Tumorigenesis and Neoplastic Progression

Caveolin-1 Deficiency (–/–) Conveys Premalignant Alterations in Mammary Epithelia, with Abnormal Lumen Formation, Growth Factor Independence, and Cell Invasiveness

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During breast cancer development, the luminal space of the mammary acinar unit fills with proliferating epithelial cells that exhibit growth factor-independence, cell attachment defects, and a more invasive fibroblastic phenotype. Here, we used primary cultures of mammary epithelial cells derived from genetically engineered mice to identify caveolin-1 (Cav-1) as a critical factor for maintaining the normal architecture of the mammary acinar unit. Isolated cultures of normal mammary epithelial cells retained the capacity to generate mammary acini within extracellular matrix. However, those from Cav-1 (–/–) mice exhibited defects in three-dimensional acinar architecture, including disrupted lumen formation and epidermal growth factor-independent growth due to hyperactivation of the p42/44 mitogen-activated protein kinase cascade. In addition, Cav-1-null mammary epithelial cells deprived of exogenous extracellular matrix underwent a spontaneous epithelial-mesenchymal transition, with reorganization of the actin cytoskeleton, and E-cadherin redistribution. Mechanistically, these phenotypic changes appear to be caused by increases in matrix metalloproteinase-2/9 secretion and transforming growth factor- β /Smad-2 hyperactivation. Finally, loss of Cav-1 potentiated the ability of growth factors (hepatocyte growth factor and basic fibroblast growth factor) to induce mammary acini branching, indicative

of a more invasive fibroblastic phenotype. Thus, a Cav-1 deficiency profoundly affects mammary epithelia by modulating the activation state of important signaling cascades. Primary cultures of Cav-1-deficient mammary epithelia will provide a valuable new model to study the spatial/temporal progression of mammary cell transformation. (Am J Pathol 2006, 168:292–309; DOI: 10.2353/ajpath.2006.050429)

Development of human breast cancers is a multistep process, arising from genetic alterations that drive the transformation of normal mammary epithelial cells into highly malignant derivatives.¹ One of the earliest premalignant lesions is hyperplasia, characterized by an increase in the number of epithelial cell layers of the mammary duct, often leading to luminal occlusion. During abnormal proliferation within the lumen, mammary epithelial cells undergo important changes that allow them to circumvent anti-proliferative signals, and to escape physical and cell contact-mediated restraints from the surrounding environment.^{2,3} As a consequence, mammary epithelial cells acquire the ability to proliferate in the absence of a growth stimulus and display defects in cell-substrate attachment. As transformation progresses, they lose their epithelial features and acquire a fibroblastic morphology, suggestive of a more undifferentiated and invasive state.⁴ Although important clues have accu-

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mulated in the last 2 decades, the molecular mechanisms underlying many of these crucial steps still await elucidation.

Hyperactivation of mitogenic signaling originating from receptor tyrosine kinases [HER-2/c-Neu and epidermal growth factor receptor (EGF-R)] and their downstream components [including the Ras-p42/44 mitogen-activated protein (MAP) kinase cascade] plays a key role in initiating the abnormal proliferation of mammary epithelial cells.⁵ Human breast cancers frequently contain increased proportions of cells with activated MAP kinase,⁶ arising from several mechanisms, including the constitutive activation of growth factor receptors, and the autocrine production of growth factors.⁷⁻⁹ A common feature of premalignant lesions *in vivo* is the perturbation of cell-substrate attachment. In the normal mammary gland, reciprocal communication between mammary epithelial cells and the surrounding microenvironment, ie, the extracellular matrix and adjacent stromal cells, modulates tissue architecture and homeostasis. Disruption of this interplay is critical for tumor invasiveness.

One of the first steps of invasion is migration, during which cancer cells detach from neighboring cells and the surrounding matrix. Cell-substrate detachment involves degradation of the basement membrane, and requires the secretion of proteases that are specific for extracellular matrix components.^{10,11} For example, two members of the matrix metalloproteinase (MMP) family, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), display the highest enzymatic activity against type IV collagen, which is the main constituent of the basement membrane. Moreover, in addition to contributing to the proteolysis of extracellular matrix components, MMP-2 activity has been shown to modulate the adhesion and spreading of human melanoma cell lines.¹² MMP activation and cellular invasion are correlated in many pathophysiological settings. Indeed, the levels of MMP expression and activity are elevated in almost every type of human cancer, and this correlates with advanced tumor stage, increased invasion and metastasis, and poor prognosis.¹³ Coincident with a more invasive phenotype, abnormally proliferating cells within a hyperplastic lesion undergo a profound morphological transformation, through which they lose their epithelial features and acquire a fibroblastic shape. Such an epithelial-mesenchymal transition (EMT) reflects the ability of cancer cells to sense and dynamically adapt to their new motility functions. Importantly, members of the transforming growth factor- β (TGF- β) superfamily have been identified as inducers of the EMT.^{14,15} Several pathways are involved in the regulation of some or all phenotypic aspects of the TGF- β -induced EMT, including Smad activation, MAP kinase, phosphatidylinositol 3-kinase (PI3-kinase), RhoA, and Rac1 small GTPases.¹⁶⁻²⁰

We and other investigators have proposed that sequestration of several growth-promoting proteins in invaginations of the plasma membrane, named caveolae, serves to potently inhibit their activity.²¹ Caveolae function as integrated platforms, to compartmentalize and concentrate signaling molecules within a discrete sub-

cellular microenvironment, thereby facilitating cross-talk among different signaling cascades.^{22,23} Consistent with this idea, the scaffolding protein necessary for caveolae formation, caveolin-1 (Cav-1), has been shown to bind and hold in an inactive state members of various signaling cascades, including certain receptor tyrosine kinases (EGF receptor and HER-2/c-Neu/Erb-B2), proteins of the prosurvival PI3-kinase/Akt pathway, as well as components of the growth factor-activated Ras-p42/44 MAP kinase cascade, which critically regulates cell proliferation and differentiation.²⁴⁻²⁷ By coordinating their binding and release, Cav-1 functions as a general negative regulator to inhibit the basal activity of many proliferative and oncogenic proteins. Mechanistically, Cav-1 tonically inhibits the activation of multiple signaling molecules, including EGF-R, ERK-1/2, MEK-1/2, and the TGF- β type I receptor, by direct binding via the caveolin-scaffolding domain.^{24,28-30} *In vitro* biochemical studies directly show that the caveolin-scaffolding domain (residues 82 to 101 in Cav-1) is a modular protein domain that acts as a generic protein kinase inhibitor.^{24,31,32} The caveolin-scaffolding domain specifically recognizes and binds to a defined aromatic amino acid sequence within the catalytic domain (namely, protein kinase subdomain IX) of both serine/threonine and tyrosine kinases.^{24,31} Thus, Cav-1 may act as a tonic inhibitor of a variety of signaling cascades, including the Ras-p42/44 MAP kinase cascade and TGF- β /Smad signaling. A prediction of this hypothesis is that a loss of Cav-1 expression may lead to hyperactivation of p42/44 MAP kinase signaling and Smad-2 hyperphosphorylation. In this study, we experimentally assess this hypothesis, using primary cultures of mammary epithelial cells derived from Cav-1 (-/-)-null mice.

