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PKC ζ Promotes Breast Cancer Invasion by Regulating Expression of E-cadherin and Zonula Occludens-1 (ZO-1) via NF κ B-p65

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Atypical Protein Kinase C zeta (PKC ζ) forms Partitioning-defective (PAR) polarity complex for apico-basal distribution of membrane proteins essential to maintain normal cellular junctional complexes and tissue homeostasis. Consistently, tumor suppressive role of PKC ζ has been established for multiple human cancers. However, recent studies also indicate pro-oncogenic function of PKC ζ without firm understanding of detailed molecular mechanism. Here we report a possible mechanism of oncogenic PKC ζ signaling in the context of breast cancer. We observed that depletion of PKC ζ promotes epithelial morphology in mesenchymal-like MDA-MB-231 cells. The induction of epithelial morphology is associated with significant upregulation of adherens junction (AJ) protein E-cadherin and tight junction (TJ) protein Zonula Occludens-1 (ZO-1). Functionally, depletion of PKC ζ significantly inhibits invasion and metastatic progression. Consistently, we observed higher expression and activation of PKC ζ signaling in invasive and metastatic breast cancers compared to non-invasive diseases. Mechanistically, an oncogenic PKC ζ -NF κ B-p65 signaling node might be involved to suppress E-cadherin and ZO-1 expression and ectopic expression of a constitutively active form of NF κ B-p65 (S536E-NF κ B-p65) significantly rescues invasive potential of PKC ζ -depleted breast cancer cells. Thus, our study discovered a PKC ζ -NF κ B-p65 signaling pathway might be involved to alter cellular junctional dynamics for breast cancer invasive progression.

Breast cancer is one of the leading causes of cancer related death in women worldwide¹. Clinically, breast cancer is considered as a heterogeneous disease and heterogeneity of breast cancer disease provides a great challenge for developing successful therapy. Comprehensive gene expression profiling indicated at least three major subtypes of breast cancer – luminal, HER2-positive, and basal-like^{2–5}. These subtypes of breast cancer are significantly different in clinical characteristics such as associated risk factors, preferable sites of metastasis, and expression of targetable surface receptors such as estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (ERBB2/HER2)⁶. While the luminal (ER/PR positive) and the HER2-positive (with amplified HER2 expression) breast cancer patients could be benefited from endocrine and HER2-targeted therapies⁷, chemotherapy is the only therapeutic option currently available for basal-like (also called triple negative breast cancers or TNBC, no expression of ER, PR, and HER2)⁸ breast tumors.

During invasive progression, breast cancer cells undergo sequential developmental alterations and eventually acquire the capacity to form metastatic growth for tumor recurrence^{9–11}. Similar to most other cancers, metastases are also considered as major reason for breast cancer-related deaths^{9,12–14} and

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development of recurrence/metastases can occur even after the initial successful therapeutic responses¹⁵. Thus, breast cancer patients are always at risk to develop recurrence/metastasis throughout their life¹⁵. As a result, identification of signaling pathways to inhibit invasive and metastatic properties of breast cancer cells is always critical for the development of successful therapies.

Invasive progression of breast cancer is initiated through the process called epithelial-to-mesenchymal transition (EMT), a developmental switch well known for tissue remodeling during normal embryonic development^{11,16,17}. The reverse process of EMT is known as mesenchymal-to-epithelial transition (MET) and characterized by the transition of mesenchymal cells to acquire epithelial characteristics¹⁸. During EMT, polarized epithelial cells transform to a highly motile mesenchymal phenotype with rearranged cytoskeleton via the loss of cell polarity. Intercellular junctions such as adherens junctions (AJ), tight junctions (TJ), gap junctions, and desmosomes are responsible to maintain cell polarity in epithelial tissues and these intercellular junctions are disrupted during the process of EMT^{17–19}. Highly conserved polarity proteins including the members of the PAR polarity complex regulate proper distributions of these cellular junctional complexes in the plasma membrane^{20,21}. The PAR polarity complexes contain PAR3, PAR6, and aPKC isozymes PKC ζ and PKC λ/ι and activation of aPKC signaling is essential for establishing functional PAR polarity complexes at the apical-lateral border in epithelial cells^{22,23}. In vertebrate epithelial cells, apical-lateral border is structurally defined by TJs, which prevents diffusion of the membrane proteins to ensure apical and basal polarity^{24–26}. Failure to maintain correct apico-basal polarity due to disruption of PAR polarity complex or down-regulation of polarity and/or junctional proteins are implicated in promoting EMT and tissue infiltration of breast and other cancers of epithelial origin^{20,27–30}.

Atypical PKCs, PKC ζ and PKC λ/ι , are the member of PKC family of serine/threonine kinases, which are involved in multiple signal transduction pathways. Activation of aPKCs is independent of both Ca²⁺ and diacylglycerol compared to conventional PKC (cPKCs; PKC α , PKC β I, PKC β II, and PKC γ) and novel PKC (nPKCs; PKC δ , PKC ϵ , PKC η , and PKC θ) subfamilies. The conventional PKC members are activated by diacylglycerol and Ca²⁺-dependent phospholipid binding to their conserved domains and the novel PKC members are activated only by diacylglycerol and phospholipids, but independent of Ca²⁺ ion^{31,32}. Although aPKC molecules play a central role to maintain epithelial cell polarity, multiple studies showed that aPKC signaling often induce invasion and metaspromotes breast cancer invasive progressing breast cancer^{33–42}. Recently, we and other laboratories showed that aPKC isozyme, PKC λ/ι promotes breast cancer invasive progression^{33,37,43,44}. On the other hand, the other aPKC isozyme, PKC ζ , has both tumor suppressive and tumor promoting functions including for breast cancer development^{45–52}. The PKC ζ found to be one of the frequently mutated genes associated with TNBC⁵³ and reported to regulate proliferation and chemokine-triggered migration of breast cancer cells^{42,54–58}. In contrast, over-expression of PKC ζ also showed growth inhibition of human MDA-MB-468 breast cancer cells⁵⁹. However, these cellular functions of PKC ζ are concluded based on the use of non-specific small molecule inhibitors and/or pseudo-substrate peptides⁶⁰ and overall, the molecular mechanisms of PKC ζ -mediated regulation of breast cancer disease progression are largely unknown.

Here, we investigated the role of PKC ζ in breast cancer development. We found that PKC ζ signaling is highly active in invasive and metastatic breast cancers compared to non-invasive ductal carcinoma *in situ* (DCIS) and depletion of PKC ζ inhibits invasion and metastasis of breast cancer cells in experimental animal models. Interestingly, we observed that loss of PKC ζ promotes MET in highly metastatic, mesenchymal-like MDA-MB-231 cells with induction of cell-cell adhesion. Our molecular analyses indicate that depletion of PKC ζ inhibits nuclear localization of NF κ B-p65 leading to elevated expressions of epithelial cell specific adherens junction protein E-cadherin and tight junction protein ZO1. We also found that ectopic expression of a constitutively active form of NF κ B-p65 (S536E-NF κ B-p65) significantly rescues invasive potential of PKC ζ -depleted breast cancer cells. Collectively, our results provide evidence for an oncogenic PKC ζ -NF κ B-p65 signaling node that suppresses E-cadherin and ZO-1 expression in breast cancer cells and might promote EMT to facilitate *in situ* to invasive transition of breast cancers.

Results

PKC ζ Regulates Cell-Cell Adhesion in the Absence of Functional PAR Polarity Complex. In epithelial cells, PKC ζ serves as an effector of the conserved PAR polarity complex, which is located at the plasma membrane domains for the regulation of apical-basal polarity by stimulating biogenesis of cell-cell junctions^{61,62}. Thus, to investigate PKC ζ signaling in breast cancer, we have tested expression and localization of PKC ζ in multiple human breast cancer cell lines including luminal MCF-7 and three basal-like cells such as MDA-MB-231, MDA-MB-468, and HCC-1937^{19,63–67}. PKC ζ is abundantly expressed in all cell lines (Supplementary Figure S1a). However, we noticed differential PKC ζ localization patterns. In basal-like cells, expression of PKC ζ indicated a diffused localization pattern without any prominent distribution at the plasma membrane domains (Fig. 1a). On the other hand, expression of PKC ζ in luminal MCF-7 cells was observed predominantly at the plasma membrane domains (Fig. 1a) and consistent with formation of PAR polarity complex as reported earlier^{20,24,26,30,49}. Interestingly, phospho-PKC ζ (phosphorylated at T410) expression also showed similar trends (Fig. 1b). Since phosphorylation at T-410 is essential for kinase activity of PKC ζ ^{49,68}, our observations indicate the presence of active PKC ζ signaling

