Tumorigenesis and Neoplastic Progression

Loss of Caveolin-3 Induces a Lactogenic Microenvironment that Is Protective Against Mammary Tumor Formation

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Here, we show that functional loss of a single gene is sufficient to confer constitutive milk protein production and protection against mammary tumor formation. Caveolin-3 (*Cav-3*), a muscle-specific caveolin-related gene, is highly expressed in muscle cells. We demonstrate that *Cav-3* is also expressed in myoepithelial cells within the mammary gland. To determine whether genetic ablation of *Cav-3* expression affects adult mammary gland development, we studied the phenotype(s) of *Cav-3*^{-/-}-null mice. Interestingly, *Cav-3*^{-/-} virgin mammary glands developed lobuloalveolar hyperplasia, akin to the changes normally observed during pregnancy and lactation. Genome-wide expression profiling revealed up-regulation of gene transcripts associated with pregnancy/lactation, mammary stem cells, and human breast cancers, consistent with a constitutive lactogenic phenotype. Expression levels of three key transcriptional regulators of lactation, namely *Elf5*, *Stat5a*, and *c-Myc*, were also significantly elevated. Experiments with pregnant mice directly showed that *Cav-3*^{-/-} mice underwent

precocious lactation. Finally, using orthotopic tumor cell implantation, we demonstrated that virgin *Cav-3*^{-/-} mice were dramatically protected against mammary tumor formation. Thus, *Cav-3*^{-/-} mice are a novel preclinical model to study the protective effects of a lactogenic microenvironment on mammary tumor onset and progression. Our current studies have broad implications for using the lactogenic microenvironment as a paradigm to discover new therapies for the prevention and/or treatment of human breast cancers. (*Am J Pathol* 2009, 174:613–629; DOI: 10.2353/ajpath.2009.080653)

Throughout the past ~40 years, many epidemiological studies have shown that early pregnancy, multiple full-term births, and extended periods of lactation are all protective against the development of human breast cancers.^{1–8} To explain this phenomenon, several theories related to i) the systemic effects of hormones induced during pregnancy and lactation (such as estrogen, progesterone, and prolactin); ii) the effects of pregnancy and lactation on the terminal differentiation of the luminal mammary epithelial cell compartment; and/or iii) local secreted factors produced in the pregnant/lactating mammary gland,⁹ such as milk protein components, have been put forth. Interestingly, early pregnancy and multiple births are only protective against estrogen-receptor-positive (ER⁺) breast cancers, whereas breastfeeding/lactation is protective against both ER⁺ and ER⁻ breast cancers, suggesting a fundamentally

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different mechanism.^{10–12} In this regard, women who breastfed for 2 years or longer showed an ~30 to 35% lower risk for developing either ER⁺ or ER[–] breast cancers.¹² Despite this intriguing epidemiological data, the mechanism(s) underlying the protective effects of pregnancy and/or lactation remain primarily unknown. Studies have been hampered in part by the lack of a suitable animal model in which to study the protective effects of pregnancy/ lactation.

Here, we describe a constitutively lactogenic mouse model that is resistant to the development of primary mammary tumors and distant lung metastases. We validated their lactogenic phenotype by genome-wide transcriptional profiling and immunohistochemical analysis. We also provide evidence that their mammary tumor resistance phenotype is attributable to the local paracrine effects of lactogenic luminal mammary epithelial cells, via implantation of mammary tumor cells within the primary ducts of their mammary glands. This resulted in a >1000-fold reduction in mammary tumor mass. Thus, our results directly support the idea that the lactogenic microenvironment is a critical factor in preventing mammary tumor onset, progression, and metastasis. Most importantly, a lactation-based therapeutic strategy would provide a more natural and non-toxic approach to the development of novel anti-cancer therapies. Finally, we propose that the secretion of anti-tumorigenic factors into breast milk may be part of a natural maternal fail-safe mechanism for the prevention of breast cancers.

Experimental Procedures

Materials

Antibodies and their sources were as follows: caveolin-3 (Cav-3) and Stat5a from BD Biosciences, Inc. (San Jose, CA); phospho-RB (Ser 807/811) from Cell Signaling, Inc. (Danvers, MA); β -casein, whey acidic protein (WAP), Sox2, Nestin, estrogen receptor- α (ER- α), progesterone receptor (PR-A/B), and p63 from Santa Cruz Biotechnology (Santa Cruz, CA); α -smooth muscle actin from Sigma (St. Louis, MO); and cytokeratin-14 (K14) from Covance, Inc. (Princeton, NJ). Samples of human breast milk were obtained commercially from Lee Biosolutions (St. Louis, MO), from several different donors.

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Animal Studies

All animals were housed and maintained in a pathogen-free environment/barrier facility at the Kimmel Cancer Center at Thomas Jefferson University under National Institutes of Health (NIH) guidelines. Mice were kept on a 12-hour light/dark cycle with *ad libitum* access to chow and water. Cav-3^{–/–}-deficient mice were generated, as we previously described.¹³ All wild-type and Cav-3 knockout (KO) mice used in this study were in the FVB/N genetic background. For most of the studies, 4-month-old virgin female mice were used, unless stated otherwise. Animal protocols used for this study were pre-approved by the institutional animal care and use committee. Interestingly, Cav-3^{–/–} mice in the C57BL/6 genetic background did not show the lobulo-alveolar hyperplasia phenotype (data not shown).

Whole Mount Analysis

Carmine dye staining of inguinal (no. 4) mammary glands was performed as we previously described.^{14–16} Briefly, fourth mammary glands (inguinal) were excised, spread onto glass slides, and fixed in Carnoy's fixative (6 parts 100% EtOH, 3 parts CHCl₃, 1 part glacial acetic acid) for 2 to 4 hours at room temperature. The samples were washed in 70% EtOH for 15 minutes and changed gradually to distilled water. Once hydrated, the mammary whole-mounts were stained overnight in carmine alum [1 g of carmine (C1022, Sigma) and 2.5 g of aluminum potassium sulfate (A7167, Sigma) in 500 ml of distilled water]. The samples were then dehydrated using step-wise ethanol concentrations and defatted in xylenes. Mammary whole-mounts were stored in methyl salicylate. Photomicrographs were generated using a Nikon stereo microscope (Nikon, Melville, NY). Quantitation of various parameters (primary branching and ductal thickness) was performed using Image J software (National Institutes of Health, Bethesda, MD). For determining the number of primary branch points, mammary glands from five mice for each genotype ($n = 5$) were subjected to detailed analysis. Similarly, for determining ductal thickness, mammary glands from five mice for each genotype were also analyzed. More specifically, the diameters of 8 to 15 ducts per genotype were measured. For each duct, the diameter was determined at 3 to 6 points and the average diameter was calculated.