Several lines of evidence support the idea that Cav-1 may function as a mammary gland tumor suppressor. Cav-1 mRNA and protein levels are down-regulated or absent in primary human cancers, in oncogenically-transformed NIH 3T3 cells, in transgenic mouse models of breast cancer, as well as in several human and mouse transformed mammary epithelial cell lines.^{25,33-39} In addition, recombinant expression of Cav-1 in transformed NIH 3T3 cells or cell lines derived from human breast cancers reduces cell proliferation and abrogates their anchorage-independent growth.^{36,40,41} Adenovirus-mediated Cav-1 expression also diminishes the metastatic potential of MTLn3 cells, a highly metastatic mammary adenocarcinoma-derived cell line, by blocking EGF-induced lamellipodia formation, and inhibiting cell migration.⁴² Importantly, in support of a tumor suppressor role, up to 16% of human breast cancers are associated with a dominant-negative mutation in Cav-1 (P132L), which results in a loss of Cav-1 function in mammary epithelial cells.^{43,44}

Analysis of the *in vivo* role of Cav-1 in mammary gland physiology and tumorigenesis has greatly benefited from the generation of Cav-1-deficient mice. Importantly, Cav-1-null mice display several significant mammary gland-specific phenotypes. First, the mammary glands of virgin Cav-1-null mice show intraductal hyperplasia, with wall thickening to three-to-four cell layers and areas of fibrosis

surrounding the epithelial tubes.⁴⁴ Consistent with the idea that tumorigenesis is a multistep process, concomitant loss of Cav-1 and a second tumor suppressor gene, ie, INK4a, further perturbs mammary gland morphology, with significant increases in the degree of intraductal hyperplasia and in the number of lateral branches, as well as surrounding ductal fibrosis.⁴⁵ Thirdly, genetic ablation of Cav-1 expression in the context of a tumor-prone mouse model, MMTV-PyMT (mouse mammary tumor virus-driven polyomavirus middle T antigen expression), accelerates the growth of multifocal dysplastic lesions during early mammary gland development, promotes mammary tumor formation at 12 to 14 weeks of age, and dramatically enhances lung metastasis.^{46,47}

Cav-1 is expressed in many different cell-types within the mammary gland, including mammary epithelia, fibroblasts, adipocytes, endothelial cells, and smooth muscle cells. As such, the contribution of each cell type to the Cav-1-null mammary gland phenotypes remains to be elucidated. Three-dimensional culture of mammary epithelial cells *in vitro* [either mammary epithelial cell lines, or freshly isolated murine mammary gland acini, ie, organoids from wild-type (WT) mice⁴⁸] recapitulates many features of the mammary epithelium *in vivo*. Once embedded in three-dimensional matrices, mammary epithelial cells form acini-like spheroids, with a single layer of polarized cells lining a hollow lumen, and the basal deposition of basement membrane components.^{49–52} As a consequence, the three-dimensional culture of mammary epithelia can be used to study the early tumor-initiating events that occur in response to oncogene activation and/or tumor suppressor inactivation.

To directly dissect the role of Cav-1 in mammary gland tumorigenesis, we isolated primary mammary epithelial cells from WT and Cav-1-deficient mice, and evaluated their phenotypic behavior either as a monolayer or in the three-dimensional Matrigel culture system. Here, we show that a Cav-1 deficiency (–/–) conveys several striking cell autonomous phenotypic defects in mammary epithelial cells, with abnormal lumen formation, EGF-independent growth, and an increased capacity for branching.

Materials and Methods

Materials

Antibodies and their sources were as follows: Cav-1 (N-20) was from Santa Cruz Biotechnology (Santa Cruz, CA), phospho-ERK-1/2, total ERK-1/2, phospho-Smad-2, and total Smad-2 were from Cell Signaling (Beverly, MA); E-cadherin, β -catenin, and Smad-2 were from BD Pharmingen (San Diego, CA); cytokeratin 5/8 and 18 were from BD Pharmingen; MMP-9 and MMP-2 were from Triple Point Biologics, Inc.; and collagen type IV was from Dako Cytomation. Other reagents were as follows: propidium iodide, Prolong gold anti-fade mounting reagent, Slow-Fade anti-fade reagent (from Molecular Probes, Eugene, OR); EGF (from Peprotech, Rocky Hill, NJ); hepatocyte growth factor (HGF) and basic fibroblast growth factor

(bFGF) (from R&D Systems, Minneapolis, MN); phalloidin-fluorescein isothiocyanate, hydrocortisone, cholera toxin, insulin, and gentamicin (from Sigma, St. Louis, MO); collagenase type I (from Life Technologies, Inc., Grand Island, NY); reduced growth factor Matrigel (from BD Biosciences and Trevigen); 3-D culture matrix rat collagen I (from Trevigen); and Lab-TekII eight-well chamber slides (from Nalgene Nunc, Naperville, IL).

Animal Studies

All animals were housed and maintained in a pathogen-free environment/barrier facility at the Institute for Animal Studies at the Albert Einstein College of Medicine under National Institutes of Health (NIH) guidelines. Cav-1-deficient mice were generated as previously described.⁵³ All WT and Cav-1 knockout (KO) mice used in this study were in the FVB/N genetic background.

Isolation of Mammary Epithelial Cells

Primary mammary gland organoids were purified from 8-week-old virgin female mice. Briefly, the fourth and fifth mammary glands from two mice of each genotype (WT versus Cav-1 KO) were removed aseptically, minced with surgical blades, incubated in a shaker (for 2 hours at 37°C) in 30 to 35 ml of growth media (Dulbecco's modified Eagle's medium/F12, 5% horse serum, 20 ng/ml EGF, 0.5 μ g/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μ g/ml insulin, Pen/Strep) containing 2 mg/ml collagenase type I, and 50 μ g/ml gentamicin. Then, the cell suspensions were spun 10 minutes at 1000 rpm to eliminate floating fat cells. Cell pellets were resuspended in 10 ml of assay media (Dulbecco's modified Eagle's medium/F12, 2% horse serum, 0.5 μ g/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μ g/ml insulin, Pen/Strep). To wash away single cells, which are mainly fibroblasts, endothelia, and smooth muscle cells, organoid pellets were subjected to repeated differential centrifugation (spun at 1000 rpm for 45 seconds; repeated for 10 cycles of pelleting and resuspension). After the last wash, organoids were resuspended in 2 ml of growth media, and disrupted by pipetting up and down 20 to 25 times with a sterile 1-ml blue pipette tip. Then, the final volume was adjusted to 6 ml. Organoids were plated either in eight-well chamber slides (~40 to 50 μ l cells in each well), or in 10-cm dishes, and organoids were allowed to attach and spread as a monolayer.

Three-Dimensional Culture of Mammary Epithelial Cells

Four to five days after purification, organoids attached to plastic dishes and grew as a mammary epithelial cell monolayer. Cell monolayers were then trypsinized, and resuspended in assay media. To obtain a single cell suspension, cells were passed 20 to 25 times through a 1-ml blue tip. This single cell suspension was then overlaid onto Matrigel, essentially as previously described for

MCF-10A cells.² Briefly, cells were diluted in assay media supplemented with 2% Matrigel and 5 ng/ml EGF. Then, 5000 cells were overlaid on top of Matrigel (ie, eight-well chamber slides, which were precoated with 40 μ l of Matrigel). Chambers were placed in a standard cell culture incubator at 37°C. Every 4 days, acini were refed with assay media supplemented with 2% Matrigel and 5 ng/ml EGF. All experiments were performed with primary mammary epithelial cells that were passage 1.

Immunofluorescence Analysis of Acini

Immunostaining of acini was performed as previously described for MCF-10A cells,² with minor modifications. Acini were fixed for 20 minutes at room temperature in 2% PFA diluted in phosphate-buffered saline (PBS), after which they were permeabilized for 10 minutes at room temperature with 0.5% Triton X-100 in PBS. After two glycine rinses (100 mmol/L glycine in PBS, 15 minutes each at room temperature), acini were incubated for 1 hour with IF buffer [PBS, containing 7.7 mmol/L NaN₃, 0.1% bovine serum albumin (BSA), 0.2% Triton X-100, 0.05% Tween-20] supplemented with 10% goat serum. Primary antibodies were incubated in IF buffer and 10% goat serum, overnight at room temperature. After washing with IF buffer (three times, 15 minutes each), acini were incubated for 45 to 50 minutes at room temperature with secondary antibodies (Jackson Laboratories, Bar Harbor, ME) diluted in IF buffer. Then, washes were performed by incubation with IF buffer (two times, 15 minutes each), and with PBS (one time, 15 minutes). To counterstain the nuclei, acini were incubated with propidium iodide in PBS (1 μ g/ml) for 30 minutes. After rinsing with PBS, three times, slides were mounted with Prolong gold anti-fade reagent (Molecular Probes). Samples were imaged with a Radiance 2000 scanning laser confocal microscope (Bio-Rad, Hercules, CA).