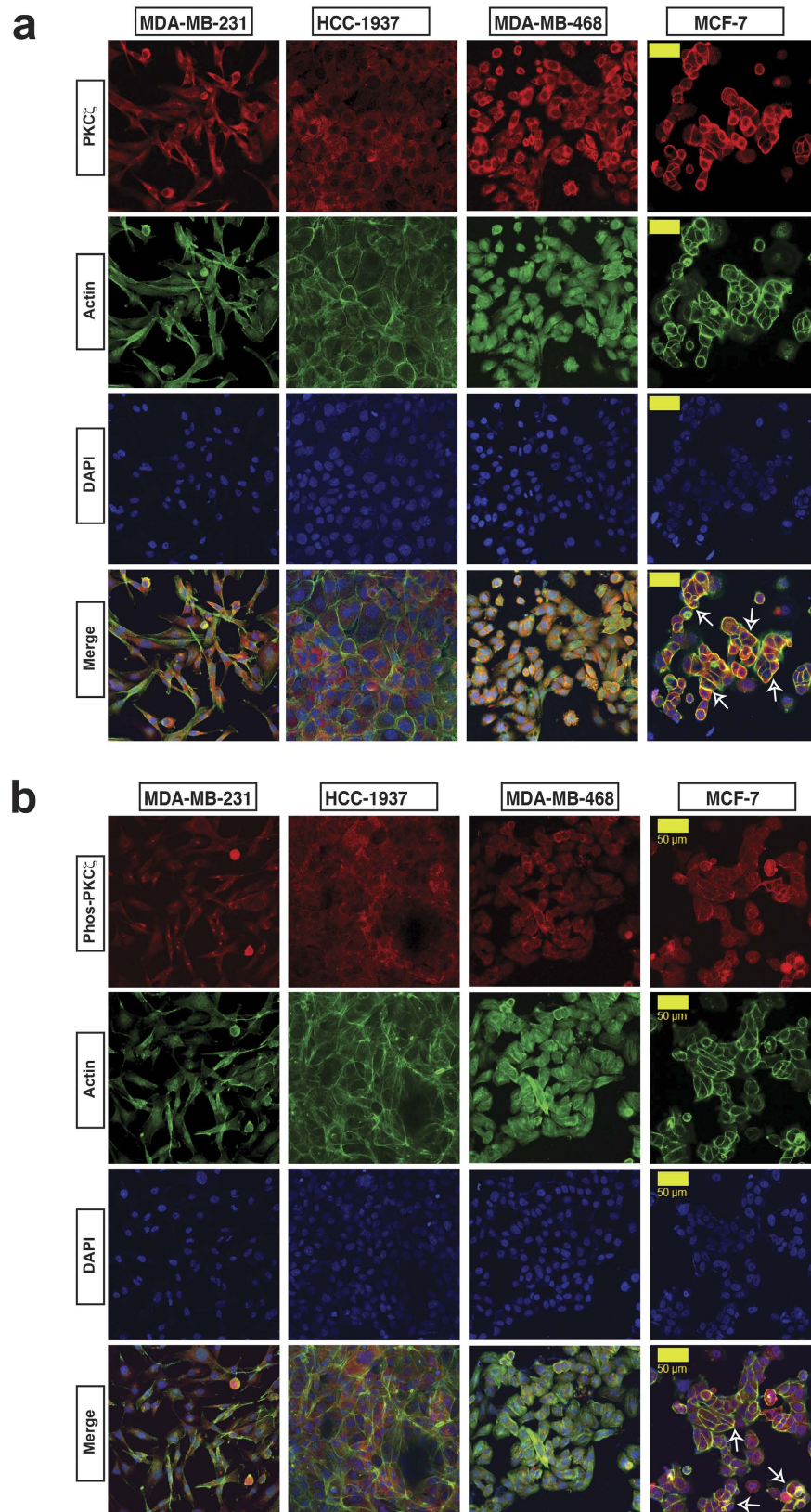


Figure 1. PKC ζ Signaling in Breast Cancer Cells. Expression of PKC ζ (a) and phospho-PKC ζ (b) in basal-like MDA-MB-231, HCC-1937, and MDA-MB-468 cells in comparison with luminal MCF-7 cells. Expression of PKC ζ and phospho-PKC ζ showed in red, actin in green and nuclear staining showed by DAPI. Yellow scale bar 50 μ m. White arrows indicated localization of PKC ζ and phospho-PKC ζ at the plasma membrane domains of MCF-7 cells.

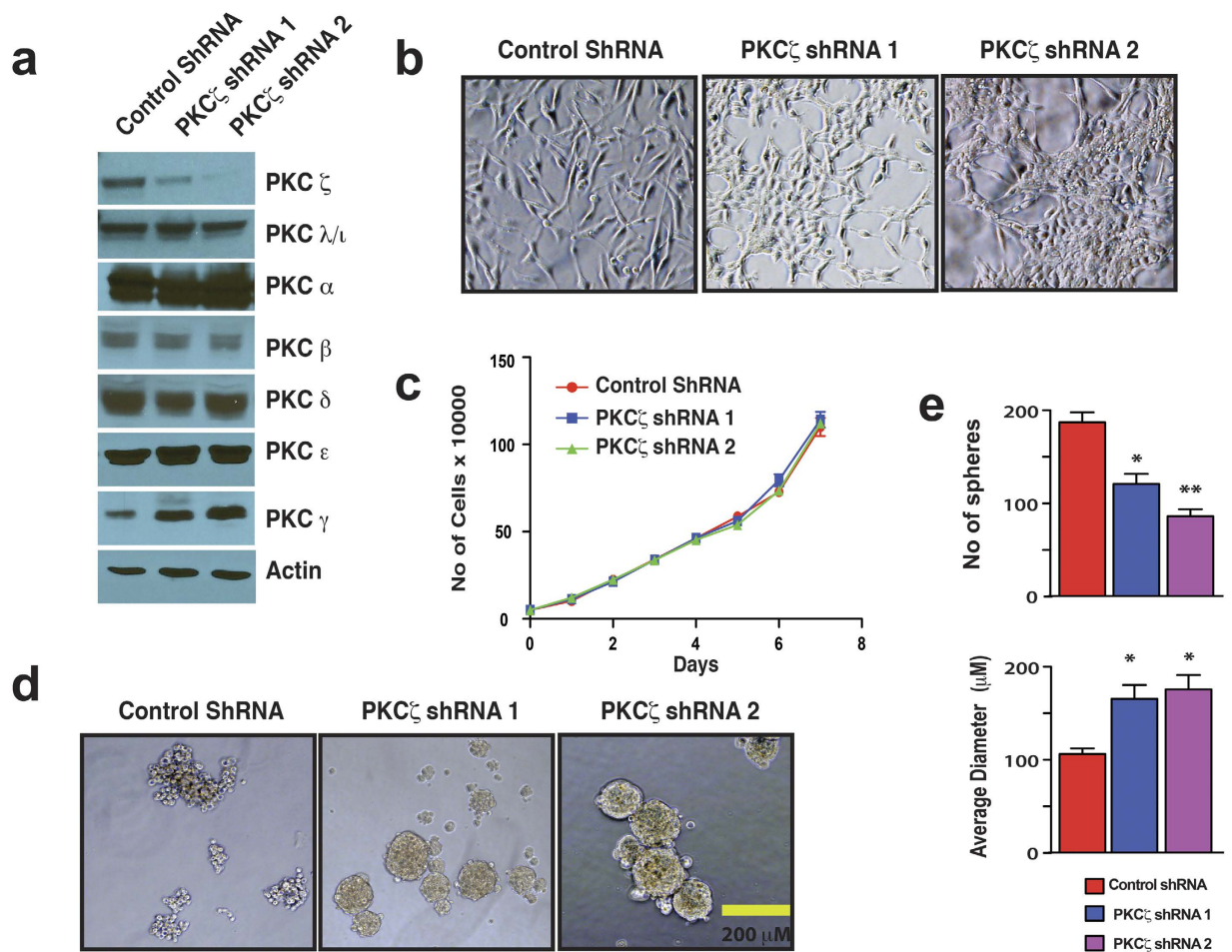


Figure 2. PKC ζ Signaling Regulates Cell-Cell Adhesion in Breast Cancer Cells. (a) Western blot analysis indicating specific knockdown of PKC ζ in MDA-MB-231 cells. (b) Morphologies of MDA-MB-231 cell with and without PKC ζ depletion. (c) Specific depletion of PKC ζ has no effect on cell proliferation. (d) Morphologies of MDA-MB-231 cell aggregates with and without PKC ζ depletion. (e) Quantification of number of MDA-MB-231 cell aggregates and their size with and without PKC ζ depletion. Results represent means \pm S.E.M. *P* values were calculated one-way ANOVA with Bonferroni post-test. **P* values \leq 0.01, ***P* values \leq 0.001, ****P* values \leq 0.0001.

in all tested breast cancer cells. However, the absence of PKC ζ and phospho-PKC ζ in the plasma membrane of basal-like cells indicate the absence of PKC ζ -containing PAR polarity complexes (Fig. 1a,b).

