Tumorigenesis and Neoplastic Progression

Stromal and Epithelial Caveolin-1 Both Confer a Protective Effect Against Mammary Hyperplasia and Tumorigenesis

Caveolin-1 Antagonizes Cyclin D1 Function in Mammary Epithelial Cells

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Here, we investigate the role of caveolin-1 (Cav-1) in breast cancer onset and progression, with a focus on epithelial-stromal interactions, ie, the tumor micro-environment. Cav-1 is highly expressed in adipocytes and is abundant in mammary fat pads (stroma), but it remains unknown whether loss of Cav-1 within mammary stromal cells affects the differentiated state of mammary epithelia via paracrine signaling. To address this issue, we characterized the development of the mammary ductal system in Cav-1^{-/-} mice and performed a series of mammary transplant studies, using both wild-type and Cav-1^{-/-} mammary fat pads. Cav-1^{-/-} mammary epithelia were hyperproliferative *in vivo*, with dramatic increases in terminal end bud area and mammary ductal thickness as well as increases in bromodeoxyuridine incorporation, extracellular signal-regulated kinase-1/2 hyperactivation, and up-regulation of STAT5a and cyclin D1. Consistent with these findings, loss of Cav-1 dramatically exacerbated mammary lobulo-alveolar hyperplasia in cyclin D1 Tg mice, whereas overexpression of Cav-1 caused reversion of this phenotype. Most importantly, Cav-1^{-/-} mammary stromal cells (fat pads)

promoted the growth of both normal mammary ductal epithelia and mammary tumor cells. Thus, Cav-1 expression in both epithelial and stromal cells provides a protective effect against mammary hyperplasia as well as mammary tumorigenesis. (Am J Pathol 2006, 169:1784–1801; DOI: 10.2353/ajpath.2006.060590)

Caveolin-1 (Cav-1) is the principal structural protein of caveolae, ~50- to 100-nm omega-shaped membrane invaginations resembling little caves.^{1,2} Cav-1 belongs to a family of three caveolins (ranging in size from 18 to 24 kd) that includes Cav-2 and Cav-3.^{3–5} Cav-1 and Cav-2 are ubiquitously co-expressed in a number of diverse cell and tissue types.⁶ In contrast, Cav-3 is a muscle-specific protein that is expressed mostly in cardiac and skeletal muscle.^{7,8} Notably, Cav-1 is expressed at high levels in terminally differentiated cells, including adipocytes, endothelia, and a variety of epithelia.⁹ Adipose tissue expresses the highest levels of Cav-1.

The mammary gland is an atypical fat pad when compared with other fat pads throughout the body. Beginning at 3 weeks of age in female mice, epithelial stem cells extend from the nipple, forming the primary duct, which then branches and grows throughout the rest of the mammary fat

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pad. These fat pads, therefore, have a high epithelial content in addition to adipocyte, endothelial, and stromal cells. It has already been established that Cav-1 is normally expressed at high levels in adipocytes and stromal cells and at moderate levels in mammary epithelia.^{10–13}

Indeed, we have demonstrated that Cav-1 has important roles in mammary gland physiology and morphology. Genetic ablation of Cav-1 in mice results in extensive mammary epithelial hyperplasia.¹⁴ During pregnancy, Cav-1 levels are normally down-regulated as the epithelium undergoes extensive proliferation and development to form milk-producing lobulo-alveolar units.¹⁵ This appears to be partly attributable to a negative regulatory pathway controlled by the hypothalamic hormone prolactin. Interestingly, we have demonstrated that complete loss of Cav-1 results in accelerated lobulo-alveolar development in the later stages of pregnancy, along with precocious milk protein production and hyperactivation of the Jak-2/STAT5a cascade.¹⁶

Cav-1 appears to have diverse functions, including vesicular transport, maintaining cellular cholesterol balance, and signal transduction.³ In addition, we and others have previously demonstrated that Cav-1 appears to act as a tumor suppressor in the mammary gland. In the majority of mammary tumor cell lines examined, Cav-1 is down-regulated, and recombinant re-expression of Cav-1 potently inhibits their proliferation, anchorage-independent growth, and invasiveness.^{10,17,18} Genetic ablation of Cav-1 in mice accelerates the appearance of dysplastic foci and mammary tumors when adenocarcinomas are induced by interbreeding Cav-1^{-/-} null mice with MMTV-polyomavirus middle T antigen (MMTV-PyMT) mice.^{19,20} MMTV-PyMT mice express the PyMT oncogene specifically in mammary epithelial cells resulting in mammary dysplasias and adenocarcinomas, closely modeling human breast cancer progression.^{21–23} In these MMTV-PyMT studies,^{19,20} we reported that the loss of Cav-1 is associated with cyclin D1 up-regulation and that the mammary tumors demonstrate extracellular signal-regulated kinase (ERK)-1/2 hyperactivation and diminished Rb activation. These molecular findings are consistent with a negative regulatory role for Cav-1 in mammary epithelial proliferation. Furthermore, a P132L mutation in CAV-1 has been identified in up to 16% of human breast cancers.²⁴ Recombinant expression of Cav-1 (P132L) is sufficient to transform NIH-3T3 cells.²⁴ This mutation appears to function in a dominant-negative manner, driving the intracellular retention of wild-type (WT) Cav-1 in the Golgi complex.¹⁴ Recently, Sloan and colleagues²⁵ have demonstrated that re-expression of Cav-1 in mammary tumor cells reduces primary tumor growth and metastasis to distant organs, such as lung and bone.

The mammary hyperplasia phenotype of Cav-1 knockout (KO) mice¹⁴ provides an underlying cause for accelerated mammary tumor development when Cav-1 KO mice are interbred with tumor-prone MMTV-PyMT mice. Indeed, hyperplasia is considered a preneoplastic lesion that, with additional genetic hits, may progress to a neoplastic state. Mammary epithelial hyperplasia can be divided into two categories depending on its location within the mammary tree: ductal hyperplasia and lobulo-alveolar hyperplasia.

Ductal hyperplasia manifests itself as ductal thickening, whereas lobulo-alveolar hyperplasia preferentially involves the terminal ductal lobular units (at the terminal ends of the mammary tree)—akin to expansion of the mammary tree during lactation.

Here, we investigate the development of epithelial hyperplasia in the mammary glands of Cav-1-null mice. We show that Cav-1 KO mice develop both ductal and lobulo-alveolar hyperplasia phenotypes, with ductal thickening and increases in the size of their terminal end buds (TEBs). In addition, we mechanistically dissect the individual contribution of epithelial and nonepithelial cells to this hyperplastic Cav-1-null phenotype. We find that overall morphogenesis of the mammary gland is not altered in Cav-1 KO mice, despite mammary epithelial hyperplasia. However, loss of Cav-1 appears to confer an increased rate of proliferation in mammary epithelial cells *in vivo*. Alterations in the expression and activation of proproliferative signaling proteins, including cyclin D1, provide molecular evidence for the observed increase in mammary proliferation in Cav-1 KO mice. Finally, using mammary reconstitution experiments, we demonstrate that loss of Cav-1 within mammary epithelial cells or nonepithelial cells (ie, stromal cells, including adipocytes) both contribute to the dysregulation of epithelial proliferation and hyperplasia. More specifically, we show that the lobulo-alveolar hyperplasia in Cav-1^{-/-} mammary glands is cell autonomous (intrinsic to the mammary epithelial cell), whereas the ductal thickening phenotype is strictly dependent on the Cav-1^{-/-} stromal microenvironment (paracrine/heterotypic signaling). Taken together, these findings demonstrate that the loss of Cav-1 in either epithelial or nonepithelial cells can independently contribute to the development of mammary hyperplasia and mammary cell transformation.

We also provide the first genetic evidence that Cav-1 functions as an antagonist of cyclin D1 in mammary epithelial cells *in vivo*. More specifically, we show that Cav-1 overexpression can suppress the transformative effects of cyclin D1, by using MMTV-cyclin D1 transgenic (Tg) mice. The cyclin D1 gene is amplified or overexpressed in ~50% of human breast cancers.^{26–30} In addition, cyclin D1 is required for Erb-B2-induced mammary tumor growth *in vivo*,³¹ and forced expression of cyclin D1, in the mammary glands of Tg mice,^{32,33} is sufficient for the induction of mammary adenocarcinoma.³⁴ Thus, Cav-1-based mimetics may represent a novel therapeutic strategy for blocking or reverting cyclin D1-induced mammary cell transformation.