Immunofluorescence Analysis of Monolayer Cells

Cells were fixed for 30 minutes at room temperature in 2% PFA diluted in PBS, after which they were permeabilized for 10 minutes at room temperature with IF buffer (PBS plus 0.2% BSA and 0.1% Triton X-100). Then, cells were incubated with NH₄Cl in PBS to quench free aldehyde groups. Primary antibodies were incubated in IF buffer overnight at room temperature. After washing with IF buffer (three times, 10 minutes each), cells were incubated for 30 minutes at room temperature with secondary antibodies (Jackson Laboratories) diluted in IF buffer. Finally, slides were washed at room temperature with IF buffer (three times, 10 minutes each), and mounted with Slow-Fade anti-fade reagent (Molecular Probes).

Electron Microscopy

Acini were grown in eight-well chamber slides coated with Matrigel. Samples were fixed within the wells using

2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer. The samples were then post-fixed with OsO₄ and *en block* stained with uranyl acetate. Before dehydration, the chamber was detached from the slide, and individual Matrigel-coated wells were removed from the slide with a beveled wooden applicator stick. The pieces were then collected, and dehydrated before being embedded in epoxy resin. Ultra-thin sections were examined with a JEOL 1200 EX transmission electron microscope.

Acinar Growth Curves

To monitor acinar growth, WT and Cav-1 KO acini were live-imaged using an Olympus 1 $\times$ 80 microscope with a $\times$ 20 objective connected with a cooled charge-coupled device camera. To count the number of acini per high-power field, acini were live-imaged using a $\times$ 10 objective. More than 50 acini for each genotype were scored at a given time point.

Western Blot Analysis

After removal of the assay media, acinar cell lysates were prepared by incubating eight-well chamber slides with lysis buffer [20 mmol/L Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 70 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 60 mmol/L octyl glucoside, 50 mmol/L NaF, 30 mmol/L sodium pyrophosphate, 10 μ mol/L orthovanadate, with a protease inhibitor tablet] on ice for 15 minutes. Proteins were collected into an Eppendorf tube, and passed five times through a 26-gauge needle. Then, the lysates were incubated on ice for an additional 15 minutes. Fifty μ l of lysates was loaded onto SDS-polyacrylamide gel electrophoresis (PAGE) gels. Equal loading was assessed by immunoblotting with epithelial markers, ie, E-cadherin and cytokeratin 18. Mammary epithelial cell monolayers were lysed in boiling sample buffer, passed five times through a 26-gauge needle, and boiled for 5 minutes. Cellular proteins were resolved by SDS-PAGE, and transferred to nitrocellulose membranes (0.2 μ m; Schleicher and Schuell, Keene, NH). Blots were blocked for 1 hour in TBST (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.2% Tween 20) containing 1% BSA and 4% nonfat dry milk (Carnation). Then, membranes were incubated for 2 hours with primary antibodies in a 1% BSA/TBST solution. Membranes were then washed with TBST, and incubated for 45 minutes with the appropriate horseradish peroxidase-conjugated secondary antibodies (diluted 5000-fold in 1% BSA/TBST; Pierce, Rockford, IL). Signal was detected with an ECL detection kit (Pierce). Nonfat dry milk was omitted from the blocking solution when we used phospho-specific antibody probes.

MMP Secretion

Low-serum assay media (containing 2% horse serum) from WT and Cav-1 KO acini was collected at 12, 16, and 20 days and concentrated fourfold using Amicon ultra

centrifugal filter devices. Equal amounts of concentrated media were then loaded onto SDS-PAGE gels and analyzed by Western blotting.

Branching Assay

For branching assays, mammary epithelial cells were prepared as for the three-dimensional assays, except that single cell suspensions were overlaid onto a matrix bed consisting of a 3:1 mixture of Matrigel and collagen I. As per the manufacturer's recommendations, the collagen I solution was equilibrated with PBS and 23 mmol/L NaOH, before mixing with Matrigel. Briefly, single-cell suspensions of mammary epithelial cells were diluted in assay media (which lacks EGF) supplemented with 2% of a 3:1 Matrigel/collagen I mixture. Then, 5000 cells were overlaid on top of 3:1 Matrigel/collagen I (ie, eight-well chamber slides, which were precoated with 40 μ l of the same mixture). Cells were left untreated or stimulated with either HGF (50 ng/ml) or bFGF (50 ng/ml). Chambers were placed in a standard cell culture incubator at 37°C. Every 2 days, acini were refed with assay media supplemented with the 2% Matrigel/collagen mixture and the appropriate growth factor. All experiments were performed with primary mammary epithelial cells that were passage 1. Quantitation of branching was performed after 5 days in culture, and was expressed as the percentage of branching structures per field. A branching phenotype was defined as an epithelial structure with at least one extension (branch) protruding from the central acinar body.

Statistical Analysis

Statistical significance was determined using the Student's *t*-test. Values of $P < 0.05$ were considered significant. Values were expressed as means $\pm$ SEM. For measuring mammary acinar diameter, more than 50 acini were scored for each condition and for each genotype. For branching assays, more than 70 acini were scored for each condition and for each genotype.

Results

Cav-1-Null Mammary Epithelial Cells Grown in Matrigel Form Three-Dimensional Acinar Structures that Lack Caveolae Organelles

Cav-1-deficient mice show several mammary gland-specific abnormalities (see Introduction). To gain insight into the mechanisms triggering these phenotypes, and to determine whether these phenotypes are cell-autonomous, ie, intrinsic to the mammary epithelial cells, we isolated primary mammary epithelial cells from WT and Cav-1-deficient mice, and evaluated their behavior in a three-dimensional system *in vitro*.

Using a combination of enzymatic treatment and differential centrifugation, organoids were purified from WT and Cav-1-deficient mammary glands, and seeded either

Figure 1. Cav-1-deficient mammary epithelial cells form three-dimensional acini-like structures but are devoid of caveolae. Primary mammary epithelial cells derived from WT and Cav-1-null mice were grown embedded in Matrigel. Under these conditions, both WT and Cav-1 KO mammary epithelial cells develop three-dimensional epithelial structures that closely resemble mammary gland acini. **A:** Day 8 WT and Cav-1-deficient acini were immunostained with anti-Cav-1 IgG and counterstained with propidium iodide to visualize cell nuclei. Single optical sections were acquired with a Bio-Rad Radiance 2000 scanning laser confocal microscope. Note that Cav-1 is properly expressed in WT mammary epithelial cells but is clearly absent in Cav-1 KO acini. **B:** Electron microscopic analysis of day 20 acini reveals that loss of Cav-1 disrupts caveolae formation in mammary epithelial cells. Note the presence of several caveolae, defined as 50- to 100-nm plasma membrane invaginations, in WT acini (arrows). On the contrary, Cav-1-deficient acini exhibit a complete loss of caveolae. ECS, extracellular space. Scale bar, 200 nm.

onto plastic dishes or onto glass chambers. After a few days in culture, organoids attached, and grew as mammary epithelial cell monolayers. Cells grown on glass were directly subjected to immunofluorescence analysis, whereas cells growing on plastic were trypsinized, and overlaid onto Matrigel as a single cell suspension. By using this approach, acini-like spheroids formed, each from a single mammary epithelial cell, thus allowing us to directly compare the morphological development and growth properties of mammary epithelial cells derived from WT and Cav-1-null mice. It is important to note that all experiments were performed with cells that were passage 1.

Figure 1A shows that, when cultured in Matrigel, WT and Cav-1 KO primary mammary epithelial cells can both form acini-like structures. As expected, immunofluorescence analysis revealed that Cav-1 expression is abrogated in Cav-1-deficient acini (Figure 1A). Cav-1 expression is essential for the formation of caveolae, 50- to

100-nm plasma membrane invaginations present in most cell types, including epithelial cells, fibroblasts, adipocytes, and endothelial cells.^{53–55} As such, we investigated whether Cav-1-deficient mammary epithelial cells still retain caveolae at their plasma membrane. Electron microscopic analysis was performed on day 20 WT and Cav-1-null three-dimensional epithelial structures. Figure 1B reveals that, although WT mammary epithelial cells have abundant caveolae at the plasma membrane, Cav-1-deficient mammary epithelial cells are devoid of caveolae, clearly demonstrating that loss of Cav-1 expression abrogates caveolae formation in mammary epithelial cells.

