Published in final edited form as:

Horm Mol Biol Clin Investig. 2010 June ; 2(2): 227–234. doi:10.1515/hmbci.2010.031.

Changes in mammary caveolin-1 signaling pathways are associated with breast cancer risk in rats exposed to estradiol in utero or during prepuberty

Ayesha N. Shajahan, Shruti Goel, Sonia de Assis, Bin Yu, Robert Clarke, and Leena Hilakivi-Clarke*

Lombardi Comprehensive Cancer Center, Georgetown University, College of Medicine, Washington, D.C., USA

Abstract

Developmental stage of rat mammary gland at the time of estrogen exposure determines whether the exposure increases or reduces later breast cancer risk. For example, in utero exposure to 17 β -estradiol (E2) increases, whereas prepubertal exposure to this hormone decreases susceptibility of developing carcinogen-induced mammary tumors. E2 mediates its actions by interacting with caveolin-1 (CAV1), a putative tumor suppressor gene in breast cancer. Mammary tissues from 2-month-old rats exposed to E2 in utero contained decreased levels of CAV1, whereas prepubertal E2 exposure increased the levels, when compared to vehicle controls. Low CAV1 expression was associated with increased cell proliferation and estrogen receptor α expression, and reduced apoptosis in the mammary glands of rats exposed to E2 in utero. In contrast, high CAV1 expression correlated with reduced cell proliferation and cyclin D1 and phospho-Akt levels, and increased apoptosis in the mammary glands of rats exposed to E2 during prepuberty. In support of the role of CAV1 as a negative regulator of a variety of pro-growth signaling proteins, we detected decreased levels of *Src* and *ErbB2* in rats exposed to E2 during prepuberty. Thus, estrogen exposure during mammary gland development affects the expression and function of CAV1 in a manner consistent with observed changes in susceptibility to mammary tumorigenesis.

Keywords

caveolin-1; estradiol; mammary gland

Introduction

Age at the time when an exposure to estrogenic compounds occurs determines how they alter susceptibility to breast cancer (1). Evidence from human studies has revealed that proxies of estrogenic stimuli in utero and before puberty onset are associated with later susceptibility to develop breast cancer (2–5). Findings obtained in animal models are supportive of the human data and show that in utero exposure to 17 β -estradiol (E2) increases, whereas an exposure during prepuberty decreases mammary cancer risk in rats (6, 7). Exposures to E2 in utero or during prepuberty alter mammary gland morphology by either increasing or decreasing, respectively, the presence of terminal end buds (TEBs), structures that (i) contain the highest number of estrogen receptor α (ER α) and proliferating

© 2010 by Walter de Gruyter

*Corresponding author: Leena Hilakivi-Clarke, PhD, Professor, Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, College of Medicine, 3970 Reservoir Road, N.W., NRB W405, Washington, D.C., 20057, USA Phone: q1-202-687-7237, Fax: q1-202-687-7505, clarkel@georgetown.edu.

cells (8), and (ii) convert to malignant tumors upon exposure to a carcinogen (9). Early life estrogenic exposures can thus affect later breast cancer risk by altering mammary gland morphology, and perhaps genes related to cell proliferation and differentiation. Other changes caused by early life exposures to E2 have not been investigated besides alterations in ER α .

E2 mediates its functions by binding to nuclear ER α and ER β followed by transactivation of target genes (10, 11). Early life estrogenic exposures have been reported to affect ER α protein levels or binding sites in the mammary gland (12), suggesting that their effects on breast cancer risk can involve alterations in these receptors. In addition to the nuclear estrogen receptors, membrane bound receptors can be important for breast cancer development. It was recently proposed that constitutive activation of membrane ER α participates in transformation of normal mammary cells to malignancy as a consequence of downregulation of caveolin-1 (CAV1) (13). CAV1, a 20–22 kDa protein that was originally identified as a tyrosine phosphorylated protein in Rous sarcoma virus-transformed fibroblasts (14), is a scaffolding protein that forms cholesterol- and sphingolipid-rich omega-shaped vesicles called caveolae at the plasma membrane. There are two isoforms of CAV1, the α and β forms that result from alternative splicing from a single gene (15). CAV1 is abundantly expressed in differentiated cells, such as adipocytes, endothelial cells and muscle cells, and are involved in various functions including cholesterol trafficking, vesicle transport and signal transduction (16). Low levels of CAV1 in human mammary gland stroma are specifically associated with early disease progression to invasive breast cancer (17). ER α expressed at the plasma membrane interacts and regulates the expression of CAV1 in epithelial breast cancer cells (18). CAV1 can sequester and regulate the function of various proteins involved in breast cancer, such as Src, eNOS, H-Ras and EGF-R (19, 20).