Comprehensive gene expression analyses indicate that MDA-MB-231 cells possess a mesenchymal-like phenotype with more stromal-like/fibroblastic character without common epithelial gene set^{19,67}. So, we predicted that functional PAR polarity complexes are absent in MDA-MB-231 cells. To confirm, we investigated expression and localization of PAR3 and PAR6. Interestingly, both PAR3 and PAR6 are expressed in MDA-MB-231 cells, however, they showed diffused expression patterns with localization both in the cytoplasm and the nuclei (Supplementary Figure S1b). The absence of localization of PKC ζ , phospho-PKC ζ , PAR3, and PAR6 at the plasma membrane or at any prominent apical/basal domains of MDA-MB-231 cells strongly supports the absence of PAR polarity complexes.

Based on the expression and cellular localization patterns in the highly invasive mesenchymal-like MDA-MB-231 and other basal-like cells, we hypothesized that PKC ζ might mediate its function independent of PAR polarity complex. Therefore, we specifically depleted PKC ζ in MDA-MB-231 cells via RNA interference (RNAi) (Fig. 2a). Depletion of PKC ζ in MDA-MB-231 cells induced a dramatic morphological alteration and organized them into a highly clustered morphology from scattered, fibroblast-like culture without any effect in cell proliferation rates (Fig. 2b,c). These alterations in morphology indicate more cell-cell contact formation. For confirmation, we tested PKC ζ -depleted cells for conventional cell aggregation assay to assess cell-cell junction formation⁶⁹. We observed that depletion of PKC ζ transformed the appearance of MDA-MB-231 cell aggregates towards more smooth and round-shaped compared to control (Fig. 2d). We also found that PKC ζ -depleted cells generated less

number of cell aggregates, however, the cell aggregates were significantly larger compared to control indicating more intercellular integrity (Fig. 2e). Overall, these results indicate that depletion of PKC ζ induces cell-cell adhesion in MDA-MB-231 cells.

Depletion of PKC ζ Induces Expression of E-cadherin and ZO-1 and Prevents Invasive Potential. In epithelial tissues, cell-cell contacts are regulated by formation of several intercellular junctions including AJs and TJs. Thus, we tested PKC ζ -depleted MDA-MB-231 cells for expression analysis of two junctional proteins - E-cadherin, localized at AJ^{17,70} and ZO-1, localized at TJ to link TJ and cytoskeleton⁷¹. Our analyses indicate that the morphological changes of PKC ζ -depleted MDA-MB-231 cells were indeed associated with upregulation of both E-cadherin and ZO-1 (Fig. 3a,b). We also tested expression of other TJ and AJ proteins such as ZO-2, ZO-3, and Afadin via western blot analysis, but did not observe any significant changes at protein levels (data not shown). Since E-cadherin and ZO-1 are considered as the epithelial markers⁷², we further tested formation of cortical actin in PKC ζ -depleted cells. Immunofluorescence staining revealed formation of cortical actin in PKC ζ -depleted cells (Fig. 3c) confirming cytoskeletal rearrangement consistent with epithelial characteristics¹⁹. Expression analysis of PAR3 in PKC ζ -depleted cells showed punctate appearance throughout the cells whereas PAR6 expression was restricted within the cytoplasm indicating the absence of functional PAR polarity complexes (Supplementary Figure S2a & S2b). These results strongly indicate that depletion of PKC ζ in MDA-MB-231 cells induce MET-like process and promotes epithelial morphology in the absence of functional PAR polarity complexes.

Since the reverse process of MET i.e. EMT is implicated in conferring invasive potential¹⁴, we tested whether induction of epithelial characteristics in PKC ζ -depleted MDA-MB-231 cells is associated with loss of invasiveness. We found that depletion of PKC ζ significantly inhibited invasive potential of MDA-MB-231 cells when tested via wound closure assays (Fig. 3d,e) and matrigel-coated transwell invasion assays (Fig. 3f,g).

Depletion of PKC ζ Inhibits Breast Cancer Metastasis. Next, we tested *in vivo* functional importance of PKC ζ signaling in breast cancer using experimental animal models utilizing MDA-MB-231 cells expressing a luciferase reporter (MDA-MB-231-luc)³³. We selected shRNA clone no 2 to deplete PKC ζ in MDA-MB-231-luc cells for better knockdown efficiency (Fig. 2a). We transplanted MDA-MB-231-luc cells with or without PKC ζ -depletion orthotopically into the 2nd mammary glands of immunodeficient mice, removed primary tumor at five weeks, and observed for spontaneous metastasis at lung for another five weeks³³. We observed that the orthotopic tumors formed by PKC ζ -depleted cells showed nearly 50% reduced primary tumor growth at five weeks compared to control (Fig. 4a,b). Notably, we observed higher expression of ZO-1 and E-cadherin in PKC ζ -depleted xenograft tumors compared to control (Fig. 4c). Both PAR3 and PAR6 polarity proteins showed diffuse expression patterns in the PKC ζ -depleted tumors (Supplementary Figure S3).

Next, we searched for spontaneous lung metastasis five weeks after resection of the primary tumors (i.e. at ten weeks after orthotopic transplantation). We observed lung metastasis in mice transplanted with control MDA-MB-231 cells at ten weeks as reported previously³³. However, no metastatic event was observed in mice transplanted with PKC ζ -depleted MDA-MB-231 cells in that time frame (Fig. 4d). To rule out the possibility that the reduced tumor growth of PKC ζ -depleted cells might be the reason for the lack of lung metastasis, we further tested metastatic potential via lung colonization after intravenous transplantation³³. We have transplanted both control and PKC ζ -depleted MDA-MB-231-luc cells via tail vein and monitored lung colonization for three weeks via bioluminescent imaging (Fig. 5a). We observed dramatic inhibition in lung metastatic colonization with the PKC ζ -depleted cells (Fig. 5b–e). These observations indicate that PKC ζ signaling is critical for breast cancer metastasis *in vivo*.

Human breast cancer shows highly active PKC ζ signaling. Prompted by our *in vitro* and *in vivo* observations, we sought to investigate PKC ζ signaling in human breast cancer samples. Previous reports indicated conflicting findings to correlate PKC ζ expression with breast cancer clinico-pathological characteristics. Studies by Whyte *et al.*⁴⁹ indicated that low levels of PKC ζ mRNA expression are more significantly associated with poor clinical outcome of breast cancer patients including for the ‘poorly differentiated’ tumors. On the other hand, studies by Yin *et al.*⁵⁰ indicated higher expression of PKC ζ associated with advanced clinical stages of breast cancer including larger tumor size, lymph node metastasis, and poor survival rates. For confirmation, we tested expression of both PKC ζ and phospho-PKC ζ in a cohort of human breast cancer samples consisting of normal breasts, ductal carcinoma *in situ* (DCIS), invasive ductal carcinomas (IDCs) of ER-positive, HER2-positive, and TNBC subtypes, and metastatic breast cancers (Fig. 6a,b). Immunohistochemical analyses showed that expression levels of PKC ζ were significantly higher in IDCs and metastatic breast cancers compared to normal breast and non-invasive DCIS samples (Fig. 6a,c,d). Notably, expression of phospho-PKC ζ (phosphorylated at T410), indicative of active PKC ζ signaling, also showed similar trends (Fig. 6b–d). These observations indicate that higher expression and activation of PKC ζ signaling significantly associated with invasive progression of breast cancer. Interestingly, the expression patterns of both PKC ζ and phospho-PKC ζ in TNBC and metastatic breast cancer samples are mainly cytoplasmic with occasional nuclear staining similar to the

