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Caveolin-1 Down-regulation Activates Estrogen Receptor Alpha Expression and Leads to 17β-estradiol-stimulated Mammary Tumorigenesis

XINTIAN ZHANG[1], PENG SHEN[2], MEGAN COLEMAN[1], WEI ZOU[3], BRIAN W. LOGGIE[1], LEIA M. SMITH[4] and ZHAOYI WANG[1]

[1]*Cancer Center, Creighton University, 2500 California Plaza, Omaha, NE 68178, U.S.A.;*
[2]*First Affiliated Hospital, Medical College, Zhejiang University, 79 QinChun Road, Hangzhou, P. R. China 310003;*
[3]*College of Life Science, Liaoning Normal University, 850 Huanghe Road, Dalian, P. R. China 116029;*
[4]*Seattle Genetics, Inc., 21823 - 30th Drive Southeast, Bothell, WA 98021, U.S.A.*

Abstract. *Constitutive activation of estrogen receptor alpha (ER-α) expression is an early event in breast cancer tumorigenesis. However, the mechanism whereby ER-α is constitutively activated during transformation of normal mammary cells has not been well established. Previously, we reported that haploinsufficiency of caveolin-1, a major structural protein that forms caveolae, resulted in anchorage-independent growth of a normal mammary epithelial cell line, MCF10A. Here, we further demonstrated that ER-α but not ER-β expression was constitutively activated in these caveolin-1 haploinsufficient cells. Transient treatment of MCF10A cells with β-methyl-cyclodextrin, a chemical that can displace caveolin-1 from the plasma membrane, also stimulated ER-α expression. We further found that the 17β-estradiol (E2) accelerated anchorage-independent growth of these cells in vitro and promoted their tumorigenesis in nude mice. These results suggest that dysregulation of caveolin-1 is one of the mechanisms by which ER-α expression is activated during initiation of breast tumorigenesis.*

The role of endogenous estrogens in breast cancer etiology has been supported by numerous studies (1). It is believed that the mitogenic activities of estrogens increase the rate of mammary cell division and thus the risk of breast cancer (2). It is also believed that the estrogen receptor-α (ER-α) mediates mitogenic functions of estrogen signaling by regulating transcription of growth-related genes in target cells (3, 4).

Correspondence to: ZhaoYi Wang, Cancer Center, Creighton University, 2500 California Plaza, Omaha, NE 68178, U.S.A. Fax: 402-280-3543, e-mail: zywang@creighton.edu

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ER-α is expressed only in approximately 15-30% of luminal epithelial cells of normal human breast (5, 6). However, ER-α expression is increased at the earliest stage of breast cancer tumorigenesis such as ductal hyperplasia, and increases even more with increasing atypia (5, 6). Furthermore, experiments with dual label immunofluorescent staining have shown that ER-α-expressing cells are not those labeled with proliferation markers in both normal human and rodent mammary glands (6, 7). However, as ER-α expression increases in hyperplasia and atypia, the inverse relationship between receptor expression and cell proliferation become dysregulated (6, 7). Approximately 70% of invasive breast carcinomas express ER-α and most of these tumors contain ER-α-positive proliferating cells (6, 7). However, answers to questions such as how ER-α expression is constitutively activated during transformation of mammary epithelial cells and why ER-α signals proliferation only in transformed cells remain largely unknown.

Caveolin-1, a 22-kDa integral membrane protein, is a major component of caveolae membranes. Accumulating evidence indicates that caveolins function as scaffold proteins for multiple membrane-initiated signaling pathways (8, 9). Through a membrane-proximal cytoplasmic domain, caveolin-1 interacts with heterotrimeric G-protein α subunits, Ha-ras, Src-family tyrosine kinases, protein kinase C isoforms, epidermal growth factor (EGF) receptor, ER-α, Neu and endothelial NO synthase, and the p85 regulatory subunit of PI3K (10, 11). The caveolin-1 gene is localized to the D7S522 locus in the q31.1 region of human chromosome 7 (12). Loss of this chromosomal region occurs in a number of human cancers, including breast cancer (12). Caveolin-1 may function as a tumor suppressor to inhibit many growth-promoting signaling pathways (11). Down-regulation or loss of caveolin-1 expression has been reported in T cell leukemia (13), breast cancer (14-16), colon (17), ovarian

