

PDZK1 Is a Novel Factor in Breast Cancer That Is Indirectly Regulated by Estrogen through IGF-1R and Promotes Estrogen-Mediated Growth

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Although a relationship between *PDZK1* expression and estrogen receptor (ER)- α stimulation has been suggested, the nature of such a connection and the function of PDZK1 in breast cancer remain unknown. Human tissue microarrays (cancer tissue: 262 cores; normal tissue: 87 cores) and breast cancer cell lines were used to conduct the study. We show that PDZK1 protein expression is tightly correlated with human breast malignancy, is negatively correlated with age and had no significant correlation with ER- α expression levels. PDZK1 exhibited an exclusive epithelial expression with mostly cytosolic subcellular localization. Additionally, 17 β -estradiol induced PDZK1 expression above its basal level more than 24 h after treatment in MCF-7 cells. PDZK1 expression was indirectly regulated by ER- α stimulation, requiring insulinlike growth factor 1 receptor (IGF-1R) expression and function. The molecular link between PDZK1 and IGF-1R was supported by a significant correlation between protein and mRNA levels ($r = 0.591$, $p < 0.001$, and $r = 0.537$, $p < 0.001$, respectively) of the two factors in two different cohorts of human breast cancer tissues. Interestingly, PDZK1 knockdown in MCF-7 cells blocked ER-dependent growth and reduced c-Myc expression, whereas ectopic expression of PDZK1 enhanced cell proliferation in the presence or absence of 17 β -estradiol potentially through an increase in c-Myc expression, suggesting that PDZK1 has oncogenic activity. PDZK1 also appeared to interact with the Src/ER- α /epidermal growth factor receptor (EGFR) complex, but not with IGF-1R and enhanced EGFR-stimulated MEK/ERK1/2 signaling. Collectively, our results clarify the relationship between ER- α and PDZK1, propose a direct relationship between PDZK1 and IGF-1R, and identify a novel oncogenic activity for PDZK1 in breast cancer.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2011.00001

INTRODUCTION

Breast cancer is the most common form of malignancy among women and is a leading cause of cancer-related deaths worldwide. It is the number one cause of cancer-related deaths in non-smoking women in the US. Estrogen is a critical regulator of growth, differentia-

tion and function in a wide array of target tissues, including the female and male reproductive tracts, the mammary gland and the cardiovascular and skeletal systems (1). Estrogen regulates both growth and differentiation of normal breast tissue; however, in pathological situations, the hormone participates in

the initiation and progression of breast cancer (1). A great deal of work has demonstrated that the steroid hormone 17 β -estradiol (E_2) induces a signal essential for the transformation of normal cells to a malignant phenotype; however, the underlying mechanism(s) by which E_2 participates in carcinogenesis is not well established (rev. in 2).

Estrogen receptor (ER) subtypes ER- α and ER- β mediate the action of E_2 as well as those of a battery of natural and synthetic chemicals that share structural features with the hormone. The ER functions as a ligand-dependent regulator of transcription upon its direct recruitment to target genes or through interactions with other transcription factors (2). In addition, ER- α can participate in cytoplasmic and membrane-

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Submitted January 1, 2011; Accepted for publication June 25, 2013; Epub (www.molmed.org) ahead of print June 26, 2013.

associated signaling processes when rapid physiological responses are required. Stimulation of the ER with E₂ can induce a rapid signaling cascade that includes important signaling molecules, such as epidermal growth factor receptor (EGFR), Raf-1, mitogen-activated protein kinase (MAPK) and Akt, as well as several others. Such induction of important signaling processes ultimately contributes to the actions of E₂ in the promotion of cancer cell proliferation and survival.

PDZK1, a 70-kDa adapter protein with four PDZ-interacting domains, which was first identified by Kocher *et al.* (3), is believed to regulate the levels of the scavenger receptor class B, type I (SR-BI) (or the scavenger receptor class B, member 1 [SCARB1]) in a posttranscriptional manner (4). PDZK1 is found primarily at the apical brush-border membrane of the proximal tubules of the kidney (3). Lower levels can be detected in the liver, small intestine, pancreas, adrenal cortex, gastrointestinal tract and testis (3). What is well established is the fact that PDZ domain proteins are known to function as adaptor/scaffold proteins that influence multiple biological functions due to their interactions with a variety of plasma membrane transporters and receptors (5). Ghosh *et al.* (6) initially reported that PDZK1 can be an estrogen-responsive gene in breast cancer cells and suggested that it may be through an indirect mechanism. Such a relationship was recently strengthened by a clinical study establishing a significant association between plasma E₂ levels and PDZK1 mRNA expression levels in ER- α -positive human breast cancer (7). These findings increased the potential importance of PDZK1 in breast cancer. However, the mechanism by which PDZK1 is induced upon E₂ stimulation and the effects of such expression have yet to be elucidated. Accordingly, the present study aimed to examine the cellular and subcellular expression patterns of PDZK1 at the protein level in human breast cancer tissues, decipher-

ing the mechanism by which ER- α stimulation leads to PDZK1 expression and defining the actual function of such protein.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin and fetal bovine serum were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). Charcoal/dextran-treated fetal bovine serum was purchased from Hyclone (Logan, UT, USA); E₂, dihydrotestosterone (DHT), tamoxifen and cycloheximide were from Sigma-Aldrich (St. Louis, MO, USA); the synthetic androgen methyltrienolone (R1881) was from PerkinElmer (Waltham, MA, USA); U0126 and PP2 were from Promega (Madison, WI, USA); ICI 182,780 was from TOCRIS (Bristol, UK); and AG1024 was from Calbiochem (San Diego, CA, USA). AG1478 was from Cell Signaling Technology (Danvers, MA, USA). Unless otherwise indicated, all other drugs were purchased from Sigma-Aldrich.

Immunohistochemistry

Breast cancer and normal tissue microarray (TMA) sections (US Biomax, Rockville, MD, USA), which included a total of 262 neoplastic tissue samples and 87 normal breast adjacent tissue cores (Supplementary Table S1), were subjected to immunohistochemistry with antibodies to PDZK1 (ProteinTech Group, Chicago, IL, USA), insulinlike growth factor 1 receptor (IGF-1R) (Santa Cruz Biotechnology, Dallas, TX, USA) or ER- α (Novus Biologicals, Littleton, CO, USA) essentially as described previously (8). TMA slides were processed by using Biocare reagents in a Biocare Nemesys 7200 automated system (Biocare Medical, Concord, CA, USA). For negative controls, the entire immunohistochemistry method was performed on sections in the absence of the primary antibody. Stained slides were scanned using the $\times 40$ objective with a Hamamatsu NanoZoomer (Hamamatsu, Bridgewater,

NJ, USA). Immunoreactivity was analyzed by using Image-Pro Plus Version 6.0 software (Media Cybernetics, Rockville, MD, USA) as described previously (9,10). The measurement parameters included density mean, area sum and integrated optical density. The software system allows a computerized assessment of the density of the staining as a sum of the values for intensity of all the pixels of a counted region in an analyzed area as well as the total area in an unbiased manner. Threshold range of the colors of positive staining was selected in such a way that both faint and strong signals were detected without a high level of background. Density of immunoreactivity was then determined for all areas with a positive signal according to the weighted histoscore method (8):

Histoscore =

$$\sum (\% \text{ negative staining} \times 0) + (\% \text{ weak staining} \times 1) + (\% \text{ moderate staining} \times 2) + (\% \text{ strong staining} \times 3).$$

In some situations, medium and high signals were combined and compared with percentage of cores with low or negative signals.