Gene Expression Profiling

These studies were performed essentially as we have previously described for other cell types.¹⁷ Total RNA (5 μ g) was reverse-transcribed using the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA) using a high performance liquid chromatography-purified T7-dT24 primer (Sigma Genosys, St. Louis, MO) that contains the T7 polymerase promoter sequence. The single-stranded cDNA was converted to double-stranded cDNA using DNA polymerase I (Promega, Madison, WI) and purified by cDNA spin-column purification using the

GeneChip sample cleanup module (Affymetrix, Santa Clara, CA). The double-stranded cDNA was used as a template to generate biotinylated cRNA using a bioarray high-yield RNA transcription labeling kit (Enzo, New York, NY) and the labeled cRNA purified by the GeneChip sample cleanup module (Affymetrix). Fifteen μg of cRNA was fractionated to produce fragments of between 35 to 200 bp using 5 $\times$ fragmentation buffer provided in the cleanup module. The sample was hybridized to mouse 430 2.0 microarray (Affymetrix) representing more than 39,000 transcripts. The hybridization and washing steps were performed in accordance with Affymetrix protocols for eukaryotic arrays. The arrays were scanned at 570 nm with a confocal scanner from Affymetrix. Analysis of the arrays was performed using the R statistics package and the limma library of the Bioconductor software package.¹⁷ Arrays were normalized using robust multiarray analysis, and a *P* value of 0.05 was applied as criteria for statistically differentially expressed genes. Mammary gland gene sets for pregnancy and parity were used for comparison purposes.^{18,19} Gene sets for embryonic stem cell-associated transcripts, as well as Nanog, Oct4, Sox2, and Myc target genes, were previously described.²⁰

Statistical Analysis of Overlapping Gene Sets

The *P* value is the probability of finding the number of overlapping genes in the two gene sets by pure chance.²¹ This is determined by the equation:

$$p = 1 - \sum_{j=0}^{i-1} \frac{c(m, j) c(t-m, n-j)}{c(t, n)}$$

where $c(n, j)$ is the number of combinations that one choose j objects from n objects, t is the total number of observable genes, i is the number of genes in the overlap, and m and n are the numbers of differentially expressed genes in the two sets. For the present comparisons, t the number of observable genes was taken as number of common genes based on the gene symbol for the two chips MU74Av2 and M430_2.0.

Pathway Analysis

Global pathway analysis of our gene expression profiling studies was performed using ASSESS (analysis of sample set enrichment scores), an unbiased computer-based gene set enrichment algorithm.²²

Immunohistochemistry

Immunohistochemical staining was performed essentially as we previously described.^{23,24} Briefly, 5- μm sections from paraffin-embedded mammary glands were deparaffinized, and then rehydrated by passage through a graded series of ethanol. Antigen retrieval was performed by microwaving the slides in 100 mmol/L sodium citrate buffer for 15 minutes. Endogenous peroxidase activity was quenched with 3% H_2O_2

for 20 to 30 minutes. Then, slides were washed with phosphate-buffered saline (PBS) and blocked with 10% goat serum in PBS for 1 hour at room temperature. Samples were incubated with the primary antibodies diluted in 10% goat serum/PBS overnight at 4°C. After washing in PBS (three times, 5 minutes each), slides were stained with the LSAB2 system kit (Dako Cytomation, Glostrup, Denmark), according to the manufacturer's recommendations. Briefly, samples were incubated with biotinylated linker antibodies for 30 minutes, washed in PBS (three times, 5 minutes each), and then incubated with a streptavidin-horseradish peroxidase-conjugated solution for 30 minutes. After washing, samples were incubated with the diaminobenzidine reagent until color production developed. Importantly, wild-type and *Cav-3*^{-/-} samples were exposed to the diaminobenzidine solution for the same amount of time. Finally, the slides were washed in PBS and counterstained with hematoxylin, dehydrated, and mounted with coverslips. Importantly, critical negative controls were performed in parallel for all of the immunohistochemical studies.

Pregnancy and Involution Studies

To compare alveolar development and milk globule content, mammary glands (no. 4) were harvested from 2-month-old wild-type and *Cav-3*^{-/-} female mice after 14 days of pregnancy. Then they were subjected to whole mount analysis and formalin-fixed, sectioned, and stained with hematoxylin and eosin (H&E). For the involution studies, lactating wild-type and *Cav-3*^{-/-} female mice (3 months of age) were subjected to forced weaning, to induce mammary gland involution (days 1, 3, and 6 after weaning). Then the mammary glands were harvested, fixed, and paraffin-embedded. Sections were used for terminal deoxynucleotidyltransferase-mediated dUTP nick-end label (TUNEL) staining to assess the onset of apoptosis.

TUNEL Staining

Briefly, 5- μm sections of involuting mammary glands were TUNEL-stained with a kit according to the manufacturer's instructions (TACs 2 TdT *in situ* apoptosis detection kit; Trevigen, Inc., Gaithersburg, MD). Then the slides were counterstained with hematoxylin to visualize overall tissue morphology. TUNEL-positive cells were scored in at least three independent $\times 20$ fields for each genotype and at each time point.

MMTV-PyMT Mammary Tumor Studies

MMTV-PyMT transgenic mice (strain 634) in the FVB/N background were generated as previously described.²⁵ Matings were performed with PyMT male hemizygous mice. PyMT/*Cav-3*^{+/+} and PyMT/*Cav-3*^{+/-} male mice were interbred with *Cav-3*^{+/+} or *Cav-3*^{-/-} female mice to generate a cohort of *Cav-3*^{+/+}, *Cav-3*^{+/-}, or *Cav-3*^{-/-} virgin female mice, all hemizygous for the PyMT transgene.

Figure 1. Cav-3 is normally expressed in the myo-epithelial cell layer in the mammary gland. Mammary glands were harvested from 2-month-old wild-type virgin female mice. Paraffin-embedded sections were prepared and immunostained with anti-Cav-3 IgG (brown color). Then, samples were counterstained with hematoxylin (blue color). Note that Cav-3 is preferentially expressed in the myo-epithelial cell layer (arrows). A negative control (omission of the primary antibody) is also shown. Scale bar = 50 μ m.

None of the PyMT transgene-negative control mice developed tumors. Early mammary lesions were operationally defined as the lesions observed in 5-week-old virgin female PyMT mice. At 14 weeks, virgin female mice were sacrificed, and all mammary tumors were carefully excised and weighed. For the male tumor study, mice were sacrificed at 22 weeks of age. All these time points were chosen based on our previous experience with the phenotypic characterization of PyMT/*Cav-1*^{-/-} mice.²⁶

MMTV-PyMT Lung Metastasis Analysis

Female PyMT mice at 14 weeks of age were sacrificed and the lungs exposed by thoracic and tracheal dissection.²⁶ The lungs were removed and insufflated with 2 ml of 15% India ink dye, washed in water for 5 minutes, and bleached in Fekete's solution (70% ethanol, 3.7% paraformaldehyde, 0.75 mol/L glacial acetic acid). Surface lung metastases were scored in a genotype-blinded manner under low power using a Nikon SMZ-1500 stereomicroscope. *P* values were determined by applying Mann-Whitney statistical analysis parameters, which does not assume a Gaussian distribution (nonparametric test).

Primary Duct and Flank Injections with Met-1 Cells

Met-1 cells were the generous gift of Dr. Robert D. Cardiff (University of California at Davis).^{27,28} For orthotopic implantation, 0.5×10^5 Met-1 cells were resuspended in 5 μ l of PBS and injected through the nipple of the inguinal (no. 4) mammary gland into 2-month-old female FVB/N mice (WT, *Cav-3*^{+/+}; HET, *Cav-3*^{+/-}; KO, *Cav-3*^{-/-}) using a Hamilton syringe with a 26-gauge needle. Met-1 cells are syngeneic to the FVB/N strain. At 6 weeks after injection, mice were sacrificed, and the tumors were carefully excised and weighed. For flank injections, 1.0×10^5 cells were resuspended in 100 μ l of PBS and injected subcutaneously into the flanks of 2-month-old FVB/N female mice. At 4 weeks after injection, mice were sacrificed, and the tumors were carefully excised and weighed.

Cell Migration Assays

The migratory potential of Met-1 cells was measured via an *in vitro* Boyden chamber assay.²⁶ Briefly, Met-1 cells in 0.5 ml of serum-free Dulbecco's modified Eagle's medium were added to the wells of 8- μ m-pore membrane Boyden chambers (for migration assays; catalog no. 354578, transwells; BD Biosciences). The lower chambers contained 10% (v/v) fetal bovine serum in Dulbecco's modified Eagle's medium to serve as a chemoattractant. The media in both chambers was supplemented with human breast milk (10% v/v). Cells were incubated at 37°C and allowed to migrate throughout the course of 6 hours. Nonmigrating cells were removed from the upper surface of the membrane by scrubbing with cotton swabs. Chambers were stained in 0.5% crystal violet diluted in 100% methanol for 30 to 60 minutes, rinsed in water, and examined under a bright-field microscope. Values for migration were obtained by counting five fields per membrane ($\times 20$ objective) and represent the average of several independent experiments performed throughout multiple days. It is important to note that when bovine serum albumin was used at the same concentration as human breast milk (as a negative control), no effects on cell migration were observed. Also, milk treatment did not appear toxic to Met-1 cells.