Cav-1-Deficient Mammary Epithelial Cells Form Larger Acini with Wall Thickening

To investigate whether loss of Cav-1 affects the growth properties of mammary epithelial cells, we next evaluated the size of WT and Cav-1 KO three-dimensional epithelial structures. To this end, we monitored growth by measuring acinar diameter at the middle optical section, at different time points, specifically at 4, 8, 12, and 16 days (Figure 2, A and B). A growth curve of WT and Cav-1 KO acini is presented in Figure 2A. Note that WT mammary epithelial cells form acini-like structures that reach a growth-arrested state by day 8. On the contrary, Cav-1-deficient acini continue to grow and enlarge for 4 additional days, until day 12, at which time they become growth-arrested. Most importantly, at any given time point, the size of Cav-1 KO acini is approximately twofold increased as compared to WT acini, obviously suggesting that loss of Cav-1 confers a growth advantage upon mammary epithelial cells.

When embedded in Matrigel, WT mammary epithelial cells develop spheroid-like structures with a hollow lumen and a regular shape. In contrast, Cav-1 KO acini appear larger, with irregular shapes, and possess an immature or filled lumen. Nuclear staining with propidium iodide supports this idea, and directly shows the wall thickening of Cav-1-deficient acini (Figure 3A). WT acini are characterized by a hollow lumen, which is surrounded by a single layer of epithelial cells. In contrast, loss of Cav-1 induces wall thickening to two or more cell layers, and, in some cases, luminal space filling.

We next quantified the extent of luminal obstruction in WT and Cav-1-deficient acini. To visualize the lumen, we incubated acini with a nuclear stain, ie, propidium iodide. Then, we scored the number of acini with a hollow lumen, with partial luminal filling, or with complete luminal filling. Partial luminal obstruction was defined as mammary acini that were more than one cell layer in thickness. Figure 3B shows that ~70% of WT acini contain a hollow lumen. In contrast, Cav-1-null acini display a ~2.3-fold increase in luminal filling (partial and complete); similarly, partial luminal filling is increased by approximately threefold. Conversely, the percentage of Cav-1-deficient acini with a hollow lumen is reduced by ~50%, as compared with WT acini. Toluidine blue-stained thin sections of WT and Cav-1 KO acini further corroborated the concept that wall thickening is increased in Cav-1 KO acini (Figure 3C). Note that, in the absence of Cav-1, acinar walls are thicker and, at times, the lumen is

Figure 2. Cav-1 KO mammary epithelial cells form dramatically larger acini. To form acini-like structures, WT and Cav-1-null mammary epithelial cells were grown in reconstituted basement membrane, ie, Matrigel. Note that loss of Cav-1 confers a growth advantage. **A:** Growth curves of WT and Cav-1 KO acini. Acinar diameter was measured at days 4, 8, 12, and 16, with the support of Image J software. Note that, at any given time point, Cav-1-null acini demonstrate an ~2- to 2.2-fold increase in size. In addition, although WT acini reach a growth-arrested state by day 8, Cav-1-deficient acini continue to enlarge and do not undergo growth arrest until day 12. More than 50 acini for each genotype were scored at a given time point. * $P \leq 0.0002$. Error bars, SEM. **B:** Phase micrographs of WT and Cav-1 KO acini. Note that WT mammary epithelial cells develop acini-like structures with a regular shape, whereas Cav-1-null acini appear larger and morphologically distinct. Representative examples at days 4, 8, 12, and 16 are shown.

filled. Indeed, wall thickening and hypercellularity of Cav-1-null acini was independently demonstrated by transmission electron microscopy (data not shown).

Figure 3. Cav-1 KO mammary acini show thickened walls with abnormal three-dimensional lumen formation. WT and Cav-1-null mammary epithelial cells were grown in reconstituted basement membrane, ie, Matrigel, to generate mammary acini. Interestingly, note that loss of Cav-1 results in wall thickening and abnormal lumen formation. **A:** Nuclear staining. Day 8 WT and Cav-1 KO acini were stained with propidium iodide to visualize nuclei (red) and appreciate lumen formation. WT acini exhibit a single layer of mammary epithelial cells lining a hollow lumen. Note that loss of Cav-1 induces wall thickening, to two or more cell layers, and in certain cases elicits luminal space filling. **B:** Quantitation of luminal obstruction. WT and Cav-1-null acini were stained with propidium iodide to visualize lumen formation. Acini were then scored based on the presence of a hollow lumen or for partial or complete luminal filling. Partial luminal obstruction was defined as mammary acini that were more than one cell layer in thickness. Note that ~70% of WT acini contain a hollow lumen. In contrast, Cav-1-null acini displayed an ~2.3-fold increase in luminal filling (partial and complete); similarly, partial luminal filling was increased by approximately threefold. Conversely, the percentage of Cav-1-deficient acini with a hollow lumen is reduced by ~50%, as compared with WT acini. More than 150 acini were scored for each genotype. **C:** Light microscopy. Thin sections of day 20 WT and Cav-1 KO acini were cut and stained with toluidine blue. Images were acquired at low magnification, and montages were assembled to illustrate the wall thickening of Cav-1-deficient acini. Note that, in certain instances, the lumen (L) appears completely filled. Original magnifications, $\times 40$ (**A**).

Cav-1 KO Acini Show Ras-p42/44 MAP Kinase Hyperactivation and EGF-Independent Growth

We next assessed whether increased acinar size and improper lumen formation in Cav-1-null acini are caused by the abnormal amplification of growth-promoting signals. Cav-1 is known to potently inhibit sig-

nals originating from certain receptor tyrosine kinases (RTKs), such as EGF-R, and some of their downstream components, including the Ras-p42/44 MAP kinase cascade, which critically regulates cell proliferation and differentiation.^{24,28, 31,56–58} As such, we first monitored the activation status of the p42/44 MAP kinase pathway by Western blot analysis of day 18 acini with

Figure 4. Cav-1-null acini show hyperactivation of the p42/44-MAP kinase signaling cascade, and EGF-independent growth. **A:** Hyperactivation of the Ras-p42/44-MAP kinase pathway in Cav-1 KO acini. Lysates from day 18 WT and Cav-1-deficient acini were subjected to Western blot analysis with antibodies against the activated phosphorylated form of ERK-1/2. Note that the Ras-p42/44-MAP kinase signaling cascade is hyperactivated in Cav-1-null acini. Equal loading was assessed by Western blot with a control phospho-independent antibody that recognizes total ERK-1/2. Immunoblotting with E-cadherin is shown as an additional control for equal epithelial protein content. **B–D:** EGF-independent growth of Cav-1-null acini. To evaluate whether loss of Cav-1 imparts growth factor independence, WT and Cav-1 KO three-dimensional epithelial structures were cultured either in the absence or in the presence of EGF, for up to 12 days. Images were acquired at days 4, 8, and 12. **B:** Phase images of EGF-deprived acini. In the absence of EGF, WT acini are very small and unable to grow. On the contrary, Cav-1-null mammary epithelial cells retain the ability to proliferate and to form acini-like spheroids without EGF. **C:** Acinar growth: size. The diameter of WT and Cav-1 KO acini, cultured with or without EGF, was measured at days 4, 8, and 12. At least 50 acini for each genotype were scored at any given time point. As expected, in the presence of EGF, Cav-1-null acini display an obvious delay in reaching a growth-arrested state (day 12 versus day 8 of WT acini), and exhibit an approximately twofold size increase, as compared to WT acini. However, in the absence of EGF, WT mammary epithelial cells develop as very small spheroids, which do not enlarge, suggesting that EGF is normally required for the growth and proliferation of three-dimensional cultures of mammary epithelial cells. In striking contrast, Cav-1-deficient acini cultured without EGF grow at a comparable rate as when they are cultured in the presence of EGF. More than 50 acini for each genotype were scored at a given time point. $P \leq 0.00004$. Error bars, SEM. **D:** Acinar growth: number of acini per high-power field. WT and Cav-1 KO acini were cultured with or without EGF in parallel experiments. The number of acini per high-power field was counted at days 4, 8, and 12. At least 20 high-power fields were scored for each genotype at any given time point. Note that, when cultured with EGF, WT and Cav-1 KO mammary epithelial cells form a similar number of acini, demonstrating that we seeded a comparable amount of WT and Cav-1-null cells into the wells. However, parallel experiments performed in the absence of EGF, show that the number of WT, but not that of Cav-1 KO acini, is greatly affected by a lack of EGF. Remarkably, in the absence of EGF, the number of Cav-1 KO acini is approximately fourfold increased, as compared to WT acini. $P \leq 0.00018$. Error bars, SEM.

phospho-specific antibodies directed against the activated form of ERK-1/2.