Cell Culture, Cell Proliferation, Transfection, Immunofluorescence Microscopy, Immunoblot Analysis and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

MCF-7, MDA-MB-231 and MCF-10A cells (all were obtained from ATCC, Manassas, VA, USA) were cultured according to ATCC specifications. These cell lines are authenticated by ATCC using short tandem repeat (STR) profiling. This PCR-based method allows the authentication of human cell lines with resolution down to the individual donor. Upon receipt from ATCC, the morphology was confirmed by microscopy and population-doubling times were determined by using the trypan blue dye exclusion method. Before treatment with E₂, medium was changed to phenol red-free DMEM supplemented with 5% charcoal/dextran-treated fetal bovine serum (CDSS). Cell proliferation and

DNA synthesis were measured by MTT assay and a 5-bromo-2-deoxyuridine (BrdU) assay kit (Roche, Indianapolis, IN, USA). MCF-7 cells were transiently transfected with specific siRNAs targeting ER- α (Santa Cruz Biotechnology), PDZK1 (AM16708 from Ambion/Life Technologies] or sc-106840 from Santa Cruz Biotechnology) or the negative control (scrambled) siRNA NEG3 (SA Bioscience, Frederick, MD, USA) by using Lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer's instructions. Cells were also infected with a control and shRNA targeting the PDZK1 lentiviral vector (sc-108080 and sc-106849-V; Santa Cruz Biotechnology). The transduced cells were selected with puromycin dihydrochloride (Santa Cruz Biotechnology). Cells were then treated as described in the figure legends before being subjected to total RNA or protein preparation. Isolated RNA was reverse-transcribed, and the resulting cDNA was subjected to conventional PCR with primer sets (IDT, San Jose, CA, USA) specific to human *PDZK1*, *IGF1-R*, β -*actin*, or *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (Supplementary Table S2). The primer sequences for β -actin were selected to avoid amplification of pseudogenes as described by Raff *et al.* (9). Protein extracts were subjected to immunoblot analysis with antibodies to phospho-MEK, MEK, phospho(T202/Y204)-ERK1/2, ERK1/2, c-Src (32G6) and EGFR (all purchased from Cell Signaling Technology); ER- α , c-Myc or GADPH (all from Santa Cruz Biotechnology); or PDZK1(EPR3751) (Novus Biologicals). Immune complexes were detected with appropriate secondary antibodies and chemiluminescence reagents (Pierce, Rockford, IL, USA). Densitometry of immunoblot signals was quantified by using the VersaDoc™ imaging system (Bio-Rad Laboratories, Hercules, CA, USA). For immunofluorescence, cells were seeded on chamber slides and treated as described in the figure legends. Cells were then fixed, permeabilized and stained with antibodies to PDZK1 (Abcam, Cambridge, MA,

USA) and with 4'-6-diamidino-2-phenylindole essentially as described (10). Cells were then examined with a Nikon fluorescence microscope.

Human Growth Factor Array

MCF-7 cells were treated with E₂ for 6 h, after which protein extracts were collected. Changes in growth factor expression were analyzed by using human growth factor antibody arrays (Ray-Biotech, Hercules, CA, USA). Each array was incubated with 1 mL E₂-treated or control cell lysates at 4°C overnight, and bound growth factors were detected by enhanced chemiluminescence according to the manufacturer's instructions. The relative signal intensity was quantified by using the VersaDoc imaging system.

Immunoprecipitation

The lysates were precleared by incubation with 20 μ L protein A/G plus-agarose beads (Santa Cruz Biotechnology) for 1 h before an overnight incubation at 4°C with indicated specific antibodies or normal IgG. Protein A/G plus-agarose beads were then added to the lysates, and the mixtures were further incubated on a rotating wheel for 4 h at 4°C. The beads were pelleted and washed three times in wash buffer (0.1% NP-40, 50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 2 μ g/mL leupeptin, 2 μ g/mL aprotinin, 1 mmol/L PMSF, 5 mmol/L NaF, 100 μ mol/L Na₃VO₄). Antibody-antigen complexes bound to the beads were eluted in the sample buffer by boiling and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11).

Statistical Analysis

Data are presented as means \pm standard error of the mean (SEM) from at least three separate experiments. Comparisons between multiple groups were performed with one-way analysis of variance with Bonferroni test. Statistical significance was considered significant at $p < 0.05$. The Pearson correlation coefficient (r) was used to examine the correlation between different variables. All sta-

tistical summaries and analyses were performed by using GraphPad software, version 5 (La Jolla, CA, USA).

All supplementary materials are available online at www.molmed.org.

RESULTS

Correlation Between PDZK1 Upregulation and Breast Malignancy

We initiated our studies by conducting a direct examination of PDZK1 expression in tissue microarray slides of both breast cancer and normal tissues. Clinical characteristics of the tissue microarray are displayed in Supplementary Table S1. Figures 1A and B show that PDZK1 expression was detectable in more than 60% of the carcinomas examined, suggesting a potentially high correlation with breast malignancy. PDZK1 immunoreactivity was largely absent or weak in both normal tissues and benign tumors. The expression of PDZK1 appeared to be exclusively epithelial, since no immunoreactivity was detected in stromal cells (Figure 1C). PDZK1 was detected in the cytoplasm, with occasional nuclear or perinuclear localization. PDZK1 immunoreactivity was significantly decreased in cancer tissues from women who were older than 55 years of age (Figure 1D). In fact, a significant negative correlation existed between PDZK1 immunoreactivity and the age of patients ($r = -0.267$, $p = 0.002$). This particular immunoreactivity remained considerably higher than that observed in normal or benign tissues. Surprisingly, however, there was no correlation between PDZK1 expression and ER- α levels ($r = -0.105$, $p = 0.821$). These results suggest a correlation between PDZK1 expression and breast carcinogenesis as well as provide a temporal window during which breast cancers would be positive for PDZK1. Interestingly, in ER- α -positive cancer tissues, PDZK1 expression was significantly higher in tissues from younger individuals (<40 years) compared with other ages ($p < 0.001$). In light of the re-

