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Breast Cancer: Basic and Clinical Research

Protein Kinase C-ε Promotes EMT in Breast Cancer

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ABSTRACT: Protein kinase C (PKC), a family of serine/threonine kinases, plays critical roles in signal transduction and cell regulation. PKCε, a member of the novel PKC family, is known to be a transforming oncogene and a tumor biomarker for aggressive breast cancers. In this study, we examined the involvement of PKCε in epithelial to mesenchymal transition (EMT), the process that leads the way to metastasis. Overexpression of PKCε was sufficient to induce a mesenchymal phenotype in non-tumorigenic mammary epithelial MCF-10 A cells. This was accompanied by a decrease in the epithelial markers, such as E-cadherin, zonula occludens (ZO)-1, and claudin-1, and an increase in mesenchymal marker vimentin. Transforming growth factor β (TGFβ) induced Snail expression and mesenchymal morphology in MCF-10 A cells, and these effects were partially reversed by the PKCε knockdown. PKCε also mediated cell migration and anoikis resistance, which are hallmarks of EMT. Thus, our study demonstrates that PKCε is an important mediator of EMT in breast cancer.

KEYWORDS: PKCε, EMT, breast cancer

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Introduction

Protein kinase C (PKC) family members are important signal transducers in cellular processes.[1] The ten isozymes in this family are classified as conventional (PKCα, -βI, -βII, and -γ), novel (PKCδ, -ε, -η, and -θ), and atypical (PKCζ and -ι) based on their structural divergence and biochemical properties.[1] Ever since the discovery of PKC as the receptors for tumor-promoting phorbol esters, PKCs are being investigated as attractive targets for cancer therapy.[2] PKCs often show altered expression and/or activity in cancers.[3]

PKCε, a novel PKC, is known for its role as a transforming oncogene.[4] It is overexpressed in many cancers, including breast cancer.[4,5] The expression of PKCε correlates with tumor grade and is predictive of poor disease outcome in breast cancer patients.[5] To exploit the therapeutic potential of PKCε, it is necessary to dissect the molecular mechanisms by which it promotes pro-tumor functions.

It is well established that PKCε promotes cell survival by inhibiting apoptosis.[4,6–12] It was also shown to promote metastasis in several cancers, including breast cancer.[4,5] The process of metastasis involves several steps, including epithelial to mesenchymal transition (EMT), local invasion, intravasation, transport through blood vessels, extravasation, and colonization at the secondary site.[13,14] Although EMT is an early step in the process of metastasis, the involvement of PKCε in EMT has not been investigated.

EMT is a process by which epithelial cells gradually lose their epithelial differentiated characteristics eg, cell–cell adhesion and apico-basal polarity, and gain mesenchymal characteristics like spindle-shaped morphology and increased migratory and invasive potential.[15] Dissemination of cells from primary tissue and migration to a different site via EMT is essential for organ formation during embryonic development.[16] Cancer cells, however, hijack this process for metastasis to distant organs.[16] EMT not only facilitates metastasis but also promotes other aspects of cancer progression.[16,17] EMT effectors were shown to inhibit oncogene-induced senescence in transformed cells.[18,19] Moreover, EMT inhibits

detachment-induced cell death (anoikis), promotes immune tolerance, and confers stem cell characteristics to cancer cells.[20–25] In addition, an increasing number of studies demonstrate EMT to be a predictor of therapy resistance in breast cancer patients.[23,26,27] Thus, inhibiting the molecular pathways that regulate EMT will provide an effective means of preventing breast cancer metastasis by targeting cell plasticity at the primary site. Therapies targeting EMT should greatly improve the clinical outcome because tumor aggressiveness is the major cause of breast cancer-related deaths.

In this study, we investigated the role of PKCε in the regulation of EMT in breast cancer. Using gain of function and loss of function approaches, we show that PKCε is an important mediator of EMT, migration, and anoikis resistance and that it partially mediates transforming growth factor β (TGFβ)-induced EMT. Thus, EMT may be the underlying mechanism by which PKCε mediates the progression of breast cancer.