Materials and Methods

Materials

Mouse monoclonal antibodies to Cav-1 (clones 2297 and 2234) were the generous gifts of Dr. Roberto Campos-Gonzalez (BD Pharmingen, Inc., San Diego, CA). Other antibodies and their sources were as follows: anti-Cav-1 (N-20) rabbit polyclonal antibody (pAb) (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti- β -actin monoclonal antibody (mAb) AC-15 (Sigma, St. Louis, MO), anti- β -tubulin mAb (Sigma), anti-cyclin D1 rabbit pAb (Lab Vision,

Inc.), anti-ERK-1/2 rabbit pAb (Cell Signaling, Beverly, MA), anti-phospho-ERK-1/2 rabbit pAb (Cell Signaling), anti-STAT5a mAb (BD Pharmingen), and anti-phospho-STAT5a mAb (BD Pharmingen). Bromodeoxyuridine (BrdU) was purchased from Calbiochem, La Jolla, CA.

Animal Studies

All animals were housed and maintained in a barrier facility at The Albert Einstein College of Medicine and the Kimmel Cancer Center at Thomas Jefferson University. Cav-1-null mice were generated as previously described.¹⁹ All mice used in this study (WT, Cav-1 KO, Cav-1 Tg, MMTV-PyMT, MMTV-cyclin D1) were in the FVB/N background, unless specifically stated otherwise. Maintenance and genotyping of Tg mice expressing the polyoma middle T antigen under the control of the MMTV LTR promoter (PyMT) were as described previously.¹⁹ MMTV-cyclin D1 (CD1) Tg mice were obtained from Dr. Andrew Arnold (University of Connecticut, Farmington, CT) and were polymerase chain reaction (PCR)-genotyped using a previously published primer set.^{32,33} All mice analyzed in this study were virgin females. In addition, all of the MMTV-CD1 and MMTV-PyMT Tg mice studied were heterozygous for the oncogenic transgene. Cav-1 Tg mice ubiquitously overexpressing a C-terminally Myc-tagged form of the Cav-1 WT cDNA (canine; in the pCAGGS vector containing a β -actin/CMV-based promoter) were generated using standard procedures, essentially as we previously described for other transgenes.³⁵ Animal protocols used for this study were approved by the Institutional Animal Care and Use Committee.

Whole Mount Analysis

Carmine dye staining of inguinal (no. 4) mammary glands was performed as we previously described.¹⁹ Photomicrographs were generated using a Nikon stereo microscope (Nikon, Melville, NY). Quantitation of various parameters (ductal thickness, TEB size, and so forth) was performed using Image J software.

BrdU Immunohistochemistry

Mice were injected intraperitoneally with BrdU in phosphate-buffered saline (PBS) (at a dosage of 100 μ g/g of body weight). Mice were sacrificed 2 hours later, and the mammary glands were isolated, formalin-fixed, and processed for paraffin-embedding. Five- μ m sections were cut and stained using a BrdU immunohistochemistry kit, according to the manufacturer's instructions (Oncogene, Inc., Boston, MA).

Mammary Reconstitution Experiments

Mammary gland transplants were performed on 3-week-old mice, using the procedure described in detail by DeOme and colleagues.³⁶ In brief, donor mice were

anesthetized with a mixture of ketamine and xylazine by intraperitoneal injections, and inguinal mammary fat pads were exposed in a sterile manner. The nipple region containing the early mammary epithelial tissue, which extends throughout the rest of the gland, was carefully excised and transplanted into the inguinal glands of recipient mice that were cleared of host mammary gland epithelial elements. After implantation of the transplant into host mice, all mice were surgically closed using staples. Mammary glands were isolated 8 weeks later and stained with carmine dye to analyze the development of transplanted mammary tissue. Both donor and recipient mice used were of the same strain (FVB/N).

Immunoblot Analysis

Cells were cultured in their respective media and allowed to reach ~90% confluency. After washing with PBS, cells were treated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer containing dithiothreitol, collected by cell scraping, and passed several times through a 26-gauge needle to completely disrupt all cells. Equal volumes were loaded on the gel. For protein isolation from tissue, inguinal mammary glands were isolated and then homogenized in an appropriate volume of lysis buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, and 60 mmol/L *n*-octyl-glucoside), containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN). Tissue lysates were then centrifuged at 12,000 $\times g$ for 10 minutes (at 4°C) to remove insoluble debris. Protein concentrations were analyzed using the BCA reagent (Pierce, Rockford, IL) and the volume required for ~20 μ g of protein was determined. Samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) and transferred to nitrocellulose. All subsequent wash buffers contained 10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 0.05% Tween 20, which was supplemented with 5% nonfat dry milk (Carnation) for the blocking solution, and 1% bovine serum albumin for the antibody diluent. Primary antibodies were used at a 1:500 dilution. Horseradish peroxidase-conjugated secondary antibodies [anti-mouse 1:10,000 dilution (Pierce) or anti-rabbit 1:5000 (Transduction Laboratories, Lexington, KY)] were used to visualize bound primary antibodies with the Supersignal chemiluminescence substrate (Pierce). When phospho-specific antibody probes were used, nonfat dry milk was omitted from the blocking and primary antibody solutions.

Immunohistochemistry

Immunohistochemical staining was performed essentially as we previously described.¹⁹

Cell Culture

The creation of both the Met-1 and hTERT-HME1 stable cell lines by retroviral-mediated transduction (using the vector pBABE-Cav-1-puro) has been previously described.^{14,20}

Cell Implantation Studies

For ectopic implantation, 10^6 Met-1 cells were resuspended in 0.1 ml of PBS and injected into the flanks of 2-month-old female mice. After 3 weeks, tumors were excised and weighed. For orthotopic implantation, 0.5×10^5 cells were resuspended in 5 μ l of PBS and injected through the nipple into 2-month-old WT FVB/N female mice using a Hamilton syringe with a 30-gauge needle. Tumors were excised, weighed, and fixed in formalin 8 weeks after injection. Met-1 cells are syngeneic to the FVB/N strain.

Mammary Tumor Implantation Studies

A large mammary adenocarcinoma from a tumor-bearing MMTV-PyMT female mouse (at ~3 months of age) was excised and cut into small ~8-mm³ cuboidal pieces before transplantation. Then, 3-month-old WT and Cav-1 KO host female mice were anesthetized with ketamine/xylazine, and one tumor transplant was embedded in a sterile manner into a small pocket made with forceps in the inguinal mammary gland. Mice were surgically closed with staples. After 3 weeks, tumors were excised, weighed, and fixed in formalin for histological analysis.

Results

Female Cav-1 KO Mammary Glands Show Dysregulated Cell Proliferation Including Ductal Hyperplasia and Enlarged TEBs

To assess whether Cav-1 has a role in mammary gland development, we examined the process of mammary morphogenesis in Cav-1 KO female virgin mice by whole mount analysis at 3, 4, 5, 6, 7, and 8 weeks of age ($n = 4$ at each age). The mammary epithelial ductal system begins developing in the embryonic stage and by 3 weeks of age extends from the nipple, forming the primary duct and several primary ductal branches, with minimal side-branching. Epithelial ductal development continues as the mouse grows older, and extends from the region of the nipple to throughout the entire mammary fat pad, until the end of development by 8 weeks of age. During ductal morphogenesis, TEBs (bulb-shaped structures at the distal ends of elongating ducts) contain the majority of continuously proliferating stem cells, which give rise to the basal and luminal epithelial cells comprising the ducts.

