Caveolin-1, Mammary Stem Cells, and Estrogen-Dependent Breast Cancers

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Abstract

Estrogen exposure is considered a significant risk factor for breast cancer development. Estrogen receptor (ER) is expressed at low levels in normal epithelia, and its expression is dramatically up-regulated as transformation progresses during mammary hyperplasia and adenocarcinoma development. The mechanism(s) driving ERα up-regulation during mammary tumorigenesis remains unclear. Caveolin-1 (Cav-1) is the structural protein of plasmalemmal invaginations, termed caveolae, which functions as a tumor suppressor gene. Interestingly, Cav-1 dominant-negative mutations are exclusively found in ERα-positive breast cancer samples. In support of these clinical findings, ERα expression is increased in Cav-1 (-/-) null mammary epithelia, and estrogen stimulation further enhances the growth of Cav-1-deficient three-dimensional epithelial structures. These phenotypes correlate with augmented levels of cyclin D1. In addition, Cav-1 gene inactivation induces the accumulation of a cell population with the characteristics of adult mammary stem cells. Primary cultures of Cav-1 (-/-) mammary epithelial cells exhibit premalignant changes, such as abnormal lumen formation, epidermal growth factor–independent growth, defects in cell substrate attachment, and increased cell invasiveness. Thus, Cav-1 gene inactivation promotes premalignant alterations in mammary epithelium and induces increased ERα expression levels and the up-regulation of cyclin D1. As tumor formation is a multihit process, Cav-1 mutations that occur during the early stages of mammary transformation may be a critical upstream/initiating event leading to increased ERα levels. (Cancer Res 2006; 66(22): 10647-51)

Introduction

Breast cancer is the most common malignancy in women and the second leading cause of cancer death in the United States (the first one being lung cancer). Because estrogens are essential mitogens of normal mammary epithelial cells (MEC), prolonged exposure to estrogen is considered an important risk factor for breast cancer development. Estrogen action is mediated by estrogen receptors (ER; ERα and ERβ), members of the nuclear receptor family and ligand-activated transcription factors that control the expression of estrogen-responsive genes. ERα is expressed at low levels in the normal breast, with increasing expression in premalignant hyperplastic lesions, and even higher levels as transformation progresses, in carcinomas (1). ERα-positive tumors account for approximately 60% to 70% of human breast cancers (2). The mechanisms that regulate ERα over-expression during transformation are still being elucidated. Interestingly, recent evidence has suggested that in the normal mammary gland a subset of ERα-positive cells represent a slowly dividing population with the characteristics of mammary stem cells (3). Through asymmetrical division, mammary stem cells possess the dual ability to self-renew and to differentiate into specific cell types. Aberrant self-renewal of ERα-positive stem cells is hypothesized to lead to an augmentation of ERα-positive cells and, thus, to be responsible for the increased ERα expression observed in many breast tumors.

Caveolins are a family of scaffolding proteins that coat 50 to 100 nm plasma membrane invaginations, termed caveolae (4, 5). Caveolae are involved in several cellular processes, such as cholesterol homeostasis, vesicular transport, and the regulation of signal transduction (for a review, see ref. 6). Caveolin-1 (Cav-1) is thought to bind and hold in an inactive state several pro-proliferative proteins [such as the epidermal growth factor (EGF) receptor, ErbB2, and members of the growth-factor-activated Ras pathway (7). The caveolin-scaffolding domain acts as a broad spectrum protein kinase inhibitor by binding an aromatic sequence within the interacting partners, thus holding them inactive and releasing this tonic inhibition on activation by the appropriate stimulus (8, 9). Cav-1 mRNA and protein are down-regulated or absent in primary human cancers, in several mouse and human breast cancer cell lines, and in oncogenically transformed NIH3T3 cells (10–13). Forced reexpression of Cav-1 in transformed mammary cell lines abrogates their oncogenic potential and inhibits their invasive properties (14–16). Cav-1 expression in the metastatic 4T1.2 mammary carcinoma cell line suppresses primary tumor growth and impedes metastasis to distant organs when these cells are orthotopically implanted into the mammary gland (17). The Cav-1 gene is located in close proximity to a genomic region, the D7S522 locus, which is frequently deleted in various human cancers, including tumors of the breast, colon, ovary, and head and neck (18). In 2001, a Japanese group reported on a Cav-1 mutation leading to a proline-to-leucine substitution (P132L) in up to 16% breast carcinoma samples (19), which behaves in a dominant-negative fashion (20). Whereas a Cav-1 deficiency in mice is not per se sufficient for cancer development, a second hit from either an environmental or genetic insult produces advanced full-blown tumors. For example, Cav-1−/− mice exhibit enhanced tumor growth and metastatic spreading, suggesting that targeted disruption of the Cav-1 gene could be a potential therapeutic approach to the treatment of breast cancer. (21)
formation (a) in the skin after treatment with the carcinogen 7,12-dimethylbenz(a)anthracene and (b) in the mammary gland by crossing them with the tumor prone mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) model (21, 22). Taken together, these data indicate that Cav-1 functions as a tumor suppressor in the mammary gland.