Materials and Methods

Materials. Human recombinant TGFβ1 was purchased from R&D Systems (Minneapolis, MN). Monoclonal antibody to E-cadherin was obtained from BD Biosciences, and monoclonal antibody to ZO-1 was from Invitrogen (Carlsbad, CA). Monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and polyclonal antibody against PKCε and Twist were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to vimentin, claudin-1, cleaved caspase-3, and cleaved caspase-7 were from Cell Signaling Technology (Danvers, MA). Monoclonal antibody against β-actin was purchased from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were purchased from Jackson Immuno Research Lab Inc (Bar Harbor, ME). Alexa Fluor® 488 goat anti-mouse and Alexa Fluor® 568 goat anti-rabbit antibodies were from Invitrogen (Carlsbad, CA), and 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR). ON-TARGETplus control and PKCε siRNAs were purchased from Dharmacon (Lafayette, CO). Polyvinylidene difluoride membrane was obtained from Millipore (Billerica, MA). Enhanced chemiluminescence detection kit was from Amersham.

Cell culture and transfection. MCF-10A cells were cultured in DMEM/F-12 supplemented with 5% horse serum, 20 ng/mL epidermal growth factor (EGF), 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin.[28] MDA-MB-231 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 5% fetal bovine serum (FBS) and 2 mM glutamine. Cells were incubated in a humidified incubator at 37 °C with 95% air and 5% CO_2. siRNA transfections were performed using Lipofectamine® RNAiMAX reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PKCε was subcloned into pLNCX2 retroviral vector, and virus particles containing pLNCX2 or pLNCX2/PKCε were generated by triple transfection of HEK-293T cells using gag–pol vector (pEQ-PAM(-E)) and envelope vector (pEQ-Env(A)). MCF-10A cells were infected with retroviral particles containing pLNCX2 or pLNCX2/PKCε and were selected using G418 (Geneticin).

Suspension culture. Poly(2-hydroxyethyl methacrylate) (polyHEMA) (Sigma, St. Louis, MO) was dissolved in 95% ethanol at a concentration of 50 mg/mL. The tissue culture plates were coated with a thin layer of polyHEMA solution and allowed to dry in an incubator for 30 minutes. The coated plates were washed with PBS before use. The cells were harvested and plated on the polyHEMA-coated plates for two days.

Boyden chamber chemotaxis assay. Control non-targeting siRNA or PKCε siRNA transfected cells were added to the top chambers of the transwell (Greiner Bio-One, Monroe, NC). A complete MCF-10 A culture medium, supplemented with 100 ng/mL EGF, was added to the lower chamber, and the plate was incubated at 37 °C for 12 hours. In the case of MDA-MB-231 cells, RPMI + 10% FBS was added to the lower chamber and cells were allowed to migrate for four hours at 37 °C. The migrated cells were fixed in 3% paraformaldehyde and stained with DAPI. The stained cells were imaged using a Zeiss Axiovert 40 inverted fluorescence microscope.

Immunocytochemistry. The cells were fixed in 3% paraformaldehyde at room temperature, permeabilized in 0.15% Triton X-100 at 4 °C, and blocked in 5% bovine serum albumin (BSA) at room temperature for one hour. The cells were incubated with primary antibodies at a dilution of 1:800 overnight at 4 °C and with secondary antibodies at a dilution of 1:600 at room temperature for one hour. Cells were mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL). Images were collected using a fluorescence microscope.

Western blot analysis. Equivalent amounts of total cellular extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a polyvinylidene fluoride membrane. Immunoblotting was performed as described previously.[29]

RT-PCR analysis. The total RNA was isolated from cells using TRI reagent RT from Molecular Research Center (Cincinnati, OH) as per the manufacturer's protocol. cDNA was synthesized from the total RNA using ImProm-II reverse transcriptase (Promega, Madison, WI). PCR reactions were performed using the following primers: *SNAI1*: forward, 5′-ACCTTCCAGCAGCCCTACGACC-3′; reverse, GTGTGGCTTCGGATGTGCATC and *ACTNB*: forward, 5′-TAC AAT GAG CTG CGT GTG GCT-3′; reverse, 5′-ATC CAC ATC TGC TGG AAG GTG GA-3′. The cycling conditions consisted of 25 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 10 minutes. The amplicons were resolved on a 2% agarose gel.