Cav-1 KO female mammary glands did not demonstrate any significant delay or acceleration of ductal branching during development. As demonstrated in Figure 1, ductal development throughout the mammary fat pad was virtually identical in WT and Cav-1 KO mice. Although the Cav-1 KO mammary fat pad is smaller because of a reduction in the amount of adipose tissue within the gland,³⁷ Cav-1 KO epithelial

ducts progressed equally well along and throughout the fat pad, when compared with age-matched WT ducts. By 4 weeks, the ducts extended just beyond the central lymph node, and, by 6 weeks, they traversed half the distance between the lymph node and end of the fat pad. By 8 weeks, ductal morphogenesis was complete and had spread throughout the entire fat pad. Furthermore, we found no significant differences in the number of side branches arising off of the main ducts (not shown). This is consistent with previous quantitative studies of branch number in the mammary glands of 3-month-old female Cav-1 KO mice.³⁷

However, there was considerable ductal hyperplasia in Cav-1 KO mice (Figure 2A). Quantitation of the thickness of carmine dye-stained glands revealed that Cav-1 KO ducts are ~2.6-fold thicker than WT ducts (WT ducts, 30 ± 3.5 μ m; Cav-1 KO ducts, 77 ± 7.7 μ m; Figure 2B). This is consistent with previous nonquantitative histological observations that most WT ductal walls are one to two cell layers thick, compared with three to five cells thick in Cav-1 KO mice.¹⁴ This hyperplasia was consistently observed at all time points, from 3 to 8 weeks of age.

Interestingly, we also noted marked differences in the size of TEBs in Cav-1 KO mice (Figure 2C). TEBs in Cav-1 KO glands were 0.098 ± 0.014 mm² in size, ~2.8-fold larger than WT TEBs (0.035 ± 0.004 mm²; Figure 2D). However, no significant differences in the total number of TEBs per gland were detected (not shown).

We also examined Cav-1^{+/-} heterozygous (HET) female mice between 3 and 8 weeks to determine whether a partial Cav-1 deficiency results in similar findings. However, we found that Cav-1 HET female mice do not differ significantly from WT female mice in terms of ductal morphogenesis, ductal thickness, or TEB size (not shown). These results indicate that complete loss of Cav-1 is required to observe ductal hyperplasia and TEB enlargement.

Male Cav-1 KO Mice Demonstrate Mammary Epithelial Compartment Development

We have previously reported that male Cav-1 KO mice develop mammary tumors at a dramatically faster rate than male WT mice, both in the MMTV-PyMT genetic background.²⁰ As depicted in Figure 3, male Cav-1 KO mice exhibited mild ductal hyperplasia. More surprisingly, however, male Cav-1 KO mice demonstrated significantly more epithelial ductal development than male WT mice. This finding suggests that the presence of Cav-1 in male mammary glands normally prevents excessive epithelial compartment development. In light of this observation, the dramatic increases in mammary tumorigenesis in male Cav-1 KO mice (observed when interbred with MMTV-PyMT mice) can be partially explained by the presence of a more extensive ductal epithelial system.

Figure 1. Morphological development of the mammary gland in female Cav-1 KO mice. Whole-mount analysis of inguinal mammary glands from female virgin WT and Cav-1 KO mice at 4, 6, and 8 weeks of age. Isolated glands were spread onto glass slides, fixed, and stained with Carmine-alum red dye overnight. Photomicrographs shown are representative images at each age. Note that there are no significant differences in the epithelial ductal progression from the primary duct (PD), past the inguinal lymph node (LN), to the medial edge of the mammary fat pad, between WT and Cav-1 KO mice. Both images at each age are the same magnification. Scale bars = 2 mm (4-week-old images); 4 mm (6- and 8-week-old images).

Mammary Fat Pads Reconstituted with Cav-1 KO Epithelial Elements Develop an Abnormal Appearance, with Areas of Focal Hyperproliferation

To investigate whether the epithelial hyperplasia observed in Cav-1 KO mammary tissue is attributable to an intrinsic

loss of Cav-1 within the mammary epithelia or whether it is a result of the loss of Cav-1 in neighboring adipocytes or stromal cells, we performed mammary reconstitution experiments. This allowed us to examine how complete loss of Cav-1 specifically in mammary epithelia affects ductal morphogenesis in a normal WT fat pad whose nonepithelial components express WT levels of Cav-1.

Figure 2. Female Cav-1 KO mammary glands show ductal hyperplasia and enlarged TEBs. **A:** Higher power Carmine dye-stained images of epithelial ducts from WT and Cav-1 KO female inguinal mammary glands from 6-week-old mice. Cav-1 KO mammary epithelial ducts exhibit pronounced hyperplasia, resulting in increased ductal thickness (brackets). **B:** Quantitation of mammary epithelial ductal thickness. Note that Cav-1 KO mammary epithelial ducts in 6-week-old female virgin mice are ~2.6-fold thicker than corresponding ducts in WT mice. This difference is highly statistically significant ($P < 0.0001$, Student's *t*-test, **asterisk**). A total of 30 ducts were measured for each genotype, from three different mice [WT ($n = 3$) and Cav-1 KO ($n = 3$)]. **C:** Representative high-power images of TEBs from WT and Cav-1 KO mammary epithelial ducts at 5 weeks of age stained with Carmine dye. **Arrows** denote TEBs. Note the dramatic increase in size of the TEBs in Cav-1 KO glands. All images are the same magnification. **D:** Quantitation of TEB size (as determined by area). Note that Cav-1 KO TEBs in 5-week-old female virgin mice are ~2.8-fold larger in size than WT TEBs. This difference is highly statistically significant ($P < 0.0003$, Student's *t*-test, **asterisk**). A total of 30 TEBs were measured for each genotype, from three different mice [WT ($n = 3$) and Cav-1 KO ($n = 3$)]. Scale bars = 0.5 mm (**A**); 0.25 mm (**C**).

We first obtained the mammary epithelial elements of 3-week-old female WT and Cav-1 KO mice by surgically isolating a small piece of the mammary gland corresponding to the nipple region. Next, we cleared the mammary fat pads of epithelial tissue in female 3-week-old WT mice, using the technique described by DeOme and colleagues.³⁶ Small pockets were then made within cleared host mammary fat pads, and the epithelial transplants were embedded into the cleared mammary fat tissue. Host mice were surgically closed, and mammary fat pads were allowed to develop for 8 weeks before their removal and whole mount analysis.

WT mice transplanted with WT epithelial elements ($n = 8$) developed normal-appearing mammary glands compared with mock surgically treated WT mice (Figure 4A). Ductal thickness, branching, and morphology were completely normal. As an additional control, we also cleared the fat pads of WT mice and surgically closed the mice without providing a transplant ($n = 2$). These mice did not

demonstrate any epithelial development after 8 weeks (not shown). However, when WT host mice were transplanted with Cav-1 KO epithelial tissue ($n = 8$), we observed dramatic morphological differences. In all cases, epithelial ducts stained more darkly, demonstrated a jagged branching appearance, and exhibited numerous areas of aberrant focal lobulo-alveolar hyperproliferation (Figure 4A, arrows, bottom right; and Figure 4B, left). These areas of focal lobulo-alveolar hyperproliferation were examined by histological analysis and determined to consist exclusively of a disordered mass of epithelial cells, which was not attributable to an infiltration of immune cells (Figure 4B, middle). Cytokeratin immunostaining confirmed the epithelial nature of these cells (not shown). Interestingly, however, we did not observe increased ductal thickness in WT mice reconstituted with Cav-1 KO tissue (Figure 4B, right). These results provide direct evidence that the intrinsic loss of Cav-1 in mammary epithelial cells results in dysregulated cell prolifer-

Figure 3. Male Cav-1 KO mice show increased mammary epithelial development. Inguinal mammary glands from WT ($n = 4$) and Cav-1 KO ($n = 4$) male mice were isolated, fixed, and stained with Carmine dye. Note that at 2 months of age (top), male KO mice demonstrate significantly more ductal development at the origin of the mammary epithelial tree (arrows). PD, primary duct. Scale bars = 2 mm.

ation *in vivo*. However, the ductal hyperplasia (increased ductal thickness) observed in Cav-1 KO mice appears to be attributable to loss of Cav-1 in the nonepithelial components of the mammary fat pad (see below).

Cav-1 KO Mammary Epithelial Cells Demonstrate Increased Rates of Proliferation in Vivo

The findings of focal hyperproliferation from the mammary reconstitution experiments suggest that the loss of Cav-1 in mammary epithelial cells may result in increased proliferative rates. To determine whether Cav-1 KO mammary epithelial cells show a tendency toward increased proliferation *in vivo*, we assessed BrdU incorporation using 6-week-old mammary glands from WT and Cav-1 KO virgin female mice (using immunocytochemistry). At this age, mammary epithelial cells are still actively dividing as they spread throughout the mammary fat pad. The majority of proliferating cells are found in the TEBs. We injected BrdU intraperitoneally into female mice, sacrificed the mice 2 hours later, isolated the mammary glands, and fixed them in formalin.