Importantly, several recent reports have now shown that Cav-1 gene inactivation affects estrogen responsiveness (see below). For a detailed discussion of both tumor suppressor and tumor-promoting functions of Cav-1, please see the following review article (18).

Cav-1 Mutations in Human Breast Cancer and ERα Signaling

To begin to dissect the role of Cav-1 in mammary tumorigenesis, our laboratories used a combination of approaches, including (a) mutational analysis on human breast cancer samples, (b) in vivo studies on Cav-1-deficient mouse models, and (c) ex vivo reconstitution of mammary acini using primary cultures of MECs derived from wild-type (WT) and Cav-1 (-/-) null mice (Fig. 1). We first examined Cav-1 mutations in human breast cancer samples and correlated them with ERα expression status. Our results showed that Cav-1 mutations associate exclusively with ERα-positive, but not with ERα-negative, breast tumors (23). More specifically, the overall incidence of Cav-1 mutations (P132L and others) in our cohort was ~19%, with a relative incidence in ERα-positive breast tumors approaching ~35% (23). This exciting clinical data opened new avenues to dissect the mechanistic interrelationship between Cav-1 mutations and ERα overexpression in breast cancer. Because the Cav-1 P132L mutation acts in a dominant-negative fashion (20), Cav-1 (-/-) null mice were used as a model system to study the effect of Cav-1 loss-of-function on ERα signaling. Interestingly, Cav-1-deficient mammary glands displayed ERα up-regulation compared with WT controls. We then cultured ex vivo primary MECs from WT and Cav-1 (-/-) mice in a three-dimensional system to mimic the formation of mammary acini-like structures. Culturing normal primary MECs on a reconstituted basement membrane extract (Matrigel) provides a system that closely mirrors the features of mammary acini in vivo, with the formation of hollow structures, with a single layer of MECs lining an epithelial lumen, and the basal deposition of basement membrane (24).

Interestingly, upon growth factor deprivation, Cav-1 (-/-) null three-dimensional epithelial structures exhibited approximately 4- to 5-fold ERα up-regulation and enhanced growth in response to estrogen stimulation. Notably, the estrogen-induced growth of EGF-deficient Cav-1 (-/-) null acini correlated with increased levels of cyclin D1. In addition, sections from ERα-positive human breast cancer samples containing Cav-1 mutations were also positive for cyclin D1, whereas no cyclin D1 immunostaining was observed in ERα-negative/Cav-1 gene normal sections (23). The cyclin D1 gene encodes the regulatory subunit of the holoenzyme that phosphorylates and inactivates the pRb protein (25). Cyclin D1 targeted to the mammary gland in mice induces mammary gland adenocarcinoma, and antisense to cyclin D1 abrogates ErbB2-induced mammary tumorigenesis (26).

In summary, these new findings define a novel pathway toward mammary tumorigenesis, in which Cav-1 gene inactivation likely occurs in the early phases of mammary transformation and induces increased sensitivity toward estrogen due to ERα up-regulation, which in turn drives augmented cyclin D1 levels. Importantly, amplification and overexpression of the cyclin D1 proto-oncogene is observed in approximately 20% and 50% of mammary tumors, respectively (25).

In direct support of our findings, Zhang et al. (27) showed that Cav-1 haploinsufficiency in an immortalized “normal” human MEC line (MCF-10A) induces the constitutive activation of ERα expression. Furthermore, they showed that estrogen stimulation is both necessary and sufficient (a) to promote the anchorage-independent growth of Cav-1 haploinsufficient MCF-10A cells and (b) to drive their tumor formation in nude mice (27). Similarly, we showed that Cav-1 gene inactivation increases ERα expression, both in primary human breast cancers and in mouse mammary epithelia, and facilitates the growth of estrogen-treated primary mammary epithelial cultures.