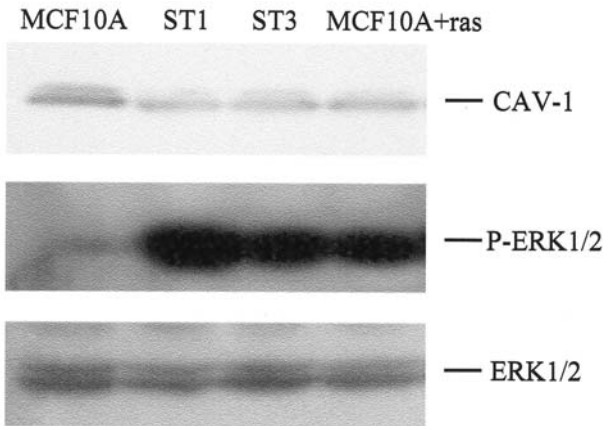

Figure 1. *Western blot analysis of caveolin-1 expression and activation of ERK1/2. Equal amounts of total cellular extracts from various cell lines were analyzed by Western blot using a rabbit anti-Cav-1 antibody, and antibodies against activated ERK1/2 and non-activated ERK1/2.*

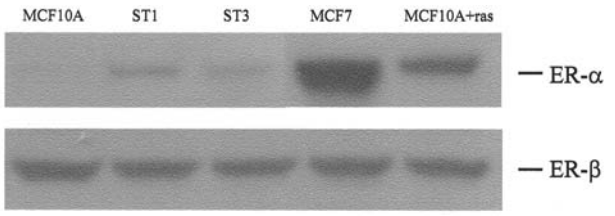

Figure 2. *Activation of ER-α expression in caveolin-1 haploinsufficient cells. Cell lysates were prepared from MCF10A, a normal mammary epithelial cell line; ST1 and ST3, two caveolin-1 haploinsufficient cell lines; MCF7, an ER-positive breast cancer cell line, and MCF10A+ras, MCF10A cells transformed by a H-ras T24 mutant. ER-α and ER-β expression were detected by immunoblotting with isoform-specific antibodies. Each lane contains equal amounts of total protein.*

(18, 19), sarcomas (20), and lung carcinoma cell lines (21). Caveolin-1 is down-regulated during transformation of NIH3T3 cells (22) and recombinant expression of caveolin-1 in transformed NIH3T3 cells (23) and cell lines derived from human breast cancers suppresses their transformed phenotypes (14-16). Targeted down-regulation of caveolin-1 expression by an antisense approach promotes anchorage-independent cell growth in soft-agar and tumorigenesis in nude mice (24).

In this study, we demonstrate that down-regulation of caveolin-1 expression in normal mammary cells functionally activates ER-α expression and promotes estrogen-stimulated transformation of normal mammary epithelial cells *in vitro* and *in vivo*.

Materials and Methods

Cells and cell culture. The MCF10A cell line was obtained from ATCC (Rockville, MD, USA). Stable cell clones (designated as ST1 and ST3), that carry an insertional mutation of the caveolin-1 gene on one allele, were derived from the gene trapped MCF10A cells (25). MCF10A+ras cells, obtained from Dr. Hyeong-Reh Kim at Wayne State University (Detroit, MI, USA), were derived from the MCF10A cells transformed by an active Ha-ras mutant. All cells were maintained in a humidified atmosphere at 37˚C in 10% CO_2 and cultured in DMEM/F12 medium supplemented with 5% horse serum, penicillin (100 U/mL), streptomycin (100 μg/mL), hydrocortisone (1.4×10^{-6} M), insulin (10 μg/mL), cholera toxin (100 ng/mL), and EGF (20 ng/mL). For methyl-β-cyclodextrin (mβCD) treatment, cells were treated with mβCD (50 μg/ml) for 30 minutes and washed with medium. Cells were harvested after different time points for Western blot analysis.