Interestingly, BrdU incorporation was significantly higher in Cav-1 KO mammary epithelial cells, compared with WT mammary epithelial cells (Figure 5, A and B). Excluding TEBs, 0.13% of WT mammary epithelial cells were BrdU⁺, compared with 2.75% of Cav-1 KO mammary epithelial cells. This ~21-fold increase in BrdU incorporation was highly statistically significant ($P < 0.002$, Student's *t*-test, asterisk) (Figure 5B).

Because BrdU is only incorporated in actively dividing cells, these results indicate that Cav-1 KO mammary epithelial cells possess increased rates of proliferation *in vivo*. We believe that a larger proportion of the cells are dividing at any given time (ie, fewer cells are in G₀/G₁), as we have previously shown that overexpression of Cav-1 leads to cell cycle arrest in the G₀/G₁,

phase of the cell cycle.³⁵ TEBs from Cav-1 KO glands also demonstrated an approximately threefold to fourfold increases in the number of BrdU⁺ cells (not shown). Cross sections and longitudinal sections of epithelial ducts stained for BrdU are shown in Figure 5A. Interestingly, note that the increase in BrdU labeling appears to be associated almost exclusively with the mammary epithelial cell population, rather than adjacent stromal cells.

Cav-1 KO Mammary Glands Demonstrate Cyclin D1 Overexpression, ERK-1/2 Hyperactivation, and Increased STAT5a Signaling

To identify the molecular basis for the increased mammary epithelial cell proliferation in Cav-1 KO mice, we performed immunoblotting on mammary tissue (isolated from 6-week-old virgin mice) to determine the activation state of a number of Cav-1-associated signaling molecules. As demonstrated in Figure 6A, significant cyclin D1 up-regulation was observed in Cav-1 KO mammary glands. This finding is consistent with our prior studies in which we showed that Cav-1 repressed the transcriptional activation of cyclin D1.³⁸ Thus, the complete absence of Cav-1 in Cav-1 KO tissue would be predicted to abolish the Cav-1-mediated transcriptional repression of cyclin D1, resulting in the overexpression of this proto-oncogene. In addition, note the ERK-1/2 hyperactivation in Cav-1 KO tissue, detected with a phospho-specific antibody probe that only recognized activated phospho-ERK-1/2. The epithelial content of the samples was normalized using a pan-cytokeratin antibody. Taken together, these results provide a molecular basis for the observed increases in cell proliferation in Cav-1 KO mammary epithelial cells.

Activated STATs induce cyclin D1 expression,^{39,40} and Cav-1 inhibits STAT signaling.^{16,41} Indeed, cyclin D1 is

required for STAT-induced cellular transformation.⁴² Consistent with the inhibition of STAT signaling by Cav-1, we observed increased STAT5a signaling, as determined by increased levels of both STAT5a and phospho-STAT5a (Figure 6A).

In addition, we performed immunohistochemistry on female Cav-1 KO mammary glands to investigate if cyclin D1 is specifically up-regulated within the mammary epithelial cells themselves and not the surrounding stroma. For this purpose, we isolated the mammary glands from 6-week-old female virgin mice and fixed them in formalin before immunostaining. Coincident with the immunoblotting results, we observed increased cyclin D1 immunostaining directly within the mammary epithelial cells of Cav-1 KO mice (Figure 6B, asterisks).

Further evidence of an inverse relationship between Cav-1 and cyclin D1 expression was achieved by extending our analysis to a nontransformed human mammary epithelial cell line, hTERT-HME1 cells. We have previously generated stable hTERT-HME1 cell lines with a pBABE (empty vector) or pBABE-Cav-1 Myc-tagged construct.¹⁴ Interestingly, forced overexpression of Cav-1 caused a dramatic reduction in cyclin D1 levels in hTERT-HME1 cells (Figure 6C). These additional findings directly show that Cav-1 expression in mammary epithelial cells negatively regulates cyclin D1 expression, both in human and mouse mammary epithelial cells.

Loss of Cav-1 Exacerbates the Lobulo-Alveolar Hyperplasia Phenotype of MMTV-Cyclin D1 Mice, Whereas Cav-1 Transgenic Overexpression Rescues Their Hyperplastic Phenotype

The identification of a negative regulatory relationship between Cav-1 and cyclin D1 prompted us to study the effect of Cav-1 on cyclin D1-induced mammary hyperplasia. We analyzed MMTV-cyclin D1 (MMTV-CD1) mice, which selectively overexpress cyclin D1 in the mammary epithelium.^{32,33} Cyclin D1 expression in these mice is sufficient to induce mammary lobulo-alveolar hyperplasia and, after a long latency period, mammary adenocarcinomas.³² Thus, we interbred MMTV-CD1 mice with Cav-1-null to generate female MMTV-CD1/Cav-1^{+/+} and MMTV-CD1/Cav-1^{-/-} mice. In addition, we generated female MMTV-CD1/Cav-1 Tg mice by crossing MMTV-CD1 mice with Cav-1 Tg mice.

At 6 months of age, we isolated mouse mammary glands and performed whole-mount analysis. As demonstrated in Figure 7A (Carmine-red staining) and Figure 7B (H&E staining), loss of Cav-1 significantly increased the degree of mammary lobulo-alveolar hyperplasia observed in MMTV-CD1 mice. In fact, genetic ablation of Cav-1 in MMTV-CD1 female mice resulted in excessive lobulo-alveolar proliferation, which appears reminiscent of a late-stage pregnant morphology in WT female mice. Loss of Cav-1 also resulted in substantially increased ductal thickening with fluid/milk

accumulation (Figure 7B, top middle, asterisk). Conversely, Tg overexpression of Cav-1 in MMTV-CD1 mice resulted in a significant reversion of the MMTV-CD1 phenotype toward a WT (non-MMTV-CD1) state. These findings provide a dramatic *in vivo* demonstration that loss of Cav-1 potentiates the formation of cyclin D1-induced preneoplastic lesions (ie, lobulo-alveolar hyperplasia) in mammary tissue. Furthermore, forced Cav-1 expression can attenuate or rescue cyclin D1-induced hyperplasia.

Cav-1-Mediated Inhibition of Mammary Tumor Growth Is Dependent on Stromal Interactions within the Mammary Fat Pad

To investigate the ability of Cav-1 to inhibit tumorigenesis in transformed mammary epithelial cells, we stably overexpressed Cav-1 in Met-1 cells through a retroviral approach.²⁰ Met-1 cells are a highly metastatic and tumorigenic cell line established from a mammary adenocarcinoma derived from a female MMTV-PyMT mouse. Met-1 cells normally express extremely low levels of Cav-1, and we have previously shown that re-expression of Cav-1 reduces their ability to undergo experimental metastasis.²⁰ Here, we used Met-1 cells to determine the effects of Cav-1 on primary tumor formation.

First, we explored the ability of Cav-1 to inhibit Met-1 tumorigenesis by performing subcutaneous injections into the flanks of female mice. After flank injection of 10⁶ cells [either Met-1/pBABE (empty vector) or Met-1/Cav-1 cells], tumors were allowed to grow for 3 weeks at these ectopic sites before their removal. Interestingly, overexpression of Cav-1 in these mammary carcinoma-derived cells did not significantly alter tumor weight (Figure 8A) or size (not shown) when the cells were injected into this ectopic site.

Next, we injected 0.5 × 10⁵ cells of the Met-1 stable cell lines through the nipple of the inguinal (no. 4) mammary gland (WT FVB/N mice) and allowed tumors to form throughout an 8-week period. Growing tumors orthotopically has the advantage of returning transformed cells to their native tissue, thereby providing cells with all of the appropriate physiological stimuli, as well as proper epithelial-mesenchymal interactions. Interestingly, we found a dramatic reduction in the size and weight of tumors derived from Met-1/Cav-1 cells, compared with Met-1/pBABE cells (vector alone control) (Figure 8B). Met-1/Cav-1 cells formed very small tumors weighing an average of 0.04 ± 0.01 g, a highly statistically significant ~23-fold reduction, compared with Met-1/pBABE tumors that weighed an average of 0.88 ± 0.15 g (*P* < 0.0005, Student's *t*-test). Histologically, these orthotopically grown tumors did not differ in their morphological appearance (data not shown). However, Met-1/pBABE tumors frequently demonstrated large central areas of necrosis, indicating that tumor angiogenesis cannot keep pace with the growth rate of these transformed cells. Met-1/Cav-1 tumors

A Mammary Transplants / Cav-1 (+/+) Fat Pads

B Cav-1 (+/+) Fat Pads, Transplanted with Cav-1 (-/-) ME Cells

were dramatically smaller and, thus, did not demonstrate these areas of necrosis.