Results and Discussion

PKCε overexpression induced EMT in MCF-10A cells. Tumor metastasis is the major cause of morbidity in

breast cancer patients.[30] Most breast tumors are of epithelial origin and therefore employ EMT as an early step during metastasis to distant organs.[17] As PKCε was associated with aggressiveness in breast cancer, we examined whether PKCε promotes EMT.

To examine the effect of PKCε on EMT, we overexpressed PKCε in non-malignant breast epithelial cell line MCF-10A. As shown in Figure 1, MCF-10A cells selected for empty vector (MCF-10A/pLNCX2) had cobblestone morphology, which is a characteristic of epithelial cells. However, PKCε-overexpressing cells (MCF-10A/PKCε) had elongated, fibroblast-type, spindle morphology. These morphological changes are reminiscent of EMT.

The phenotypical changes in EMT are associated with a corresponding change in molecular markers such as a loss of adhesion proteins E-cadherin, zonula occludens (ZO)-1, and claudin-1, and a gain in proteins abundant in mesenchymal cells such as vimentin, *N*-cadherin, and fibronectin.[16] In particular, the loss of E-cadherin from cell to cell contacts is a prominent feature of EMT.[16] Given the morphological alterations apparent upon PKCε overexpression, we compared the immunostaining of E-cadherin in MCF-10A/pLNCX2 versus MCF-10A/PKCε cells. As shown in Figure 2, E-cadherin was enriched at the cell–cell contacts in MCF-10A/pLNCX2 cells. However, E-cadherin expression was markedly reduced in PKCε-overexpressing cells.

We further determined the effect of PKCε overexpression on several EMT markers using Western blot analysis. As shown in Figure 3, PKCε overexpression resulted in a marked decrease in epithelial markers, namely E-cadherin, ZO-1, and claudin–1. There was a concomitant increase in vimentin, which is a marker for mesenchymal cells. These results show that PKCε overexpression is sufficient to trigger EMT in MCF-10A cells.

PKCε knockdown inhibited cell migration. Increased cell motility is the hallmark of EMT.[16] Because PKCε promoted EMT in MCF-10A cells, we examined whether it is also required for migration in these cells. MCF-10A cells show a strong migratory response to EGF and therefore are suited for migration studies.[31] We examined the effect of PKCε knockdown on EGF-induced migration of MCF-10A cells. We used ON-TARGETplus siRNAs, which are reported to be highly specific to the target sequence. We compared the ability of four different PKCε siRNAs (6, 7, 8, and 9) to deplete PKCε in different cell lines and found that siRNA 6 caused the maximum depletion of PKCε, followed by siRNA 8 (data not shown). We therefore used these two siRNAs for our study. EGF-induced cell migration was examined using a Boyden chamber chemotactic assay. As seen in Figure 4, PKCε knockdown (Fig. 4A) substantially reduced MCF-10A cell migration (Fig. 4B).

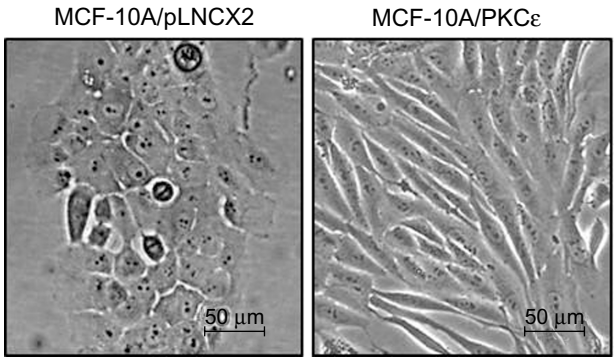

Figure 1. PKCε overexpression led to spindle-shaped morphology in MCF-10A cells. MCF-10A cells stably transfected with pLNCX2 or pLNCX2/PKCε were grown in monolayer culture. Phase contrast images were collected using inverted light microscope.