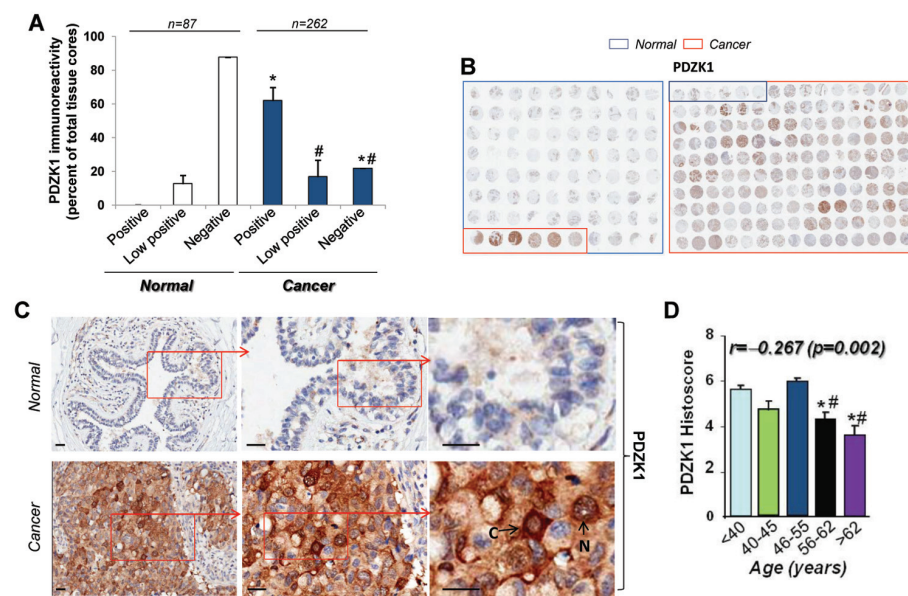


Figure 1. Association between PDZK1 upregulation and breast malignancy. (A) The extent of PDZK1 immunoreactivity was expressed as a percentage of total tissues. Medium and high signals were combined and compared with percentage of cores with low or negative signals. *Difference from the number of PDZK1-negative cancerous tissues; $p \leq 0.005$. (B) Represents scanned TMA sections (mostly cancer, left panel, or mostly normal, right panel, tissues) subjected to immunohistochemical staining with antibodies to human PDZK1. (C) Tissue cores representing normal breast and cancer tissue. Note the mostly cytoplasmic distribution and the occasional nuclear or perinuclear localization of PDZK1. Bar: 4 μ m. Nuc, Nuclear staining; Peri-Nuc, perinuclear staining. (D) Histocores of PDZK1 immunoreactivity according to age of women from which tumors were collected. *Difference from the number of PDZK1-positive cancerous tissues collected from women younger than 40 years of age; $p \leq 0.005$. #Difference from the number of PDZK1-positive tissues from women between 46–55 years of age; $p \leq 0.005$.

cent report identifying a significant association between plasma E_2 and PDZK1 mRNA levels in ER- α -positive breast cancers (7), our results suggest a more complex relationship between ER- α and PDZK1. Accordingly, it becomes imperative that the mechanism by which estrogen regulates PDZK1 expression is clarified and, more importantly, that the exact function of PDZK1 in breast cancer is explored.

PDZK1 is not an immediate early gene product of ER- α stimulation, but is critically required for E_2 -dependent growth of MCF-7 cells. Figure 2A shows that treatment of MCF-7 cells with 1 nmol/L E_2 promoted a time-dependent increase in PDZK1 expression, as assessed by conventional PCR and immunoblot analysis. Notably, the accu-

mulation of PDZK1 was rather slow, unlike ER- α -dependent genes such as *GREB* and *pS2* (genes regulated by estrogen in breast cancer) that tend to exhibit much faster kinetics (6). Immunofluorescence analysis revealed that E_2 -induced PDZK1 exhibited a primarily cytosolic localization (Figure 2B) with some nuclear staining (Figure 2C). Such a pattern was similar to that observed in epithelial cells of breast cancer tissues (Figure 1C). Figure 2D shows that blockage of E_2 -mediated signaling by the ER- α antagonist fulvestrant (ICI182.780) or the selective estrogen modulator tamoxifen markedly blocked the induction of PDZK1 expression in response to the hormone, demonstrating the link between E_2 , ER- α and PDZK1. Surprisingly, in contrast to the reducing

effect of the ER- α antagonists on E_2 -induced PDZK1 expression, inhibition of EGFR, a receptor that is rapidly activated upon ER- α stimulation (12), by its antagonist AG1478, did not exert any noticeable effect (Figure 2D). Additionally, treatment of the ER- α -deficient cell lines MDA-MB-231 and MCF-10A with E_2 did not result in any detectable PDZK1 expression (Supplementary Figure S1), supporting the notion that E_2 -induced PDZK1 expression is specific for ER- α stimulation. Neither a natural (DHT) nor synthetic (R1881) androgen induced PDZK1 expression in MCF-7, MDA-MB-231 or MCF-10A cells (Supplementary Figure S1).

A critical outcome of ER- α signaling is its promotion of cellular growth. To determine whether a relationship exists between the E_2 -induced increase in PDZK1 expression and cell growth, we examined whether PDZK1 knockdown by siRNA would affect E_2 -promoted growth of MCF-7 cells. Treatment of MCF-7 cells with PDZK1-specific siRNAs, which markedly reduced the expression of the PDZK1 protein (Figure 2E, inset), coincided with the inhibition of E_2 -induced cell growth, as assessed by the MTT assay (Figure 2E) and confirmed by BrdU incorporation (Supplementary Figure S2). The approximate 40% increase in growth in response to E_2 is typical and has been reported previously by others (13). The effect of PDZK1 knockdown on E_2 -promoted growth of MCF-7 cells was also confirmed by using a lentiviral vector encoding shRNA targeting PDZK1 (Supplementary Figure S3). Figure 2F shows that PDZK1 knockdown significantly reduced the number of cells in the S phase upon E_2 treatment, suggesting a potential involvement of the protein in E_2 -induced entry into the S phase of the cell cycle. Such an effect on the cell cycle coincided with a marked decrease in E_2 -induced c-Myc expression (Figure 2G). The connection among ER- α , PDZK1 and c-Myc was subsequently confirmed by knocking down ER- α , which resulted in a major decrease in PDZK1 (Supplementary Figure S4). Such decrease coincided, as expected,

