met-1 cells (Fig. 11C, bottom panel). Even overnight treatment with EGF or FGF-2, two growth factors that up-regulate MMP production and secretion (32), did not restore MMP-9 secretion in Met-1/Cav-1 cells. Since secretion of MMPs accounts for the majority of their matrix-degrading activities, this finding provides a molecular mechanism for the reduced invasion and experimental metastasis observed in Met-1/Cav-1 cells.

To more definitively demonstrate that reductions in the invasive potential of Met-1/Cav-1 cells are due to inhibition of MMPs, we treated Met-1/pBABE cells with three commercially available MMP inhibitors: GM6001, a potent broad spectrum MMP inhibitor (53), and two MMP-2/MMP-9 specific inhibitors (N-sulfonylamino acid derivatives I and II, Ref 54). These inhibitors were all used at a concentration that inhibits 95% of their MMP enzymatic activity. As depicted in Fig. 11D (top panel), all three MMP inhibitors dramatically reduced the invasion of Met-1/pBABE cells by –2.45-fold. Interestingly, these
reductions in invasion were significantly smaller than the previously observed inhibition induced by Cav-1 re-expression in these Met-1 cells (~4.5–4.8-fold). Furthermore, treatment of Met1/Cav-1 cells with these same MMP inhibitors only slightly reduced invasion, and the data was not statistically significant (Fig. 11D, bottom panel).

Taken together, these findings suggest that the metastasis-and invasion-inhibiting effects of Cav-1 expression in metastatic mammary tumor cells appears to be mediated in part through inhibition of MMP-2 and MMP-9 activities.

Met-1/Cav-1 Cells Demonstrate Diminished ERK-1/2 Activation in Response to Serum Activation—While MMP inhibition appears to be one of the main mechanisms by which Cav-1 expression operates in reducing invasion and metastasis, it does not appear to be the only mechanism. Inhibition of Met-1/pBABE invasion with chemical MMP inhibitors reduced invasion by only ~2.5-fold, as compared with ~4.5-fold reduced invasion observed in Met-1/Cav-1 cells. Therefore, there must be other additional mechanisms operating which function to inhibit invasion better in Met-1/Cav-1 cells.

We decided to focus on intracellular signaling pathways, particularly ERK-1/2, based on our findings that tumors lacking Cav-1 expression displayed ERK-1/2 hyperactivation. The ERK-1/2 (p42/44 MAP kinase) pathway is a well characterized pathway controlling proliferation that is also implicated in transformation, invasion, tumor progression, and metastatic processes (55, 56). First, we examined the constitutive activation state of Met-1/pBABE and Met-1/Cav-1 cells in complete medium (DMEM/10% serum) and found no alterations in the constitutive activation state of ERK-1/2 (data not shown).

Next, we tested whether the initiation of ERK-1/2 activation in response to extracellular signals was affected. Therefore, we serum-starved Met-1/pBABE and Met-1/Cav-1 cells overnight, then stimulated them with complete medium for various periods of time, and examined their level of ERK-1/2 activation by immunoblotting whole cell lysates with a phosphospecific ERK-
1/2 antibody. As shown in Fig. 12, Met-1/Cav-1 cells demonstrate a blunted ERK-1/2 response to serum stimulation beginning as early as 10 min and up to 3 h of continued stimulation. These results indicate that the presence of Cav-1 in metastatic mammary tumor cells results in a defect in their ERK-1/2 signaling axis, causing these cells to have a diminished response to serum or other growth factor stimuli.

In summary, we have identified another potential mechanism to account for the reduced metastasis and invasion of metastatic mammary tumor cells expressing Cav-1. These cellular findings parallel the hyperactivation of ERK-1/2 observed in tumor tissue lacking endogenous expression of Cav-1 in vivo. Taken together, they suggest that alterations in the ERK signaling pathway by Cav-1 may be largely responsible for the differences in transformation, tumorigenesis, invasion, and metastasis that we observe.