Western blot analysis. Cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 0.25 mM EDTA pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF and protease inhibitor cocktail). Equal amount of cell lysates were analyzed by Western blot analysis. Thirty μg of proteins were boiled for 5 minutes in SDS gel loading buffer and separated on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membranes were probed with primary antibodies. Primary antibodies against ER-α (F10), ER-β (H-150) and caveolin-1 (N-20) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phospho- and non-phospho ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Western blots were visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham, Piscataway, NJ, USA). The same membranes were stripped and reprobed with an antibody against β-actin (Sigma, St. Louis, MO, USA) to confirm equal loading.

Soft-agar colony formation assay and tumor formation in nude mice. To determine anchorage-independent growth in soft-agar, 1×10^5 cells from each of the cell lines were suspended in 3 ml of 3.5% (W/V) agar containing phenol red-free DMEM/F12 medium plus 10% E2-free fetal calf serum. The cells were then overlaid onto a 0.7% (W/V) agar containing phenol red-free DMEM/F12 medium plus 10% E2-free fetal calf serum in five replica 60mm dishes. Cells on soft agar were covered with medium plus 10% E2-free fetal calf serum with or without 1 nM E2. After three weeks, colonies were photographed using an inverted microscope.

For tumor formation assay, a total of 1×10^7 cells pooled from ST1 and ST3 caveolin-1 haploinsufficient cells were resuspended in 0.2 ml of Matrigel (Collaborative Research, Bedford, MA, USA) and inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice (6-week-old athymic nude mice, strain Ncr nu/nu; Sprague-Dawley, Indianapolis, IN, USA) 5 days after subcutaneous implantation of 1.7 mg/60-day release E2 (Innovative Research, FL, USA) (treated; 12 mice) or placebo pellets (control; 12 mice). Animals were monitored weekly, beginning at two weeks after the injections, to measure the rate of tumor growth. Large (D) and small (d) diameters of growing tumors were measured with vernier calipers every week and the corresponding volumes (V) were estimated using the equation $V = d^2 \times D \times \pi/6$. All pellets were replaced at 7 weeks and all mice were sacrificed after 10 weeks.

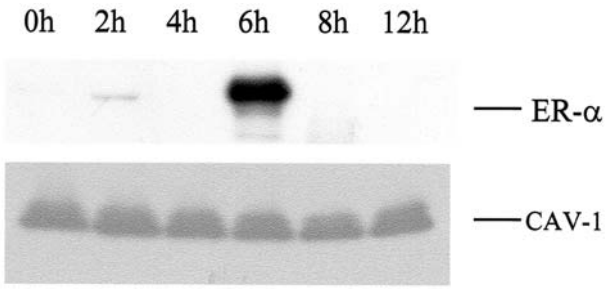

Figure 3. *Delocalization of caveolin-1 activates ER-α expression in MCF10A cells. MCF10A cells were treated with methyl-β-cyclodextrin (50 μg/ml) for different time points. Cells were then lysed and cell lysates were subjected to immunoblotting using anti-ER-α and anti-caveolin-1 antibodies.*