Results

Cav-3 Is Preferentially Expressed in the Myo-Epithelial Cell Layer in the Adult Mammary Gland

Cav-3 is a muscle-specific gene that is expressed in striated and smooth muscle cells.^{29,30} However, its potential role in mammary gland development has never been formally evaluated. We speculated that *Cav-3* might be expressed in the myo-epithelial cell layer in the adult mammary gland. To test this hypothesis, mammary glands were harvested from 2-month-old wild-type virgin female FVB/N mice. After paraffin-embedding, 5- μ m tissue sections were immunostained with a well-characterized monospecific antibody that

Figure 2. *Cav-3*^{-/-} mice develop extensive lobulo-alveolar hyperplasia. Mammary glands (no. 4) were harvested from 4-month-old wild-type (WT) and *Cav-3*^{-/-} virgin female mice and subjected to whole-mount analysis. The distribution of the mammary tree was visualized by staining with carmine alum dye. **A:** Note that *Cav-3*^{-/-} mice demonstrate a significant increase in both ductal thickness and mammary branching, reminiscent of lobulo-alveolar hyperplasia seen in pregnant/lactating mice. Scale bar = 1 mm. **B:** Increasing levels of magnification ($\times 1.25$, $\times 2$, $\times 4$, and $\times 10$) are shown to better appreciate the detailed morphology of this mammary branching phenotype. Scale bars are as indicated. **C:** The number of primary branch points and ductal diameters were quantitated using NIH Image J software. Note the more than fourfold increase in primary branching and the near threefold increase in ductal thickness. $n = 5$ mice were examined for each genotype. * $P < 0.001$.

selectively recognizes Cav-3,³⁰ but does not recognize other caveolins, such as Cav-1 and Cav-2. Slides were then counterstained with hematoxylin to visualize tissue morphology. Figure 1 shows that, as predicted, antibodies directed against Cav-3 selectively stained the myo-epithelial cell layer.

Cav-3^{-/-} Mice Develop Extensive Lobulo-Alveolar Hyperplasia, Akin to the Changes Normally Observed During Pregnancy and Lactation

To determine whether loss of Cav-3 expression has any functional consequences for mammary gland architecture, we next assessed the status of mammary gland

morphology in *Cav-3*^{-/-} mice. For this purpose, we first crossed our *Cav-3*^{-/-} mice onto the FVB/N genetic background because most mouse mammary gland studies related to mammary tumorigenesis are done in the FVB/N genetic background. Then, we surgically isolated the mammary glands (no. 4) from 4-month-old wild-type and *Cav-3*^{-/-} virgin female mice and subjected them to a detailed analysis using whole-mounts and paraffin-embedded tissue sections.

Figure 2A shows the results of our whole mount analysis. The mammary tree was visualized by staining with carmine dye. Note that there is a striking alteration in mammary gland morphology. There are three significant changes that are immediately apparent. These include an increase in ductal thickness and primary branching, as well as the onset of lobulo-alveolar hyperplasia, akin to

Table 1. *Cav-3^{-/-}* Virgin Mammary Glands Express Members of a Parity-Induced Gene Signature (6 of 17)

Symbol	Gene name	Fold up-regulation in Cav-3 KO
<i>Car2</i>	Carbonic anhydrase 2	4.1
<i>Csn2</i>	Casein β	38.4
<i>Lbp</i>	Lipopolysaccharide binding protein	3.5
<i>Pde4b</i>	Phosphodiesterase 4B, cAMP-specific	2.4
<i>Pigr</i>	Polymeric immunoglobulin receptor	22.5
<i>Tgfb3</i>	Transforming growth factor, β 3	2.1

what is normally observed in pregnant/lactating mice. These interesting morphological changes are also shown at higher power in Figure 2B. Most importantly, quantitation revealed a more than fourfold increase in primary branching and a near threefold increase in ductal thickness (Figure 2C). Interestingly, the *Cav-3^{-/-}* hyperplastic mammary phenotype is quantitatively more severe than what we have previously observed in *Cav-1^{-/-}* mice at the same age and in the same genetic background.³¹ For example, *Cav-1^{-/-}* mice only show an approximately twofold increase in ductal thickness,¹⁶ with no apparent increase in primary branching.³²

Transcriptional Gene Profiling of *Cav-3^{-/-}* Virgin Mammary Glands Reveals a Constitutive Lactogenic Phenotype

To mechanistically dissect the lobulo-alveolar hyperplastic phenotype of *Cav-3^{-/-}* mice, we surgically isolated the mammary glands from virgin female mice and subjected them to genome-wide transcriptional profiling. The expression of 863 known genes and transcripts was changed in *Cav-3^{-/-}* mammary glands, as compared with wild-type glands; 530 transcripts were up-regulated and 333 transcripts were down-regulated. All of these genes and transcripts changed by more than or equal to twofold and achieved statistical significance ($P < 0.05$) (see Supplemental Table S1 at <http://ajp.amjpathol.org>; for full details, see GEO accession no. GSE12881).

Consistent with our morphological observations of lobulo-alveolar hyperplasia (Figure 2), we see a number of genes that are normally up-regulated in the mammary gland during pregnancy and lactation (see Supplemental Table S2 at <http://ajp.amjpathol.org>). Expression levels of three key transcriptional regulators of lactation, namely *Elf5* (approximately fivefold), *Stat5a* (approximately twofold), and *c-Myc* (approximately threefold) are also significantly elevated. It is important to note that these DNA microarray data are derived from nonpregnant virgin female *Cav-3^{-/-}* mice. In addition, several genes involved in mammary stem cell self-renewal are up-regulated, as are a num-

Table 2. RB/E2F Signature Genes Are Up-Regulated in *Cav-3^{-/-}* Virgin Mammary Glands (28 Genes)

Symbol	Gene name	Fold up-regulation in Cav-3 KO
<i>5730507H05Rik</i>	RIKEN cDNA 5730507H05 gene	3.3
<i>Brca1</i>	Breast cancer 1	3.3
<i>Brrn1</i>	Barren homolog (Drosophila)	2.5
<i>Ccnb1</i>	Cyclin B1	2.8
<i>Cdca5</i>	Cell division cycle-associated 5	3.3
<i>Cdca8</i>	Cell division cycle-associated 8	3.3
<i>Chaf1b</i>	Chromatin assembly factor 1, subunit B (p60)	3.1
<i>Chek1</i>	Checkpoint kinase 1 homolog (<i>S. pombe</i>)	3.1
<i>Ect2</i>	Ect2 oncogene	3.1
<i>Exo1</i>	Exonuclease 1	3.4
<i>Fanca</i>	Fanconi anemia, complementation group A	2.0
<i>Fen1</i>	Flap structure specific endonuclease 1	2.2
<i>Hmmr</i>	Hyaluronan-mediated motility receptor (RHAMM)	2.8
<i>Kntc1</i>	Kinetochore associated 1	2.9
<i>Mcm2</i>	Minichromosome maintenance-deficient 2 mitotin (<i>S. cerevisiae</i>)	2.6
<i>Mcm5</i>	Minichromosome maintenance-deficient 5, cell division cycle 46 (<i>S. cerevisiae</i>)	3.8
<i>Mcm6</i>	Minichromosome maintenance-deficient 6 (MIS5 homolog, <i>S. pombe</i>) (<i>S. cerevisiae</i>)	2.4
<i>Mki67</i>	Antigen identified by monoclonal antibody Ki 67	3.4
<i>Mphosph1</i>	M phase phosphoprotein 1	3.0
<i>Nasp</i>	Nuclear autoantigenic sperm protein (histone-binding)	2.0
<i>Pole</i>	Polymerase (DNA directed), epsilon	2.5
<i>Prim1</i>	DNA primase, p49 subunit	2.4
<i>Rfc5</i>	Replication factor C (activator 1) 5	2.2
<i>Skp2</i>	S phase kinase-associated protein 2 (p45)	2.4
<i>Smc2</i>	Structural maintenance of chromosomes 2	2.2
<i>Stmn1</i>	Stathmin 1	3.2
<i>Tripl3</i>	Thyroid hormone receptor interactor 13	2.9
<i>Tyms</i>	Thymidylate synthase	2.2