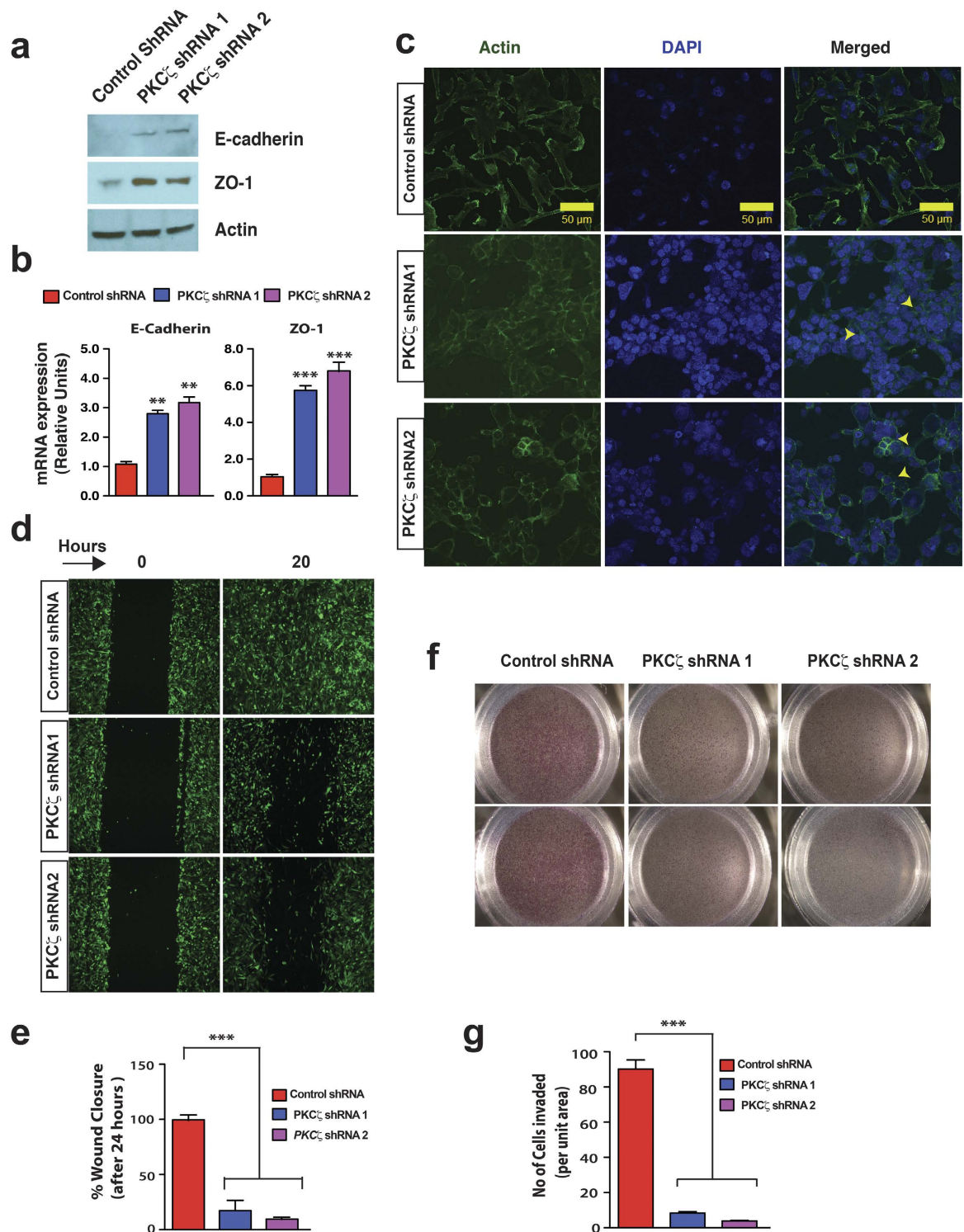


Figure 3. PKC ζ Signaling Regulates Cell-Cell Junction Dynamics and Invasion. (a) Western blot analysis of E-cadherin and ZO-1 after specific knockdown of PKC ζ in MDA-MB-231 cells. (b) Quantitative RT-PCR measurements of E-cadherin and ZO-1 after specific knockdown of PKC ζ in MDA-MB-231 cells. Results represent means \pm S.E.M. *P* values were calculated by two-tailed unpaired Student's *t* test. ***P* values \leq 0.01, ****P* values \leq 0.001. (c) Rearrangement of actin in MDA-MB-231 cells with and without PKC ζ depletion. Specific knockdown of PKC ζ in MDA-MB-231 cells induced appearance of cortical actin showed by yellow arrowheads. Scale bar 50 μ M. (d) Wound closure assays of PKC ζ -depleted MDA-MB-231 cells. (e) Quantification of wound closure assays (*n* = 3). (f) Transwell invasion of PKC ζ -depleted MDA-MB-231 cells. (g) Quantification of invasion assays (each field was divided into 9 unit areas and 3 fields per condition). For all quantifications, results represent means \pm S.E.M. *P* values were calculated by two-tailed unpaired Student's *t* test. ****P* values \leq 0.001.

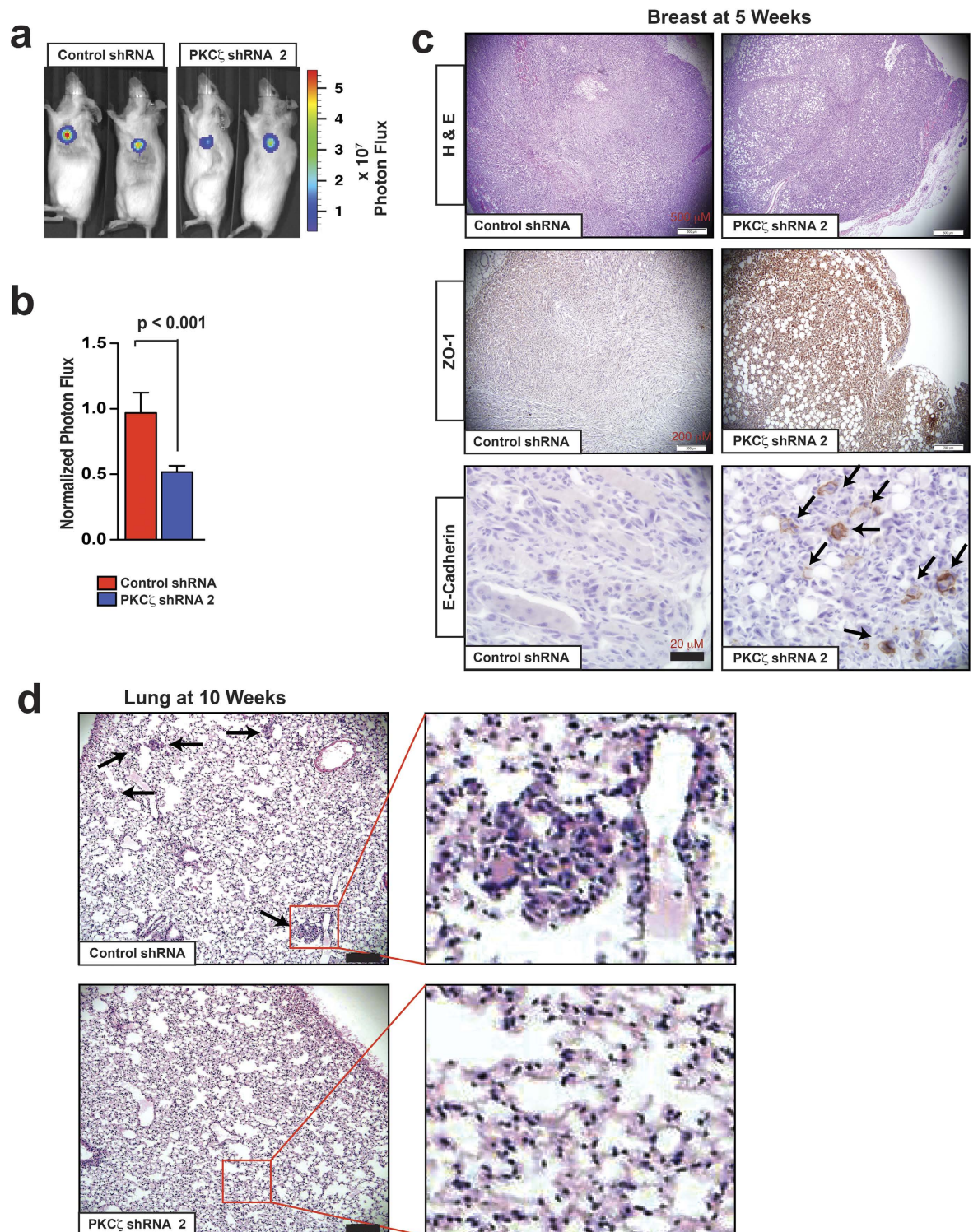


Figure 4. Depletion of PKC ζ Inhibits Breast Cancer Metastasis. (a) Representative whole-animal images at five weeks after orthotopic transplantation of MDA-MB-231-luc cells with and without PKC ζ depletion. (b) Quantification of tumor growth via luminescence measurements at five weeks after orthotopic transplantation ($n = 8$). Results represent means \pm S.E.M. P values were calculated one-way ANOVA with Bonferroni post-test. (c) H&E and immunohistochemical analysis of ZO-1 and E-cadherin expression in xenograft breast tumors removed at five weeks after orthotopic transplantation. The mice were kept alive for another five weeks for spontaneous metastasis to lung. Black arrows indicate expression of E-cadherin. (d) H&E staining of lung tissues at 10 weeks after orthotopic transplantation. Black arrows showed lung colonization and outlined areas by indicated red squares represent the higher magnification images in right panels.