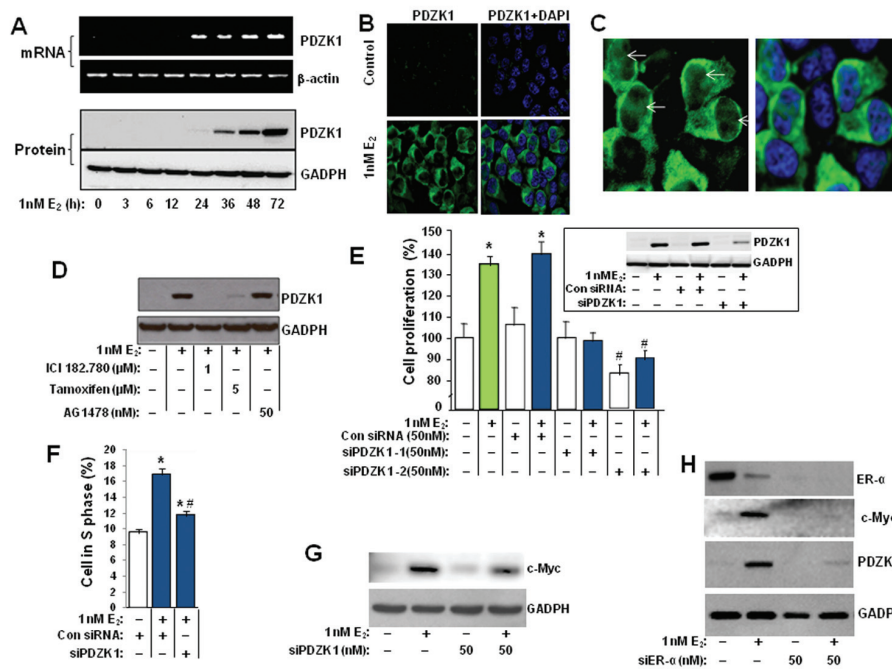


Figure 2. PDZK1 is not an immediate early ER- α -dependent gene product but is required for E₂-dependent growth of MCF-7 cells. (A) MCF-7 cells were treated with 1 nmol/L E₂ for different time intervals or left untreated. Total RNA was subjected to RT-PCR analysis with primers specific to PDZK1 or β -actin (upper two panels). Protein extracts were subjected to immunoblot analysis with antibodies against PDZK1 or GAPDH (lower two panels). (B) MCF-7 cells were treated with 1 nmol/L E₂ for 48 h or left untreated. Cells were subjected to immunofluorescence with antibodies against PDZK1. Note the partial nuclear localization of PDZK1 in E₂-treated MCF-7 cells (C). (D) MCF-7 cells were treated with 1 nmol/L E₂ in the presence or absence of the ER- α antagonist ICI 182,780, the selective estrogen modulator tamoxifen or the EGFR kinase inhibitor AG1478 for 48 h, after which protein extracts were subjected to immunoblot analysis with antibodies against PDZK1 or GAPDH. (E) MCF-7 cells were transiently transfected with siRNA targeting PDZK1 (siPDZK1-1) or control siRNA and treated with 1 nmol/L E₂ or left untreated for 48 h. Protein extracts were prepared and subjected to immunoblot analysis with antibodies against PDZK1 or GAPDH (inset). MCF-7 cells were subjected to PDZK1 knockdown with siPDZK1-1 (sequence 1) or PDZK1-2 (sequence 2), after which cell proliferation was assessed by using the MTT assay. *Difference from respective untreated controls; #difference from E₂-treated cells; $p < 0.05$. (F) MCF-7 cells were subjected to PDZK1 knockdown with the siPDZK1, after which cells in the S phase were assessed by fluorescence-activated cell sorter analysis. *Difference from untreated control; #difference from E₂-treated cells; $p < 0.05$. (G) MCF-7 cells were subjected to PDZK1 knockdown with the siPDZK1-1 sequence and treated with 1 nmol/L E₂ for 48 h. Protein extracts were subjected to immunoblot analysis with antibodies against c-Myc or GAPDH. (H) MCF-7 cells were subjected to ER- α knockdown with siRNA and treated with 1 nmol/L E₂ for 48 h. Protein extracts were subjected to immunoblot analysis with antibodies against c-Myc, PDZK1 or GAPDH. Note that the immunoblot for ER- α was run on a different gel and its GAPDH loading control is depicted in Supplementary Figure S4; the latter figure also depicts immunoblot for ER- α in protein extracts from cells treated with control siRNA.

with a reduction in c-Myc (Figure 2H). Together, these results strongly suggest that PDZK1 is not only an estrogen-responsive protein, but also plays a critical role in the induction process of cell growth upon ER- α stimulation by E₂.

PDZK1 Expression Is Not Directly Controlled by E₂ and Requires Expression of an Intermediate ER- α -Regulated Gene Product

As noted above, the kinetics of PDZK1 expression was relatively slow and not re-

flective of the rapid signal transduction normally induced by ER- α stimulation. This relationship was complicated even more by the lack of correlation between the two factors in human breast cancer tissues. Accordingly, it is conceivable that ER- α stimulation may be inducing an intermediate factor, which, in turn, induces PDZK1 expression. To test such a hypothesis, we examined the effects of inhibiting protein synthesis, a cycloheximide-sensitive event, on PDZK1 mRNA expression, a cycloheximide-resistant event. Figure 3A shows that E₂-induced PDZK1 mRNA expression was almost completely blocked by cycloheximide, suggesting that E₂ induces a protein or combination of proteins required for PDZK1 expression. These results confirm the observation made by Weigel's group, who made the original observation on the dependence of PDZK1 on estrogen and actually suggested that the relationship between estrogen and PDZK1 might be indirect (6). To determine the critical time window during which this factor(s) was produced, we subjected cells to treatment with cycloheximide at different time intervals after E₂ stimulation, as depicted in Figure 3B. Figure 3C shows that inhibition of protein synthesis 3–6 h after stimulation with E₂ is a critical time window during which the unknown factor is synthesized. Later exposure to the drug could not effectively block PDZK1 expression upon E₂ exposure.