ASSESS: Pathway Analysis

Figure 4. Results from pathway analysis with ASSESS. Pathway analysis was performed using ASSESS (analysis of sample set enrichment scores). Note that stem cell-associated genes, E2F target genes, c-Myc target genes, and TGF- β /Wnt/HGF/Rho signaling all appear to be activated/up-regulated, as expected during pregnancy and lactation. Blue indicates down-regulated; red indicates up-regulated. For example, note that genes down-regulated in stem cells (STEMCELL_COMOM_DN) are also down-regulated in *Cav-3*^{-/-} mammary glands. Conversely, note that genes up-regulated in stem cells (HSC_INTERMEDIATEPROGENITORS_SHARED; FETAL_LIVER_ENRICHED_TRANSCRIPTION_FACTORS; WNTPATHWAY; LIN-WNT_UP) are also up-regulated in *Cav-3*^{-/-} mammary glands. WT, *Cav-3*^{+/-}; KO, *Cav-3*^{-/-}.

ber of established breast cancer biomarkers and genes associated with the transforming growth factor (TGF)- β and hepatocyte growth factor (HGF) signaling pathways. All of these changes in gene expression are consistent with a constitutive lactogenic phenotype.

Pregnancy/Lactation Genes

Several genes that are up-regulated have been shown to be selectively increased in the mammary gland during pregnancy (lobulo-alveolar expansion), and/or are associated with lactation/milk production, including *Csn* 1, 2, and 3 (the caseins); *Car6* (carbonic anhydrase 6); *Gjb6* (gap junction membrane channel protein β 6; Cx-30); *Gjb2* (gap junction membrane channel protein β 2; Cx-26); *Muc4* (mucin 4); *Muc15* (mucin 15); *Vdr* (vitamin D receptor); *Hunk* (hormonally up-regulated Neu-associated kinase); *Wfdc12* (WAP four-disulfide core domain 12); *ErbB4* [v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)]; and *Stat5a*. *ErbB4* also acts as a nuclear chaperone for *Stat5a*, a master regulator of lactation.³³ *Muc4* also functions as a membrane-bound ligand for *ErbB2*.³⁴ Similarly, *Pigr* (polymeric immunoglobulin receptor; secretory component) is normally up-regulated during pregnancy and lactation for IgA transport into milk. *Wnt5b* is normally selectively expressed only during pregnancy.³⁵ *Elf5* is an epithelial-specific ETS family transcription factor that is a master regulator of lobulo-alveolar outgrowth and is a downstream target of prolactin signaling.³⁶ Finally, *WAP*, *Csn1/2* (α - and β -caseins), *Gjb6* (Cx-30), *Gjb2* (Cx-26), and *Elf5* are all

phospho-RB (Ser 807/811)

Figure 5. *Cav-3*^{-/-} mammary epithelia show increased levels of phospho-RB (Ser 807/811), consistent with increased cell cycle progression. We immunostained paraffin-embedded sections derived from wild-type (WT) and *Cav-3*^{-/-} virgin female mice with phospho-specific antibodies directed against the cell cycle regulatory protein, RB. Interestingly, our results indicate that *Cav-3*^{-/-} mice show a dramatic increase in RB phosphorylation in their luminal mammary epithelial cells. **A**, **C**, and **E** are from WT mice; **B**, **D**, and **F** are *Cav-3*^{-/-} mice. Similar results were obtained with young (2 months old) and older mice (4 and 8 months old). Scale bars: 100 μ m (**A**, **B**); 50 μ m (**C**–**F**). Original magnifications: $\times 20$ (**A**, **B**); $\times 60$ (**C**–**F**).

thought to be *Stat5* target genes that are transcriptionally up-regulated on *Stat5* activation.³⁷

Mammary Stem Cell Genes

Several genes that are up-regulated are known to control the self-renewal of stem cells, such as *Rspo1* [R-spondin homolog (*Xenopus laevis*)], *Wnt5b* (wingless-related MMTV integration site 5B), *Fzd5* [frizzled homolog 5 (Drosophila)], *Sox4*, *Sox9*, and *Sox10* (SRY-box containing genes). *Irx2* [Iroquois-related homeobox 2 (Drosophila)] is selectively expressed in mammary terminal end buds (TEBs) that are known to be enriched in mammary stem cells.³⁸ Also, *Krt5* (keratin 5) is a putative mammary stem cell marker. *Par6* (partitioning defective 6) is an important gene for maintaining the asymmetrical division of stem cells. Other well-established stem cell genes that are up-regulated include: *Aldh18a1*, *Cd24a*, *Cdcp1*, *Cyr61*, *Klf5*, *Lgr6*, and *c-Myc*. Mammary stem cell numbers are known to be increased during pregnancy and lactation in the mammary gland.³⁹

Figure 6. *Cav-3*^{-/-} mammary epithelia show increased levels of Sox2 and Nestin, consistent with an increased stem/progenitor cell population. We immunostained paraffin-embedded sections derived from wild-type (WT) (left) and *Cav-3*^{-/-} (right) virgin female mice with antibodies directed against the progenitor/stem cell markers, Sox2 (A) and Nestin (B). Interestingly, our results indicate that *Cav-3*^{-/-} mice show a dramatic increase in Sox2 expression in luminal epithelial cells, myo-epithelial cells, and their stromal compartment. Nestin levels were also elevated in *Cav-3*^{-/-} mice, but it showed a different cellular distribution than Sox2 (arrows point at the basal/myo-epithelial cell layer). Scale bars = 50 μ m. Original magnifications, $\times 40$.

TGF- β and HGF Signaling

TGF- β signaling regulates the epithelial-mesenchymal transition that is associated with branching morphogenesis, as occurs during pregnancy and lactation.^{40,41} Interestingly, we observed that the ligands Tgfb2 and Tgfb3 (TGF- β 2 and - β 3) and Tieg3 (TGF- β -inducible early growth response 3), a TGF- β target gene, are significantly increased. Thbs1 (thrombospondin 1) is an activator of TGF- β ligands. Also, several genes that are up-regulated are proteases/inhibitors that function in the regulation of HGF (also known as scatter factor) processing, a key epithelial morphogen involved in branching morphogenesis.^{40,41} These include Prss8 [protease, serine, 8 (prostasin)], St14 (MTSP1/Matriptase/PRSS14/Prostamin/SNC19/TADG15), and Spint2 (serine protease inhibitor, Kunitz type; hepatocyte growth factor activator inhibitor 2).