**DISCUSSION**

The goal of these studies was to establish whether caveolin-1 plays a protective role in suppressing mammary tumorigenesis and metastasis. Regarding the importance of caveolin-1 in the mammary gland, we have previously reported several phenotypes present in Cav-1 (−/−)-null mice. First, as early as 6 weeks of age, female Cav-1 (−/−)-null mice demonstrate mammary epithelial ductal hyperplasia (27). Secondly, genetic disruption of both Cav-1 alleles results in premature development

![Graph showing the variation in antibody activity](image)
of the lobulo-alveolar compartment during pregnancy (57). Finally, complete absence of Cav-1 accelerates the appearance of oncogene-induced dysplasias (pre-malignant lesions) in the mammary epithelium (28). Although these results are suggestive, it remains unknown whether loss of caveolin-1 has any consequences for the development of advanced and aggressive mammary tumors and resulting metastatic disease.

Here, we have shown that the presence of Cav-1 in mammary tumor cells suppresses tumor growth and metastasis. Utilizing the PyMT transgenic model of breast cancer, we examined the formation of early palpable tumors through to the development of late carcinomas, as well as lung metastasis. Tumor latency was decreased in PyMT/Cav-1 (−/−) mice, with the initial early palpable tumors requiring an average of 12% less time to appear. Additionally, tumor multiplicity was increased in PyMT/Cav-1 (−/−) mice. All mice eventually exhibited 100% involvement of all 10 mammary glands by 12 weeks of age, demonstrating the multifocal efficiency and high penetrance of the PyMT transgenic model. Moreover, tumor burden was substantially increased in PyMT/Cav-1 (−/−) mice, with up to ~200% increases in combined tumor weights per mouse. Accordingly, male PyMT/Cav-1 (−/−) mice also showed significant increases in tumor incidence, multiplicity, and tumor burden, albeit with more dramatic differences as compared with the female cohort. These differences are likely due to the much longer tumor latency normally observed in male PyMT mice, enabling moderate differences to become compounded over time. Morphologically, tumors derived from PyMT mice either expressing or lacking Cav-1 do not show any differences, indicating that Cav-1 does not affect histopathological progression of these tumors at the ages examined (12 and 14 weeks). We have also established, using mammary adenocarcinoma cells (Db7) and PyMT-transformed MEFs, that Cav-1 exerts transformation/tumor suppressive functions intrinsically from within the tumor cell.

The amounts of phosphorylated active ERK-1/2 (p42/44 MAP kinase) were increased in PyMT/Cav-1 (−/−) tumors, suggesting the dysregulation of cell proliferation. ERK-1/2, or extracellular-regulated kinase, is part of a well-characterized receptor signaling cascade that relays the effects of extracellular signaling to the nucleus. Upon activation, ERK-1/2 translocates to the nucleus and activates a host of transcription factors including Elk-1 (58–60), and c-Ets family proteins (61). Activation of the Ras-p42/44 MAP kinase cascade positively regulates proliferation and sustained activation appears to be required for fibroblasts to progress beyond the G1 restriction point and to enter the S-phase (62, 63). In addition, activation of Ras or the downstream ERK-1/2 pathway results in the up-regulation of cyclin D1 expression, an important regulatory component of cyclin-dependent kinase (CdKd4/6) complexes that control cellular proliferative checkpoints (64–67). Concordant with these findings, we observe increases in cyclin D1 expression in PyMT/Cav-1 (−/−) tumors. Interestingly, up to ~40% of human breast cancers demonstrate overexpression or amplification of cyclin D1 (68, 69). The potency of cyclin D1 as a mammary proto-oncogene was definitively demonstrated by transgenic overexpression of cyclin D1 in the mammary epithelium using the MMTV promoter, as these mice spontaneously develop mammary hyperplasia and full-blown adenocarcinomas (70).