We performed immunohistochemistry to determine whether the orthotopically grown tumors would demonstrate any differences in cyclin D1 expression. Notably, Met-1/pBABE tumors expressed substantially more cyclin D1 compared with Met-1/Cav-1 tumors, especially at the leading edge of the tumors (Figure 8C). These results indicate that Cav-1 also functions as a suppressor of cyclin D1 in transformed mammary epithelial cells.

Mammary Reconstitution of Cav-1 KO Mice Reveals that the Ductal Hyperplasia Is Attributable to Nonepithelial/Stromal Components

The results of the aforementioned mammary reconstitution in WT mice revealed that the loss of Cav-1 in mammary epithelial cells resulted in dysregulation of cell proliferation and focal lobulo-alveolar hyperplasia (Figure 4). However, the earlier finding that Cav-1 KO female mice also exhibit mammary ductal hyperplasia (increased ductal thickness) was not observed when Cav-1 KO mammary epithelial elements were transplanted into WT mice. Because the inhibition of Met-1 tumor growth by Cav-1 appears to be a stromal-dependent phenomenon, we postulated that the loss of Cav-1 in the nonepithelial/stromal compartment of the mammary fat pad may be responsible for this ductal hyperplasia phenotype.

Therefore, we transplanted WT ($n = 4$) and Cav-1 KO ($n = 4$) mammary epithelial elements from 3-week-old female mice into epithelial-cleared mammary glands of 3-week-old female Cav-1 KO mice (Figure 9, A and B). Transplants were once again allowed to reconstitute the fat pads for 8 weeks before whole-mount analysis. As expected mock surgically treated WT mice (Figure 9B, bottom right) exhibited no ductal thickening, whereas mock surgically treated Cav-1 KO mice (Figure 9B, bottom left) demonstrated the usual ductal thickening. As an additional control, Cav-1 KO mice that were cleared and surgically closed with no transplants developed no epithelial ducts after 8 weeks ($n = 2$) (not shown). Likewise, Cav-1 KO epithelial transplants into Cav-1 KO cleared mammary fat pads (Figure 9B, top left) appeared identical to Cav-1 KO control glands. Interestingly, when WT epithelial elements were transplanted into epithelial-cleared Cav-1 KO mammary fat pads, ductal thickening was also observed (Figure 9B, top right). The degree of ductal thickening in these mice was the same as in nor-

mal nontransplanted Cav-1 KO mice or in Cav-1 KO mice transplanted with Cav-1 KO epithelial elements.

These results demonstrate that the complete loss of Cav-1 in the adipocytes and/or other stromal elements of the fat pad is directly responsible for the observed ductal thickening phenotype. Taken together, our mammary reconstitution experiments (Figures 4 and 9) directly show that the epithelium and stromal elements both contribute to the mammary epithelial hyperplasia phenotype(s), which results from the whole-body genetic ablation of Cav-1.

Orthotopic Transplantation of PyMT Tumors into Mature Cav-1 KO Fat Pads Enhances Mammary Tumor Growth

Because it appears that the stromal components of the Cav-1 KO fat pad appear to promote mammary ductal hyperplasia, we also investigated whether Cav-1 KO fat pads can facilitate mammary tumor growth. Therefore, we isolated a mammary adenocarcinoma from a 3-month-old MMTV-PyMT female mouse and transplanted small pieces of the tumor into the mammary fat pads of 3-month-old WT and Cav-1 KO mice. These orthotopic tumor transplants were allowed to grow for 3 weeks, at which point the tumors were isolated and weighed. As depicted in Figure 10A, histological characterization of the tumors from WT and Cav-1 KO mice did not demonstrate any significant morphological differences. However, mammary tumors grown in Cav-1 KO fat pads weighed an average of 1.56 ± 0.33 g, an ~66% increase compared with WT tumors, whose average weight was 0.94 ± 0.11 g. This difference was statistically significant ($P < 0.05$, Student's *t*-test, asterisk). These results indicate that the Cav-1 KO mammary fat pad promotes mammary tumor growth. In combination with the mammary reconstitution experiments, these findings reveal that the loss of Cav-1 in nonepithelial/stromal components of the mammary fat pad has both hyperplastic and tumor-promoting effects.

Discussion

Here, we have characterized the development of the mammary gland in the setting of complete Cav-1 genetic ablation. We have learned that while overall morphogenesis of the epithelial compartment is not affected in Cav-1 KO female mice, there is significant ductal hyperplasia in the form of ductal thickening that appears as early as 3 weeks of age. This ductal hyperplasia is apparent

Figure 4. Reconstitution of WT fat pads with Cav-1 KO mammary epithelial elements reveals that loss of Cav-1 results in focal lobulo-alveolar hyperproliferation. **A:** Mammary transplants were performed on 3-week-old WT host mice using transplant tissue derived from WT ($n = 8$) or Cav-1 KO ($n = 8$) female virgin mice. Inguinal glands were isolated after 8 weeks and stained with carmine dye to assess mammary epithelial development. Top row: lower power images, bottom row: higher power images. Note that WT transplants (middle) appear morphologically similar to normal, nontransplanted WT mammary epithelial tissue (left). Cav-1 KO donor tissue, however, demonstrates areas of focal hyperproliferation and tissue disorganization. **Arrows** at the top denote the sites of transplanted tissue. **Arrows** at the bottom right highlight several areas of focal hyperproliferation. The boxed area at the top right is also shown as a higher power image in **B**. **ME,** mammary epithelia; Cav-1^{+/+}, WT; Cav-1^{-/-}, KO. **B:** Left: higher power carmine-stained image of focal hyperproliferation observed in WT mammary fat pads reconstituted with Cav-1 KO mammary epithelial tissue. Middle and right: Hematoxylin-stained sections of WT mammary fat pads reconstituted with Cav-1 KO mammary epithelial tissue. The middle panel shows a representative region of epithelial hyperproliferation often seen at ductal branch points. The right panel shows that general ductal thickness and morphology appear unaltered. Scale bars = 2 mm (**A**); 50 μ m (**B**).

Figure 5. Cav-1 KO mammary epithelial cells demonstrate increased rates of proliferation. Inguinal mammary glands from WT ($n = 4$) and Cav-1 KO ($n = 4$) 6-week-old virgin female mice were isolated and formalin-fixed 2 hours after injection with BrdU. After tissue processing, 5- μ m sections were cut and processed for BrdU immunohistochemistry. **A:** H&E-stained photomicrographs from WT and Cav-1 KO mammary glands stained for BrdU. Note the increased number of BrdU⁺ cells (brown) in Cav-1 KO mammary epithelial cells in both cross-sections and longitudinal sections. **B:** The number of BrdU⁺ cells was ~21-fold greater in Cav-1 KO glands compared with WT glands. At least 1300 mammary epithelial cells were counted for each genotype. TEBs were excluded from the counting process. The increase in number of BrdU⁺ cells is highly statistically significant (asterisk; $P < 0.002$, Student's t -test). Scale bars = 50 μ m (**A**).

throughout the remainder of glandular development (8 weeks) and does not regress in the adult stage. Quantitation of the ductal hyperplasia reveals that Cav-1 KO ducts are ~2.6-fold thicker than WT ducts. This is consistent with previous histological findings that the walls of Cav-1 KO ducts are typically three to five cells thick,

compared with WT ducts that are one to two cells thick. In addition, we have observed TEB enlargement during ductal morphogenesis. Because TEBs harbor mammary stem cells, these findings are consistent with the idea that Cav-1 KO mice have an increase in their mammary stem cell population.⁴³ The increase in growth of these contin-