IGF-1R Expression Is Critical for PDZK1 Expression on E₂ Stimulation of MCF-7 Cells

To identify the potential factor that is responsible for PDZK1 expression, we subjected protein extracts from MCF-7 cells treated with E₂ for 6 h to a growth factor array. Analysis of these results revealed that one of the most prominent candidates was IGF-1R (Figure 4A). Indeed, E₂ induced IGF-1R expression above basal levels as early as 3 h after treatment in MCF-7 cells; IGF-1R reached maximum levels at approximately 12 h (Figure 4B), which remained high and sustained for at least 48 h after treatment. Figure 4C shows the depen-

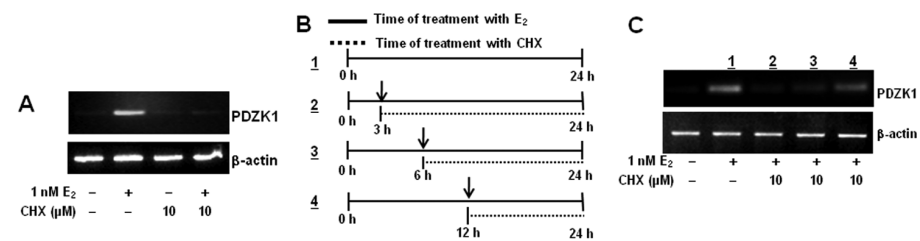


Figure 3. Protein synthesis is required for PDZK1 expression upon E_2 stimulation in MCF-7 cells. (A) MCF-7 cells were treated with 1 nmol/L E_2 in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) for 24 h. Total RNA was subjected to RT-PCR with primers specific to *PDZK1* or β -actin. (B) Diagram of cell treatment with CHX and E_2 for the results depicted in (C). (C) MCF-7 cells were treated with 1 nmol/L E_2 followed by the addition of CHX 3, 6 or 12 h after treatment with E_2 as illustrated in (B). Total RNA was prepared 24 h after E_2 treatment and subjected to RT-PCR with primers specific to *PDZK1* or β -actin.

dence of IGF-1R expression above basal levels on ER- α stimulation, since knock-down of ER- α by siRNA drastically reduced expression of the receptor after E_2 -treatment in MCF-7 cells. Inhibition of ER- α activity by ICI182.780 or tamoxifen also markedly reduced IGF-1R expression in response to E_2 treatment; however, treatment of cells with the IGF-1R antagonist AG1024 did not have any

effect on E_2 -induced IGF-1R expression (Supplementary Figure S5).

To establish the involvement of IGF-1R in the E_2 -induced expression of PDZK1, we examined whether inhibition of IGF-1R-associated signaling by its potent inhibitor AG1024 would block PDZK1 expression in response to E_2 treatment in MCF-7 cells. Further, we determined whether direct stimulation of IGF-1R

would result in PDZK1 expression in the absence of E_2 . Figure 4E shows that AG1024 markedly reduced PDZK1 expression in a dose-dependent manner. More importantly, stimulation of IGF-1R with IGF-1 promoted rather fast expression of PDZK1; a noticeable expression level was observed after 3–6 h of treatment. Together, these results provide additional support for the involvement of IGF-1R in E_2 -induced expression of PDZK1.

Correlation Between IGF-1R and PDZK1 Expression in Human Breast Cancer at the Protein and mRNA Levels

We tested whether the relationship between PDZK1 and IGF-1R expression that was observed in the cell culture model was also present in human breast cancer tissues using tissue microarray slides. Analysis of the immunoreactivity to the two proteins in serial sections revealed a significant correlation ($r = 0.591$; $p < 0.001$), thus supporting the association between IGF1-R expression and PDZK1 up-regulation (Figure 5A). Figure 5B depicts two examples of breast cancer tissue cores that are either positive or negative, which are respectively positive or negative for PDZK1. Using data deposited in the public domain Gene Expression Omnibus (GEO, GDS3097) Profiles database (14) and conducted by Boersma *et al.* (15), we examined the correlation between *IGF1-R* (203628_at) and *PDZK1* (205380_at) mRNA levels in 48 fresh-frozen excised breast cancer tissues procured from a different cohort of patients. Figure 5C shows a significant correlation between the two transcripts ($r = 0.537$; $p < 0.001$), which lends additional independent support to the positive relationship between PDZK1 and IGF-1R in breast cancer.

Ectopic Expression of PDZK1 Stimulates Growth and Enhances E_2 -Promoted Growth of MCF-7 Cells

Given the correlation between PDZK1 expression and human breast cancer and the potential role of PDZK1 in ER- α -stimulated cell growth, we investigated the mechanism of action of PDZK1 in

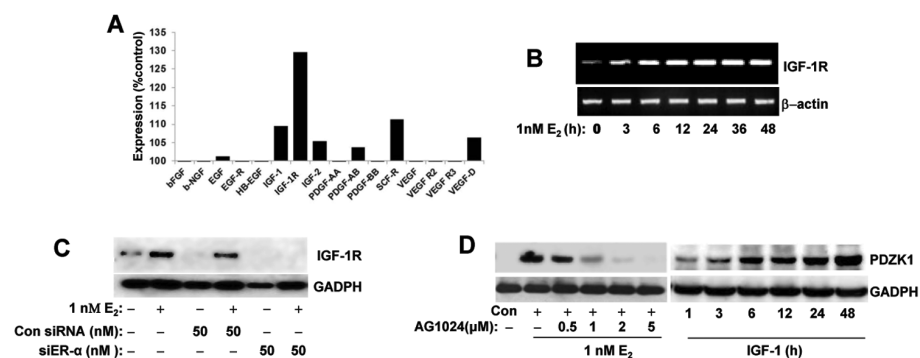


Figure 4. Expression of IGF-1R is critical for PDZK1 expression upon E_2 stimulation in MCF-7 cells. (A) MCF-7 cells were treated with 1 nmol/L E_2 for 6 h, after which cell lysates were prepared and incubated at 4°C overnight with a membrane containing fixed antibodies against the indicated growth factor. Bound growth factors were detected by enhanced chemiluminescence according to the manufacturer's instructions. The relative signal intensity was quantified by using the VersaDoc imaging system. (B) MCF-7 cells were treated with 1 nmol/L E_2 for different time intervals or left untreated. Total RNA was subjected to RT-PCR analysis with primers specific to human *IGF-1R* or β -actin. (C) MCF-7 cells were transiently transfected with siRNA targeting ER- α or control siRNA (Supplementary Figure S4). Cells were treated with 1 nmol/L E_2 or left untreated for 48 h (left panel), after which protein extracts were prepared and subjected to immunoblot analysis with antibodies against IGF-1R or GAPDH. (D) MCF-7 cells were treated with 1 nmol/L E_2 in the presence or absence of different doses of the IGF-1R antagonist AG1024 for 48 h or with human IGF-1 (10 ng/mL) for the indicated time intervals (right panel), after which protein extracts were prepared and subjected to immunoblot analysis with antibodies against PDZK1 or GAPDH.