Cav-1 and Adult Mammary Stem Cells

How does Cav-1 loss-of-function induce increased ERα expression? The increase of ERα-positive cells is believed to occur at an early stage during mammary transformation and is presumed to represent the expansion of a mammary stem cell population (3). As a consequence, our laboratories evaluated recently whether Cav-1 deficiency, which leads to ERα up-regulation, is also associated with the amplification of mammary stem cells (28). In direct support of this notion, in vivo expression of two stem cell markers, Sca-1 and keratin-6, is enhanced in Cav-1 (-/-) null mammary glands. Moreover, three-dimensional cultures of primary MECs derived from Cav-1 (-/-) null mice exhibit 2- to 3-fold increases in the expression of three stem cell markers (i.e., Sca-1, keratin-6, and keratin-5) compared with WT controls. Mechanistically, members of the Wnt/β-catenin pathway, which controls stem cell self-renewal (i.e., β-catenin and TCF-4), are increased in Cav-1-deficient mammary glands. These results suggest that loss of Cav-1 induces the accumulation of a population of adult mammary stem cells by up-regulating Wnt/β-catenin signaling and that this event may induce ERα overexpression. Thus, we speculate that Cav-1 gene inactivation may constitute a critical initiating step during mammary tumorigenesis.

Although we appreciate that the ER status of adult mammary stem cells is still a hotly debated topic (29), ERα (but not ERβ) knockout (KO) mice lack functional mammary glands due to a loss of mammary epithelial development (30, 31)—consistent with the idea that mammary stem cells express ERα.

Loss of Cav-1 Confers Premalignant Properties: Hyperproliferation, Growth Factor Independence, and Cell Invasiveness

Hyperplasia is a premalignant disorder, in which MECs undergo abnormal proliferation with the thickening of ductal and acinar walls to three-to-four cell layers. Aberrantly proliferating cells within the hyperplastic lesion undergo phenotypic and molecular changes in their growth characteristics and differentiated state. As a consequence, they achieve the ability to proliferate in the absence of an appropriate growth signal and to detach from the basement membrane and other surrounding cells. In addition, to adapt to their new motility functions, hyperplastic mammary cells may lose their epithelial morphology and acquire a more invasive fibroblastic shape.
To mechanistically determine how Cav-1 loss-of-function affects the progression of mammary tumorigenesis, our laboratories studied ex vivo the morphogenesis and three-dimensional development of primary MECs derived from WT and Cav-1 (−/−) null mice (32). When embedded in Matrigel, Cav-1-deficient MECs formed acini that grew 2-fold larger than WT control cells, with wall thickening to three-to-four cell layers. Remarkably, we quantified luminal occlusion and showed that loss of Cav-1

Figure 1. Cav-1 gene inactivation drives premalignancy (mammary epithelial hyperplasia) and tumor initiation. Genetic inactivation of Cav-1 correlates with ER<sub>α</sub> up-regulation and with increased levels of cyclin D1, likely due to the amplification of an ER<sub>α</sub>-positive stem cell population. In addition, Cav-1 loss-of-function induces ligand-independent hyperactivation of the Ras-p42/44 MAPK and Smad signaling pathways as well as enhanced MMP-2/9 secretion (A). Each of these pathways likely contributes to the development of mammary epithelial hyperplasia and tumor initiation (B).
induces a ~2.3-fold increase in luminal filling. These results are consistent with previous findings showing that Cav-1 KO mice exhibit intraductal hyperplasia, with wall thickening to three- to four-cell layers and areas of fibrosis surrounding the epithelial ducts (20). Enhanced growth of Cav-1 (−/−) null three-dimensional epithelial structures was attributed to hyperactivation of the Ras-p42/44 MAPK cascade. Hyperactivation of the p42/44 MAPK pathway might trigger self-sufficient growth, even in the absence of a proper stimulatory signal. In fact, when cultured in the absence of EGF, Cav-1 (−/−) null acini grow at a similar rate as when they are grown in the presence of EGF. Both the size and the number of acini/field are greatly increased in EGF-deprived Cav-1 (−/−) null acini compared with WT controls (32). This is the first time that a loss of Cav-1 was shown to induce EGF-independent growth, with ligand-independent hyperactivation of the Ras-p42/44 MAPK pathway. Cav-1 deficiency and hyperactivation of the p42/44 MAPK pathway are correlated in several pathologic settings. For example, p42/44 MAPK hyperphosphorylation is observed in both carcinogen-induced epidermal tumors and oncogenically induced mammary tumors derived from Cav-1-deficient mice (21, 22), in cardiac fibroblasts of hypertrophic Cav-1 (−/−) null hearts (33), as well as in neoinflammatory lesions of the common carotid artery in Cav-1 KO mice (34).