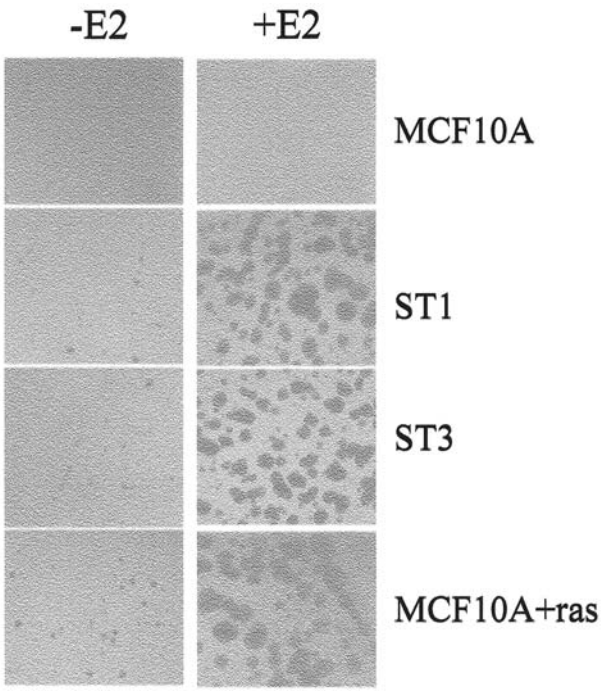

Figure 4. *Caveolin-1 down-regulation promotes E2-stimulated colony formation in soft agar. Parental MCF10A cells, caveolin-1 haploinsufficient cells and ras-transformed MCF10A cells were compared for their ability to undergo anchorage-independent growth in soft agar in the presence or absence of E2.*

Results

We have previously employed a retrovirus-mediated poly-A gene trapping approach to identify genes that may be involved in mammary cell transformation using a spontaneously immortalized mammary epithelial cell line, MCF10A. We found that two isolated cell clones, that expressed reduced levels (~50%) of caveolin-1 mRNA and protein caused by a retrovirus-mediated insertional mutation in the caveolin-1 gene, were able to grow on soft agar but failed to form tumors in nude mice (25), indicating that down-regulation of caveolin-1 expression can lead to partial transformation of normal mammary epithelial cells.

To investigate the mechanism by which caveolin-1 down-regulation induces transformation of normal mammary cells, we further analyzed the caveolin-1 haploinsufficient cells obtained from the gene trapping approach (25). Figure 1 shows a Western blot analysis of caveolin-1 expression in parental MCF10A cells, two caveolin-1 haploinsufficient cell lines (ST1 and ST3) as described before (25), and MCF10A+ras cells, derived from MCF10A cells transformed by an active Ha-Ras mutant, as a positive control. In ST1 and ST3 cells, caveolin-1 protein levels were about 50% of the levels of parental MCF10A cells (Figure 1), consistent with our previous report that caveolin-1 expression is decreased to ~50% when only one functional allele of the caveolin-1 gene is functional (25). The MCF10A+ras cells also expressed less caveolin-1 protein compared to parental MCF10A cells (Figure 1).

We then checked the MAPK pathway activation in these cells by examining the phosphorylation levels of ERK1/2 using a phospho-specific antibody. Lysates from parental MCF10A cells, ST1 and ST3 cells, and MCF10A+ras cells were first probed with an antibody against phosphorylated ERK1/2, and then with an ERK phospho-independent antibody to assess equal loading. We found that ERK1/2 are highly and constitutively phosphorylated in ST1 and ST3 cells but not in MCF10A cells (Figure 1), consistent with the previous report that down-regulated caveolin-1 expression leads to constitutive activation of the MAPK pathway (24). As expected, the MAPK pathway is highly activated in MCF10A+ras cells.

Previously, it was reported that the ER-negative MCF10A cells expressing a Ha-ras mutant exhibit spontaneous gain in expression of functionally active ER-α (26, 27). We decided to examine the expression of ER-α and-β using Western blot analysis in ST1 and ST3 cells. MCF10A was again used as a negative control, and MCF10A+ras and MCF7 cells, an ER-positive cell line, were used as positive controls. We found that ST1 and ST3 cells expressed ER-α, whereas parental MCF10A cells expressed undetectable levels of ER-α (Figure 2). As positive controls, MCF10A+ras and MCF7 cells expressed high levels of ER-α. However, ER-β expression was without any change in all of these cells (Figure 2). This data indicated that expression of ER-α but not ER-β is activated in these caveolin-1 haploinsufficient cells.