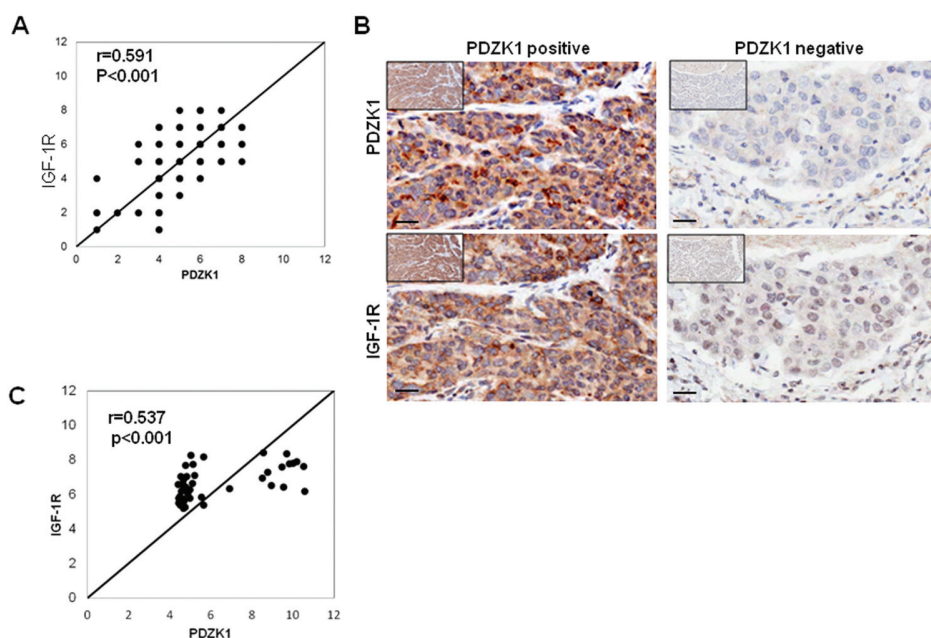


Figure 5. Correlation between PDZK1 upregulation and IGF-1R in breast cancer. (A) Correlation of PDZK1 protein expression with that of IGF-1R as assessed by immunohistochemistry by using TMA serial sections prepared from the same tissues where the Pearson correlation coefficient was $r = 0.591$ with $p < 0.001$. (B) Examples of PDZK1 in a positive or negative core with respective positive or negative expression of IGF-1R; insets represent a lower magnification but larger area of each sample. (C) Correlation between *PDZK1* and *IGF-1R* transcripts ($r = 0.537$; $p < 0.001$) in breast cancer tissues determined using data deposited in the GEO Profiles database (14) and conducted by Boersma *et al.* (15).

breast cancer cell proliferation. Initially, we addressed whether mere expression of PDZK1 was sufficient to promote growth of MCF-7 cells. To this end, MCF-7 cells were transiently transfected with an expression vector encoding human PDZK1 or GFP-expressing control vector (pcDNA3.1; Figure 6A), and cell growth was monitored in the absence of E_2 . Figure 6B shows that ectopic expression of PDZK1 significantly enhanced growth of MCF-7 cells (~35%; $p < 0.005$), as assessed by an MTT assay. Interestingly, the level of increase in cell growth was similar to that achieved by E_2 treatment (Figure 2E). Given that PDZK1 knock-down reduced the E_2 -stimulated increase in c-Myc expression, we postulated that PDZK1 overexpression may consequently increase c-Myc in MCF-7 cells in the absence of E_2 . Indeed, ectopic expression of PDZK1 promoted a marked elevation in the level of c-Myc (Figure 6C). When growth of PDZK1-transfected MCF-7

cells was monitored in the presence of E_2 , proliferation of the cells was substantially enhanced (~80%; Figure 6D). The latter results were confirmed by direct cell counting with Trypan blue (Supplementary Figure S6). The substantial increase in the growth of MCF-7 cells transfected with the *PDZK1*-expressing vector did not appear to be associated with a greater elevation in *PDZK1* upon E_2 treatment, since the cumulative expression of *PDZK1* was similar between untreated and E_2 -treated cells (Figure 6E). Together, these results clearly suggest an oncogenic function for PDZK1 in breast cancer cells and indicate that PDZK1 can enhance the growth-promoting activity of E_2 .

PDZK1 potentially stimulates growth of MCF-7 cells through interaction with the Src/ER- α /EGFR complex and enhancement of EGF-mediated signal transduction. Given PDZK1-mediated enhancement of cell growth, especially in response to ER- α stimulation, the question of how such protein influences E_2 -associated signaling events became important. It is noteworthy that PDZK1 was reported to interact with membrane receptors, including

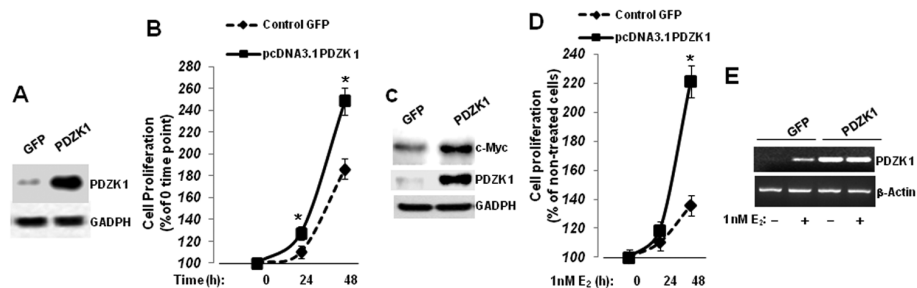


Figure 6. Ectopic expression of PDZK1 stimulates growth and enhances E_2 -promoted growth of MCF-7 cells. (A) MCF-7 cells were transfected with an expression vector encoding human PDZK1 or control empty vector (pcDNA3.1). Protein extracts from these cells were subjected to immunoblot analysis with antibodies to PDZK1 or GAPDH. (B) Growth rates of PDZK1-expressing or control vector transfected MCF-7 cells were determined 24 or 48 h after plating in the absence of E_2 by using the MTT assay. Data are expressed as percentage of growth at time 0. *Difference from control vector-transfected cells; $p < 0.05$. (C) Exponentially growing PDZK1-expressing or control vector transfected MCF-7 cells were collected, and protein extracts were prepared and subjected to immunoblot analysis with antibodies to c-Myc, PDZK1 or GAPDH. (D) Growth rates of the cells assessed in the presence of 1 nmol/L E_2 . Data are expressed as percentage of growth of untreated cells. *Difference from control vector-transfected cells; $p < 0.05$. (E) PDZK1-expressing or control vector transfected MCF-7 cells were treated with 1 nmol/L E_2 or left untreated for 24 h. Cells were collected and total RNA was prepared and subjected to conventional RT-PCR with primers specific to human *PDZK1* or *β -actin*.