When deprived of exogenous extracellular matrix (i.e., by direct culture on glass coverslips), Cav-1 null MECs exhibit defects in cell substrate attachment (32). Cell substrate detachment is an essential step during migration and invasion. Migration requires the localized degradation of the basement membrane as well as the expression and secretion of proteases, such as the matrix metalloproteinases (MMP), which are specific for extracellular matrix components. Mechanistically, Cav-1-deficient MECs show increased MMP-2/9 synthesis and secretion. Consistent with these findings, Cav-1 expression also inhibits migration and tumor invasion. For example, Cav-1 expression in the MTLn3 metastatic cell line was shown to prevent EGF-induced lamellipodia formation and invasion in vitro (14). Conversely, Cav-1 deficiency in the context of a tumor-prone mouse model (the MMTV-PyMT mouse) increases tumor formation and promotes lung metastasis (22). In addition, Cav-1 functions as a suppressor of MMP secretion in a PyMT-derived metastatic cell line, (i.e., Met-1 cells; ref. 22).

After prolonged culture, Cav-1 null MECs undergo a spontaneous epithelial-mesenchymal transition, as evidenced by changes in their morphology, reorganization of their actin cytoskeleton, and the redistribution of E-cadherin to an intracellular location (32). However, these cells continued to express epithelial keratins (5/8 and 18), suggesting that Cav-1 may be involved in the early stages of epithelial transdifferentiation. Mechanistically, these phenotypic changes seemed to be due to Smad-2/3 hyperactivation. Finally, Cav-1-deficient acini also displayed increased branching in response to hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) stimulation. Branch formation requires the degradation of basement membrane, induction of cell migration, loss of cell polarity, and the acquisition of a fibroblastic phenotype. Interestingly, when grown in a branching-permissive microenvironment (i.e., in a mixture of collagen and Matrigel), Cav-1 (−/−) null acini exhibited approximately 1.7- and 1.9-fold increases in branch formation in response to HGF and basic FGF stimulation, respectively (32).

Taken together, these *ex vivo* experiments with primary cultures of MECs show that loss of Cav-1 drives premalignant alterations in mammary epithelia, with abnormal lumens formation, EGF-independent growth, defects in cell substrate attachment, and increased cell invasiveness.

**Cav-1, Janus-Activated Kinase-2/Signal Transducer and Activator of Transcription 5a Signaling, and Mammary Tumorigenesis.**

The mammary glands of Cav-1 null mice also displayed constitutive activation and hyperphosphorylation of the transcription factor signal transducer and activator of transcription (STAT) 5a (35, 36). STAT5a is an established regulator of differentiation, growth, and survival of mouse MECs and is critical for terminal differentiation during lactogenesis. Consistent with the associated hyperproliferative and hyperplastic phenotype of mammary epithelia in Cav-1 (−/−) mice, several reports have indicated a tumor-promoting role for STAT5a in mouse mammary glands (37, 38). On the other hand, we and others have reported associations between STAT5a activity and cellular differentiation of human breast cancer (39) and that activation of STAT5a/b was associated with a favorable prognosis in human breast cancer (40).

Future studies will focus on defining the interactions between caveolin and STAT5a/b and the prosurvival and prodifferentiation roles of STAT5a/b that may affect mammary breast tumorigenesis and progression. One hypothesis is that sustained STAT5 activation, via Cav-1 gene inactivation, may provide another important proliferative signal driving mammary tumor initiation and progression. Finally, epithelial-stromal “paracrine” interactions (the tumor microenvironment) may also play a critical role in this process (41).

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Due to space limitations, we regret that many important primary articles could not be cited.

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