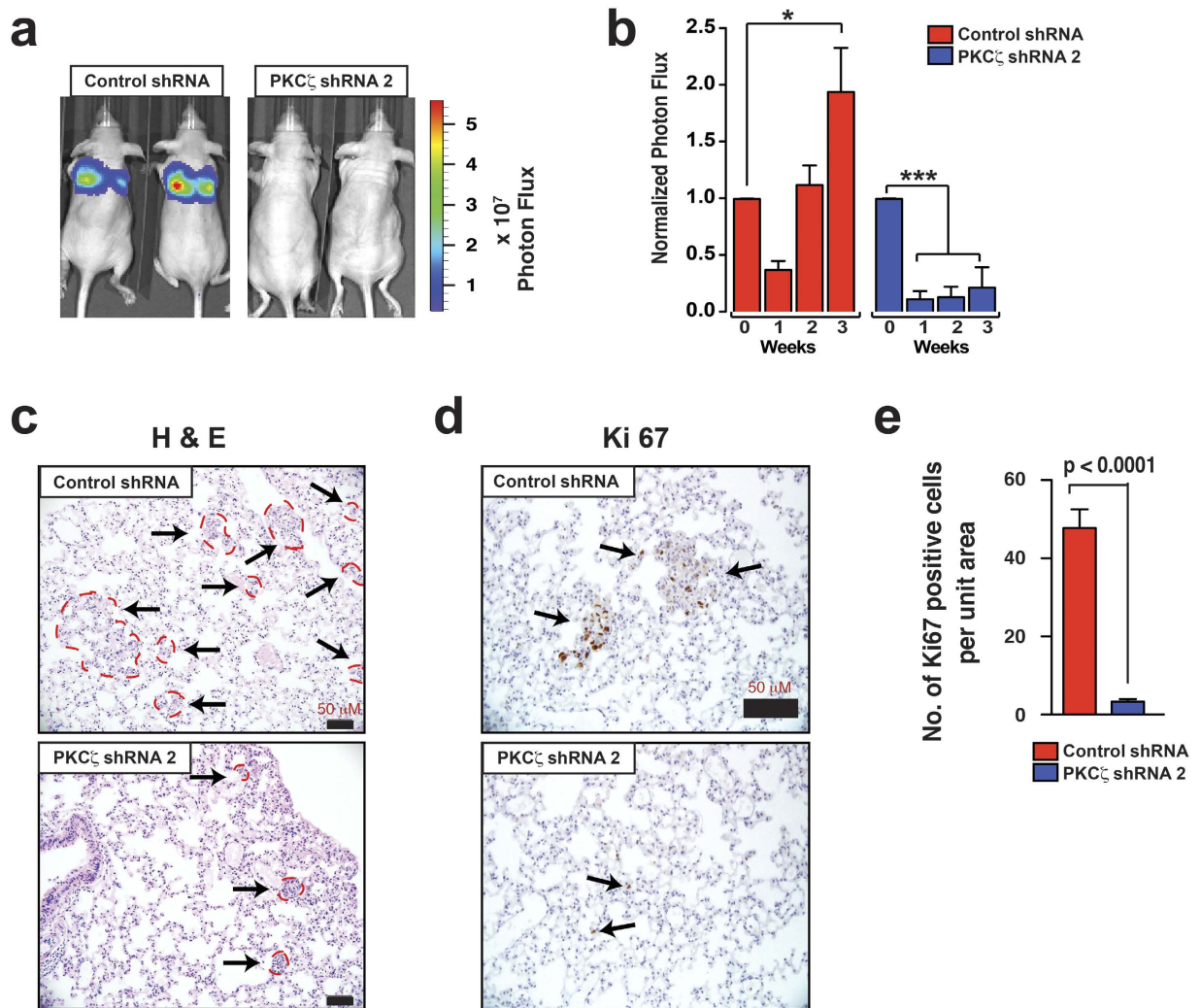


Figure 5. Depletion of PKC ζ Inhibits Lung Metastatic Colonization. (a) Representative whole-animal images at three weeks after intravenous transplantation (via tail vein) of MDA-MB-231-luc cells with and without PKC ζ depletion. (b) Quantification of metastatic lung colonization via luminescence measurements ($n = 5$). Results represent means \pm S.E.M. P values were calculated using one-way ANOVA with Bonferroni post-test. * P values ≤ 0.01 , ** P values ≤ 0.001 , *** P values ≤ 0.0001 . (c) H&E staining of lung tissues three weeks after intravenous transplantation. Indicated regions by perforated red lines and arrows showed lung colonization. Scale bar 50 μ M. (d) Ki-67 staining of lung tissues at three weeks post-transplantation. Scale bar 50 μ M. (e) Quantification of Ki67 staining ($n = 12$). Results represent means \pm S.E.M. P values were calculated by one-way ANOVA with Bonferroni post-test.

immunostaining patterns as observed in xenograft tumor formed by MDA-MB-231 cells (Supplementary Figure S4) and consistent with pro-oncogenic role of PKC ζ signaling in breast cancer.

PKC ζ regulates NF κ B-p65 (RelA) Nuclear Translocation and Transcriptional Activity to Regulate Expression of E-Cadherin and ZO-1. To understand the mechanism of PKC ζ signaling mediated regulation of breast cancer progression in the absence of functional PAR complex, we searched for transcription factors known to be modulated by PKC ζ signaling. We hypothesized that the transcription factor(s), regulated by PKC ζ signaling, might link how depletion of PKC ζ resulted in significant increase of E-cadherin and ZO-1 expression. In multiple cell types, PKC ζ signaling has been shown to regulate NF κ B-p65 (RelA) transcriptional activity^{73–75}. In addition, NF κ B-p65 is known to repress expression of both E-Cadherin and ZO1^{76,77}. Thus, to understand the possible molecular mechanism of PKC ζ -mediated regulation of human breast cancer development, we focused on NF κ B-p65. We performed NF κ B reporter gene analysis and observed repression of endogenous NF κ B transcription activity in the PKC ζ -depleted MDA-MB-231 cells compared to control (Fig. 7a). Furthermore, we observed a dramatic reduction of NF κ B-p65 nuclear localization (Fig. 7b–d). Importantly, depletion