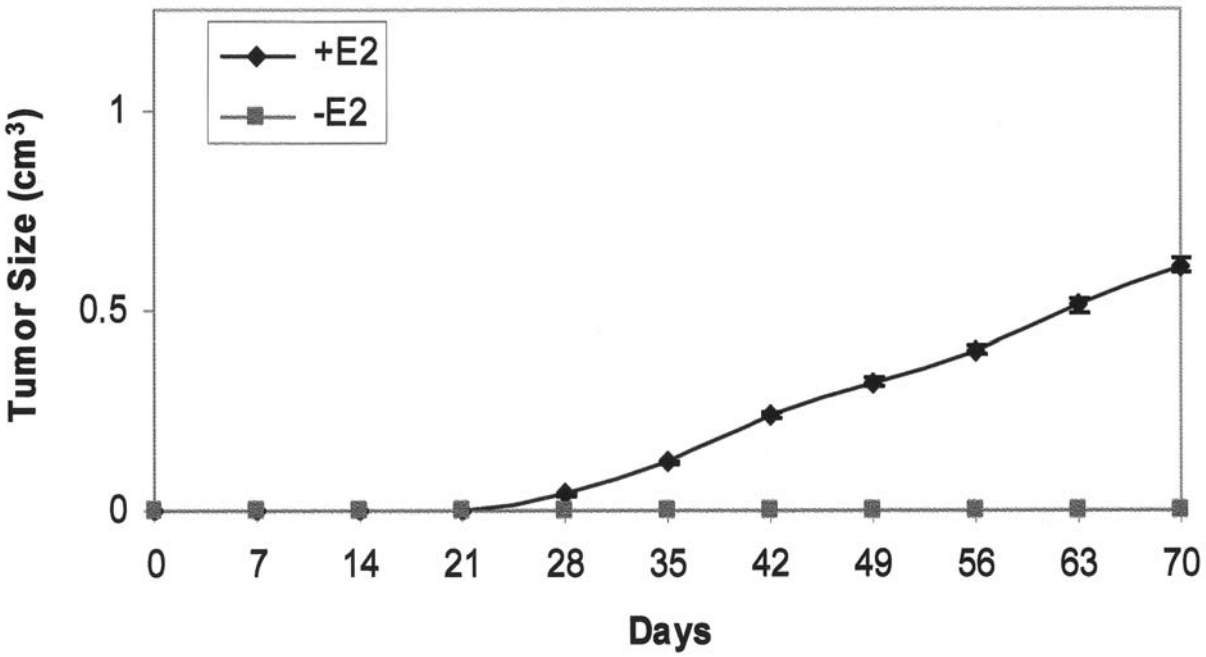

Figure 5. *Caveolin-1 down-regulation promotes E2-stimulated tumor formation in nude mice. Ovariectomized female immunodeficient mice were inoculated with 1 X 10^7 cells subcutaneously into the mammary fatpad 5 days after subcutaneous implantation of 1.7 mg/60-day release E2. Animals were monitored weekly, beginning at two weeks after the injections, to measure the rate of tumor growth. Large (D) and small (d) diameters of growing tumors were measured with vernier calipers every week and the corresponding volumes (V) were estimated using the equation V = d^2 X D X π/6. All pellets were replaced at 7 weeks and all mice were sacrificed after 10 weeks.*

Caveolin-1 is a cholesterol binding protein. It has been shown that extraction of plasma membrane cholesterol with methyl-β-cyclodextrin (mβCD), a chemical which can delocalize caveolin-1 from caveolae on the plasma membrane, activates the Ras/MEK/ERK signaling pathway (28). We treated MCF10A cells with the mβCD to examine whether caveolin-1 relocalization from the plasma membrane results in activation of ER-α expression. The levels of ER-α expression were determined by Western blot analysis in the lysates of MCF10A cells treated with mβCD for different time points. We found that mβCD treatment strongly induced ER-α expression at 6 hours, but ER-α protein was reverted back to undetectable level at 8 hours (Figure 3). The protein levels of caveolin-1 were without any change after mβCD treatment (Figure 3), consistent with the previous report that mβCD treatment leads to relocalization of caveolin-1 from the plasma membrane but has no effect on its expression levels (28). Again, mβCD treatment had no effect on ER-β expression (data not shown). This data further confirmed that dysregulation of caveolin-1 activates ER-α expression in normal mammary epithelial cells.

