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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 nontoxic 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.

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 $CHCl_3$, 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 stepwise 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 μg) 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

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