The role of CAV1 in cancer remains to be determined: whereas some studies indicate CAV1 to be a tumor suppressor (21, 22), others relate high CAV1 expression levels to invasive phenotype in some tumors (23–25). Here we investigated whether in utero E2 exposure that increases later mammary tumorigenesis (7), or prepubertal E2 exposure that provides a strong protection against breast cancer (6), might alter CAV1 expression and function. We found that CAV1 was differentially expressed in the rat mammary gland in response to early life E2 exposures: the levels were reduced in 2-month-old rats following in utero exposure and increased following prepubertal exposure, supporting the role of CAV1 as a tumor suppressor. In addition, protein levels of activated *Src*, ErbB2 and Akt, as well as cell proliferation and apoptosis were affected consistently with the changes in CAV1 expression. These findings provide novel insights into the signaling mechanism of in utero or prepubertal estrogenic exposures in increasing or reducing, respectively, the susceptibility to develop breast cancer.

Materials and methods

Animals and estrogen exposure

Female Sprague-Dawley rats (Charles River Laboratories) were purchased on day 10 of gestation. The animals were housed singly and were fed AIN93G semi-purified laboratory chow. Two days after the offspring were born, the females were crossfostered, weaned on postnatal day 22 and thereafter housed in groups of 3–5 animals. For the in utero treatment group, pregnant rats (on gestation day 10) and for the prepubertal group, rat pups (on postnatal day 7) received either 10 μ g E2 (Sigma Chemical Co., St. Louis, MO, USA) or vehicle (peanut oil), administered as s.c. injections in a volume of 0.05 mL. The injections were repeated daily between gestation days 11 and 20 or postnatal days 8 and 20. Animals were sacrificed at 3 weeks (n=6–7/group) and at 8 weeks of age (n=6–7/group). Fourth

abdominal mammary glands were collected to study changes in protein expression, cell proliferation and apoptosis.

Western blot analyses

Mammary gland tissue samples were homogenized in standard RIPA buffer containing protease inhibitor mixture (Sigma). Protein concentration was estimated using the BCA Protein Assay (Pierce, IL, USA) and equal protein amounts were subjected to Western blot analysis (A) using specific antibody to CAV1 (BD Transduction Laboratories, San Jose, CA, USA). The equivalences of loading and transfer were checked by reprobing with β -actin (Sigma) antibody and the density of bands were quantified/measured using Scion Image (National Institutes of Health). The relative expression levels of proteins here and elsewhere were determined by dividing the band intensities of the protein by the respective band densities of β -actin for each sample group and the mean ratios were plotted.

Immunohistochemistry

Formalin-fixed tissue sections (5 μ m) obtained from five rats per group were deparaffinized in xylene, hydrated through graded alcohols and incubated with 3% H_2O_2 for 10 min to block endogenous peroxidases to assess the expression of CAV1. Non-specific binding was blocked with normal rabbit serum from the Vectastain Elite ABC Kit (Vector Laboratories, Inc, Burlingame, CA, USA) for 20 min, blocked, incubated with CAV1 antibody (1:200) (BD Biosciences, Mountain View, CA, USA) washed, treated with biotinylated goat anti-serum to mouse IgG followed by incubation with streptavidin-peroxidase conjugate (Dako Cytomation, The Ark Kit, Carpinteria, CA, USA). Antigen-antibody complexes were visualized by 3',3'-diaminobenzidine and counterstained with hematoxylin stain, dehydrated and mounted. Control slide was incubated with normal mouse serum. Cell proliferation was assessed using proliferating cell nuclear antigen (PCNA). Tissue sections were incubated overnight with the primary antibody against PCNA (Santa Cruz Biotechnology, Inc, CA, USA). After several washes, sections were treated with the secondary antibody (biotinylated anti-goat IgG from the Vectastain Elite ABC Kit; Vector Laboratories, Inc) for 30 min, followed by treatment with avidin and biotinylated horseradish peroxidase complex from the Vectastain Elite ABC Kit (Vector Laboratories, Inc) for 30 min at room temperature. Sections were washed and stained with 3,3'-diaminobenzidine (Vector Laboratories, Inc) for 1 min, washed and counterstained with Vector's Hematoxylin QS Nuclear Counterstain (Vector Laboratories, Inc) for 45 s. Proliferation index was determined by calculating the percentage of cells that had positive PCNA staining (only dark stained cells were counted) in 1000 cells per mammary gland section. Slides were blindly evaluated with the aid of Metamorph software.

TUNEL staining

For paraffin embedded sections, five rats per group were used to measure apoptosis with the ApopTag Kit (Serologicals Corporation, Norcross, GA, USA) following the manufacturer's protocol. Sections were deparaffinized and rehydrated in a series of graded alcohols followed by pretreatment with 20 μ g/mL of Proteinase K for 15 min. Endogenous peroxidases were quenched with 3% H_2O_2 for 5 min. Sections were washed with equilibration buffer and incubated with the terminal deoxynucleotidyl transferase TdT enzyme. The reaction was stopped and the sections were incubated with a digoxigenin peroxidase conjugate. Sections were washed, incubated with the peroxidase substrate for 6 min, rewashed and counterstained with 0.5% methyl green (Vector Laboratories, Inc) for 10 min, and finally washed in water followed by washes in 100% butanol, dehydrated. Staining in 1000 ductal, 1000 lobuloalveolar and 1000 TEB cells were counted. Apoptotic index was determined by calculating the percentage of cells that were apoptotic through both positive

staining and histological evaluation. Slides were blindly evaluated by two independent investigators.