To determine whether E2 will drive transformation of these caveolin-1 haploinsufficient cells, we subjected these cells to soft-agar assay in the presence or absence of E2. Parental MCF10A cell line served as a negative control. Both ST1 and ST3 cell lines exhibited accelerated growth in soft-agar in the presence of E2 by increasing both number and size of colonies, whereas MCF10A cells failed to grow on soft agar (Figure 5). E2 also stimulated growth of MCF10A+ras cells on soft-agar (Figure 5), consistent with the previous report that estrogen promotes transformed growth of MCF10A cells+ras (26, 27). These results indicate that estrogen signaling exerts a growth-promoting effect on these caveolin-1 haploinsufficient cells that leads to an accelerated anchorage-independent growth.

Previously, we found that the caveolin-1 haploinsuffcient cells failed to form tumors in nude mice without exogenous E2 supplementation (25). We reasoned that the endogenous estrogens may be insufficient to promote these cells to form tumors *in vivo*. We decided to examine the effects estrogen exerts on the neoplastic progression of these caveolin-1 haploinsufficient cells *in vivo*. A total of 1 X 10^7 cells, pooled from two caveolin-1 haploinsufficient cell lines (ST1 and ST3), were inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice 5 days after subcutaneous implantation of slow release E2 or placebo pellets. Twelve mice were used for the experimental

and control groups. The tumor formation was monitored every week for 10 weeks. From 4 weeks, detectable tumor formation was observed in the E2-supplemented group, but not in the control group. At 10 weeks, E2 significantly accelerated tumor formation of these caveolin-1 haploinsufficient cells in nude mice (Figure 6). In contrast, the control group did not exhibit any tumor. This experiment provides further evidence to demonstrate that down-regulation of caveolin-1 activates functional estrogen signaling and promotes estrogens stimulated breast tumorigenesis.

Discussion

Accumulating evidence suggests a role of caveolin-1 in breast cancer development. Using differential display and subtractive hybridization techniques, Sager and colleagues identified caveolin-1 as one of the 26 genes that were down-regulated during mammary tumorigenesis (30). Caveolin-1 expression was severely reduced and absent in several breast cancer cell lines compared to normal human mammary epithelial cells (30). Reintroduction of caveolin-1 into breast cancer cells inhibits transformed phenotypes of these cells (14-16). Recently, it was reported that the human caveolin-1 gene is heterozygously mutated at residue P132 (P132L) in up to 16% human breast cancer samples examined (31), and that this caveolin-1 mutant functions in a dominant-negative manner to cause mislocalization and intracellular retention of wild-type caveolin-1 (32). Taken together, these data suggest that caveolin-1 acts as a tumor suppressor in mammary tumorigenesis.

Previously, we established a gene-trapped cell library from the normal mammary cell line MCF10A, and identified clones which had acquired anchorage–independent growth and expressed reduced levels (50%) of caveolin-1 mRNA and protein, consistent with knocking out one allele of caveolin-1 gene (25). However, caveolin-1 haploinsufficiency did not cause significant tumor formation in nude mice, indicating that caveolin-1 down-regulation only leads to partial transformation but does not result in a fully malignant phenotype. These findings are consistent with the previous report that caveolin-1 null mice developed mammary epithelial cell hyperplasia in 6-week-old virgin female mice (32). Thus, in the absence of other genetic alterations or growth-promoting signals, dysregulation of caveolin-1 alone is unable to promote malignant transformation. Indeed, when caveolin-1 null mice were interbred with tumor prone transgenic mice (MMTV-PyMT), loss of caveolin-1 expression dramatically stimulated the development of dysplastic mammary lesions in these mice (33).

In dissecting the mechanisms underlying the transformation induced by caveolin-1 haploinsufficiency, we demonstrated that ER-α expression is functionally activated in caveolin-1 haploinsufficient cells and that estrogen signaling dramatically stimulates malignant transformation of these caveolin-1 haploinsufficient cells.