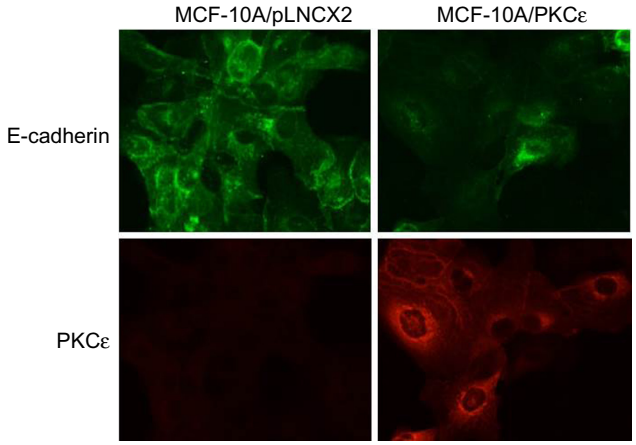

Figure 2. PKCε overexpression decreased E-cadherin in MCF-10A cells. MCF-10A/pLNCX2 and MCF-10A/PKCε cells were subjected to immunofluorescence using antibodies against E-cadherin (green) and PKCε (red) and visualized using Zeiss Axiovert 40 inverted fluorescence microscope as described under Materials and Methods.

Overexpressed PKCε protected MCF-10A cells from anoikis. Another important characteristic of EMT is anoikis resistance.[25] Adherent cells, when deprived of matrix attachment, undergo apoptosis, which is referred to as anoikis.[32] Metastatic cells, however, acquire the ability to resist anoikis to survive in the absence of a matrix attachment.[32] EMT helps cells not only to disseminate from the primary tissue and to migrate through the stroma but also to promote anoikis resistance.[25,33] Because PKCε promoted EMT, we examined its ability to protect MCF-10A cells from detachment-induced apoptosis. We compared the extent of apoptosis in MCF-10A/pLNCX2 versus MCF-10A/PKCε cells in a suspension culture on poly-HEMA-coated plates while cells plated on normal tissue culture plates served as the adherent control. Apoptosis was determined by the extent of PARP, caspase-3, and caspase-7 cleavage. As seen in Figure 5, the cleavage of caspase-3, caspase-7, as well as PARP is substantially reduced in PKCε-overexpressing cells as compared to MCF-10 A/pLNCX2 cells. Thus, PKCε overexpression protected MCF-10 A cells from anoikis.

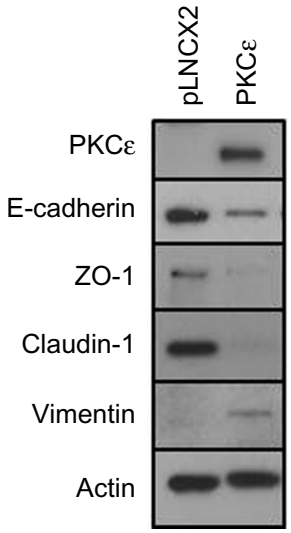

Figure 3. PKCε overexpression led to increase in mesenchymal and decrease in epithelial markers in MCF-10A cells. MCF-10A/pLNCX2 and MCF-10A/PKCε cells were grown in monolayer culture for 48 hours. Total cell lysates were subjected to Western blot analysis using indicated antibodies. Actin was used as the loading control. The results are representative of two to five independent experiments.

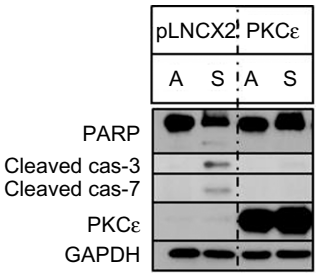

Figure 5. PKCε overexpression protected MCF-10A cells from anoikis. MCF-10A/pLNCX2 and MCF-10A/PKCε cells were grown in adherent (A) or in suspension (S) culture conditions for two days as described under Materials and Methods. Total cell lysates were subjected to Western blotting using indicated antibodies. GAPDH was used as the loading control.