The major target for active cyclin D1-cdk4/cdk6 complexes is the retinoblastoma protein, or Rb (71). A large majority of proliferative or anti-proliferative signals are mediated through Rb at the molecular level. Hypophosphorylated Rb blocks proliferation by disrupting the function of E2F transcription factors, which normally transcribe a host of genes necessary for entry into S phase. Phosphorylation of Rb by cyclin D-cdk4/6 complexes renders Rb inactive, thus preventing it from blocking proliferation. A number of Rb residues that become phosphorylated by various cyclin-dk complexes have been identified (40). Interestingly, cyclin D1-cdk4/6 complexes target Ser-780 and cyclin D1 is required for phosphorylation at this position (41). Here, we have demonstrated that the absence of Cav-1 (−/−) results in hyper-phosphorylated Rb (Ser-780) in tumors derived from PyMT mice. This suggests that these tumor cells are more predisposed to transitioning through G1 into the S phase, with subsequently increased proliferative rates. Hyperphosphorylation of Rb at serine 780 is also consistent with increased cyclin D1-Cdk4/6 activity, resulting from the overexpression of cyclin D1.

Our findings that ERK-1/2 is hyperactivated in mammary tumors and a mammary tumor cell line lacking Cav-1 expression provide further evidence of a negative regulatory relationship between Cav-1 and the ERK signaling cascade. While we have previously demonstrated that Cav-1 overexpression in different cell types can inhibit cyclin D1 transcription and ERK-1/2 activation (12, 19, 35, 72), this is the first report documenting that the downstream effects of this inhibition are mediated through the Rb tumor suppressor protein. These findings of ERK-1/2 hyperactivation, cyclin D1 overexpression, and Rb hyperphosphorylation in tumors derived from PyMT/Cav-1 (−/−) mice provide novel molecular insights into the observed acceleration of tumor onset and increased tumorigenesis. However, these altered molecular profiles may also have implications for our metastatic findings. For instance, it has recently been shown that a cyclin D1 deficiency dramatically suppresses cell motility and invasiveness, suggesting that overexpression may augment these prometastatic processes (73). Furthermore, inhibition of ERK-1/2 has been shown to reduce migration in breast cancer cell lines (74). In addition, sustained activation of the ERK-pathway, through growth factor stimulation, results in matrix metalloproteinase expression and cellular invasion (75) (76). Concordant with this, we find that in mammary tumor cells re-expressing Cav-1 (Met-1/Cav-1), there is reduced ERK-1/2 activation in response to serum stimulation. These molecular findings may assist in explaining the observed decreases in migration, invasion, and experimental
metastasis in Cav-1 re-expressing metastatic mammary tumor cells.

A controversial role for caveolin-1 in metastasis has emerged from investigations into various types of cancers. We have previously shown that adenoviral-driven recombinant expression of caveolin-1 in MTLn3 cells (a rat metastatic mammary adenocarcinoma-derived cell line) inhibits EGF-induced lamellipod extension and cell migration in vitro (77). Here, we have identified a similar decrease in motility in Met-1 cells expressing Cav-1, but not to the same extent. Similarly, Fiucci et al. (78) reported that overexpression of Cav-1 in human breast cancer MCF-7 cells inhibits soft agar growth, matrix invasion, and collagenolytic activity, features characteristic of highly metastatic cells. More recently, it was shown that down-regulation of Cav-1 in human tumor cells causes the down-regulation of E-cadherin, an important cell-cell adhesion protein, increased β-catenin-TCF/LEF-1 transcriptional activity, and promotes invasion into collagen gels (79). Despite this evidence linking down-regulation of Cav-1 to increased metastasis, caveolin-1 overexpression has been found to be associated with metastasis-derived prostate cancer cells in mice and humans, and appears to clinically correlate with prostate cancer progression in humans (80–83). Additionally, Cav-1 up-regulation correlates with increased metastatic capability in lung adenocarcinoma cells in culture and the presence of lung adenocarcinoma metastases in patients (84). These seemingly disparate observations may be explained partly by cell type-specific roles for Cav-1. Conversely, these findings may suggest a biphasic expression pattern, where Cav-1 is down-regulated during cellular transformation/tumorigenesis and, then, up-regulated during late metastasis.