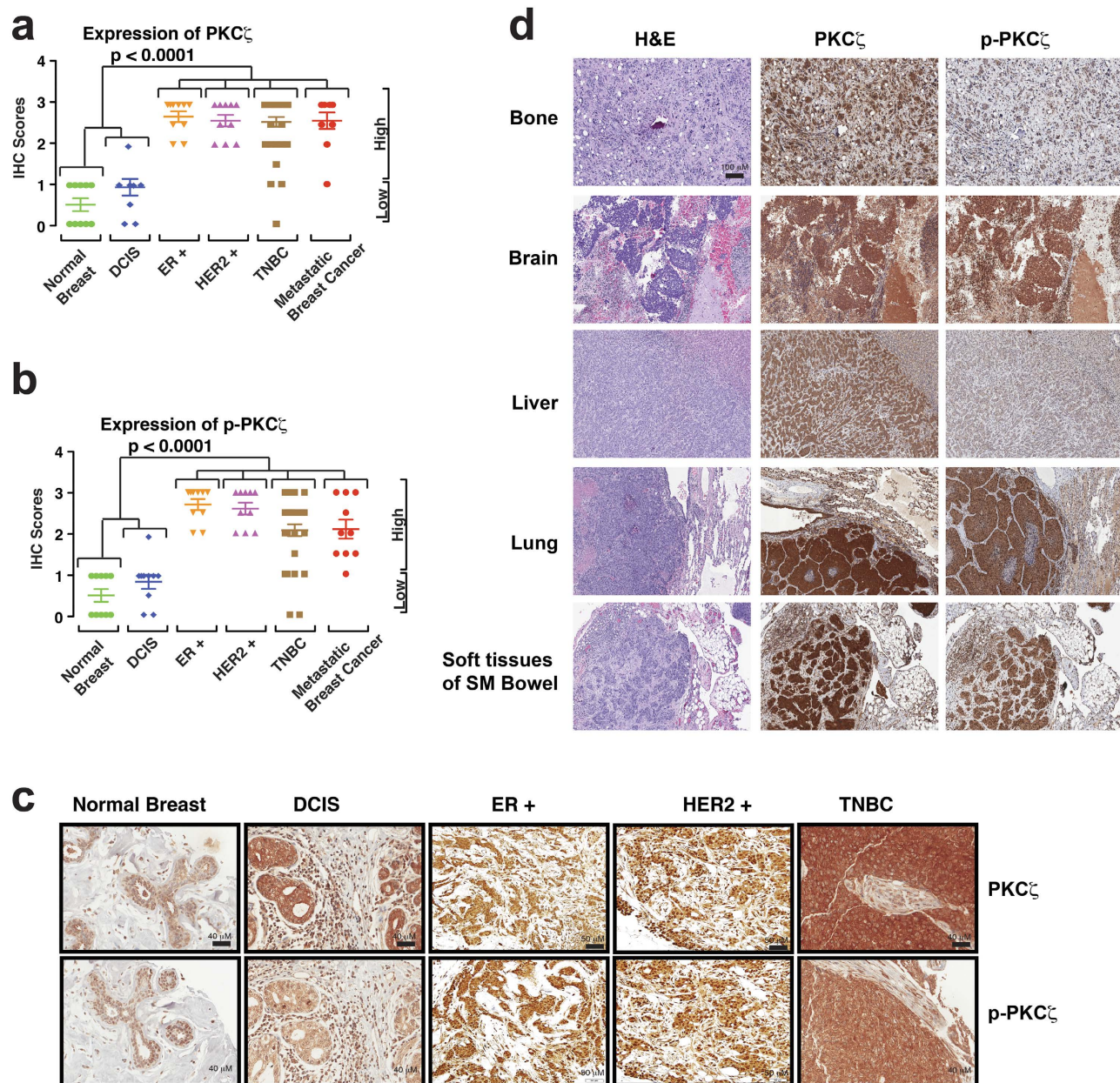


Figure 6. Aggressive Breast Cancers Are Associated With Higher Expression and Activation of PKC ζ . Expression of PKC ζ (a) and phospho-PKC ζ (b) in human normal breast, DCIS, IDCs (ER+, HER2+, and TNBC), and metastatic breast cancer samples. Results were expressed as IHC scores of individual samples (by two independent pathologists) using a scale 0 to 3. IHC scores in between 0 to 1 considered as low expression whereas IHC scores >1 considered as high expression. Majority of IDCs and metastatic breast cancer samples showed high expression of PKC ζ and phospho-PKC ζ . P-values were calculated by two-way ANOVA with Bonferroni post-test. (c) Representative images showing expression and localization of PKC ζ and phospho-PKC ζ in normal breast, DCIS, and IDCs with ER+, HER2+, and triple negative status. Expression of PKC ζ and phospho-PKC ζ gradually increased from normal breast to DCIS, and significantly increased in IDC and metastatic breast cancer samples. (d) Expression of PKC ζ and phospho-PKC ζ in human metastatic breast cancers. Scale bar 100 μ M.

of PKC ζ in HCC-1937 cells also showed repressed NF κ B transcription activity and impaired nuclear localization of NF κ B-p65 (Supplementary Figure S5a-S5c). For further confirmation, we tested invasive potential of PKC ζ -depleted MDA-MB-231 cells after ectopic expression of a constitutively active S536E RelA construct⁷⁸. We observed significant rescue of invasive phenotype as well as repressed expression of both ZO-1 and E-cadherin in PKC ζ -depleted MDA-MB-231 cells (Fig. 7e-h). These results indicate

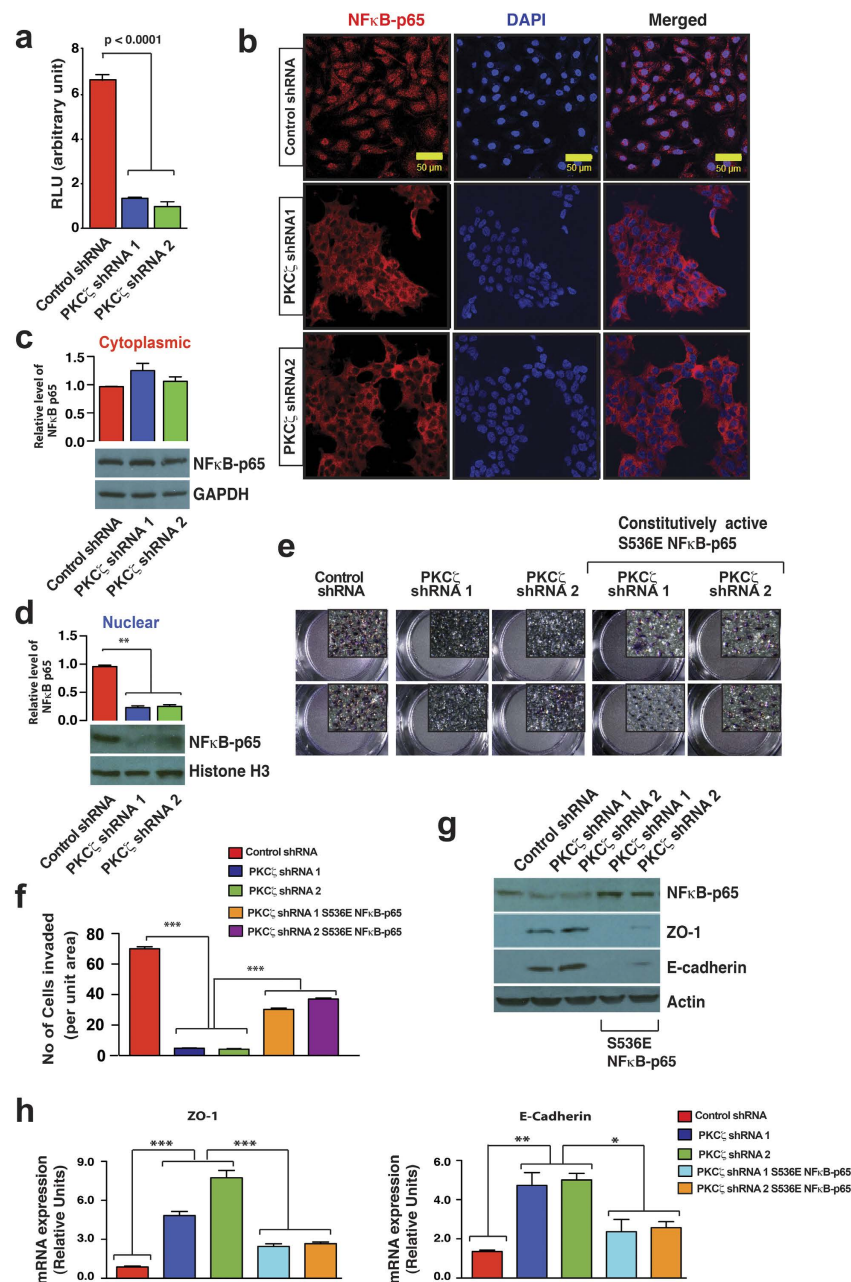


Figure 7. Involvement of PKC ζ -NF κ B Regulatory Axis. (a) NF κ B reporter gene assay of MDA-MB-231 cells with and without PKC ζ depletion ($n = 3$). Results represent means \pm S.E.M. P values were calculated using two-tailed unpaired Student's t test. (b) Localization of NF κ B-p65 in MDA-MB-231 cells with and without PKC ζ depletion. Expression of NF κ B-p65 showed in red and nuclear staining showed by DAPI. Scale bar, 50 μ M. Western blot analysis of cytoplasmic NF κ B-p65 (c) and nuclear NF κ B-p65 (d) expression level after PKC ζ depletion indicating impaired NF κ B-p65 nuclear translocation. For quantification, results represent means \pm S.E.M. ($n = 3$) and P value was calculated using one-way ANOVA with Bonferroni post-test. (e) Rescue of invasive potential in PKC ζ -depleted MDA-MB-231 cells via ectopic expression of constitutively active S536E NF κ B-p65 mutant. Inset represents higher magnification image of the corresponding transwell filter. (f) Quantification of invasion assay performed by counting cells present per unit area (each field was divided into 9 unit areas and 3 fields per condition) indicating significant rescue of invasion. For all quantifications, results represent means \pm S.E.M. ($n = 3$). P values were calculated by one-way ANOVA with Bonferroni post-test. * P values ≤ 0.05 , ** P values ≤ 0.01 , *** P values ≤ 0.001 . (g) Western blot analysis of NF κ B-p65, ZO-1, E-cadherin, and Actin after ectopic expression of a constitutively active S536E NF κ B-p65 mutant in PKC ζ -depleted MDA-MB-231 cells. (h) Quantitative RT-PCR measurements of ZO-1 and E-cadherin after ectopic expression of a constitutively active S536E NF κ B-p65 mutant in PKC ζ -depleted MDA-MB-231 cells. Results represent means \pm S.E.M. P values were calculated by two-tailed unpaired Student's t test. * P values ≤ 0.05 , ** P values ≤ 0.01 , *** P values ≤ 0.001 .

that PKC ζ -NF κ B signaling node certainly linked to regulate breast cancer progression by regulating expression of cellular junctional proteins such as E-cadherin and ZO-1.