PKCε knockdown reversed TGFβ-induced EMT in MCF-10A cells. A prominent inducer of EMT during embryonic development as well as in cancer cells is TGFβ.[34] TGFβ mediates the aggressiveness of breast tumors and is indicative of poor disease outcome.[34] Because PKCε positively regulated EMT, we examined if it had a role in TGFβ-induced EMT. We therefore examined the consequence of PKCε knockdown on increase in EMT markers by TGFβ. As shown in Figure 6A, treatment with TGFβ increased vimentin in control siRNA transfected MCF-10A cells. However, the ability of TGFβ to increase vimentin was compromised in PKCε-depleted MCF-10A cells, indicating that PKCε, at least partially, mediated TGFβ-induced EMT.

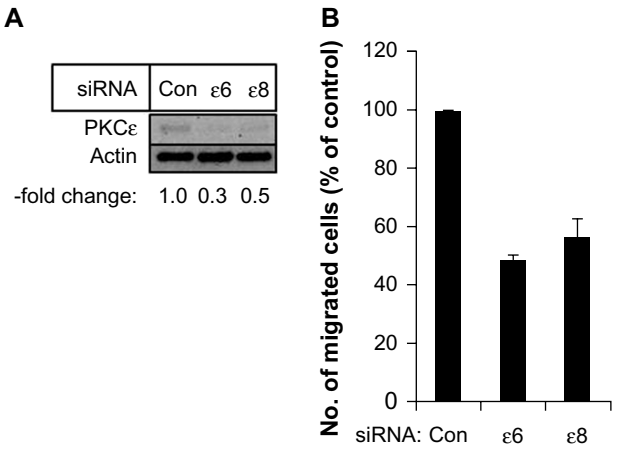

Figure 4. PKCε knockdown decreased cell migration in MCF-10A cells. MCF-10A cells were transfected with two different siRNAs (ε6 and ε8) against PKCε or with non-targeting control siRNA (con). (**A**) Total cell lysates were subjected to Western blot analysis using PKCε antibody. Actin was used as the loading control. Intensity of PKCε was quantified using Image J software and normalized to actin. The numbers represent the fold change in PKCε levels as compared to control. (**B**) Cell migration was examined using the Boyden chamber chemotaxis assay described under Materials and Methods. The bar graphs represent percentage of cells migrated with respect to control siRNA transfected cells. The results are representative of two independent experiments.

EMT induction causes a drastic change in a cell's cytoskeleton, and therefore this process is associated with major transcriptional reprogramming. Most EMT induction pathways, however, converge onto a handful of transcription factors.[16] The key transcriptional regulator of TGFβ-induced EMT is Snail.[35,36] Snail is a zinc finger-containing transcription factor that represses the transcription of E-cadherin and other adhesion-related genes but causes induction of vimentin and other fibroblast-specific genes.[35] Depending on the cellular context, the basic helix–loop–helix transcription factor Twist may also be induced by TGFβ.[37] However, Twist was reported to have a role in EMT maintenance rather than the induction of EMT by TGFβ in breast epithelial cells.[37]

Because PKCε mediated EMT in response to TGFβ, we examined the effect of PKCε depletion on the induction of the transcription factors that execute TGFβ-induced EMT. As shown in Figure 6A, Snail was undetectable in MCF-10A cells but TGFβ treatment caused a marked induction in Snail. However, the depletion of PKCε inhibited the ability of TGFβ to induce Snail protein levels (Fig. 6A and B). To determine if PKCε affects Snail expression at the transcriptional level, we examined the consequence of PKCε knockdown on *Snail* mRNA by RT-PCR. As shown in Figure 6C, knockdown of PKCε decreased *Snail* mRNA expression by approximately 40%. We did not see a noticeable change in Twist protein levels by TGFβ treatment in our experimental conditions. This is consistent with the previous reports that Twist is dispensable for EMT induction by TGFβ.[37] These results show that PKCε plays an important role in the induction of EMT by TGFβ.

The effect of PKCε on EMT was corroborated by its effect on TGFβ-induced morphological changes in MCF-10A cells.