Figure 4A shows that ERK-1/2 is hyperphosphorylated in Cav-1 KO acini, suggesting that hyperactivation of MAP kinase-dependent growth signals is involved in the hyperplastic-like phenotype of Cav-1-deficient three-dimensional epithelial cultures. One intriguing implication

of these findings is that steady-state hyperactivation of the Ras-p42/44 MAP kinase cascade could initiate proliferation, even in the absence of an appropriate growth stimulus, such as EGF, and trigger self-sufficiency in growth signals.

To test whether loss of Cav-1 would confer EGF-independent growth, we cultured WT and Cav-1 KO mam-

mary epithelia either in the absence (–) or in the presence (+) of EGF, for up to 12 days. Figure 4B shows representative phase images of EGF-deprived WT and Cav-1-deficient three-dimensional mammary epithelial structures. Note that in the absence of EGF, WT mammary epithelial cells fail to grow, but still form small organized acini. On the contrary, even without EGF, Cav-1 KO mammary epithelial cell acini retain the ability to develop large three-dimensional epithelial structures.

Quantitation of acinar growth, in the presence or absence of EGF, is shown in Figure 4, C and D. For this purpose, we scored both acinar diameter and number of acini per high-power field. Figure 4C shows that, in the absence of EGF, WT mammary epithelial cells form small acini, which do not have the ability to grow. When cultured in the presence of EGF, they form mature acini, which undergo growth-arrest at day 8 (consistent with the data presented in Figure 2A).

In contrast, Cav-1-deficient mammary acini undergo EGF-independent growth. Note that without EGF, Cav-1-deficient mammary epithelial cells form large acini-like structures, which grow almost at the same rate as when they are cultured in the presence of EGF (Figure 4C). In support of these results, Figure 4D shows that the number of WT, but not Cav-1 KO acini, is greatly affected by the lack of EGF. Importantly, a parallel experiment performed in the presence of EGF reveals that there is no difference in the number of WT and Cav-1 KO acini, demonstrating that, indeed, we seeded an equal amount of cells in each well.

Cav-1 Deficiency Induces Defects in Cell-Substrate Attachment and Increased MMP Secretion with Loss of Collagen Type IV

During the development of a hyperplastic lesion, mammary epithelial cells undergo a series of phenotypic changes, which include a loss of cell-substrate attachment and increased secretion of matrix-degrading enzymes, such as matrix metalloproteinases (MMPs).¹⁰ To evaluate whether Cav-1-deficient mammary epithelial cells harbor a defect in cell-substrate attachment, we deprived mammary epithelial cells of exogenously added extracellular matrix. For this purpose, freshly isolated mammary acini—organoids—were plated directly on glass coverslips, without the addition of Matrigel.

Figure 5 shows that, after 4 days of culture on glass, WT organoids were able to attach and spread, forming a monolayer of mammary epithelial cells. Under identical conditions, however, Cav-1 KO organoids display an obvious delay in attaching to glass, such that after 4 days they did not start to spread. These results clearly suggest that loss of Cav-1 impairs the cell-substrate attachment properties of mammary epithelial cells. [However, after 7 to 8 days on glass, both WT and Cav-1 KO organoids were able to grow, and formed an epithelial monolayer (data not shown).]

Several factors have been shown to modulate cell-matrix adhesion, but a major role is thought to be played

Figure 5. Cav-1-null organoids show impaired attachment/spreading on glass. Intact mammary acini, ie, organoids, were purified from WT and Cav-1 KO mice and seeded onto glass coverslips. Note that after 4 days in culture, WT organoids were able to attach and grow out as a mammary epithelial cell monolayer. On the contrary, at the same 4-day time point, Cav-1 KO organoids attached but did not spread out into a cell monolayer. However, after 7 to 8 days in culture, both WT and Cav-1 KO organoids were able to grow as a mammary epithelial cell monolayer on glass (not shown). Original magnifications, $\times 10$.

by a family of enzymes involved in the cleavage of various components of the extracellular matrix, the MMP family.¹⁰ To gain mechanistic insights into the defects in attachment and spreading of Cav-1-null organoids, we first evaluated MMP expression in Cav-1-deficient mammary epithelial cells (grown for 8 to 9 days on glass), by immunofluorescence analysis with antibodies directed against MMP-9 and -2.

Figure 6, A and B, shows that MMP-9 and MMP-2 expression, respectively, are greatly increased in Cav-1-null mammary epithelial cells. Note that MMP-9 and MMP-2 are highly expressed in the extracellular matrix as well as in the intracellular compartments of Cav-1 KO cells, suggesting that loss of Cav-1 enhances MMP synthesis and secretion. These results were independently confirmed by Western blot analysis with antibodies directed against MMP-9 and -2. Figure 6C reveals that MMP-2 and -9 expression levels are increased in Cav-1-deficient acini. Interestingly, Western blot analysis on media collected from WT and Cav-1-null three-dimensional epithelial cultures on days 12, 16, and 20, shows that MMP-9 secretion is significantly increased in Cav-1-null acini (Figure 6D). Taken together, these results demonstrate that loss of Cav-1 leads to defects in cell-substrate attachment/spreading, and induces significant increases in MMP expression and secretion.

Because three-dimensional epithelial cultures have the ability to deposit basal membrane components, such as type IV collagen, we next asked whether the increase in MMP secretion we observe in Cav-1-deficient acini would lead to the abnormal degradation of the extracellular matrix. To this end, we performed immunofluorescence analysis on organoids—ie, intact freshly isolated mammary acini—with antibodies directed against collagen IV.

Figure 6. Cav-1 KO mammary epithelial cells display increased expression and secretion of MMP-9 and MMP-2. **A** and **B**: WT and Cav-1-deficient organoids, ie, intact mammary acini, were seeded onto chamber slides, cultured for 8 to 9 days, and allowed to spread as a monolayer of mammary epithelial cells. Cells were then immunostained with antibodies against MMP-9 (**A**) and MMP-2 (**B**). Note that both MMP-9 and MMP-2 expression levels are dramatically increased in Cav-1-null mammary epithelial cells. Note the strong staining within the extracellular matrix and intracellularly. **C**: Western blot analysis of day 18 WT and Cav-1-null acini was performed with antibodies against MMP-9 and MMP-2. Note that MMP-9 and MMP-2 expression levels are increased in Cav-1 KO mammary epithelial cells, as compared to their WT counterparts. Immunoblotting with E-cadherin is also shown as a control for equal epithelial protein content. Regarding the apparent molecular weight of MMP-2, the band that we observe is ~66 kD, which is more consistent with the activated form. **D**: Increased MMP-9 secretion in Cav-1 KO acini. Media from WT and Cav-1-null acini was collected at days 12, 16, and 20 and concentrated fourfold using a centrifugal filter device. Equal amounts of concentrated media were resolved by SDS-PAGE, followed by Western blot analysis with antibodies against MMP-9. Note that Cav-1-deficient acini secrete higher levels of MMP-9 than WT acini, at any given time point. It is important to note that these experiments were performed with medium containing reduced serum content (2% horse serum; see Materials and Methods). However, the mobility of MMP-9 may still be shifted upward because of the presence of serum albumin.

Figure 7 reveals the abundant basal deposition of collagen IV in WT organoids. On the contrary, Cav-1-deficient organoids display a loss of basement membrane type IV collagen, very likely associated with increased MMP-2 and -9 expression. Taken together, these results suggest that Cav-1 deficiency increases MMP expression and secretion, which impairs cell-substrate adhesion via the loss (most likely digestion) of basement membrane components (collagen IV), all essential steps for tumor invasion.