Discussion

Functional importance of PKC ζ in epithelial cells is often assigned to the establishment of a PAR polarity complex, which has been implicated in breast cancer metastasis^{29,79,80}. Interestingly, our findings in this study indicate that a PAR polarity complex-independent function of PKC ζ contributes to invasive progression of breast cancer. Depletion of PKC ζ induced intercellular adhesion of mesenchymal-like MDA-MB-231 cells and transformed them toward epithelial phenotypes in the absence of a functional PAR polarity complex. Importantly, depletion of PKC ζ significantly induced expression of junctional proteins E-cadherin and ZO-1. Downregulation of both E-cadherin and ZO-1 are associated with cancer progression including breast cancer^{81,82}. Consistently, both *in vitro* as well as *in vivo* functional assays indicate that depletion of PKC ζ inhibits invasive potential of mesenchymal-like MDA-MB-231 cells and significantly reduce breast tumor metastasis. Our study with human patients samples further supports the importance of PKC ζ during invasive progression of breast cancer. Expression and phosphorylation analyses confirmed that aggressive forms of breast cancers i.e. IDCs and metastatic breast cancers are associated with higher expression and activation of PKC ζ . Thus, our results indicate an oncogenic PKC ζ signaling node involved in breast cancer invasion and metastasis.

Clinical relevance of PKC ζ expression and enzymatic activity in different human cancers has been reported previously^{45–50}. However, both up- and down-regulation of PKC ζ were observed in human cancers indicating tissue specific role of this enzyme as an oncogene or as a tumor suppressor^{45–51}. In fact, conflicting findings of *Whyte et al.*⁴⁹ and *Yin et al.*⁵⁰ in breast cancer also indicated that function of PKC ζ is context dependent and thus, detailed research is required to dissect molecular mechanism for these two opposite functions. Previous reports indicate that PKC ζ can directly phosphorylate S311 residue of NF κ B-p65 to regulate transcription activity and this mechanism is associated with stress included metabolic reprogramming where PKC ζ acts as a tumor suppressor^{45,47}. On the other hand, our results indicate a possible mechanism by which PKC ζ can function as an oncogene. We found that PKC ζ signaling regulates cell-cell junctional dynamics also through an NF κ B-p65-related mechanism and phosphorylation of S536 residue of NF κ B-p65 is involved in this process.

Constitutive NF κ B activity is often involved in proliferation of basal-like breast cancer cells^{83–85}. In our study, we observed that knockdown of PKC ζ significantly reduced endogenous NF κ B transcription activity in multiple basal-like breast cancer cells including MDA-MB-231 and HCC-1937 cells. In many cancers, constitutive nuclear NF κ B activity has emerged as a hallmark for cancer progression including breast cancer and constitutive NF κ B activity often linked to drug resistance and increased cell survival in response to genotoxic stress⁸⁶. Furthermore, constitutive NF κ B activity has been reported to induce EMT program in breast cancer cells and development of metastatic disease^{83,87}. Supportive to these notions, inhibition of NF κ B sensitizes many tumor cells to chemotherapeutic drugs⁸⁵. However, constitutive activation of NF κ B is regulated through multiple signaling cascades in a context dependent manner⁸⁸ and identification of signaling nodes responsible for constitutive activation of NF κ B always provide putative therapeutic target for cancer progression. In fact, NF κ B-p65 can repress expression of both E-cadherin and ZO-1 in multiple cell types including mammary epithelial cells^{76,77,89,90} and we observed that depletion of PKC ζ significantly upregulated expression of both E-Cadherin and ZO1. Moreover, we also observed repression of both E-cadherin and ZO-1 expression and restoration of invasive potential of PKC ζ -depleted cells after ectopic expression of constitutively active NF κ B-p65. Thus, it is plausible that PKC ζ signaling might be important for constitutive NF κ B activity responsible for breast cancer progression and this observation is further supported by our observation that both PKC ζ and functionally active phospho-PKC ζ expression is significantly higher in aggressive forms of breast cancer.

Our study indicates that downstream to oncogenic PKC ζ signaling, NF κ B-p65 represses expressions of ZO-1 and E-cadherin in breast cancer cells. Importantly, several EMT-associated transcription regulators such as Snail, Twist 1, ZEB1 and ZEB2 have been reported previously as NF κ B-p65 target genes and these transcription factors are known to repress expression of E-cadherin^{77,89,91}. Thus, the repression of ZO-1 and E-cadherin by PKC ζ -NF κ B-p65 signaling might be mediated indirectly via these transcription regulators. In general, NF κ B-p65 is primarily considered a transcriptional activator and S536 phosphorylation of NF κ B-p65 has been thought to be associated with transcriptional activation⁹². However, multiple studies also indicated emerging role of NF κ B-p65 in transcriptional repression via direct interaction with histone deacetylase (HDAC) co-repressor proteins such as HDAC1^{93–95}. Thus, it is tempting to propose that oncogenic PKC ζ signaling might be involved in certain posttranslational modification(s) of NF κ B-p65 to favor its interaction with HDAC1 or other unknown factors to repress target genes expression such as ZO-1 and E-cadherin. Importantly, the dominant negative S536A mutant of NF κ B-p65 has been reported to significantly less effective in repressing gene expression in other system⁹⁶. Consistently, we also observed restoration of ZO-1 and E-cadherin expressions in PKC ζ -depleted cells after ectopic expression of dominant active S536E mutant of NF κ B-p65. However, we do not know the detailed molecular mechanism yet and future research in this direction will provide new mechanistic information to understand oncogenic PKC ζ -NF κ B-p65 signaling and to develop potential therapeutic strategy for breast cancer.

Previously, inhibition of PKC ζ has been linked to EGFR-induced chemotactic migration of breast cancer cells⁵⁴. In our study, we used MDA-MB-231 cells that express higher level of EGFR⁸⁵. Our observations indicate that PKC ζ played a significant role to dictate migration and invasiveness utilizing NF κ B-p65 as one of the possible downstream transcription factors. In fact, EGFR induced NF κ B activation has been reported to play an inductive role for breast cancer cell migration⁸⁵, however, the downstream mechanism to active NF κ B transcription activity is not fully understood. Based on our data, it is tempting to propose that PKC ζ might acts as the necessary kinase required for EGFR induced NF κ B activation during breast cancer progression and future research in this direction will provide more detailed mechanism of breast cancer growth, invasion, and metastasis.

In summary, our observations indicate an oncogenic PKC ζ -NF κ B signaling node, which is responsible to regulate intercellular junctional dynamics and facilitates breast cancer cells to achieve invasiveness and metastatic capability. Thus, targeting the oncogenic PKC ζ -NF κ B signaling node might be beneficial for breast cancer treatment.

Experimental Procedures

Cell lines. The MCF-7, MDA-MB-231, MDA-MB-468, and HCC1937 cells were purchased from the American Type Culture Collection. For bioluminescent imaging, the MDA-MB-231-luc cells were generated as described earlier³³. For knockdown of PKC ζ , cells were transduced with lentiviral pGIPZ shRNAmir vector containing short hairpins and GFP reporter (Open biosystem). For isolation of PKC ζ -depleted MDA-MB-231-luc cells, transduced dual positive cells were enriched by fluorescence-activated cell sorting using a BD FACSAria™ cell sorter equipped with BD FACSDiva™ software (BD biosciences) with purity of the population more than 85%.

Lentiviral Particle Generation, Transduction, and Puromycin Selection. Lentiviral particles were prepared as described earlier³³. The target sequences were summarized in Supplementary Table 1. For transduction, cells were plated in 6-well tissue culture plates (BD Bioscience, Catalog No 353046, 250000 cells per well) and after 24 hours, media were replaced. The cells were transduced with viral particles (at MOI of 10 using the formula [(No. cells X MOI)/Viral Titer] × 1000) in serum free, antibiotic free growth media. Two days after transduction, the media were replaced with normal media supplemented with 2 μ g ml⁻¹ of puromycin (Fisher Scientific, Catalog No 100552) and the cells continued to grow in the presence of puromycin for successive passages to get nearly 100% GFP positive stable cell lines. For transduction of MDA-MB-231-luc cells, 100000 cells were spin-infected in serum free, antibiotic free growth media containing 8 μ g ml⁻¹ Polybrene (Sigma-Aldrich) with virus particles at MOI of 25.