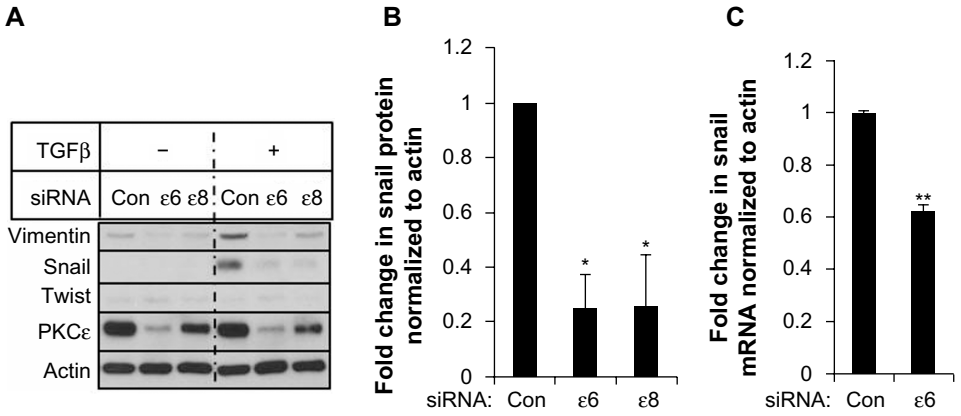

Figure 6. PKCε depletion reversed TGFβ-induced EMT in MCF-10A cells. MCF-10A cells transfected with either non-targeting control (con) or PKCε siRNAs (ε6 and ε8) were treated with or without 5 ng/mL TGFβ for 16 hours. (**A**) Total cell lysates were subjected to Western blot analysis using indicated antibodies. Actin was used as the loading control. (**B**) Intensity of Snail was quantified using Image J software and normalized to actin. The data represents fold decrease in TGFβ-induced Snail protein levels in PKCε siRNA cells as compared to control siRNA cells. (**C**) TGFβ-induced *Snail* mRNA levels were examined in cells transfected with control (con) or PKCε (ε6) siRNAs using RT-PCR as described in Materials and Methods. Intensity of *Snail* mRNA was quantified using Image J software and normalized to actin. The data represent fold change in TGFβ-induced *Snail* mRNA levels with respect to control siRNA samples. Each bar represents mean ± SD of three independent experiments.
Notes: **P* < 0.05 and ***P* < 0.01 using paired Student's *t*-test.

As shown in Figure 7, TGFβ treatment resulted in spindle/mesenchymal morphology in MCF-10A cells transfected with control siRNA. The induction of mesenchymal features by TGFβ was however compromised in PKCε-depleted cells. Thus, PKCε is an important mediator of TGFβ-induced EMT.

As MDA-MB-231 breast cancer cells exhibit a mesenchymal phenotype and express high levels of PKCε, we examined if a PKCε knockdown would reverse EMT in these cells. The silencing of PKCε by siRNA 6 caused a significant increase in the epithelial marker E-cadherin (Fig. 8A). We were unable to detect a change in mesenchymal to epithelial morphology. It is conceivable that other factors in addition to PKCε contribute to the maintenance of mesenchymal morphology in these cells and therefore the depletion of PKCε alone was not sufficient to cause a morphological reversion. However, PKCε knockdown decreased cell migration, another characteristic of EMT (Fig. 8C). Given that the overexpression of PKCε in non-tumorigenic MCF-10A cells increases cell migration and that the knockdown of PKCε in highly aggressive metastatic MDA-MB-231 cells inhibits cell migration suggest that PKCε can be targeted to inhibit metastatic potential. However, to establish clinical significance, future studies should examine the role of PKCε in EMT using xenograft models.