Figure 7. *Cav-3*^{-/-} mice show significant increases in lobulo-alveolar development during pregnancy, with enhanced milk droplet production. Mammary glands (no. 4) were harvested from 2-month-old wild-type (WT) and *Cav-3*^{-/-} female mice after 14 days of pregnancy. **A:** Whole-mount analysis. The distribution of the mammary tree was visualized by staining with carmine alum dye. Note that *Cav-3*^{-/-} mice demonstrate a significant increase in lobulo-alveolar development during early pregnancy, at day 14. **B:** H&E staining. Samples were fixed, paraffin-embedded, sectioned, and subjected to H&E staining. Note that *Cav-3*^{-/-} mice show a significant increase in milk droplet production during early pregnancy, at day 14. Scale bars: 2 mm (A); 50 μ m (B). Original magnifications: $\times 2$ (A); $\times 40$ (B).

Comparison with Other Pregnancy/Lactation/Parity Gene Sets

We also compared our results with previously published mouse mammary gland gene sets associated with pregnancy, lactation, and involution. Gene transcript levels altered in *Cav-3*^{-/-} mammary glands showed the most overlap with pregnancy-associated genes (comparing pregnancy day 1 with pregnancy day 19).¹⁸ Supplemental Table S3 (available at <http://ajp.amjpathol.org>) shows that the transcriptional expression of more than 170 pregnancy-associated genes is changed in *Cav-3*^{-/-} virgin mammary glands ($P = 0.007$). Hierarchical clustering of these gene changes is shown in Figure 3A. Similarly, gene changes in the *Cav-3*^{-/-} mammary glands also showed significant overlap with a highly-conserved parity-induced gene signature (6 of 17 genes) (Table 1).¹⁹

A recent article suggests that RB inactivation (hyperphosphorylation) and E2F activation (release from RB sequestration) are key signaling events that occur during

Table 3. ES Cell-Related Genes Up-Regulated in *Cav-3*^{-/-} Virgin Mammary Glands

ES cell-expressed genes (32 genes)
<i>Aass, Ap1m2, Atp1a2, Aut5, Ccna1, Cdca5, Chek1, Cobl, Crabp2, Cxadr, Dnmt3b, Dsg2, Ect2, Fen1, Fzd5, Gylt1b, Hells, Hmnr, Hspa8, Lsr, Mal2, Mcm2, Mcm5, Mcm6, Nasp, Nfe2l3, Prim1, Rbm35a, Rcc2, St6gal1, Tacstd1, Tia1</i>
Nanog targets (32 genes)
<i>Acsf4, Bclaf1, Bmp7, Cdh1, Col4a5, Col4a6, Cxadr, Cyp1b1, Cyr61, Exph5, Fanca, Grhl2, Has2, Hells, Igfbp2, Irx2, Klf5, Loh11cr2a, Marveld2, Nebl, Nfe2l3, Rab15, Rab17, Rab25, Rbp1, Ror1, Rpl17, Slc7a5, Tia1, Tnc, Tpx2, Ube2t</i>
Oct4 targets (13 genes)
<i>Bmp7, Cdh1, Grhl2, Has2, Irx2, Klf5, Nebl, Nfe2l3, Ror1, Tcf12, Tjp3, Tnc, Trps1</i>
Sox2 targets (28 genes)
<i>Atf3, Bclaf1, Bmp7, Cyr61, Exph5, Grhl2, Has2, Hells, Igfbp2, Irx2, Klf5, Kntc1, Loh11cr2a, Mphosph1, Myo9a, Nebl, Nfe2l3, Prss8, Rab15, Rab17, Rab25, Ror1, Sap30, Slc7a5, Tia1, Tjp3, Tnc, Ube2t</i>
Myc targets (27 genes)
<i>Aldh18a1, Ammcr1, Arhgef16, Ccna1, Cib1, Exo1, Fkbp11, Fzd5, Gmcs, Hmnr, Hook2, Hspa8, Lsr, Mcm5, Mphosph1, Muc1, Pterf, Pterf, Pycr1, Sap30, Slc39a8, Spag5, Stmn1, Tcf12, Tfr, Tgfb3, Trip13</i>
ES cell common transcriptional regulators (15 genes)
<i>Elf5, Etv6, Foxc1, Id4, Irx2, Klf5, Lmo4, Myc, Nfe2l3, Sap30, Sox10, Sox4, Sox9, Trip13, Whsc1</i>

Underlined genes were up-regulated more than or equal to fivefold.

pregnancy-induced hyperproliferation in the mammary gland.⁴² Consistent with these findings, the levels of 28 RB/E2F target genes are transcriptionally elevated in *Cav-3*^{-/-} mammary glands (Table 2).

To independently verify that many of these signaling pathways are indeed activated in *Cav-3*^{-/-} mammary glands, we performed pathway analysis using ASSESS (analysis of sample set enrichment scores), an unbiased computer-based gene enrichment algorithm (Figure 4). Based on this type of analysis, stem cell-associated genes, RB/E2F target genes, c-Myc target genes, and TGF- β /Wnt/HGF/Rho signaling all appear to be activated/up-regulated, as expected during the epithelial remodeling (proliferation and branching morphogenesis) normally associated with pregnancy and lactation.

Validation of the Constitutive Lactogenic Phenotype of *Cav-3*^{-/-} Virgin Mammary Glands

To validate our results from gene transcriptional profiling, we next subjected the mammary glands from virgin female *Cav-3*^{+/+} and *Cav-3*^{-/-} mice to immunohistochemical analyses. A key hallmark of the pregnant/lactating mammary gland is the production of milk proteins. Figure 3, A–C, shows that *Cav-3*^{-/-} mammary glands overexpress a number of gene products associated with lactation and milk production, including mouse milk proteins (β -casein and WAP) and Stat5a. These genes were all transcriptionally up-regulated, as seen in Supplemental Tables S1, S2, and S3 at <http://ajp.amjpathol.org>. These results provide direct validation of the lactogenic phenotype of *Cav-3*^{-/-} virgin mammary glands.

The RB (retinoblastoma tumor suppressor) protein negatively controls cell proliferation by regulating the G₁ restriction point. Cyclin-dependent kinases (CDKs) phosphorylate RB, thereby inactivating RB function and allowing cell cycle progression via the release of sequestered E2F family transcription factors. Figure 5 shows that *Cav-3*^{-/-} mammary glands have elevated levels of phospho-RB (Ser 807/811), consistent with the up-regulation of a significant number of RB/E2F target genes (Table 2) and the hyperproliferation of luminal epithelial cells normally associated with lobuloalveolar hyperplasia and pregnancy.

Sox2 is a transcription factor that is essential to maintain self-renewal of undifferentiated pluripotent embryonic stem cells.⁴³ The SOX (SRY-related HMG-box) gene family is involved in the regulation of embryonic development and in the cell fate determination.⁴³ Together with Oct4 and Nanog, Sox2 is necessary for the maintenance of pluripotent potential. Thus, we used Sox2 as a stem/progenitor cell marker to assess the predicted up-regulation of the adult mammary stem cell population in *Cav-3*^{-/-} mammary glands. Figure 6A shows that the levels of Sox2 expression are clearly elevated in *Cav-3*^{-/-} luminal epithelia, myo-epithelia, and the stromal compartment. Similarly, other putative stem cell markers were up-regulated in *Cav-3*^{-/-} mammary glands, including Nestin (Figure 6B). This pattern of Nestin immunostaining is consistent with its up-regulation and association with the basal (triple-negative) breast cancer phenotype.⁴⁴ Taken together, these findings support the idea that loss of *Cav-3* can increase the adult mammary stem cell population, as normally occurs during pregnancy and lactation. Similarly, a number of embryonic stem cell-associated genes are up-regulated in *Cav-3*^{-/-} mammary glands, including Nanog, Oct4, Sox2, and Myc target genes (Table 3). For example, *Nfe2l3* is a common target of Nanog, Oct4, and Sox2, and is dramatically up-regulated by 8.9-fold in *Cav-3*^{-/-} mammary glands.