Statistical analyses

The data obtained for Western blots, PCNA staining and TUNEL staining were analyzed with SigmaStat software using two-way analysis of variance (ANOVA), with age and treatment as independent variables. In addition, PCNA and TUNEL staining in 8-week-old rats exposed to E2 (a) in utero were also analyzed using two-way ANOVA, with epithelial structure and treatment as independent variables, and (b) prepubertally were analyzed using the t-test. Where appropriate, between-group comparisons were done using Tukey's multiple comparisons test. The differences were considered significant if the p-value was <0.05. All probabilities were two-tailed.

Results

Exposure to E2 in utero or during prepuberty results in differential expression CAV1 in the mammary gland

To investigate the correlation between CAV1 expression and early life estrogen exposure, we obtained protein samples from the 4th abdominal mammary glands of 3- (day 21) or 8-week-old (day 50) Sprague-Dawley rats that were exposed to 17- β estradiol (E2) in utero or during prepuberty. In utero exposed rats were treated daily with 10 μ g E2 via s.c. injections through a pregnant dam between gestation days 10–20, and prepubertally exposed rats were given 10 μ g E2 daily between postnatal days 7 and 20. E2 was dissolved in peanut oil and thus the control animals received oil-vehicle treatment.

CAV1 expression was detected mainly in the myoepithelial cells and to a much lesser extent in other types of epithelial cells of the mammary gland (Figure 1). In addition, CAV1 was expressed in the stromal cells and adipose cells. Total CAV1 expression in the mammary gland was quantified by Western blotting and subsequent densitometric analyses (Figure 2A). Using two-way ANOVA, with treatment and time as independent variables, the expression level of CAV1 in the mammary glands of in utero as well as prepubertal rats showed significant differences from the control rats at both 3 and 8 weeks (Figure 2B). At 3 weeks of age, the mammary glands of rats treated with E2 in utero showed increased CAV1 expression in comparison to control rats ($p=0.03$) or rats treated with E2 during prepuberty ($p=0.007$). At 8 weeks, rats treated with E2 during prepuberty showed increased CAV1 expression compared to control rats ($p=0.04$) or in utero E2 exposed rats ($p=0.003$), whereas rats treated with E2 in utero showed decreased ($p=0.04$) CAV1 expression compared to controls.

ER α and cyclin D1 protein expression levels are modified by E2 exposure

Findings of cell culture studies indicate that disruption of CAV1 expression is one of the mechanisms by which an increase in ER α expression and activation contributes to breast tumorigenesis (13). However, the physiological relevance of the correlation between CAV1 and ER α in breast development and tumorigenesis remains unknown. In our rat model, the levels of ER α were significantly higher in the mammary glands of 8-week-old rats treated with E2 in utero than in the controls ($p=0.04$) or rats treated with E2 during prepuberty ($p=0.025$) (Figure 2C). Prepubertal E2 exposure tended to reduce the expression, but the data did not reach significance.

Upon E2 stimulation, expression of cyclin D1, an important regulator of cell transformation, can occur via the non-genomic pathways (26). Moreover, expression levels of cyclin D1 inversely correlate with CAV1 expression in transformed cells (27). In our rat model,

expression level of cyclin D1 was significantly altered in the mammary of animals treated with E2 during prepuberty, but the direction of the change was different at 3 weeks (before puberty onset) and at 8 weeks (Figure 2D). The expression level of cyclin D1 at 3 weeks was significantly higher in the animals treated with E2 during prepuberty than in the controls ($p=0.004$) or in utero E2 treated rats ($p=0.004$), but at 8 weeks of age significantly lower ($p=0.002$) compared to controls. Because CAV1 expression in these rats followed a similar pattern, i.e., it was reduced at 3 weeks and increased at 8 weeks, our findings are consistent with the inversed association reported between cyclin D1 and CAV1 expression. Levels of cyclin D1 levels in the in utero samples at 8 weeks compared to control were not significant.

Activation levels of progrowth signaling proteins are altered in response to E2 exposure

CAV1 interacts with various essential signaling proteins that reside within caveolae through its scaffolding domain and is generally known to negatively regulate their function (19, 28). To examine whether the differential expression of CAV1 upon E2 exposure in utero or during prepuberty had an effect on the activation of essential progrowth signaling proteins in the mammary gland, we examined the expression levels of phospho-(Y1139)ErbB2, phospho-(Y418)*Src* and phospho-(S473)Akt by Western blot analysis (Figure 2E–H). The relative level of phospho-(Y1139)ErbB2, in the mammary gland tissues of rats exposed to estradiol in utero, from both 3 and 8 weeks, were similar to that of the control rats, respectively, suggesting that this kinase was not affected by reduced CAV1 expression and might not be involved in affecting the increased risk of breast cancer following in utero estradiol treatment (Figure 2E). However, the level of phospho-(Y1139)ErbB2 in mammary gland tissue of animals treated with estradiol during prepuberty and sacrificed at 8 weeks of age was significantly reduced ($p=0.04$). Reduced expression of ErbB2 could thus be associated with reducing breast cancer risk in mammary glands of rats exposed to E2 during prepuberty.