Experimental data strongly suggest that estrogens contribute to the initiation and development of human breast cancer (34). Although the precise roles of estrogens in the biology of breast tumorigenesis remain to be fully elucidated, estrogens exert both direct and indirect proliferative effects on target mammary cells. These effects are mediated mainly by liganded-ER through transactivation of specific target genes. This process stimulates DNA synthesis, cell division and production of growth–promoting proteins such as tumor growth factor-α and epidermal growth factor.

In the normal human breast tissue, ER-α is expressed only in a small number of luminal epithelial cells and not at all in any of the other cell types. However, ER-α expression is increased at the very early stages of breast cancer tumorigenesis such as ductal hyperplasia (5), indicating that activation of ER-α expression contributes to the initiation of breast cancer. Dual label immunofluorescence techniques revealed that ER-α-expressing cells are separate from those cells labeled with proliferation markers in both normal human and rodent mammary glands (6, 7). When ER-α was overexpressed in ER-negative CHO cells (35), and human cervical cancer HeLa cells (36), E2 did not stimulate cell growth. On the contrary, E2 inhibited cell growth and even induced cell death. Likewise, the ER-negative immortal MCF10A breast epithelial cells (37) and MDA-MB-231 breast cancer cells (38) were both growth inhibited when stably transfected with ER-α. These experimental results argue against a positive function of ER-α alone in cell proliferation stimulated by estrogen signaling and suggest these cells may lack certain signaling cascades to complement the estrogen signaling pathway to stimulate cell growth. Approximately 70% of invasive breast carcinomas express ER-α and most of these tumors contain ER-α-positive proliferating cells (6, 7). Thus, certain genetic programs may be reprogrammed or some signaling pathways may be derailed during transformation of mammary cells in order for ER-α expression to be constitutively activated and for ER-α to signal cell proliferation in response to estrogens. Our data from this study suggest that caveolin-1 down-regulation is one of the mechanisms by which ER-α is constitutively activated and estrogen signaling exerts a proliferative effect on mammary epithelial cells.

It has been reported before that estrogen signaling regulates the production of caveolin-1 (39, 40). E2 functions to either activate or suppress expression of caveolin-1, depending on the cell context (39). The importance of caveolin-1 in estrogen signaling has been highlighted by the finding that ER-α physically interacts with caveolin-1, and this association is regulated by E2 (39). Furthermore, re-

expression of caveolin-1 in MCF7 cells translocates ER-α to the plasma membrane and inhibits ERα-induced ERK activation (39).

Schlegel *et al.* reported that caveolin-1 functions as a specific co-activator of ER-α (41) and that ER-α binds to caveolin-1 directly *via* its AF-1 domain, which then results in a ligand-independent activation of ER-α-mediated transcription (42). Here, we report that caveolin-1 down-regulation results in activation of ER-α expression, further suggesting that there is a loop of transcriptional regulation of ER-α and caveolin-1. These two important molecules may regulate each other's expression and activity.

It is not clear as to how caveolin-1 down-regulation leads to activation of ER-α expression. Previously, it has been reported that the MCF10AT cells, MCF10A cells transformed by the T24 Ha-ras mutant, activate ER-α expression, and estrogen stimulates malignant progression of ER-positive MCF10AT cells *in vivo* (26, 27). The Ras/MAPK pathway may be involved in the activation of ER-α expression, as suggested by the results from our study and previous ones. However, treatment of caveolin-1 haploinsufficient cells with PD 98059, a potent inhibitor of the MAPK pathway, was unable to block the expression of ER-α (data not shown), indicating that signaling pathways other than the MAPK pathway may contribute to activated ER-α expression.

Taken together, our results demonstrate that caveolin-1 down-regulation activates ER-α expression. This suggests that caveolin-1 may play an important regulatory function in the maintenance of normal cell growth in breast tissue. Our study also described a model to study the direct action of estrogen on the sequence of neoplastic progression of mammary epithelial cells.

Acknowledgements

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