Activation of TGF- β signaling and fibrosis/collagen production are tightly-linked events.⁴⁵ Numerous procollagen genes were up-regulated (*Col4a5*, *Col4a6*, *Col9a1*, *Col9a3*, *Col13a1*, *Col16a1*) between approximately two-fold to six-fold (see Supplemental Table S2 at <http://ajp.amjpathol.org>). Consistent with this finding, *Cav-3*^{-/-} mammary glands show dramatic increases in trichrome-staining, indicative of increased collagen deposition (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Finally, estrogen receptor (ER- α) and progesterone receptor (PR) expression are normally down-regulated in luminal mammary epithelial cells during late pregnancy and lactation.^{9,46,47} Similarly, *Cav-3*^{-/-} luminal mammary epithelial cells show a loss of ER- α and PR-A/B-positive cells (see Supplemental Figures S2 and S3 at <http://ajp.amjpathol.org>), as would be predicted.

Cav-3^{-/-} Mammary Glands Undergo Premature Lactation and Show Reduced Apoptosis During Involution

Given the observed lactogenic phenotype of *Cav-3*^{-/-} mice, we suspected that they may also show accelerated

Figure 8. *Cav-3*^{-/-} mammary glands are resistant to involution-induced apoptosis. **A:** Lactating wild-type (WT) and *Cav-3*^{-/-} female mice (3 months of age) were subjected to forced weaning, to induce mammary gland involution (days 1, 3, and 6 after weaning; **D1**, **D3**, and **D6**). Then, the mammary glands were harvested, fixed, and paraffin-embedded. Sections were used for TUNEL staining to assess the onset of apoptosis (brown color). Slides were counterstained with hematoxylin (blue color) to visualize overall tissue morphology. Importantly, note that *Cav-3*^{-/-} mammary epithelia appear more resistant to involution-induced apoptosis at all three time points. **B:** Bar graph depicts the mean value and the SEM. Note that *Cav-3*^{-/-} mammary glands show a threefold to sixfold reduction in TUNEL⁺ cells per ×20 field, depending on the time point. (**P* < 0.05, Student's *t*-test). Scale bars = 200 μm.

mammary gland development and milk production during pregnancy. To test this hypothesis directly, we mated a cohort of 2-month-old virgin female mice to examine wild-type and *Cav-3*^{-/-} mammary glands at various stages during pregnancy and lactation. Interestingly, our results demonstrate that, at pregnancy day 14, *Cav-3*^{-/-} mammary glands show a clear premature lactation phenotype (Figure 7A), with the morphological appearance of well-defined milk droplets (Figure 7B). Given these results, we would predict that *Cav-3*^{-/-} mammary epithelia will also show dramatic defects during involution. Thus, we next examined the extent of apoptosis in wild-type and *Cav-3*^{-/-} mammary glands during involution (forced weaning) at days 1, 3, and 6. Apoptosis was detected using a standard TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling) assay.

Figure 8A clearly shows that wild-type mice undergo significant apoptosis during involution at days 1, 3, and 6 after weaning (left panels), as expected. In striking contrast, *Cav-3*^{-/-} mammary glands show a dramatic reduction in TUNEL staining (right panels), consistent with the idea that loss of Cav-3 confers resistance to apoptotic cell death during involution. Thus, Cav-3 is normally required for the proper onset of apoptotic cell death during mammary gland involution. Quantitation of these results is presented in Figure 8B. Note that *Cav-3*^{-/-} mammary glands show a threefold to sixfold reduction in TUNEL⁺ cells, depending on the time point examined. These re-

sults are consistent with our observations that *Cav-3*^{-/-} mammary epithelial cells constitutively overexpress Elf5 and Stat5a, two critical positive transcriptional regulators of lactation. Similar resistance to involution-induced apoptosis has been previously observed with *Stat5a* and *Elf5* transgenic mice.^{36,48,49}

Loss of Cav-3 Inhibits the Growth of Mammary Tumors and Dramatically Reduces Lung Metastases

Epidemiological data have shown that lactation (early and increased duration) in humans is protective against the development of breast cancers. However, the mechanism(s) by which lactation prevents breast cancer remains unknown.⁹ Progress has been hampered by the absence of a constitutively lactating mouse model. To begin to address this issue, we crossed *Cav-3*^{-/-} mice with MMTV-PyMT mice (polyomavirus middle T antigen transgenic). MMTV-PyMT mice are an established mouse model of human breast cancer that undergoes spontaneous progression to metastatic disease, such as lung metastasis.^{25,50,51} In MMTV-PyMT mice, the luminal mammary epithelial cell population is selectively targeted for cell transformation.²⁵

MMTV-PyMT mice provide an ideal model to study the effect of a given gene product on early tumorigen-

Figure 9. Loss of Cav-3 is protective against the formation of early mammary lesions. Mammary glands were harvested from virgin female PyMT mice at 5 weeks of age, fixed in ethanol/acetic acid for 2 to 4 hours, and stained overnight with carmine dye. **A:** To quantify the growth of mammary lesions, digital images were acquired and the total area occupied by dysplastic lesions was measured for each mammary gland examined using NIH Image J software. Note that PyMT/Cav-3^{-/-} mice showed an ~1.6-fold reduction in lesion area when compared with matched PyMT/Cav-3^{+/+} and PyMT/Cav-3^{+/-} mice. **B:** Representative examples of early mammary lesions are shown. PD, primary duct; LN, lymph node. WT, PyMT/Cav-3^{+/+}; HET, PyMT/Cav-3^{+/-}; KO, PyMT/Cav-3^{-/-}. $n = 10$ to 14 mice were analyzed for each genotype. Scale bar = 1 mm.

esis, because multifocal dysplastic foci develop in the mammary epithelium of MMTV-PyMT mice as early as 3 weeks of age and eventually progress to adenocarcinomas.^{25,51} Whole-mount preparations are a well-established and recommended method to identify early premalignant lesions of the mammary epithelium. These lesions have also been termed mammary intraepithelial neoplasia (MIN). These dysplastic lesions are present in the majority of mammary glands by 3 weeks of age in MMTV-PyMT mice and spread throughout the entire mammary fat pad by 7 weeks of age.

We first assessed the development of early mammary lesions in PyMT/Cav-3^{+/+}, PyMT/Cav-3^{+/-}, and PyMT/Cav-3^{-/-} mice at 5 weeks of age. Interestingly, PyMT/Cav-3^{-/-} mice showed an ~1.6-fold reduction in lesion area when compared with matched PyMT/Cav-3^{+/+} and PyMT/Cav-3^{+/-} mice (Figure 9, A and B). As such, loss of both Cav-3 alleles is required for this protective effect

($n = 10$ to 14 mice for each group). After a longer 14-week observation period, PyMT/Cav-3^{+/+}, PyMT/Cav-3^{+/-}, and PyMT/Cav-3^{-/-} mice were next evaluated for mammary tumor burden and lung metastasis. Representative images are shown in Figure 10, A and B, and quantitation is presented in Figure 10, C and D. Note that PyMT/Cav-3^{+/-} and PyMT/Cav-3^{-/-} mice show ~1.3-fold and ~2.2-fold reductions in tumor mass, respectively ($n = 8$ to 14 mice for each group). This corresponds to 25% and 55% reductions in tumor mass.