Figure 6. Cyclin D1 expression in the mammary gland and in a human mammary epithelial cell line is inversely related to Cav-1 expression. **A:** Immunoblot analysis. Mammary glands were harvested from 6-week-old WT and Cav-1 KO mice. Tissue lysates were prepared, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Normalization of epithelial cell content was performed using a pan-cytokeratin antibody. Relative levels of total ERK-1/2 are unchanged. However, Cav-1 KO tissue demonstrates cyclin D1 up-regulation and ERK-1/2 hyperactivation, as well as changes in the levels of phospho-STAT5a and total STAT5a. **B:** Immunohistochemistry. To visualize the tissue distribution of cyclin D1, we performed immunohistochemical analysis on mammary tissue derived from 6-week-old female WT and Cav-1 KO mice. Paraffin-embedded mammary glands were sectioned at 5 μ m and immunostained with a rabbit polyclonal antibody to cyclin D1. Note that Cav-1 KO mammary epithelial cells demonstrate increased cyclin D1 immunostaining (brown/black color; asterisks). The boxed area is shown at higher magnification. **C:** hTERT-HME1 stable cell lines. hTERT-HME1 cells (a human mammary epithelial cell line, immortalized with telomerase) were stably transfected with pBABE alone (empty vector) or pBABE-Cav-1 (C-terminally Myc-tagged). These cell lines were then characterized by immunoblotting with a rabbit polyclonal antibody directed against Cav-1 (N-20). Note that hTERT-HME1-Cav-1 cells express both endogenous (lower band) and exogenous (Myc-tagged, upper band) Cav-1 (arrow). Interestingly, recombinant expression of Cav-1 in hTERT-HME1 cells causes a dramatic reduction in the steady-state expression levels of cyclin D1 (asterisk). Blotting with β -tubulin is shown as a control of equal protein loading. Scale bar = 50 μ m (**B**).

uously extending and proliferating structures offers a potential explanation for the observed increase in ductal thickness, because ductal epithelium is the by-product of TEB extension and growth.

In addition, we have clearly established a relationship between Cav-1 and mammary epithelial proliferation *in vivo*. We show that Cav-1 KO ducts demonstrate increased proliferative rates because these ducts incorporate significantly more BrdU during development. Once development is completed, however, Cav-1 KO ducts do not continue to become enlarged throughout adulthood (unpublished observations). One can therefore assume that proper developmental signals are in place to restrict further growth of Cav-1 KO mammary epithelial cells in the virgin state. During the natural process of pregnancy, however, the epithelial compartment once again undergoes rapid expansion and proliferation. In this situation, we have observed that Cav-1 KO mammary epithelial

cells demonstrate precocious development of the lobulo-alveolar compartment, as well as accelerated milk protein production and STAT5a hyperactivation.¹⁶

The initial discovery that Cav-1 can transcriptionally repress cyclin D1 was made using luciferase-based promoter studies in cultured CHO cells.³⁸ This initial observation led us to investigate the interrelationship between Cav-1 and cyclin D1 in nontransformed mammary epithelial cells, both *in vitro* and *in vivo*. Cyclin D1 forms the regulatory component of the cyclin D1-cdk4/6 holoenzyme that phosphorylates the retinoblastoma, Rb, tumor suppressor protein.^{30,34} Inactivation of Rb by phosphorylation allows cells to exit the G₁ phase of the cell cycle and progress into S phase (DNA synthesis).³⁰ The importance of cyclin D1 in human breast cancer is supported by the findings that ~15 to 20% of breast tumors demonstrate *Cyclin D1* gene amplification, with a much larger proportion (>50%) exhibiting cyclin D1 overexpres-

Figure 7. Loss of Cav-1 exacerbates the mammary hyperplasia phenotype of MMTV-cyclin D1 mice, whereas transgenic overexpression of Cav-1 abrogates this hyperproliferative phenotype. **A:** Representative images of Carmine dye-stained inguinal mammary glands from 6-month-old [MMTV-CD1 ($n = 8$), MMTV-CD1/Cav-1 KO ($n = 8$), and MMTV-CD1/Cav-1 Tg ($n = 4$)] female virgin mice are shown. Top: Low-power images, bottom: higher magnification images. Note the dramatic increase in mammary epithelial hyperplasia in MMTV-CD1/Cav-1 KO mice that completely lack Cav-1 expression. There is extensive lobulo-alveolar development resembling a pseudo-pregnant state. In contrast, transgenic overexpression of Cav-1 (Cav-1 Tg) in MMTV-CD1 mice strongly diminishes the mammary epithelial cell hyperplasia, almost returning the morphology of the epithelium to a WT (non-MMTV-CD1) state. **B:** Histological analysis (H&E staining) of inguinal mammary glands from MMTV-CD1, MMTV-CD1/Cav-1 KO, and MMTV-CD1/Cav-1 Tg mice. MMTV-CD1/Cav-1 KO mice demonstrate significantly increased side-branching, lobulo-alveolar development, and ductal thickening compared with MMTV-CD1 mice. Substantially enlarged ducts often were fluid filled in MMTV-CD1/Cav-1 KO mice (**asterisk**). On the other hand, MMTV-CD1/Cav-1 Tg mice showed almost complete rescue of the mammary hyperplasia phenotype in MMTV-CD1 mice. Top: Low-power images; Bottom: high-power images. Scale bar = 2 mm (**A**, top); 0.5 mm (**A**, bottom); 200 μ m (**B**, top); 50 μ m (**B**, bottom).

Figure 8. The ability of Cav-1 to inhibit tumor growth in transformed mammary epithelial cells is dependent on mammary gland stromal interactions. **A:** Ectopic Implantation. Met-1 cell lines were injected subcutaneously into the flanks of female mice ($n = 10$ per cell line) and allowed to form tumors. Note that there are no significant differences in tumor weight between Met-1/pBABLE cells or Met-1 cells re-expressing Cav-1. This experiment was performed multiple times with virtually identical results. **B:** Orthotopic Implantation. Met-1 cell lines were injected through the nipple of female FVB/N mice ($n = 5$ per cell line) and allowed to form tumors. Note that Cav-1-overexpressing Met-1 cells demonstrate a dramatic reduction (~ 25 -fold) in tumor weight, compared with Met-1/pBABLE cells. This difference is highly statistically significant ($P < 0.0005$, Student's *t*-test, **asterisk**). This experiment was performed multiple times with virtually identical results. **Inset** depicts representative gross photos of tumors derived from Met-1/pBABLE ($n = 2$, left) and Met-1/Cav-1 ($n = 2$, right) cells. **C:** Immunohistochemistry. Met-1/pBABLE orthotopic tumors (top left) demonstrate marked increases in cyclin D1 expression at the leading edge of the tumors, compared with Met-1/Cav-1 orthotopic tumors (top right). This is consistent with the notion that Cav-1 negatively regulates cyclin D1 expression and/or function during mammary tumorigenesis. Bottom panels demonstrate Cav-1 expression. All images were taken at the same magnification. Scale bar = 25 μ m.

sion.^{26–30,34} Furthermore, in mice, cyclin D1 expression is required for the oncogenic transformation of mammary epithelial cells by activated c-Neu/ErbB2.^{31,44} Definitive proof of the importance of cyclin D1 in murine mammary tumorigenesis was demonstrated through the generation of MMTV-cyclin D1 mice. This Tg mouse model develops significant mammary epithelial cell hyperplasia that progresses to full-blown mammary adenocarcinomas, albeit after a long latency period.^{32,33}

Here, we have provided data suggesting that Cav-1 normally antagonizes cyclin D1 function in 1) normal nontransformed mammary epithelia (hTERT-HMEC cells; Figure 6C), 2) preneoplastic (hyperplastic) mammary epithelial cells (MMTV-CD1 mice; Figure 7, A and B), and 3) transformed mammary epithelial cells (Met-1 cell derived tumors; Figure 8C). These data provide definitive molecular support for the hypothesis that Cav-1 normally exerts

both anti-proliferative and transformation-suppressive activity in mammary epithelial cells.

Despite these results, Cav-1 does not meet the classical criteria as a tumor suppressor protein (such as BRCA-1, Rb, or p53) because Cav-1 KO mice do not spontaneously develop mammary tumors. On the other hand, we have previously demonstrated that Cav-1 KO mice are more susceptible to mammary epithelial transformation and tumorigenesis when interbred with a tumor-prone Tg mouse model (MMTV-PyMT).^{19,20} We also demonstrate here that intrinsic expression of Cav-1 in a mammary carcinoma cell line (Met-1 cells) reduces mammary tumorigenesis. Therefore, Cav-1 may be more suitably termed a “mammary tumor modulator.”