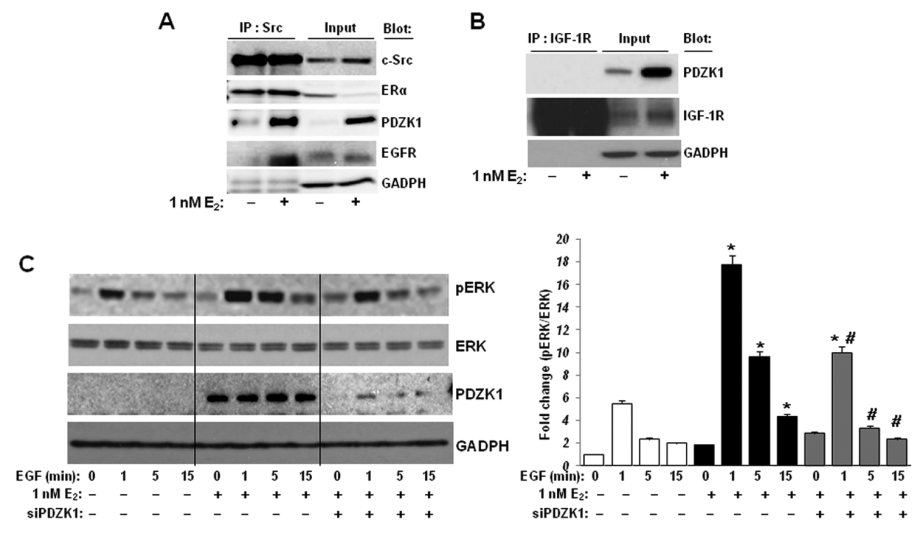


Figure 7. PDZK1 interacts with the Src/ER- α /EGFR complex and enhances EGF-mediated signal transduction. (A) MCF-7 cells were treated with 1 nmol/L E₂ for 48 h or left untreated, after which protein extracts were subjected to immunoprecipitation with antibodies against c-Src. The immunoprecipitates, along with 10% input, were subjected to immunoblot analysis with antibodies against c-Src, ER- α , PDZK1, EGFR or GAPDH. The same protein extracts were subjected to immunoprecipitation with control IgG followed by immunoblot analysis with antibodies against c-Src, PDZK1 or GAPDH (Supplementary Figure S8). (B) The same protein extracts were subjected to immunoprecipitation with antibodies against IGF-1R followed by immunoblot analysis with antibodies against PDZK1, IGF-1R or GAPDH. (C) MCF-7 cells were transiently transfected with siRNA targeting PDZK1 and were treated with 1 nmol/L E₂ or left untreated for 48 h. In the absence of E₂, cells were treated with 20 ng/mL EGF for different time intervals. Cells were collected and protein extracts were prepared and subjected to immunoblot analysis with antibodies against phospho-ERK1/2 (pERK), ERK, PDZK1 or GAPDH. Immunoblots for pERK were quantified by using Adobe Photoshop CS, and data are expressed as the fold-change from control. *Difference from untreated WT control; #difference from similarly E₂-treated nontransfected cells; $p < 0.05$.

SR-BI, which appears to be essential for the initiation of its associated signal transduction in endothelium (16). c-Src is considered to be a critical factor that mediates signal transduction initiated by a number of receptor molecules including ER- α , IGF-1R and EGFR to ultimately promote cell growth (17). Inhibition of c-Src by PP2 was as efficient in inhibiting ERK1/2 phosphorylation in response to E₂ stimulation as the MEK inhibitor U0126 (Supplementary Figure S7). For this purpose, we targeted c-Src to investigate whether PDZK1 influences E₂-induced growth of MCF-7 cells by interacting with such key ER- α -associated receptor complex(es). Figure 7A demonstrates, as expected, an interaction among c-Src, ER- α and EGFR by using a coimmunoprecipitation assay. Interestingly, PDZK1 was also part of that

complex in extracts from E₂-treated cells, suggesting a potential interaction among the protein, c-Src and the receptors. c-Src and PDZK1 were not immunoprecipitated with control IgG (Supplementary Figure S8A). The interaction of PDZK1 and c-Src with IGF-1R was also tested but not detected (Supplementary Figure S8B), which prompted us to verify such lack of interaction by immunoprecipitation with antibodies against the receptor. Figure 7B shows that IGF-1R appeared not to bind to PDZK1 despite the large amount of the protein that was induced by E₂ treatment.

In a recent comprehensive study, Santen's group (18) reported that IGF-1R functions upstream of EGFR in a linear pathway, promoted as a result estrogen-mediated activation of MAPK and subsequent cell growth stimulation. Accord-

ingly, we next wished to determine the consequence of the interaction of PDZK1 with the c-Src/ER- α /EGFR complex in EGFR-stimulated signal transduction. Figure 6C shows that stimulation of MCF-7 cells by EGF induced a fast and transient activation of ERK1/2. Such activation was greatly enhanced in cells that were treated with E₂ for 48 h. PDZK1 knockdown by siRNA greatly dampened such ERK1/2 stimulation of E₂-treated cells, strongly suggesting a causal relationship between expression of PDZK1 and enhancement of ERK1/2 activation. This enhancement of the ERK1/2 pathway may, in part, explain the involvement of PDZK1 in enhancing E₂-mediated growth of MCF-7 cells. To define the exact underlying mechanism by which PDZK1 influences EGFR signal transduction and cell growth requires much more extensive and detailed studies.

DISCUSSION

The connection between estrogen and PDZK1 in breast cancer has recently received additional support from a clinical study conducted by Dunbier *et al.* (7), which showed a significant association between plasma E₂ and PDZK1 mRNA levels in ER- α -positive breast cancers. Our results lend support for this association by providing the specific characteristics of protein distribution and its exclusive epithelial expression pattern with a primarily cytosolic and occasional nuclear localization in cancerous tissues, as assessed by immunohistochemistry. Interestingly, the expression profile of PDZK1 in human breast cancer appears to be prominent in younger women but decreased with age. Our findings become even more important in light of our mechanistic studies using an *in vitro* system with the estrogen-responsive breast cancer cell line MCF-7. Surprisingly, however, our study found no correlation between PDZK1 and ER- α expression levels, suggesting a more complex relationship. A more robust correlation was identified between PDZK1 and IGF-1R expression. The *in vitro* studies clarified this relationship and demonstrated that a potential link between E₂/ER- α and PDZK1 expres-

sion may be IGF-1R, which functions as an ER- α -dependent intermediate factor that is required for PDZK1 expression. A critical finding of the current study was the determination of an actual function for PDZK1 as a growth-promoting factor in breast cancer cells. Such function is clearly different from its well-known scaffolding role in SR-BI protein stability and function in HDL signaling (5). The ability of PDZK1 to contribute to cell growth appears to be linked to c-Myc expression and enhancement of EGFR-associated signal transduction.