Here, we have provided clear in vivo evidence that Cav-1 suppresses pulmonary metastasis from primary mammary tumors. We observed an ~4.2-fold increase in the number of metastases present in PyMT/Cav-1 (−/−) mice. We have also established that this increase in metastasis is not due to host-mediated effects, such as defects in the lung, using the highly metastatic PyMT/mammary epithelial cell line (Met-1). Additionally, Cav-1 re-expression in Met-1 cells markedly reduced experimental metastasis, providing further evidence that down-regulation of Cav-1 in mammary tumor cells favors metastasis. Matrigel invasion, an assay used to measure the invasive/metastatic potential of cells, was also markedly reduced in Cav-1 re-expressing Met-1 cells. These invasion inhibiting properties of Cav-1 appear to be mediated by the caveolin-scaffolding domain (CSD; residues 82–101). Furthermore, we have established a relationship between Cav-1 and MMP activation and function. The reductions in invasion resulting from Cav-1 expression in mammary tumor cells appear to be due, in part, to reduced secretion of the gelatinases MMP-2 and MMP-9. Interestingly, Cav-1 expression more potently inhibits invasion compared with specific chemical inhibition of MMPs, indicating that Cav-1-mediated inhibition of invasion and metastasis likely operates through multiple mechanisms. As MMP chemical inhibitors have undergone clinical trials for the treatment of human cancer, these findings suggest that Cav-1 gene therapy for the treatment of metastatic human breast cancer might be a worthwhile pursuit.

Taken together, these findings definitively demonstrate that down-regulation of Cav-1 in mammary tumor cells is a metastasis-promoting event. This does not rule out the possibility that caveolin-1 possesses other tumor cell-type or tissue-specific roles. Indeed, Cav-1 may have distinct and non-overlapping functions in prostate, lung, and mammary tumor cells.

In conclusion, we show here that Cav-1 functions to suppress the processes of tumorigenesis and metastasis in the mammary gland. Complete ablation of Cav-1 (−/−) clearly accelerates tumor onset and growth, as well as the appearance of metastatic disease. As human tumors demonstrate sporadic dominant-negative mutations (Cav-1 P132L) or significant down-regulation of caveolin-1 expression, these alterations may confer a form of acquired resistance to the intracellular effects of this protein on inhibiting cellular proliferation, tumorigenesis, invasion, and other anti-metastatic functions. Thus, our findings suggest that forced endogenous or recombinant re-expression of Cav-1 in breast cancer cells may serve to therapeutically reduce their tumorigenic and/or metastatic potential in vivo.

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Note Added in Proof—While this paper was under review, another report appeared online (85) showing that caveolin-1 plays an important role in breast cancer onset and metastasis. In this “short report,” the authors used a completely different model system to test the hypothesis that caveolin-1 functions as a mammalian gland tumor suppressor gene. Briefly, they stably transfected a transformed metastatic mouse mammary adenocarcinoma cell line (T4-2 cells) with the caveolin-1 cDNA. Then, they implanted these cells within the mammary fat pads of wild-type female BALB/c mice. Using this orthotopic model of spontaneous breast cancer metastasis, they showed that recombinant expression of caveolin-1 inhibits both primary tumor growth and metastasis. More specifically, they observed an ~3-fold reduction in tumor weight, and an ~50-fold reduction in lung metastasis. Caveolin-1-transfected T4.2 cells also showed clear reductions in anchorage-independent growth and a loss of invasiveness, using in vitro cell culture assays. However, these authors did not examine the role of caveolin-1 in MMP secretion. Thus, both reports (our paper and Ref. 85) provide important new evidence that caveolin-1 expression exerts a positive protective effect against the development of mammary tumors and that caveolin-1 can also slow or prevent progression to metastatic disease.

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