The observation presented here that expression of Cav-1 in mammary tumor cells inhibits tumor growth in orthotopic sites but not in ectopic sites provides an

A Mammary Transplants / Cav-1 (-/-) Fat Pads

B Mammary Transplants / Cav-1 (-/-) Fat Pads

Figure 9. Reconstitution of Cav-1 KO mammary fat pads with WT mammary epithelial elements results in mammary ductal hyperplasia. **A:** Mammary transplants were performed on 3-week-old Cav-1 KO host female mice (Cav-1^{-/-} fat pads) using epithelial transplant tissue derived from WT ($n = 4$) or Cav-1 KO ($n = 4$) female virgin mice. Inguinal glands were isolated after 8 weeks and stained with Carmine-dye to study mammary epithelial development. Transplant sites are indicated by **arrows** (top row). Note the presence of ductal hyperplasia in Cav-1 KO mice transplanted with WT mammary epithelial tissue (right). Mock-transplanted Cav-1 KO glands (left) or Cav-1 KO glands transplanted with Cav-1 KO mammary epithelial tissue (middle) are shown as controls. Top: Low-power images; bottom: higher-power images. ME, mammary epithelia; Cav-1^{+/+}, WT; Cav-1^{-/-}, KO. **B:** Higher magnification views of the ductal hyperplasia observed in Cav-1 KO female mice reconstituted with mammary epithelial tissue from WT female virgin mice. Mock-transplanted Cav-1 KO glands (bottom left) and Cav-1 KO fat pads reconstituted with Cav-1 KO epithelial tissue (top left) both demonstrate ductal thickening. Note that Cav-1 KO fat pads transplanted with WT mammary epithelium also exhibit increased ductal thickness (top right). An image of a mock-transplanted gland from WT female mice, without ductal thickening, is shown for reference (bottom right). All images were taken at the same magnification. Scale bars = 2 mm.

Figure 10. Orthotopic transplantation of PyMT tumors into mature Cav-1 KO fat pads enhances mammary tumor growth. **A:** Representative photomicrographs of tumors derived from WT (left) or Cav-1 KO (right) female mice orthotopically transplanted with small portions of a mammary carcinoma derived from a MMTV-PyMT/Cav-1^{+/+} female mouse. No significant morphological differences were detected. Cav-1^{+/+}, WT; Cav-1^{-/-}, KO. **B:** PyMT mammary tumor transplants were excised after 3 weeks and weighed. Note that the ~66% increase in tumor weight from PyMT-transplanted tumors derived from Cav-1 KO female mice ($n = 10$) compared with WT mice ($n = 10$). Data represent the average of multiple experiments and are statistically significant ($P < 0.05$, Student's *t*-test, **asterisk**). Scale bar = 25 μ m.

explanation for a previous report documenting Cav-1 up-regulation in breast cancer metastasis.⁴⁵ Once the tumor is established in an ectopic site, the expression of Cav-1 can no longer serve to inhibit tumor growth, and stochastic up-regulation of Cav-1 by the tumor cell would no longer serve as a negative selective pressure. In addition, these results provide further evidence for the tissue- or cell-specific roles of Cav-1 with regard to the development of cancer. In fact, in other tissues, such as the prostate, bladder, and esophagus, Cav-1 up-regulation is associated with carcinogenesis.⁴⁶ Therefore, it appears that the transformation-suppressive effects of Cav-1 on mammary tumorigenesis are limited directly to the epithelium, within the mammary fat pad. This is not entirely surprising because the mammary fat pad is an extremely distinctive organ, exposing the epithelial cells to a unique set of signals (ie, growth factors, hormones, and cytokines) that are not present in the same combination or degree in any

other tissue. Indeed, recent studies have documented the importance of the extracellular matrix, stroma, and other nonepithelial components (eg, adipocytes) in modulating the progression of human breast cancer and murine mammary tumorigenesis.^{47,48}

Here, we have demonstrated that Cav-1 expression in the mammary fat pad (namely the stromal microenvironment) is critically important for controlling epithelial morphogenesis, proliferation, and tumorigenesis. Our findings show that the specific absence of Cav-1 in the nonepithelial compartment is directly responsible for the increased epithelial ductal thickening in the Cav-1 KO mouse. This observation clearly demonstrates that the Cav-1 KO stromal environment is dramatically different from that of WT mice. Furthermore, we have demonstrated that loss of Cav-1 in male mice results in marked overdevelopment of the epithelial compartment. Possible explanations for this observation include, but are not limited to, the following: 1) loss of Cav-1 within male mammary epithelial cells affects their proliferative or differentiated state, 2) loss of Cav-1 affects the secretion of certain factors from stromal cells or adipocytes, or 3) the reduction in fat pad size (attributable presumably to a reduction in the adipocyte compartment) prevents sufficient encasement or anti-proliferative signals normally resulting from epithelial-mesenchymal interactions. Finally, we have demonstrated that the loss of Cav-1 in the mammary fat pad also facilitates mammary tumorigenesis using the orthotopic transplantation of small pieces of MMTV-PyMT mammary tumors.

In summary, we have established a direct inverse relationship between Cav-1 and cyclin D1 function in normal and transformed mammary epithelial cells. In addition, we have shown that loss of Cav-1 in either epithelial or nonepithelial cells results in both proproliferative and protumorigenic effects in the mammary gland. Therefore, Cav-1 expression in either of these two mammary gland compartments normally serves to inhibit excessive mammary epithelial cell proliferation, morphogenesis, and transformation.

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References

1. Lisanti MP, Scherer P, Tang Z-L, Sargiacomo M: Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends Cell Biol* 1994, 4:231-235
2. Okamoto T, Schlegel A, Scherer PE, Lisanti MP: Caveolins, a family of scaffolding proteins for organizing "pre-assembled signaling complexes" at the plasma membrane. *J Biol Chem* 1998, 273:5419-5422
3. Cohen AW, Hnasko R, Schubert W, Lisanti MP: Role of caveolae and caveolins in health and disease. *Physiol Rev* 2004, 84:1341-1379
4. Razani B, Woodman SE, Lisanti MP: Caveolae: from cell biology to animal physiology. *Pharmacol Rev* 2002, 54:431-467