Next, we examined the activation of *Src*, Akt and ERK1/2 (Figure 2F–H) that are essential in estrogen-mediated signaling in cultured cells and have been reported to interact with or are regulated by CAV1 (29–33). Levels of phospho-(Y418)*Src* were significantly increased ($p=0.012$) in the mammary gland of 8-week-old rats treated with E2 in utero, whereas they were decreased ($p=0.01$) in the mammary glands of animals exposed to E2 during prepuberty compared to the control rats. Before puberty onset at 3 weeks of age, the level of phospho-(S473)Akt was significantly higher ($p<0.01$) in the mammary glands of animals exposed to E2 in utero or prepuberty than in the control rats. At 8 weeks of age, Akt activation was decreased in the mammary glands of prepubertally E2 exposed rats ($p=0.02$). There were no differences in the levels of phospho-(T¹⁸⁵Y¹⁸⁷)ERK1/2 mammary glands of rats at 3 weeks for all treatment groups or at 8 weeks between the in utero and control rats (Figure 2H). However, there was a significant increase in ERK1/2 activation in mammary tissue of rats exposed to E2 prepubertally at 8 weeks compared to control rats ($p<0.05$) or to rats treated with E2 in utero. The expression level of total ErbB2, *Src*, Akt and ERK1/2 were not significantly altered (data not shown).

Cell proliferation and apoptosis are modified by E2 exposure

Increased cell proliferation plays a key role in affecting susceptibility to breast cancer. Several studies have shown that CAV1 plays a critical role in the regulation of cell proliferation by inhibiting signaling molecules activating cell cycle (34–36). Cell proliferation was determined by measuring PCNA staining (37) and apoptosis was measured by TUNEL assay. The numbers of proliferating and apoptotic cells were counted separately in the TEBs, lobuloalveolar structures and ducts in the in utero exposed rats. In the rats that were exposed to prepubertal treatments, PCNA stain and apoptotic cells were counted in the

lobuloalveolar structures and ducts only, because no TEBs were present in the blocks obtained from the E2 exposed rats.

In utero exposure to E2 significantly increased the number of cells that stained positive for PCNA (Figure 3A). The increase was seen in the TEBs but not in the ducts or lobuloalveolar structures. In addition to the differences between the treatments, the level of cell proliferation was higher in the lobuloalveolar structures than in the ducts. Prepubertal E2 exposure reduced the number of cells that stained positive for PCNA (Figure 3B). In contrast, the level of apoptosis was reduced in all three epithelial structures studied, but particularly in the TEBs in the in utero E2 exposed rats (Figure 4A): there were no apoptotic cells observed in the TEBs of these rats. The proportion of apoptotic cells was significantly higher in the prepubertally E2 exposed rat mammary glands, when compared to the vehicle treated control group (Figure 4B).

Discussion

Function of CAV1 is strictly tumor-specific and CAV1 expression levels are downregulated in ovarian, lung and mammary carcinomas but upregulated in bladder, esophagus, thyroid and prostate carcinomas (24). During normal mammary gland development, CAV1 can function to regulate proliferation: CAV1 knockout mice display mammary gland hyperplasia within 6 weeks of age (38). Consistent with the concept that CAV1 functions as a tumor suppressor in a normal mammary gland, expression of CAV1 was reduced in the mammary glands of rats exposed to E2 in utero (Figure 2A) and these rats exhibit increased mammary tumorigenesis (7). CAV1 expression was elevated in prepubertally E2 exposed rats which are at a reduced mammary cancer risk (6). These changes were observed at the time when the mammary gland is most susceptible for malignant transformation. However, before puberty onset, the association between early life E2 exposures and mammary gland CAV1 expression was reversed: increased CAV1 expression was observed in the mammary glands of 3-week-old rats exposed to E2 in utero, whereas reduced CAV1 expression was observed in the prepubertally E2 exposed rats. These findings suggest that although changes in gene expression, induced by early life E2 exposures, are observed before puberty, onset of ovarian estrogen production at puberty can further modify the expression of certain genes and perhaps determine the long-term gene expression patterns. It should be noted that we have determined plasma levels of estrogen in female Sprague-Dawley rats in similar studies before, and the serum E2 levels during week 8 were not significantly different between the rats exposed to vehicle (67.4 ± 7.4 pg/mL, $n=4$) or E2 (74.4 ± 7.4 pg/mL, $n=4$) during the prepubertal period (6). In addition, E2 levels have been measured in adult rats exposed to either 20 ng (7) or 10 μ g (B. Yu et al., unpublished data) E2 in utero and no significant differences compared to control animals were found. Therefore, we did not repeat these studies here.