Our study shows that PDZK1 protein expression was undetectable in normal breast tissues or in benign tumors. In contrast, levels of PDZK1 protein is elevated in breast cancer tissues from women younger than 55 years of age. The levels of PDZK1 decrease in tissues from women older than 55 years of age. It is tempting to speculate that this result may be linked to the decreasing levels of plasma estrogen (19,20). This potential connection becomes even more important in light of the report that intratumoral estrogen levels could reach levels as high as 20 times that detected in the plasma of affected individuals (21). What renders the *PDZK1* gene responsive to estrogen is also unclear. Our *in vitro* studies suggest that a potential requirement for PDZK1 expression is an increase in IGF-1R expression and activity. It is important to note that IGF-1R may not be the sole intermediate factor. Many epidemiological and mechanistic studies are necessary to identify the reason(s) behind the observed expression pattern of PDZK1 and whether these changes are critical determinants in breast carcinogenesis.

For more than a decade now, PDZK1 was believed to be a direct product of ER- α stimulation (22–24), although Weigel's group suggested the potentially indirect relationship between estrogen and PDZK1 (6). Our results confirm the indirect relationship between PDZK1 and ER- α and showed that an intermediate E₂-stimulated factor was required for PDZK1 induction. This factor was identified as IGF-1R. Indeed, direct stimulation of IGF-1R by IGF-1 in MCF-7 cells resulted in a fast and per-

sistent expression of PDZK1 mRNA and protein. Additionally, expression of PDZK1 in E₂-stimulated MCF-7 cells was completely blocked upon IGF-1R inhibition. The relationship between IGF-1R and PDZK1 was further supported by the strong correlation that existed between the proteins in human breast cancer tissues. This relationship was strengthened by the significant correlation between the mRNA levels of the two factors, as assessed in data deposited in the GEO Profiles database (14) and conducted by Boersma *et al.* (15). Additional support for the relationship between PDZK1 and IGF-1R in breast cancer comes from the finding that 100% of the samples tested in the latter study with high levels of *PDZK1* mRNA exhibited moderate to high levels of *IGF-1R*. Conversely, 100% of the samples with low levels of *IGF-1R* mRNA exhibited low levels of *PDZK1*.

The correlation between plasma E₂ and PDZK1 in ER- α -positive breast cancer tissues ($r = 0.310$, $p = 0.0014$) reported by Dunbier *et al.* (7) was lower than that between PDZK1 and IGF-1R ($r = 0.559$, $p = 0.0001$) observed in our study. Our assessment of ER- α was not limited to positivity but rather to protein levels, which we believe is more reflective of the potential response of breast cancer cells to estrogen. However, it is also possible that the intratumoral or plasma estrogen levels may influence the correlation (21). ER- α -positive breast cancer cells would not elicit an ER- α response if estrogen levels are low. It is important to acknowledge that the assessment of ER- α protein levels in tissues that come into contact with estrogen may be complicated by the fact that stimulation of ER- α by estrogen downregulates its receptor. This result was observed in MCF-7 cells (Supplementary Figure S4) and originally reported by Saceda *et al.* (25). Accordingly, the lack of correlation between ER- α and PDZK1 in the present study may be connected to such an event. Interestingly, MacKay *et al.* (23) recently showed that profound changes in *PDZK1* gene expression in addition to other ER- α -responsive genes can be observed in ER- α -positive

breast cancer tissues isolated from patients who were treated with aromatase inhibitors for 2 wks before surgery. A closer examination of the supplementary data presented in the latter study revealed a pronounced effect on IGF-1R expression levels, suggesting that the effect on PDZK1 by aromatase inhibitors may be associated with a reduction in IGF-1R. It is noteworthy that our results do not exclude a role for ER- α in PDZK1 expression but rather establish a stronger and direct link with IGF-1R and may explain why PDZK1 can be expressed in ER- α -negative breast cancers (6).

A remarkable feature of PDZK1 revealed by our study is that, although the expression of the protein is not directly regulated by ER- α stimulation, PDZK1 can influence the function of ER- α through its interaction with EGFR and Src. c-Src, a non-receptor tyrosine kinase, promotes the transduction of signaling receptors (such as IGF-1R, ER- α and EGFR) that regulate proliferation, survival, cell adhesion and migration (17,26). The involvement of c-Src in extra-nuclear signaling of ER- α is well established (26). The ability of PDZK1 to promote cell growth may be associated with the induction of c-Myc. Such a relationship was supported by the fact that knockdown of PDZK1 led to reduced c-Myc expression upon E₂ treatment and ectopic PDZK1 overexpression correlated with an increase in c-Myc levels. The mechanism by which PDZK1 induces c-Myc expression is unclear. However, the association of PDZK1 with key signaling molecules, including EGFR, c-Src and ER- α , suggests that growth factor signaling is involved. Such a possibility is supported by the finding that E₂-induced PDZK1 expression substantially increased ERK1/2 phosphorylation upon treatment with EGF. Given that ERK1/2 activation is required for c-Myc expression (27), the enhanced signal might be responsible for the involvement of PDZK1 in c-Myc expression. However, such a possibility remains to be determined with more detailed experimentation. Interestingly, Zhu *et al.* (28) reported that although PDZK1 is uniquely required for

signaling by HDL through SR-BI, the interaction between c-Src and SR-BI is not influenced by PDZK1 in endothelial cells, suggesting a more complex role for the protein in breast cancer cells. Although EGFR signaling is enhanced by PDZK1, EGFR signaling does not appear to be involved in PDZK1 expression, since direct stimulation of cells with EGF did not result in PDZK1 expression and inhibition of EGFR activity by AG1478 did not suppress E₂-stimulated PDZK1 expression (Figure 2).

CONCLUSION

Together, our findings provide further evidence for a relationship between E₂, ER- α and PDZK1 in breast cancer cells and clarify the molecular mechanisms that underlie PDZK1 gene and protein expression. Our findings do not challenge the relationship between ER- α and PDZK1 but, instead, reveal an indirect association between these proteins. Interestingly, PDZK1 appears to have growth-promoting activity in the absence of ER- α stimulation. How PDZK1 promotes c-Myc expression without an external stimulus is puzzling to us but may be due to continuous interaction with c-Src and the promotion of persistent signaling, leading to expression of the oncogene and subsequent growth promotion. Much work remains to decipher the exact mechanism by which PDZK1 overexpression promotes growth of breast cancer cells. Taken together, our results suggest that PDZK1 may be a promising therapeutic target for inhibiting the growth of breast cancer cells.

ACKNOWLEDGMENTS

This work was supported in part by grant RSG-116608 from the American Cancer Society and grant HL072889 from the National Institutes of Health (NIH) as well as funds from the Louisiana Cancer Research Consortium (New Orleans, LA, USA) to AH Boulares. This work was also supported in part by grant P20GM103501 (NIH/COBRE) to AS Naura (overall principal investigator: A Ochoa) and by grant PJ008047 from the Next-Generation BioGreen 21 Program Rural Development

Administration (Republic of Korea) to JG Kim.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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