However, much more dramatic reductions in spontaneous lung metastases were observed. To quantitate metastasis, the number of metastases per lung lobe was scored. Interestingly, PyMT/Cav-3^{-/-} mice possessed only 0 to 7 metastases, whereas PyMT/Cav-3^{+/+} mice had up to 115 metastases. In contrast, PyMT/Cav-3^{+/-} showed an intermediate phenotype, with an average of ~18.6 metastases (Figure 10). As such, PyMT/Cav-3^{+/-} and PyMT/Cav-3^{-/-}

Figure 10. Genetic ablation of Cav-3 significantly reduces mammary tumor mass and dramatically inhibits lung metastasis. PyMT virgin female transgenic mice were sacrificed at 14 weeks of age, and all tumors were carefully excised and weighed. **A** and **B:** Representative images of the primary mammary tumors (**A**) and lung metastases (**B**) derived from PyMT/Cav-3^{+/+} and a PyMT/Cav-3^{-/-} mice. Boxed areas of lung metastases are shown at higher magnification. **C** and **D:** Note that PyMT/Cav-3^{+/+} and PyMT/Cav-3^{-/-} mice show ~1.3-fold and ~2.2-fold reductions in tumor mass, as compared with PyMT/Cav-3^{+/-} mice (**C**). Similarly, PyMT/Cav-3^{+/-} and PyMT/Cav-3^{-/-} mice showed ~2.6-fold and ~20-fold reductions in metastasis, respectively (**D**). For scoring metastasis, we measured the number of metastases per lung lobe for each mouse. WT, PyMT/Cav-3^{+/+}; HET, PyMT/Cav-3^{+/-}; KO, PyMT/Cav-3^{-/-}. $n = 8$ to 14 mice were analyzed for each genotype.

Figure 11. Male *Cav-3*^{-/-} mammary glands are also protected against mammary tumor formation. PyMT male transgenic mice were sacrificed at 22 weeks of age, and all tumors were carefully excised and weighed. Male mice were analyzed at 22 weeks of age because they show a longer tumor latency period. Note that male PyMT/*Cav-3*^{-/-} mice show an ~2.5-fold reduction in tumor mass. WT, PyMT/*Cav-3*^{+/+}; HET, PyMT/*Cav-3*^{+/-}; KO, PyMT/*Cav-3*^{-/-}. $n = 9$ to 22 mice were analyzed for each genotype.

mice showed ~2.6-fold and ~20-fold reductions in metastasis, respectively ($n = 8$ to 14 mice for each group). In summary, loss of both *Cav-3* alleles inhibited the development of early mammary lesions (1.6-fold), frank mammary tumors (2.2-fold), and distant lung metastases (20-fold)—all in the context of the MMTV-PyMT mouse model.

Similar results were also obtained in male PyMT/*Cav-3*^{-/-} mice, with an ~2.5-fold reduction in mammary tumor mass (Figure 11). Male mice were analyzed at 22 weeks of age because they show a longer tumor latency period. Thus, the tumor-resistance phenotype associated with PyMT/*Cav-3*^{-/-} mice appears to be independent of ovarian hormones ($n = 9$ to 22 mice for each group), suggesting instead a role for the local mammary microenvironment.

Cav-3^{-/-} Mammary Glands Are Protected Against Orthotopic Tumor Implantation: Role of Paracrine versus Systemic Factors

We speculated that exposure to secreted factors from *Cav-3*^{-/-} luminal mammary epithelial cells, such as milk proteins, could be responsible for the mammary tumor resistance phenotype of *Cav-3*^{-/-} mice. To test this hypothesis, we injected mammary tumor cells (Met-1 cells) directly into the primary duct, to maximize their exposure to lumenally secreted factors, including milk protein components. Met-1 cells are a highly tumorigenic mammary cell line established from a mammary adenocarcinoma derived from a female MMTV-PyMT mouse.²⁸ It is important to note that Met-1 cells are syngeneic to the FVB/N strain.

We orthotopically implanted Met-1 cells in the primary mammary duct of *Cav-3*^{+/+}, *Cav-3*^{+/-}, and *Cav-3*^{-/-} virgin female FVB/N mice, via nipple injection. Figure 12A demonstrates that tumor cells implanted in *Cav-3*^{-/-} mammary glands show a >1000-fold reduction in tumor mass, as compared with *Cav-3*^{+/+} mammary glands injected in parallel. Interestingly, *Cav-3*^{+/-} mammary glands are also protected against tumor formation, showing an ~10-fold

Figure 12. *Cav-3*^{-/-} mammary glands are protected against orthotopic tumor implantation: role of paracrine versus systemic factors. **A:** Loss of *Cav-3* prevents mammary tumor formation. For orthotopic implantation, 0.5×10^5 cells were resuspended in 5 μ l of PBS and injected through the nipple of the inguinal (no. 4) mammary gland into 2-month-old FVB/N female mice using a Hamilton syringe with a 26-gauge needle. Met-1 cells are syngeneic to the FVB/N strain. At 6 weeks after injection, mice were sacrificed, and the tumors were carefully excised and weighed. Note that *Cav-3*^{+/-} (HET) and *Cav-3*^{-/-} (KO) mice are protected against mammary tumor formation. In contrast, *Cav-3*^{+/+} (WT) show the development of large mammary tumors. **B:** Flank injections: role of the local tumor microenvironment. For injections, 1.0×10^5 cells were resuspended in 100 μ l of PBS and injected subcutaneously into the flanks of 2-month-old FVB/N female mice. At 4 weeks after injection, mice were sacrificed, and the tumors were carefully excised and weighed. Note that all three genotypes show the development of mammary tumors. WT, *Cav-3*^{+/+}; HET, *Cav-3*^{+/-}; KO, *Cav-3*^{-/-}.

reduction in tumor mass ($n = 14$ to 24 mammary glands injected for each group). *Cav-3*^{-/-} mice also showed dramatic reductions in tumor incidence, with only 9% of the mammary glands (2 of 22) injected developing tumors. In contrast, ~80% of *Cav-3*^{+/+} mammary glands (19 of 24) developed tumors after injection. This represents an approximately ninefold reduction in tumor incidence.

Importantly, when Met-1 cells were injected into the flanks of *Cav-3*^{+/+}, *Cav-3*^{+/-}, and *Cav-3*^{-/-} mice, tumors of equivalent sizes formed in all three genotypes (Figure 12B) ($n = 8$ mammary glands injected for each group). As such, the mammary tumor-resistance phenotype observed in the mammary glands of *Cav-3*^{+/-} and *Cav-3*^{-/-} mice is not related to systemic factors. Rather, it appears to be attributable to local factors, indicative of a tumor-resistant mammary microenvironment. Thus, local secreted paracrine factors and/or cell-cell interactions are likely to mediate this tumor-resistance phenotype.

Figure 13. Growth media supplemented with human breast milk inhibits the migration of mammary tumor cells. Transwells (8- μ m pore size; noncoated) were used to measure cell migration. Met-1 cells in serum-free media (containing 0.1% bovine serum albumin) were placed in the upper chamber. Media containing 10% FCS (the chemoattractant) was placed in the lower chamber. Human breast milk (10%) was added to both the upper and lower chambers. Then, Met-1 cells were allowed to migrate through the 8- μ m filter throughout a 6-hour period. Samples of human breast milk were obtained from four independent donors (numbered 1 to 4). Note that when Met-1 cells are incubated for 6 hours in media supplemented with 10% (v/v) human breast milk, there is up to a >40-fold reduction in cell migration (see donor 4), depending on the donor. * $P < 0.05$, Student's *t*-test.

Human Breast Milk Reduces the Migration of Mammary Tumor Cells

Because one of the key features of the lactogenic microenvironment is milk production, we also examined the effects of human breast milk on the migratory behavior of Met-1 cells. Figure 13 shows that human breast milk dramatically inhibits the migratory behavior of Met-1 cells. Samples of human breast milk were obtained from four independent donors. Note that when Met-1 cells are incubated for 6 hours in media supplemented with 10% (v/v) human breast milk, there is up to a >40-fold reduction in cell migration, depending on the donor. Virtually identical results were obtained with lower concentrations of human breast milk, as low as 0.5%. Importantly, these results provide direct evidence that Met-1 cells are indeed responsive to the effects of secreted factors present within breast milk.