In utero exposure to E2 increases the number of TEBs (7), i.e., targets for carcinogen-induced malignant transformation (39). Similar structures, terminal ductal lobular units, give rise to 90% of human breast cancer (40). Prepubertal E2 exposure, by contrast, reduces the number of TEBs (6). Here we show that E2 exposures alter cell proliferation and the rate of apoptosis in the mammary epithelium, particularly in the TEBs. Following the in utero E2 exposure, all mammary epithelial structures expressed significantly lower number of apoptotic cells than the controls. In contrast, the number of proliferating cells was increased in the TEBs of these rats. Opposing changes were observed in the mammary epithelial structures of rats exposed to E2 during prepuberty. Because CAV1 increases during cell differentiation (41), and loss of CAV1 is associated with increased epithelial proliferation (38), it is possible that the reduced CAV1 expression in the rats treated with E2 in utero and the increased expression in rats exposed to E2 during prepuberty correlate with the reduced

and increased differentiation of the mammary epithelial tree, respectively. The differential activation of ErbB2, *Src* and Akt in the rats treated in utero or during prepuberty with E2 could reflect changes in cell proliferation and differentiation mediated by CAV1.

CAV1 regulates the function of several genes that have been linked to increased breast cancer risk, including ER α and cyclin D1 (13, 27, 42). ER α and cyclin D1 expression levels are correlated with increased risk of developing breast cancer (43). CAV1 interacts with ER α at the plasma membrane in breast epithelial cells (33) and downregulation of CAV1 is one of the mechanisms by which membrane ER α expression is thought to be activated during initiation of breast tumorigenesis (13). Cyclin D1 is a key target in E2 signaling and inhibition of cyclin D1 function inhibits proliferation in breast cancer cells (44). Cyclin D1 is transcriptionally reduced by CAV1 leading to inhibition of transformation (27). We have previously noted that prepubertal E2 exposure reduces the expression of ER α , but the reduction was significant only in older rats (16 and 22 weeks of age) (6): in the present study we determined ER α levels at the ages of 21 and 50 days. Our data suggest that, in addition to the reduction in CAV1 expression in the 8-week-old rats exposed to E2 in utero, ER α levels were increased in their mammary glands. In the mammary glands of prepubertally E2 exposed rats, where CAV1 expression was increased (Figure 2B), cyclin D1 levels were reduced. It is not clear why both ER α and cyclin D1 were not affected simultaneously.

CAV1 regulates mitogenic pathways in breast carcinogenesis such as those mediated by *Src*, Akt, ErbB2 and ERK1/2 (28). Most of this research has been done in vitro in cell culture systems. Our data show that E2 exposures at crucial developmental stages not only modulate CAV1 expression but also alter the phosphorylation/activation of ErbB2, *Src*, Akt and ERK1/2. ErbB2, a tyrosine kinase, belonging to a family of growth factor receptors, is overexpressed in 20–30% of human breast cancers (45). ErbB2 has been localized within caveolae (19), where it is regulated by CAV1 (46). The oncogenic potential of ErbB2 depends on this autophosphorylation (47) and one such site is on tyrosine 1139 (48). *Src* interacts with, and is regulated by, CAV1 within caveolae (32, 49) through its scaffolding domain (20). Moreover, *Src* interacts with ErbB2 and increases tyrosine phosphorylation of its non-autophosphorylation sites (50). CAV1 at the plasma membrane is involved in E2 induced non-genomic signaling involving ERK1/2 and Akt (29). Phosphorylation of ErbB2, *Src* and Akt were significantly reduced, and CAV1 expression was significantly increased in rats, which were exposed to E2 prepubertally compared to control animals. Because rats exposed to E2 during prepuberty display reduced susceptibility to carcinogen-induced breast tumors (6), increased expression of CAV1 and decreased activation of ErbB2, *Src* and Akt could play a role protecting the breast from malignant transformation by negatively regulating phosphorylation of these proteins.

Although it is becoming increasingly evident that early life hormonal environment can determine later susceptibility to breast cancer (51), the mechanisms remain unknown. In this study, we show that CAV1 was differentially expressed in the rat mammary gland in response to early life estradiol exposures that either increase or reduce later breast cancer risk: the levels were reduced following in utero exposure and increased following prepubertal exposure, when measured after puberty onset, supporting the role of CAV1 as a regulator of breast tumorigenesis. In addition, putative downstream targets of CAV1, such as *Src*, ErbB2, Akt and ERK1/2, were affected. Thus, CAV1 could be an essential component of signaling cascades that are associated with cell proliferation, differentiation and transformation, and an essential biomarker that can assist in determining susceptibility to breast cancer.