5. Williams TM, Lisanti MP: The caveolin proteins. *Genome Biol* 2004, 5:214
6. Scherer PE, Lewis RY, Volonte D, Engelman JA, Galbiati F, Couet J, Kohtz DS, van Donselaar E, Peters P, Lisanti MP: Cell-type and tissue-specific expression of caveolin-2. Caveolins 1 and 2 co-localize and form a stable hetero-oligomeric complex in vivo. *J Biol Chem* 1997, 272:29337-29346
7. Tang Z-L, Scherer PE, Okamoto T, Song K, Chu C, Kohtz DS, Nishimoto I, Lodish HF, Lisanti MP: Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J Biol Chem* 1996, 271:2255-2261
8. Song KS, Scherer PE, Tang Z-L, Okamoto T, Li S, Chafel M, Chu C, Kohtz DS, Lisanti MP: Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J Biol Chem* 1996, 271:15160-15165
9. Razani B, Lisanti MP: Caveolin-deficient mice: insights into caveolar function and human disease. *J Clin Invest* 2001, 108:1553-1561
10. Lee SW, Reimer CL, Oh P, Campbell DB, Schnitzer JE: Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. *Oncogene* 1998, 16:1391-1397
11. Sager R, Sheng S, Anisowicz A, Sotiropoulou G, Zou Z, Stenman G, Swisshelm K, Chen Z, Hendrix MJC, Pemberton P, Rafidi K, Ryan K: RNA genetics of breast cancer: maspin as a paradigm. *Cold Spring Harbor Sym Quant Biol* 1994, LIX:537-546
12. Engelman JA, Lee RJ, Karnezis A, Bearss DJ, Webster M, Siegel P, Muller WJ, Windle JJ, Pestell RG, Lisanti MP: Reciprocal regulation of Neu tyrosine kinase activity and caveolin-1 protein expression in vitro and in vivo. Implications for mammary tumorigenesis. *J Biol Chem* 1998, 273:20448-20455
13. Engelman JA, Zhang XL, Lisanti MP: Sequence and detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1). Methylation of a CpG island in the 5' promoter region of the caveolin-1 gene in human breast cancer cell lines. *FEBS Lett* 1999, 448:221-230
14. Lee H, Park DS, Razani B, Russell RG, Pestell RG, Lisanti MP: Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (-/-) null mice show mammary epithelial cell hyperplasia. *Am J Pathol* 2002, 161:1357-1369
15. Park DS, Lee H, Riedel C, Hult J, Scherer PE, Pestell RG, Lisanti MP: Prolactin negatively regulates caveolin-1 gene expression in the mammary gland during lactation, via a Ras-dependent mechanism. *J Biol Chem* 2001, 276:48389-48397
16. Park DS, Lee H, Frank PG, Razani B, Nguyen AV, Parlow AF, Russell RG, Hult J, Pestell RG, Lisanti MP: Caveolin-1-deficient mice show accelerated mammary gland development during pregnancy, premature lactation, and hyperactivation of the Jak-2/STAT5a signaling cascade. *Mol Biol Cell* 2002, 13:3416-3430
17. Fiucci G, Ravid D, Reich R, Liscovitch M: Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. *Oncogene* 2002, 21:2365-2375
18. Zhang W, Razani B, Altschuler Y, Bouzahzah B, Mostov KE, Pestell RG, Lisanti MP: Caveolin-1 inhibits epidermal growth factor-stimulated lamellipod extension and cell migration in metastatic mammary adenocarcinoma cells (MTLn3). Transformation suppressor effects of adenovirus-mediated gene delivery of caveolin-1. *J Biol Chem* 2000, 275:20717-20725
19. Williams TM, Cheung MW, Park DS, Razani B, Cohen AW, Muller WJ, Di Vizio D, Chopra NG, Pestell RG, Lisanti MP: Loss of caveolin-1 gene expression accelerates the development of dysplastic mammary lesions in tumor-prone transgenic mice. *Mol Biol Cell* 2003, 14:1027-1042
20. Williams TM, Medina F, Badano I, Hazan RB, Hutchinson J, Muller WJ, Chopra NG, Scherer PE, Pestell RG, Lisanti MP: 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. *J Biol Chem* 2004, 279:51630-51646
21. Guy CT, Cardiff RD, Muller WJ: Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* 1992, 12:954-961
22. Maglione JE, Moghanaki D, Young LJ, Manner CK, Ellies LG, Joseph SO, Nicholson B, Cardiff RD, MacLeod CL: Transgenic polyoma middle-T mice model premalignant mammary disease. *Cancer Res* 2001, 61:8298-8305
23. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, Pollard JW: Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol* 2003, 163:2113-2126
24. Hayashi K, Matsuda S, Machida K, Yamamoto T, Fukuda Y, Nimura Y, Hayakawa T, Hamaguchi M: Invasion activating caveolin-1 mutation in human scirrhous breast cancers. *Cancer Res* 2001, 61:2361-2364
25. Sloan EK, Stanley KL, Anderson RL: Caveolin-1 inhibits breast cancer growth and metastasis. *Oncogene* 2004, 23:7893-7897
26. Lammie GA, Fantl V, Smith R, Schuurings E, Brookes S, Michalides R, Dickson C, Arnold A, Peters G: D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. *Oncogene* 1991, 6:439-444
27. Bartkova J, Lukas J, Muller H, Lutzhoft D, Strauss M, Bartek J: Cyclin D1 protein expression and function in human breast cancer. *Int J Cancer* 1994, 57:353-361
28. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G: Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* 1994, 54:1812-1817
29. Zukerberg LR, Yang W, Gadd M, Thor AD, Koerner FC, Schmidt EV, Arnold A: Cyclin D1 (PRAD1) protein expression in breast cancer: approximately one-third of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene. *Mod Pathol* 1995, 8:560-567
30. Sherr CJ: Cancer cell cycles. *Science* 1996, 274:1672-1677
31. Lee RJ, Albanese C, Fu M, D'Amico M, Lin B, Watanabe G, Haines III GK, Siegel PM, Hung MC, Yarden Y, Horowitz JM, Muller WJ, Pestell RG: Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol Cell Biol* 2000, 20:672-683
32. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV: Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 1994, 369:669-671
33. Sakamaki T, Casimiro M, Ju X, Quong AA, Katiyar S, Liu M, Jiao X, Li A, Zhang X, Lu Y, Wang C, Nicholson R, Link T, Shemluck M, Yang J, Fricke ST, Novikoff P, Papanikolaou A, Arnold A, Albanese C, Pestell R: Cyclin D1 determines mitochondrial function in vivo. *Mol Cell Biol* 2006, 26:5449-5469
34. Fu M, Wang C, Li Z, Sakamaki T, Pestell RG: Cyclin D1: normal and abnormal functions. *Endocrinology* 2004, 145:5439-5447
35. Galbiati F, Volonte D, Liu J, Capozza F, Frank PG, Zhu L, Pestell RG, Lisanti MP: Caveolin-1 expression negatively regulates cell cycle progression by inducing G(0)/G(1) arrest via a p53/p21(WAF1/Cip1)-dependent mechanism. *Mol Biol Cell* 2001, 12:2229-2244
36. DeOme KB, Faulkin Jr LJ, Bern HA, Blair PB: Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res* 1959, 19:515-520
37. Razani B, Combs TP, Wang XB, Frank PG, Park DS, Russell RG, Li M, Tang B, Jelicks LA, Scherer PE, Lisanti MP: Caveolin-1-deficient mice are lean, resistant to diet-induced obesity, and show hypertriglyceridemia with adipocyte abnormalities. *J Biol Chem* 2002, 277:8635-8647
38. Hult J, Bash T, Fu M, Galbiati F, Albanese C, Sage DR, Schlegel A, Zhurinsky J, Shtutman M, Ben-Ze'ev A, Lisanti MP, Pestell RG: The cyclin D1 gene is transcriptionally repressed by caveolin-1. *J Biol Chem* 2000, 275:21203-21209
39. Matsumura I, Kitamura T, Wakao H, Tanaka H, Hashimoto K, Albanese C, Downward J, Pestell RG, Kanakura Y: Transcriptional regulation of cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J* 1999, 18:101-111
40. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Albanese C, Pestell RG, Darnell JE: Stat3 as an oncogene. *Cell* 1999, 98:295-303
41. Sotgia F, Schubert W, Pestell RG, Lisanti MP: Genetic ablation of caveolin-1 in mammary epithelial cells increases milk production and hyper-activates STAT5a signaling. *Cancer Biol Ther* 2006, 5:292-297
42. Leslie K, Lang C, Devgan G, Azare J, Berishaj M, Gerald W, Kim YB,

- Paz K, Darnell JE, Albanese C, Sakamaki T, Pestell R, Bromberg J: Cyclin D1 is transcriptionally regulated by and required for transformation by activated signal transducer and activator of transcription 3. *Cancer Res* 2006, 66:2544–2552
43. Sotgia F, Williams TM, Cohen AW, Minetti C, Pestell RG, Lisanti MP: Caveolin-1-deficient mice have an increased mammary stem cell population with upregulation of Wnt/beta-catenin signaling. *Cell Cycle* 2005, 4:1808–1816
 44. Yu Q, Geng Y, Sicinski P: Specific protection against breast cancers by cyclin D1 ablation. *Nature* 2001, 411:1017–1021
 45. Yang G, Truong LD, Timme TL, Ren C, Wheeler TM, Park SH, Nasu Y, Bangma CH, Kattan MW, Scardino PT, Thompson TC: Elevated expression of caveolin is associated with prostate and breast cancer. *Clin Cancer Res* 1998, 4:1873–1880
 46. Mouraviev V, Li L, Tahir SA, Yang G, Timme TM, Goltsov A, Ren C, Satoh T, Wheeler TM, Ittmann MM, Miles BJ, Amato RJ, Kadmon D, Thompson TC: The role of caveolin-1 in androgen insensitive prostate cancer. *J Urol* 2002, 168:1589–1596
 47. Iyengar P, Combs TP, Shah SJ, Gouon-Evans V, Pollard JW, Albanese C, Flanagan L, Tenniswood MP, Guha C, Lisanti MP, Pestell RG, Scherer PE: Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. *Oncogene* 2003, 22:6408–6423
 48. Wiseman BS, Werb Z: Stromal effects on mammary gland development and breast cancer. *Science* 2002, 296:1046–1049