Discussion

Here, we present several independent lines of evidence that loss of Cav-3 expression induces a lactogenic phenotype in the virgin mammary gland. First, Cav-3^{-/-} mammary glands morphologically undergo lobulo-alveolar hyperplasia, with ductal thickening and increased primary branching, akin to the changes normally observed during pregnancy/lactation. Second, unbiased genome-wide transcriptional profiling directly shows the up-regulation of gene transcripts normally associated with the pregnant/lactating mammary gland. Included in this gene set are three critical transcription factors that are known to regulate the lacto-

genic differentiation program in the mammary gland, including Elf5, Stat5a, and c-Myc.^{36,52-54} Several other key signaling pathways linked to cell proliferation (RB/E2F) and branching morphogenesis (Wnt/TGF- β /HGF) are up-regulated/activated, as would be expected during pregnancy/lactation. Third, validation studies based on the immunohistochemical analysis of Cav-3^{-/-} mammary glands provide direct evidence for milk protein production, such as β -casein and WAP, and Stat5a overexpression. In addition, Cav-3^{-/-} mammary glands show an increase in their mammary stem cell population and a loss of ER/PR expression, that is characteristic of mammary gland development during late pregnancy/early lactation. Finally, in accordance with the presence of a basal lactogenic phenotype, Cav-3^{-/-} mice undergo precocious mammary gland development during pregnancy and show resistance to the onset of apoptosis during involution. For example, at pregnancy day 14, Cav-3^{-/-} mammary glands already show extensive lobulo-alveolar hyperplasia and the presence of abundant mature milk droplets within their luminal mammary epithelia. Thus, one of the normal functions of Cav-3 is to suppress the onset of the lactogenic differentiation program in mammary epithelial cells.

We can use the expression levels of certain lactational-specific transcripts in an attempt to estimate the lactational stage that Cav-3^{-/-} virgin mammary glands have achieved. For example, Locke and colleagues⁵⁵ carefully followed the fold-induction of the mRNA encoding the gap junction protein, connexin 30, during adult mammary gland development using DNA microarray analysis. Based on this analysis, because Cav-3^{-/-} mammary glands have an ~30-fold increase in the connexin 30 transcript, this corresponds to a point between pregnancy day 8.5 and pregnancy day 12.5. Similarly, the expression of β -casein and WAP normally begins on pregnancy days 9 and 14, respectively.^{56,57} However, both are well-expressed in virgin Cav-3^{-/-} mammary glands, based on transcriptional profiling and the immunostaining of tissue sections.

What is the mechanism by which the loss of Cav-3 expression leads to lactogenic mammary gland development? We show here that Cav-3 is normally expressed in the myo-epithelial cell compartment in wild-type virgin mammary glands. Thus, one possibility is that the hyperproliferation and/or improper differentiation of Cav-3^{-/-} myo-epithelial cells transmits a lactational signal to the luminal mammary epithelial cells, related to secreted factors and/or attributable to a loss of normal myo-epithelial barrier functioning. To assess the differentiation state of the myo-epithelial cell layer, we immunostained paraffin-embedded sections derived from 4-month-old wild-type and Cav-3^{-/-} virgin female mice with a panel of myo-epithelial markers: i) α -smooth muscle actin, ii) cytokeratin-14 (K-14), and iii) p63 (a p53 family member). Supplemental Figure S4 (see <http://ajp.amjpathol.org>) shows that all three myo-epithelial markers are down-regulated or fail to be expressed in the Cav-3^{-/-} myo-epithelial cell layer. These results indicate that loss of Cav-3 results in a

failure in the proper differentiation of myo-epithelial cells, thereby compromising their normal barrier function. This is consistent with the notion that Cav-3 expression is required for the proper terminal differentiation of myo-epithelial cells because Cav-3 is known to function as a broad-spectrum kinase inhibitor that induces cell cycle arrest in the G₀/G₁ phase of the cell cycle.^{58,59} In further support of the idea that Cav-3^{-/-} myo-epithelial/basal cells are not fully differentiated, these cells show the up-regulation of Nestin, a stem/progenitor cell marker (Figure 6B). Nestin immunostaining is characteristic of the basal cell (triple-negative) breast cancer phenotype.⁴⁴

Importantly, epidemiological data have demonstrated that lactation (early and increased duration) in humans is protective against the development of breast cancer. The mechanism(s) by which lactation prevents breast cancer remains unknown.⁹ In many cases, this protective effect has been attributed to systemic increases in pregnancy-induced lactogenic hormones, including estrogen, progesterone, and/or prolactin.^{60–63} However, each of these hormones, either individually or in combination, has also been implicated in the pathogenesis of human breast cancer. This has primarily prevented the evaluation of hormone-induced lactation as a differentiation-based therapy for breast cancer.

Here, we show that Cav-3^{-/-} mammary glands are resistant to primary mammary tumor formation and to the development of distant lung metastases (Figures 10 and 12). When mammary tumor cells were implanted in the primary duct of Cav-3^{-/-} mammary glands, a >1000-fold reduction in tumor mass was observed. However, no such tumor resistance effects were observed when the same tumor cells were implanted in the flank of Cav-3^{-/-} mice. These results indicate that the tumor resistance phenotype of Cav-3^{-/-} mice is not a systemic effect, but rather is related to the local microenvironment of the Cav-3^{-/-} mammary gland. Implantation of mammary tumor cells in the primary duct of Cav-3^{-/-} mammary glands would bathe tumor cells in apically secreted factors produced by lactogenic luminal mammary epithelial cells, including milk components. Thus, we believe that the tumor resistance phenotype of Cav-3^{-/-} mammary glands is attributable to the local paracrine effects of lactogenic luminal mammary epithelial cells.

Human breast milk contains numerous mediators of innate immunity (such as the defensins), as well as growth factors, cytokines, and chemokines, that all affect mammary epithelial cell signaling.^{64–67} Several independent laboratories have also suggested that components of breast milk may have anti-tumorigenic properties, including MDGI (mammary-derived growth inhibitor), lactalbumin, lactoferrin, and WAP.^{68–72} Unfortunately, these studies were mostly conducted *in vitro* with cultured breast cancer cells. This was attributable mainly to the absence of a well-characterized constitutively lactating mouse model. It may very well be that multiple components of breast milk have anti-tumor properties, and that the sum of these factors has the most potent effects. Thus, we analyzed the effects of human breast milk on

the migratory behavior of mammary tumor cells. Interestingly, our results show that the addition of human breast milk to the culture media (at the same concentration as serum), resulted in up to a >40-fold reduction in cell migration.

As such, the secretion of anti-tumorigenic factors into milk may be part of a natural fail-safe mechanism to prevent the onset of breast cancers in the mammary gland during pregnancy, a time of extensive cell proliferation and developmental remodeling. Although relatively rare (an incidence of 1 in 3000 pregnancies), pregnancy-associated breast cancers do occur and this type of breast cancer is thought to have a worse prognosis.⁷³ One possibility is that these patients might have defects in this putative milk-based fail-safe mechanism. Future studies are necessary to determine whether patients with pregnancy-associated breast cancers harbor specific protein sequence variations (either polymorphisms or mutations) in milk protein components. Interestingly, naturally occurring polymorphisms in the bovine casein genes (α -, β -, and κ -) are known to be associated with dramatic changes in overall milk production traits.^{74–77} Similarly, gene-silencing mutations in both caprine and bovine milk proteins (α -, β - and κ -casein, lactalbumin, WAP) have been described.^{78–83}

In summary, our current studies have broad implications for using the lactogenic microenvironment to discover new differentiation-based therapies for the prevention and/or treatment of human breast cancers. A lactation-based therapeutic strategy should provide a more natural and nontoxic approach to the development of novel anti-cancer therapies. In this regard, targeted reduction of Cav-3 levels in the mammary gland may represent a new therapeutic strategy for preventing the onset of human breast cancers.

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