Acknowledgments

We thank Dr. Gloria Chepko for helpful suggestions and technical assistance. This research was supported by grants to L.H.-C. from the NCI (1 U54 CA00100971, 5 RO1 CA89950), the Susan G. Komen for the Cure Foundation, and the American Institute for Cancer Research and by a Fellowship (PDF0600477) to A.N.S. by Susan G. Komen for the Cure Foundation.

References

1. Hilakivi-Clarke L, Cabanes A, Olivo S, Kerr L, Bouker KB, Clarke R. Do estrogens always increase breast cancer risk? *J Steroid Biochem Mol Biol*. 2002; 80:163–74. [PubMed: 11897501]
2. Ahlgren M, Melbye M, Wohlfahrt J, Sorensen TI. Growth patterns and the risk of breast cancer in women. *N Engl J Med*. 2004; 351:1619–26. [PubMed: 15483280]
3. Hilakivi-Clarke L, Cho E, DeAssis S, Olivo S, Ealley E, Bouker KB, et al. Maternal and prepubertal diet, mammary development and breast cancer risk. *J Nutr*. 2001; 131:154S–7S. [PubMed: 11208953]
4. Michels KB, Trichopoulos D, Robins JM, Rosner BA, Manson JE, Hunter DJ, et al. Birthweight as a risk factor for breast cancer. *Lancet*. 1996; 348:1542–6. [PubMed: 8950880]
5. Trichopoulos D. Hypothesis: does breast cancer originate in utero? *Lancet*. 1990; 335:939–40. [PubMed: 1970028]
6. Cabanes A, Wang M, Olivo S, DeAssis S, Gustafsson JA, Khan G, et al. Prepubertal estradiol and genistein exposures up-regulate BRCA1 mRNA and reduce mammary tumorigenesis. *Carcinogenesis*. 2004; 25:741–8. [PubMed: 14729590]
7. Hilakivi-Clarke L, Clarke R, Onojafe I, Raygada M, Cho E, Lippman M. A maternal diet high in n-6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. *Proc Natl Acad Sci USA*. 1997; 94:9372–7. [PubMed: 9256489]
8. Russo J, Russo IH. Biological and molecular bases of mammary carcinogenesis. *Lab Invest*. 1987; 57:112–37. [PubMed: 3302534]
9. Russo J, Russo IH. Toward a unified concept of mammary carcinogenesis. *Prog Clin Biol Res*. 1997; 396:1–16. [PubMed: 9108586]
10. Klinge CM. Estrogen receptor interaction with co-activators and co-repressors. *Steroids*. 2000; 65:227–51. [PubMed: 10751636]
11. Pearce ST, Jordan VC. The biological role of estrogen receptors alpha and beta in cancer. *Crit Rev Oncol Hematol*. 2004; 50:3–22. [PubMed: 15094156]
12. Hilakivi-Clarke L, Stoica A, Raygada M, Martin MB. Consumption of a high-fat diet alters estrogen receptor content, protein kinase C activity, and mammary gland morphology in virgin and pregnant mice and female offspring. *Cancer Res*. 1998; 58:654–60. [PubMed: 9485017]
13. Zhang X, Shen P, Coleman M, Zou W, Loggie BW, Smith LM, et al. Caveolin-1 down-regulation activates estrogen receptor alpha expression and leads to 17beta-estradiol-stimulated mammary tumorigenesis. *Anticancer Res*. 2005; 25:369–75. [PubMed: 15816560]
14. Glenney JR Jr, Zokas L. Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. *J Cell Biol*. 1989; 108:2401–8. [PubMed: 2472406]
15. Razani B, Lisanti MP. Caveolins and caveolae: molecular and functional relationships. *Exp Cell Res*. 2001; 271:36–44. [PubMed: 11697880]
16. Anderson RG. The caveolae membrane system. *Annu Rev Biochem*. 1998; 67:199–225. [PubMed: 9759488]
17. Witkiewicz AK, Dasgupta A, Nguyen KH, Liu C, Kovatich AJ, Schwartz GF, et al. Stromal caveolin-1 levels predict early DCIS progression to invasive breast cancer. *Cancer Biol Ther*. 2009; 8:1071–9. [PubMed: 19502809]
18. Razandi M, Pedram A, Merchenthaler I, Greene GL, Levin ER. Plasma membrane estrogen receptors exist and functions as dimers. *Mol Endocrinol*. 2004; 18:2854–65. [PubMed: 15231873]
19. Couet J, Sargiacomo M, Lisanti MP. Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. *J Biol Chem*. 1997; 272:30429–38. [PubMed: 9374534]