Matrigel Invasion Assay. Matrigel Invasion assays were performed as described earlier³³. Briefly, Both sides of the transwell filters (8 μ M pore size, Costar, Catalog No 3422) were coated with 1 μ g ml⁻¹ of Matrigel™ (BD Bioscience, Catalog No 354234) at 37 °C (500 μ l underside and 200 μ l in the topside) for 1 hour. Cells were starved for 24 hours in serum free media and plated in serum free media on the upper chamber of the filter wells (200 μ l volume, 50,000 cells per well) and the wells were placed on the top of serum containing complete media (600 μ l per well) (i.e. with growth factor in the lower bottom of the transwell filters). After incubation, the cells on the transwells were fixed in 10% formalin, stained with 0.1% crystal violet to take pictures with a Nikon SMZ 1500 Stereo Microscope and quantification performed by counting the number of cells present per unit areas (9 unit areas in each field and three fields/well at magnification 8).

Wound Closure Assay. Wound closure assays were performed as described previously³³. Briefly, confluent cells were plated in a 24 well plate (BD Bioscience, Catalog No 353043) and were starved for 24 hours using serum-free media. Wounds were generated on the monolayer of cells and images were at T₀ before switched to serum containing normal media. Pictures of the same position were taken at appropriate time point and areas of the wounds were measured using ImageJ software.

Cell Aggregation Assay. Single cells were plated on ultralow attachment 6 well tissue culture plates (Costar, Catalog No 3471) at a density of 10000 cells/ml and cultured in serum-free mammary epithelial basal medium media (Lonza, Walkersville, MD, Catalog No CC-3150) supplemented with 20 ng/mL EGF (Chemicon, Catalog No EA140), 5 μ g/mL insulin (Sigma-Aldrich, Catalog No 15500), 1 μ g/mL hydrocortisone (Stem Cell Technologies, Catalog No 07904), 20 ng/mL bFGF (Invitrogen; Catalog No 13256), B27 (Invitrogen; Catalog No 17504), 4 μ g/mL hePARin (Stem Cell Technologies, Catalog No 07980), 100 IU/mL penicillin, and 100 μ g/mL streptomycin (Stem Cell Technologies, Catalog No 07500). Cells were fed every three days by adding additional media to wells. After 7 days, diameters and numbers of aggregated cell spheres were measured using Celigo Cytometer (Cytellect, San Diego, CA).

NF κ B Luciferase Reporter Gene Assay. The *cis*-reporter construct pNF κ B luc (Stratagene, Catalog No 219078), containing a luciferase cDNA under a regular TATA box and an enhancer element with five NF- κ B binding sites, was transiently transfected into MDA-MB-231, HCC-1937, and BT-20 cells with or without PKC ζ depletion using lipofectamine following manufacturer's recommended protocol. Luciferase activities were measured 48 hours after transfection using Dual-Glo® Luciferase Assay System

following manufacturer's recommended protocol (Promega, Madison, WI, Cat. No. E2920). The experiments were repeated in triplicate.

Extraction of Nuclear and Cytoplasmic Proteins. Harvested cells were subjected to isolation of nuclear and cytoplasmic protein extraction using a commercially available kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Cat # 78833, Thermo Scientific) following manufacturer's protocol. Separated nuclear and cytoplasmic fractions were analyzed by western blot as described earlier³³. Histone H3 and GAPDH were used as the markers for nuclear and cytoplasmic fractions, respectively.

Constitutively Active RelA Expression. The constitutively active NF κ B-p65 (RelA) construct T7-RelA536E⁷⁸ (Addgene, Catalog No 24156) harboring mutation at Serine 536 and substituted with glutamic acid (S536E), was transiently transfected into PKC ζ -depleted MDA-MB-231 cells using lipofectamine 2000 following manufacturer's protocol. Expression of constitutively active RelA was confirmed by western blot analysis 48 hours after transfection and subjected to MatrigelTM Invasion Assay as described earlier.

RNA Isolation and Quantitative RT-PCR. Total RNA from cells was isolated using RNeasy Mini Kit (Qiagen, Catalog No 74104) using manufacturer's protocol. Complementary DNA was synthesized as described earlier³³. Briefly, 1 μ g of total RNA and 5:1 mixture of random and oligo(dT) primers were heated at 68 °C for 10 min. This was followed by incubation with moloney murine leukemia virus reverse transcriptase (50 units) (Invitrogen) combined with 10 mM dithiothreitol, RNasin (Promega, Madison, WI), and 0.1 mM dNTPs at 42 °C for 1 hour. Reactions were diluted to a final volume of 100 μ l and heat-inactivated at 97 °C for 5 min. 20- μ l PCR reactions contained 1 μ l of cDNA, 10 μ l of 2X SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and 100–300 nM of corresponding primer sets. Primers were listed in Supplementary Table S2. Reactions, lacking reverse transcriptase, were used as control. Product accumulation was monitored by SYBR Green fluorescence using Step-one Plus real time PCR system (Applied Biosystems, Carlsbad, CA). Control reactions using water yielded very low signals. Relative expression levels were determined from a standard curve of serial dilutions of cDNA samples of human universal RNA (Stratagene, Santa Clara, CA, Catalog No 740000) and were normalized to the expression of HPRT1.

Western Blotting. Whole cell lysates were prepared with a lysis buffer and the western blot analyses were performed as described earlier³³. The primary antibodies for western blot analysis were listed in Supplementary Table 3. The membranes were stripped by incubating with stripping buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β -mercaptoethanol) at 50 °C for 30 min followed by washing with TBST 3–4 times (15 minutes each) and reprobed whenever necessary. Quantifications performed by measuring intensities of the bands of interest using ImageJ software.

Immunofluorescence. Cells were cultured on glass cover slip, washed with PBS and fixed with 4% paraformaldehyde to perform immunofluorescence analysis as described earlier³³. The primary antibodies and their dilutions for immunofluorescence analysis were listed in Supplementary Table 3.

Immunohistochemistry (IHC). Harvested tissues from transplanted mice were fixed in 4% paraformaldehyde at 4 °C overnight and 5 μ m thick tissue sections were subjected to immunohistochemistry after deparaffinization at 56 °C for 1 h followed by treatment with 1% hydrogen peroxide for ten minutes. Antigen retrieval were performed using Reveal Decloaker (Biocare Medical, CA, USA) following manufacturer's protocol and incubated with a blocking buffer followed by primary antibody of interest as listed in Supplementary Table 3. Biotinylated secondary antibodies and an ABC avidin- biotin-DAB detection kit (Vector laboratories, CA, USA) were used for visualization following manufacturer's protocol. Stained slides were analyzed under Olympus Imaging microscope.

Animal Studies and Bioluminescent Imaging. All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Female NOD-SCID NSG mice (Charles River) of 4–6 weeks old were used in xenograft studies for both spontaneous metastasis development and lung metastatic colonization assays. PKC ζ knockdown MDA-MB-231-luc cells (shRNA clone 2) were harvested in PBS and subsequently injected into the mammary fat pad or lateral tail vein in a volume of 0.1 ml as described earlier³³. Following isoflurane-induced anesthesia, mice were imaged for luciferase activity immediately after injection to exclude any that were not successfully xenografted as described earlier³³. Imaging were performed with a Xenogen IVIS[®] system coupled to Living Image[®] acquisition and analysis software version 4.0 (Xenogen) and described earlier³³.

Analysis of human primary breast tumor samples by IHC. The tissue microarray slides consist of 55 IDCs (10 ER positive, 10 HER2 positive, 35 TNBC), 10 DCIS, 10 normal breast tissues, and 10 metastatic breast cancers were prepared by the University of Kansas Medical Center Department of Pathology from archival material following IRB approval. Expression levels of both PKC ζ and phospho-PKC ζ were

analyzed by immunohistochemistry. Digital images of the stained slides were taken using Aperio® TMA software and expression of both PKC ζ and phospho-PKC ζ were analyzed by two independent pathologists in a double-blind fashion. The expression levels (IHC scores) were indicated in a scale of 0 to 3, where 3 indicates highest and 0 indicates lowest expressions. IHC scores 0 to 1 and >1 were considered as low and high expression respectively. Expression level value greater than 1 is considered as high and the significance were calculated by two-way ANOVA with Bonferroni post-test.

Statistical Analysis. All statistical analyses were performed using GraphPad Prism5 statistical software (GraphPad Software Inc., San Diego, CA). All data are expressed as means \pm S.E.M. *P*-values were calculated by two-tailed unpaired Student's *t* test and one-way or two-way ANOVA with Bonferroni post-test. *P* < 0.05 was considered as significant.

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Author Contributions

Conceived and designed the experiments: A.P. and S.P. Performed the experiments: A.P., M.D., B.S. Contributed reagents/materials/analysis tools: A.P., O.T. and S.P. Wrote the manuscript: A.P.

Additional Information

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