Cav-1 KO Epithelial Cells Undergo a Spontaneous EMT

During the process of abnormal proliferation within a hyperplastic lesion, mammary epithelial cells often undergo an EMT, resulting in a more invasive fibroblastic

phenotype. Hallmarks of the mesenchymal transdifferentiation of epithelial cells include acquisition of a spindle-shape, formation of actin stress fibers, and delocalization of E-cadherin from cell-cell contacts.⁴ It is well-established that TGF- β signaling is crucially involved in triggering the EMT.⁵⁹

Previous research has demonstrated that Cav-1 expression inhibits TGF- β signaling in fibroblasts. For example, in NIH 3T3 cells and in 293T cells, Cav-1 directly interacts with the TGF- β type I receptor, and suppresses the TGF- β -stimulated phosphorylation of Smad-2 and subsequent transcriptional activation. Interestingly, antisense-mediated down-regulation of Cav-1 was sufficient to increase TGF- β 1-induced Smad-2 phosphorylation.³⁰ However, it remains unknown whether Cav-1 plays an *in vivo* role in any of the cellular processes mediated by TGF- β . We reasoned that, in mammary epithelial cells,

Collagen Type IV Immuno-staining

Figure 7. Loss of type IV collagen in Cav-1-null organoids. Intact mammary acini, organoids, isolated from WT and Cav-1 KO mammary glands were cultured in Matrigel for 5 days and immunostained with a monoclonal antibody that recognizes type IV collagen (green). Nuclei were counterstained with propidium iodide (red). Note that WT organoids exhibit the basal deposition of extracellular matrix components, such as collagen type IV. On the contrary, Cav-1 KO organoids display a loss of collagen IV, probably because of the increased secretion of MMPs, which possess collagenase activity.

genetic ablation of Cav-1 could hyperactivate TGF- β 1 signaling, and lead to a spontaneous EMT.

To evaluate whether Cav-1 plays a role in the EMT, WT and Cav-1 KO mammary epithelial cells were cultured on glass for prolonged periods of time, and monitored daily for morphological changes. Figure 8A shows that, after 7 and 9 days in culture, WT mammary epithelial cells display typical cobblestone-like morphology, whereas Cav-1 KO mammary epithelial cells undergo a spontaneous change in morphology acquiring a more spindle-shaped fibroblast-like appearance. Concomitant with these morphological changes, Cav-1-null mammary epithelial cells form actin stress fibers, and show the mislocalization of E-cadherin to an intracellular compartment (Figure 8B), suggestive of cell-cell junction remodeling, all key features of the EMT. Interestingly, the localization of β -catenin is also profoundly affected, revealing changes in its plasma membrane appearance, assuming a jagged-edge distribution pattern (Figure 8B).

Interestingly, immunofluorescence analysis with two epithelial-specific markers, cytokeratins 5/8 and 18, reveals that Cav-1 KO cells still maintain epithelial features, and continue to express epithelial keratins (Figure 8C), suggesting that Cav-1 might be involved in early stages of the epithelial-to-mesenchymal conversion. However, Figure 8C shows that Cav-1-null mammary epithelial cells display striking changes in the organization of their cytokeratin filaments, which indeed assemble into thicker fibers, as compared to WT cells, again indicative of morphological transformation. Electron microscopic examination independently demonstrated that Cav-1-deficient mammary epithelial cells possess larger and more numerous intermediate filament bundles (Figure 8D).

To further evaluate whether Cav-1-null mammary epithelial cells are still epithelial after prolonged culture (9 days), we next assessed the expression levels of β -catenin, E-cadherin, and cytokeratin 18 by Western blot analysis on lysates from WT and Cav-1 KO mammary epithelial cells. As shown in Figure 8E, E-cadherin, and cytokeratin 18 expression levels remain unchanged in Cav-1-deficient mammary epithelial cells, while β -catenin levels are mildly decreased (by ~30 to 50%). Taken together, these results directly show that loss of Cav-1 induces profound morphological changes in mammary epithelial cells, suggestive of a spontaneous transition to a fibroblastic phenotype, including the acquisition of a spindle-shape, formation of actin stress fibers, and the disruption of cell-cell junctions. However, despite all these clear morphological signs of an EMT, Cav-1 KO mammary epithelial cells still express epithelial markers, strongly suggesting that Cav-1 might be involved in the very early stages of epithelial *trans*-differentiation.

To gain mechanistic insight into the regulation of this spontaneous EMT, we next evaluated the activation status of TGF- β signaling in Cav-1-null mammary epithelial cells. In cultured cell models, Cav-1 negatively regulates TGF- β signaling, and inhibits Smad-2 phosphorylation and its activity.³⁰ As such, we first evaluated whether loss of Cav-1 induces baseline Smad-2 hyperactivation. After TGF- β -induced phosphorylation, Smad-2 normally translocates to the nucleus, and promotes transcription of various target genes.^{60–62} As shown in Figure 9A, Smad-2 is mainly localized within the nucleus in Cav-1 KO mammary epithelial cells, suggesting that loss of Cav-1 induces the constitutive hyperactivation of one of the main mediators of TGF- β signaling. These results were independently validated by Western blot analysis with anti-phospho Smad-2 polyclonal antibodies by probing lysates derived from WT and Cav-1 KO mammary epithelial cells. Figure 9B clearly demonstrates that Smad-2 is hyperphosphorylated in Cav-1-deficient mammary cells, but total Smad-2 levels remain unchanged. Thus, loss of Cav-1 induces a spontaneous EMT, with constitutive hyperactivation of Smad signaling.

Cav-1-Deficient Acini Show Increased Branching in Response to Growth Factor Stimulation

The acinar branching assay is a well-established approach to study the EMT in a three-dimensional physiological environment. Branch formation requires degradation of the basement membrane, a loss of cell polarity, and acquisition of a fibroblastic phenotype. An interplay between MMPs and growth factor stimulation, such as HGF (also known as scatter factor) and bFGF, normally regulates the branching of mammary epithelial cells.⁴⁸

As we show here that Cav-1-null mammary epithelial cells display increased MMP-2/-9 expression and an early-stage spontaneous EMT, we next evaluated whether culturing Cav-1-null acini in a branching-permissive microenvironment would augment branch formation. To this end, we overlaid single-cell suspensions of WT

Figure 8. Cav-1-deficient mammary epithelial cells undergo a spontaneous epithelial mesenchymal transition (EMT). Organoids from WT and Cav-1 KO mammary glands were seeded onto glass chamber slides. During prolonged culture on glass, Cav-1-null mammary epithelial cells underwent spontaneous morphological changes, resembling an epithelial mesenchymal transition (EMT). **A:** Phase images. Note that after 7 and 9 days in culture, WT mammary epithelial cells retained a clear epithelial phenotype. On the contrary, at the same time points, Cav-1 KO mammary epithelial cells lost their epithelial morphology and acquired a more fibroblastic shape. **B:** Immunofluorescence with phalloidin, E-cadherin, and β -catenin on WT and Cav-1 KO mammary epithelial cells demonstrates that Cav-1 KO mammary epithelial cells undergo profound morphological changes. Phalloidin staining reveals that actin is no longer membrane-bound but rather is organized in stress fibers. The distribution of E-cadherin and β -catenin is profoundly altered in Cav-1 KO mammary epithelial cells. In WT cells, E-cadherin and β -catenin are localized to the plasma membrane, at areas of cell-cell contact. On the contrary, in Cav-1-null cells, E-cadherin is redistributed to an intracellular compartment, whereas β -catenin staining reveals a ruffled or jagged-edge membrane morphology. **C:** Immunofluorescence with cytokeratin 5/8 and 18 on WT and Cav-1 KO mammary epithelial cells shows an abundance of epithelial-specific intermediate filaments in both cell types, suggesting that Cav-1-null cells still retain an epithelial nature. However, the cytokeratin organization is strikingly different in Cav-1-deficient cells, as compared to WT cells, with an abundance of densely compacted intermediate filaments. **D:** Electron microscopy. Cav-1-deficient mammary epithelial cells possess larger and more numerous intermediate filament bundles, compared to WT cells. Although intermediate filaments (**arrows, a**) can be observed in WT cells, the overall size and number of filaments in the Cav-1 KO cells is substantially increased (**arrows, c**). Scale bar, 200 nm. **b** and **d:** Higher magnification views of boxed areas in WT and Cav-1 KO cells, respectively. **E:** Western blot analysis of WT and Cav-1-deficient mammary epithelial cells. Loss of Cav-1 does not affect the expression levels of E-cadherin and cytokeratin 18 but induces a mild decrease (~ 30 to 50% reduced) in β -catenin levels. Immunoblotting with a polyclonal antibody against Cav-1 demonstrates that Cav-1 expression is indeed abrogated in Cav-1 KO mammary epithelial cells. Immunoblotting with β -actin is included as an additional control for equal protein loading.