20. Li S, Couet J, Lisanti MP. Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *J Biol Chem.* 1996; 271:29182–90. [PubMed: 8910575]
21. Belanger MM, Roussel E, Couet J. Caveolin-1 is down-regulated in human lung carcinoma and acts as a candidate tumor suppressor gene. *Chest.* 2004; 125(Suppl 5):106S. [PubMed: 15136443]
22. Zou W, McDanel L, Smith LM. Caveolin-1 haploinsufficiency leads to partial transformation of human breast epithelial cells. *Anticancer Res.* 2003; 23:4581–6. [PubMed: 14981899]
23. Sunaga N, Miyajima K, Suzuki M, Sato M, White MA, Ramirez RD, et al. Different roles for caveolin-1 in the development of non-small cell lung cancer versus small cell lung cancer. *Cancer Res.* 2004; 64:4277–85. [PubMed: 15205342]
24. Williams TM, Lisanti MP. The Caveolin genes: from cell biology to medicine. *Ann Med.* 2004; 36:584–95. [PubMed: 15768830]
25. Yang G, Timme TL, Frolov A, Wheeler TM, Thompson TC. Combined c-Myc and caveolin-1 expression in human prostate carcinoma predicts prostate carcinoma progression. *Cancer.* 2005; 103:1186–94. [PubMed: 15712208]
26. Marino M, Acconcia F, Bresciani F, Weisz A, Trentalance A. Distinct nongenomic signal transduction pathways controlled by 17beta-estradiol regulate DNA synthesis and cyclin D(1) gene transcription in HepG2 cells. *Mol Biol Cell.* 2002; 13:3720–9. [PubMed: 12388769]
27. Hulit J, Bash T, Fu M, Galbiati F, Albanese C, Sage DR, et al. The cyclin D1 gene is transcriptionally repressed by caveolin-1. *J Biol Chem.* 2000; 275:21203–9. [PubMed: 10747899]
28. Bouras T, Lisanti MP, Pestell RG. Caveolin-1 in breast cancer. *Cancer Biol Ther.* 2004; 3:931–41. [PubMed: 15539932]
29. Acconcia F, Ascenzi P, Bocedi A, Spisni E, Tomasi V, Trentalance A, et al. Palmitoylation-dependent estrogen receptor alpha membrane localization: regulation by 17beta-estradiol. *Mol Biol Cell.* 2005; 16:231–7. [PubMed: 15496458]
30. Engelman JA, Lee RJ, Karnezis A, Bearss DJ, Webster M, Siegel P, et al. Reciprocal regulation of neu tyrosine kinase activity and caveolin-1 protein expression in vitro and in vivo. Implications for human breast cancer. *J Biol Chem.* 1998; 273:20448–55. [PubMed: 9685399]
31. Lee H, Woodman SE, Engelman JA, Volonte D, Galbiati F, Kaufman HL, et al. Palmitoylation of caveolin-1 at a single site (Cys-156) controls its coupling to the c-Src tyrosine kinase: targeting of dually acylated molecules (GPI-linked, transmembrane, or cytoplasmic) to caveolae effectively uncouples c-Src and caveolin-1 (TYR-14). *J Biol Chem.* 2001; 276:35150–8. [PubMed: 11451957]
32. Minshall RD, Tiruppathi C, Vogel SM, Niles WD, Gilchrist A, Hamm HE, et al. Endothelial cell-surface gp60 activates vesicle formation and trafficking via G(i)-coupled Src kinase signaling pathway. *J Cell Biol.* 2000; 150:1057–70. [PubMed: 10973995]
33. Zivadinovic D, Watson CS. Membrane estrogen receptor-alpha levels predict estrogen-induced ERK1/2 activation in MCF-7 cells. *Breast Cancer Res.* 2005; 7:R130–44. [PubMed: 15642162]
34. Fiucci G, Ravid D, Reich R, Liscovitch M. Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. *Oncogene.* 2002; 21:2365–75. [PubMed: 11948420]
35. Williams TM, Lee H, Cheung MW, Cohen AW, Razani B, Iyengar P, et al. Combined loss of INK4a and caveolin-1 synergistically enhances cell proliferation and oncogene-induced tumorigenesis: role of INK4a/CAV-1 in mammary epithelial cell hyperplasia. *J Biol Chem.* 2004; 279:24745–56. [PubMed: 15044451]
36. Xie Z, Zeng X, Waldman T, Glazer RI. Transformation of mam-mary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 activates beta-catenin and c-Myc, and down-regulates caveolin-1. *Cancer Res.* 2003; 63:5370–5. [PubMed: 14500370]
37. Aaltomaa S, Lipponen P, Syrjanen K. Prognostic value of cell proliferation in breast cancer as determined by proliferating cell nuclear antigen (PCNA) immunostaining. *Anticancer Res.* 1992; 12:1281–6. [PubMed: 1380227]
38. Lee H, Park DS, Razani B, Russell RG, Pestell RG, Lisanti MP. Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative

- manner and caveolin-1 (-/-) null mice show mammary epithelial cell hyperplasia. *Am J Pathol.* 2002; 161:1357–69. [PubMed: 12368209]
39. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, van Zwieten MJ. Comparative study of human and rat mammary tumorigenesis. *Lab Invest.* 1990; 62:244–78. [PubMed: 2107367]
 40. Hilakivi-Clarke L. Nutritional modulation of terminal end buds: its relevance to breast cancer prevention. *Curr Cancer Drug Targets.* 2007; 7:465–74. [PubMed: 17691906]
 41. Scherer PE, Lisanti MP, Baldini G, Sargiacomo M, Mastick CC, Lodish HF. Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J Cell Biol.* 1994; 127:1233–43. [PubMed: 7962086]
 42. Schlegel A, Wang C, Katzenellenbogen BS, Pestell RG, Lisanti MP. Caveolin-1 potentiates estrogen receptor alpha (ERalpha) signaling. Caveolin-1 drives ligand-independent nuclear translocation and activation of ERalpha. *J Biol Chem.* 1999; 274:33551–6. [PubMed: 10559241]
 43. Hui R, Cornish AL, McClelland RA, Robertson JF, Blamey RW, Musgrove EA, et al. Cyclin D1 and estrogen receptor messenger RNA levels are positively correlated in primary breast cancer. *Clin Cancer Res.* 1996; 2:923–8. [PubMed: 9816251]
 44. Lukas J, Bartkova J, Bartek J. Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb-controlled G1 checkpoint. *Mol Cell Biol.* 1996; 16:6917–25. [PubMed: 8943347]
 45. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science.* 1987; 235:177–82. [PubMed: 3798106]
 46. Engelman JA, Chu C, Lin A, Jo H, Ikezu T, Okamoto T, et al. Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain. *FEBS Lett.* 1998; 428:205–11. [PubMed: 9654135]
 47. Heffelfinger SC, Lower EE, Miller MA, Fenoglio-Preiser CM. Plasma membrane phosphotyrosine, Her2-NEU, and epidermal growth factor receptor in human breast cancer. A comparative study. *Am J Clin Oncol.* 1996; 19:552–7. [PubMed: 8931669]
 48. Hazan R, Margolis B, Dombalagian M, Ullrich A, Zilberstein A, Schlessinger J. Identification of autophosphorylation sites of HER2/neu. *Cell Growth Differ.* 1990; 1:3–7. [PubMed: 1706616]
 49. Shajahan AN, Tiruppathi C, Smrcka AV, Malik AB, Minshall RD. Gbetagamma activation of Src induces caveolae-mediated endocytosis in endothelial cells. *J Biol Chem.* 2004; 279:48055–62. [PubMed: 15345719]
 50. Stover DR, Becker M, Liebetanz J, Lydon NB. Src phosphorylation of the epidermal growth factor receptor at novel sites mediates receptor interaction with Src and P85 alpha. *J Biol Chem.* 1995; 270:15591–7. [PubMed: 7797556]
 51. Hilakivi-Clarke L, Cho E, Cabanes A, DeAssis S, Olivo S, Helferich W, et al. Dietary modulation of pregnancy estrogen levels and breast cancer risk among female rat offspring. *Clin Cancer Res.* 2002; 8:3601–10. [PubMed: 12429652]

Figure 1.

Expression pattern of CAV1 in the mammary gland was analyzed by immunocytochemistry (B, C, E, F). Control slide was incubated with normal mouse serum (A and D). CAV1 was predominantly expressed in the myoepithelial cells and in some epithelial cells of the mammary glands. Expression was also observed in the stroma and adipocytes. No staining was detected when slides were incubated with normal mouse IgG (A and D). Magnification, 20 $\times$.

Figure 2.

(A) Western blot analysis of CAV1, ERα, cyclin D1, phosphor-ErbB2(Y1139), phospho-Src(Y418), phospho-Akt(S473) and phospho-ERK1/2 in mammary gland tissue in control and E2 exposed rats (in utero and prepuberty). The equivalences of loading and transfer were checked by reprobing with β-actin antibody. (B–H) Density of bands from Western blot analysis from three sets of rats per treatment and time group were quantified measured using Scion Image (National Institutes of Health). The relative expression levels of proteins were determined by dividing the band intensities of the protein by the respective band densities of β-actin (loading control). Columns show mean from three different samples ±SEM. *p<0.05 compared to the controls.

Figure 3.

Cell proliferation in the mammary glands of 8-week-old rats exposed daily to 10 μ g E2 either during (A) in utero or (B) prepuberty. Proliferation was evaluated via immunohistochemistry for PCNA in the ductal cells, the lobuloalveolar cells and the TEB cells. Data are illustrated as a percentage of proliferating cells per 1000 cells per structure counted. Values represent the mean of five animals per group $\pm$ SEM. Significantly different from the controls: * $p < 0.05$, the Tukey test.

Figure 4.

Apoptosis in the mammary glands of 8-week-old rats exposed daily to 10 μ g E2 either during (A) in utero or (B) prepuberty. Apoptosis was evaluated via a TUNEL assay and confirmed by histological examination in the ductal cells, the lobuloalveolar cells and the TEB cells. Data are illustrated as a percentage of apoptotic cells per 1000 cells counted. Values represent the mean of five rats per group $\pm$ SEM. Significantly different from the controls: * $p < 0.05$, the Tukey test.