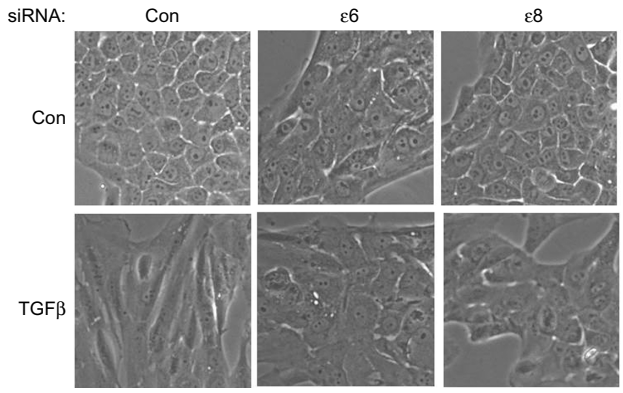

Figure 7. PKCε knockdown reversed TGFβ-induced morphological changes in MCF-10A cells. MCF-10A cells were transfected with siRNAs as in Figure 6 and treated with 5 ng/mL TGFβ for three days. Phase contrast images were collected using inverted light microscope.

In summary, we have shown that PKCε is an important inducer of EMT in breast cancer cells. Although we showed PKCε to be working downstream of TGFβ, an earlier study demonstrated PKCε's role in the production of active TGFβ.[38] Thus, there is a possibility of PKCε and TGFβ working in a positive feedback loop to promote EMT. We have also shown that PKCε positively regulates the expression of Snail, an important mediator of EMT. We have previously shown that PKCε acts upstream of Akt,[39] which is known to upregulate Snail via NF-κB.[40] Thus, PKCε may increase Snail transcription via the Akt/NF-κB pathway. Future studies should discern the mechanism(s) by which PKCε regulates Snail transcription. Other likely mechanisms by which PKCε could induce EMT are via its downstream targets RhoC[5] and STAT3,[39] which have been shown to regulate EMT.[40,41] A recent study demonstrated a role of PKCε in the regulation of stem cell marker Nanog.[42] Because EMT is closely associated with the stem cell phenotype,[22,24] PKCε's role in stemness could be examined in the future. Thus, PKCε-mediated regulation of EMT underscores the importance of PKCε in breast cancer and its potential as a therapeutic target for cancer treatment.

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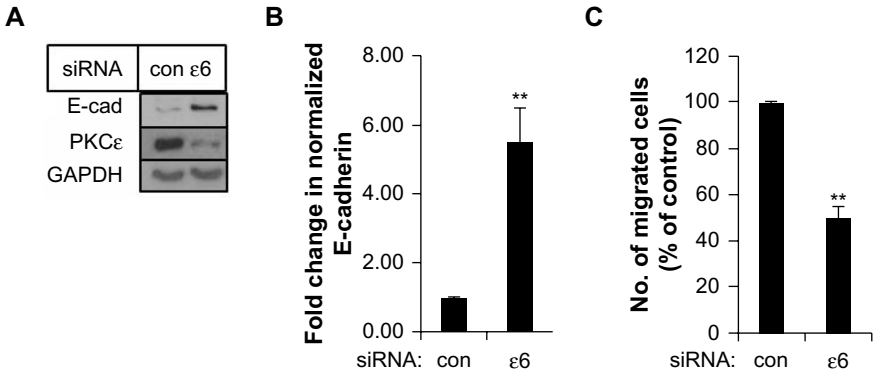

Figure 8. PKCε depletion increased E-cadherin and reduced cell migration in MDA-MB-231 cells. MDA-MB-231 cells were transfected with control non-targeting (con) or PKCε (ε6) siRNA. (**A**) Total cell lysates were subjected to Western blot analysis using E-cadherin antibody. GAPDH was used as the loading control. (**B**) The intensity of E-cadherin levels was examined using Image J software and normalized to GAPDH. The bar graphs represent fold change in E-cadherin protein levels as compared to control siRNA transfected cells. The data are representative of four independent experiments. (**C**) Migration was examined using Boyden chamber chemotaxis assay as described under Materials and Methods. The bar graphs represent the percentage of cells migrated with respect to the control siRNA transfected cells. The data are representative of three independent experiments. **, $P < 0.01$ using paired Student's *t*-test.

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Author Contributions

AB and KJ conceived and designed the experiments. AB and KJ analyzed the data. KJ wrote the first draft of the manuscript. AB and KJ contributed to the writing of the manuscript. AB and KJ agreed with manuscript results and conclusions, jointly developed the structure and arguments for the paper, and made critical revisions. All authors reviewed and approved the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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