Figure 9. Smad-2 hyperactivation in Cav-1-null mammary epithelial cells. To assess the molecular mechanisms underlying the spontaneous EMT of Cav-1-deficient mammary epithelial cells, we evaluated the activation status of Smad-2. Cav-1 was previously shown to negatively regulate TGF- β signaling and to suppress Smad-2 phosphorylation in fibroblasts. After TGF- β stimulation, Smad-2 normally undergoes phosphorylation and translocates to the nucleus, resulting in gene activation. **A:** Immunofluorescence analysis with a monoclonal antibody raised against Smad-2 reveals that Smad-2 is localized to the nucleus in Cav-1-null mammary epithelial cells, suggesting that ablation of Cav-1 induces the constitutive activation of TGF- β signaling. **B:** WT and Cav-1 KO mammary epithelial cells were subjected to Western blot analysis with a phospho-specific probe directed against Smad-2. Note that Smad-2 is constitutively hyperphosphorylated in Cav-1-null cells. Equal loading was assessed by immunoblotting with a polyclonal antibody that recognizes total Smad-2. Immunoblotting with E-cadherin is shown as an additional control for equal epithelial protein content.

and Cav-1-deficient mammary epithelial cells onto a Matrigel/collagen I mixture, and stimulated them with HGF and bFGF for a period of 5 days. Then, we scored the number of branching epithelial structures, ie, acini having at least one process (also known as branch) protruding from the central body.

Interestingly, Figure 10A shows that either HGF or bFGF stimulation induces an increase in branching of ~ 1.7 - and ~ 1.9 -fold, respectively, in Cav-1-null three-dimensional cultures, as compared to their WT counterparts. However, no statistically significant differences were observed when acini were cultured in the absence of growth factors (no GF). Representative images of WT and Cav-1-null epithelial structures grown in the absence

of growth factor (Figure 10B), in the presence of HGF (Figure 10C), and in the presence of bFGF (Figure 10D) are shown to illustrate the differences in branching between WT and Cav-1-null acini. Thus, loss of Cav-1 clearly potentiates the effects of growth factors, such as HGF and bFGF, on epithelial branching and the EMT.

Discussion

Here, we demonstrate that Cav-1-null mammary epithelial cells 1) form larger acini-like structures, with wall thickening and improper lumen formation; 2) exhibit EGF-independent growth, with hyperactivation of p42/44-MAP kinase signaling; 3) show defects in cell-substrate attachment and spreading, with increased MMP secretion, and a loss of basement membrane components; and 4) undergo a spontaneous early-stage EMT, with Smad-activation, and increased branching of mammary acini. We propose that all of these cellular and molecular phenotypes (summarized in Table 1) contribute to the pathogenesis of the mammary epithelial cell hyperplasia and fibrosis observed in the Cav-1 KO mammary gland *in vivo*.

Also, these results clearly show that three-dimensional cultures of Cav-1-null primary epithelial cells recapitulate several features of the Cav-1-null mammary epithelium *in vivo*, strongly suggesting that Cav-1-null mammary gland phenotypes are cell autonomous, ie, intrinsic to the mammary epithelia themselves. These results clearly corroborate the idea that Cav-1 actively regulates a plethora of physiological and pathological processes in the mammary gland. These phenotypic changes in the behavior of Cav-1-null mammary epithelial cells have important implications for understanding the normal role of Cav-1 in regulating the proliferation, development, and differentiation of mammary epithelial cells, and for comprehending how loss of Cav-1 functionally contributes to the development of human breast cancers. To our knowledge, this is one of the first times that mammary epithelial cells derived from genetically engineered mice have been used to reconstitute mammary acini formation *in vitro*.

Cav-1 Deficiency Conveys a Cell Autonomous Proliferation Defect in Mammary Epithelial Cells with Luminal Filling

Several genetic and molecular lines of evidence suggest that Cav-1 functions as a breast cancer tumor suppressor gene. The human CAV-1 gene is localized to the D7S522 locus on chromosome 7q31.1, a hot spot that is frequently deleted in a variety of human epithelial cell malignancies, including breast cancer.^{63–71} Indeed, up to 16% of human breast cancers harbor a dominant-negative mutation in Cav-1 (P132L), which results in the loss-of-function of Cav-1 in mammary epithelial cell lines.^{43,44} In this regard, the Cav-1 KO mouse is a valuable animal model for understanding the role of Cav-1 gene inactivation in the pathogenesis of human breast cancers.

Figure 10. Cav-1-deficient acini exhibit increased branching in three-dimensional collagen cultures. To induce branching, single-cell suspensions of WT and Cav-1-null mammary epithelial cells were overlaid onto a mixture of Matrigel/collagen I (see Materials and Methods), where they formed acini. Starting from day 0, cells were either left untreated [no growth factor (GF) addition] or stimulated with HGF or with bFGF. It is important to note that EGF was omitted from the media. **A:** Quantitation of the percentage of branching epithelial structures. Note that in Cav-1 KO acini, HGF and bFGF stimulation induces an ~1.7-fold and ~1.9-fold increase in branching, respectively, as compared with WT controls. More than 70 acini were scored for each condition and for each genotype. * $P \leq 0.00003$. Error bars, SEM. **B–D:** Phase images of WT and Cav-1-deficient acini grown in the absence of growth factors (**B**), in the presence of HGF (**C**), or in the presence of bFGF (**D**). Note that loss of Cav-1 increases the branching of these three-dimensional cultures of mammary epithelial cells. In **C** and **D**, three representative examples of the Cav-1 KO branching phenotype are shown. **Arrows** point at branching epithelial structures. Original magnifications, $\times 10$.

To mechanistically dissect how loss of Cav-1 function affects mammary epithelial cell growth and differentiation, we studied the phenotypic behavior of isolated Cav-1-null mammary epithelial cells, either as a monolayer or using three-dimensional Matrigel cultures. When cultured in Matrigel, Cav-1-null mammary epithelial cells form acini-like spheroids, which present with several features of immature development, closely resembling mammary epithelial cell hyperplasia. Cav-1-deficient acini displayed increased size (approximately twofold), wall thickening, and partial or complete lumen filling. These results recapitulate previous *in vivo* findings, demonstrating that Cav-1-null mammary glands exhibit hyperplasia, with an increased number of ducts per field and increased intraductal thickness.^{44,45}

EGF-Independent Growth and p42/44 MAP Kinase Hyperactivation

Cav-1-null acini displayed basal hyperactivation of the Ras-p42/44 MAP kinase cascade, which allows them to grow in an EGF-independent manner. Several mechanisms are thought to induce hyperactivation of the MAP kinase pathway, including autocrine growth factor overproduction, amplification of growth factor receptors, such as EGF-R/ErbB2, and structural alterations in any of the components of the cascade itself, such as Ras mutations.^{72–74}

Herein, we provide evidence that functional inactivation of Cav-1 constitutes an independent strategy for imposing a flux of mitogenic signals upon mammary ep-

Table 1. Premalignant Alterations Observed in Cav-1 KO Mammary Epithelial Cells.

Phenotype	Detailed explanation	Culture conditions
Increased growth	Twofold increase in acinar diameter; Growth arrest on day 8 (WT) versus day 12 (Cav-1 KO)	3D in Matrigel
Defective lumen formation	Increase in luminal filling (2.3-fold overall); Partial luminal filling (threefold increase); Complete luminal filling (1.7-fold increase)	3D in Matrigel
EGF-independent growth	Acinar diameter (twofold increase); Acinar number (fourfold increase); Hyper-activation of the Ras-p42/44 MAP kinase cascade	3D in Matrigel
Defective attachment and spreading	Increased MMP expression and secretion (MMP-2/9); Decreased collagen type IV	2D on glass
Spontaneous early-stage EMT	Fibroblastic appearance after 7 to 9 days Develop actin stress fibers; Changes in E-cadherin/ β -catenin localization; Continue to express epithelial markers (keratins 8/18; E-cadherin); Hyperactivation of TGF- β /Smad signaling	2D on glass
Increased branching/invasiveness	Increased EMT response to growth factor stimulation (both HGF and bFGF)	3D in Matrigel/collagen mixture

ithelial cells. Cav-1 suppresses several proproliferative pathways, by directly binding, and holding in an inactive state key members of the MAP kinase cascade, such as MEK-1/2 and ERK-1/2.^{25,28} Indeed, we show here that loss of this inhibitory interaction between Cav-1 and such crucial components, results in constitutive hyperactivation of the Ras-p42/44 MAP kinase cascade. These observations are consistent with previous *in vitro* and *in vivo* studies, demonstrating that Cav-1 negatively regulates p42/44 MAP kinase activity. For example, anti-sense-mediated down-regulation of Cav-1 in NIH 3T3 cells leads to constitutive activation of p42/44 MAP kinase (ERK-1/2) and oncogenic transformation.²⁹ In *Caenorhabditis elegans*, Cav-1 gene silencing by an RNA interference-based approach, advances meiotic cell-cycle progression, a phenotype that mirrors hyperactivation of Ras signaling.⁷⁵ Similarly, genetic ablation of Cav-1 in mice induces hyperactivation of the p42/44 MAP kinase pathway in certain cell types, for example cardiac fibroblasts.⁷⁶ Moreover, combined loss of Cav-1 and INK4a produces a similar hyperactivation of p42/44 MAP kinase in mouse embryo fibroblasts.⁴⁵ Finally, MMTV-PyMT-induced mammary tumors from Cav-1-null mice display p42/44 MAP kinase hyperactivation.⁴⁷ However, this is the first time it has been shown that loss of Cav-1 confers growth-factor independence, which then dramatically affects mammary acinar morphogenesis. In light of this evidence, we speculate that loss of Cav-1 disrupts critical homeostatic mechanisms that normally ensure the proper proliferation and growth of mammary epithelial cells.

To assess whether the EGF-independent growth of Cav-1-deficient acini is dependent on hyper-activation of the Ras-p42/44 signaling cascade, we cultured EGF-deprived Cav-1 KO acini either in the presence of a specific inhibitor of the MAP kinase pathway, namely PD98059, or in the presence of the vehicle alone, DMSO. Interestingly, treatment with two different concentrations of PD98059 (either 5 μ M or 10 μ M) effectively blocked the EGF-independent growth of Cav-1-deficient acini (data not shown). Both the diameter and the number of

Cav-1-null acini were greatly reduced by inhibitor treatment, and restored to normal levels observed with WT acini grown in the absence of EGF. These results strongly suggest that the EGF-independent growth of Cav-1-null mammary epithelial cells is dependent on baseline hyper-activation of the Ras-p42/44 signaling cascade.

Cav-1 Regulates Cell Attachment/Spreading and MMP Secretion

Although growth factor independence is a key event in the early stages of cellular transformation, a hallmark of tumor progression is the disruption of communications between neighboring cells and between cells and the extracellular matrix.¹ In this regard, breakdown of the basement membrane is believed to be an essential step in initiating tumor invasiveness. Interestingly, we show here that, when Cav-1-null mammary epithelial cells were deprived of exogenously added extracellular matrix (ie, by direct culture on glass coverslips or tissue-culture plastic), they reveal defects in cell-substrate attachment and spreading, with increased expression and secretion of at least two members of the MMP protein family, ie, MMP-2 and -9, which are normally involved in matrix degradation and remodeling.

Freshly isolated Cav-1-deficient mammary acini—organoids—exhibit a loss of basement membrane components, such as type IV collagen. Interestingly, an association between loss of basement membrane type IV collagen and increased expression of MMP-2 and -9 has been established in various human cancers, such as colorectal tumors.⁷⁷ Our current results are in agreement with previous findings, demonstrating that ablation of Cav-1 expression in MMTV-PyMT mice greatly increases mammary tumor formation and enhances lung metastasis.⁴⁷ Interestingly, Cav-1 functions as a negative regulator of matrix metalloproteinase (MMP) secretion, and inhibits cell invasiveness in a highly metastatic PyMT mammary carcinoma-derived cell line, namely Met-1

cells.⁴⁷ Independent studies have shown that forced re-introduction of Cav-1 into highly metastatic 4T1.2 mammary cells suppresses primary tumor formation, after implantation into the mammary gland, and inhibits subsequent metastasis to distant organs (bone and lung).⁷⁸ In support of these findings, we show here that Cav-1-null acini grown in a more permissive three-dimensional environment, such as a Matrigel/collagen I mixture, display invasive potential, with increased branching in response to HGF and bFGF stimulation. Interestingly, MMP expression was recently shown to promote directly the growth of tumor cells cultured in three-dimensional matrices by eliminating structural constraints imposed by the basal membrane and by overcoming growth-inhibitory signals embedded within the extracellular matrix.⁷⁹

The EMT and Smad Signaling

During tumor formation, mammary epithelial cells often undergo an EMT, resulting in a more invasive, less-differentiated fibroblastic phenotype. Interestingly, here, we provide evidence that loss of Cav-1 induces a spontaneous EMT in primary cultures of mammary epithelial cells. After prolonged culture on glass (7 to 9 days), Cav-1-deficient cells show several functional features of an EMT, including changes in cell morphology, the formation of actin stress fibers, and the loss of E-cadherin from cell-cell contacts.

However, Cav-1-null cells still express epithelial-specific markers, suggesting that Cav-1 may be involved in the early stages of epithelial *trans*-differentiation. Mechanistically, we demonstrate here that loss of Cav-1 induces Smad-2 hyperactivation in mammary epithelial cells. Interestingly, we have previously shown that in NIH 3T3 and 293T cells, Cav-1 functions as a negative regulator of TGF- β signaling,³⁰ which is thought to trigger the EMT *in vivo*. The role of Smad-dependent signaling in the TGF- β -mediated EMT remained controversial for several years,^{17,80} and other pathways, including p42/44-MAP kinase, PI-3K, RhoA, and Rac1 small GTPases, have been shown to contribute to some or all phenotypic aspects of the EMT.^{18–20,81} Yet, recent studies have convincingly shown that Smad activation is required for the immediate-early stages of the EMT, such as cellular separation and disassembly of E-cadherin adherens junctions.⁸² Consistent with these findings, we show here that loss of Cav-1 function hyperactivates Smad signaling, and induces an early-stage EMT. Of course, because we also show that Cav-1-null mammary epithelial cells display hyperactivation of the p42/44 MAP kinase cascade, we cannot rule out that this pathway may also contribute. To our knowledge, our current results are the first to show a physiologically relevant role for Cav-1 in a TGF- β -mediated cellular process.

The occurrence of a full EMT, with loss of epithelial markers and activation of the mesenchymal program, often requires that cells are cultured in three-dimensional collagen gels and occurs on prolonged growth factor exposure.⁸³ We show here that culturing Cav-1-null acini in a three-dimensional collagen-rich environment in-

creases mammary epithelial branching, in response to the addition of exogenous HGF and bFGF. In this regard, increased branching is considered a sign of invasiveness and is indicative of a complete EMT. Cav-1-null mammary glands also show significant areas of ductal fibrosis surrounding the epithelia.^{44,45} We speculate that this mammary ductal fibrosis phenotype may reflect constitutive TGF- β /Smad signaling and a spontaneous EMT in Cav-1 (–/–)-null mammary epithelial cells *in vivo*.

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