The Polarity Protein Par6 Induces Cell Proliferation and Is Overexpressed in Breast Cancer

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Abstract

The polarity protein complex Par6/atypical protein kinase (aPKC)/Cdc42 regulates polarization processes during epithelial morphogenesis, astrocyte migration, and axon specification. We, as well as others, have shown that this complex is also required for disruption of apical-basal polarity during the oncogene ErbB2-induced transformation and transforming growth factor β–induced epithelial-mesenchymal transition of mammary epithelial cells. Here, we report that expression of Par6 by itself in mammary epithelial cells induces epidermal growth factor–independent cell proliferation and development of hyperplastic three-dimensional acini without affecting apical-basal polarity. This is dependent on the ability of Par6 to interact with aPKC and Cdc42, but not Lgl and Par3, and its ability to promote sustained activation of MEK/ERK signaling. Down-regulation of Cdc42 or aPKC expression suppresses the ability of Par6 to induce proliferation, demonstrating that Par6 promotes cell proliferation by interacting with aPKC and Cdc42. We also show that Par6 is overexpressed in breast cancer–derived cell lines and in both precancerous breast lesions and advanced primary human breast cancers, suggesting that Par6 overexpression regulates tumor initiation and progression. Thus, in addition to regulating cell polarization processes, Par6 is an inducer of cell proliferation in breast epithelial cells. [Cancer Res 2008;68(20):8201–9]

Introduction

Par6 is a scaffolding molecule identified in Caenorhabditis elegans as a regulator of asymmetric cell division during embryonic development (1). In mammals, Par6 localizes to tight junctions (2, 3), axon tips (4), and nuclear speckles (5) and regulates diverse cellular processes. Par6 regulates establishment of apical-basal polarity (2, 3), inhibition of cell death (6), directional migration of astrocytes and keratinocytes (7, 8), and axon specification in neurons (4, 9). Thus, Par6 regulates diverse biological processes likely in a cell type–specific and context-specific manner.

Par6 is a scaffolding molecule that is involved in multiple protein-protein interactions (10, 11). The most prominent interactions are those made with the members of the Par polarity complex, which consists of Par3, an additional scaffolding molecule, atypical protein kinase (aPKC), and the GTP binding proteins Cdc42/Rac (12–15). These interactions are required for Par6 regulation of cell biological processes. For instance, Par6 regulates aPKC-induced phosphorylation of Lgl (16), Par1 (17), and Numb proteins (18) during establishment of apical-basal polarity in epithelial cells. Likewise, Par6-associated aPKC regulates glycogen synthase kinase 3β (GSK3β) activity to induce polarized migration of astrocytes (19) and promote cell death during three-dimensional epithelial morphogenesis (6). Thus, the Par6 complex can serve as a signaling node that regulates diverse biological processes by controlling activation of downstream pathways.

In addition to its role during normal cell biology, Par6 also regulates initiation and progression of cell transformation. For example, Par6 cooperates with Rac1/Cdc42 to transform fibroblasts (12). We recently found that Par6 interacts with the oncogene receptor tyrosine kinase ErbB2, and this interaction is required for ErbB2-induced disruption of cell polarity and inhibition of cell death in three-dimensional mammary epithelial structures (20). Par6 also cooperates with transforming growth factor β (TGFβ) receptor to promote TGFβ–induced epithelial-to-mesenchymal transition (21). Interestingly, among the three isoforms of Par6 (gene name Pard6a, Pard6b, and Pard6g, the Pard6g isoform is located in a region of the genome that is frequently amplified and overexpressed in breast cancer (22–25). It is likely that Par6 not only cooperates with regulators of cell transformation but may also directly regulate cell transformation processes.

Here, we show that expression of Par6 induces epidermal growth factor (EGF)–dependent proliferation of normal mammary epithelial cells by promoting activation of mitogen-activated protein kinase (MAPK) signaling. This function of Par6 was dependent on its ability to interact with aPKC/Cdc42, demonstrating that Par6 regulates cell proliferation pathways, in addition to its previously shown role as a regulator of cell polarity and cell migration. Furthermore, we show that Par6 is overexpressed both in human precancerous breast lesions and in estrogen receptor (ER)–positive breast cancers, suggesting that Par6 pathways are likely to play critical roles during initiation and progression of breast cancer. Thus, the polarity protein Par6 promotes transformation of epithelial cells not only by regulating cell polarity pathways but also by inducing cell proliferation.

Materials and Methods

Cell culture and stable cell line generation. MCF-10A cells were maintained, as previously described (26). Comma-1D β-galactosidase cells were kindly provided by Daniel Medina (Baylor College of Medicine) and maintained in DMEM/F12 supplemented with 2% fetal bovine serum (FBS), 10 μg/mL insulin, and 5 ng/mL EGF (27). HC11 cells were obtained from the Rosen laboratory and cultured according to Xian and colleagues (28). Preparation of virus and infection was performed as previously described (29). All cells lines were selected with 1 μg/mL of puromycin.
Cell growth and S-phase assays. MCF-10A (Vector, Par6α, K19A, ΔPro136, M235W, and Par6β) cells were grown to confluency under normal growth conditions and then placed in assay medium (DMEM/F12 supplemented with 0.5% FBS and 5 μg/mL insulin). 1 × 10⁵ cells were plated, and cells numbers were counted on day 3 using a hemacytometer. Three-dimensional morphogenesis assay. MCF-10A stable cell lines (Vector, Par6α, K19A, and Par6β) were grown to confluency under normal growth conditions and then placed in assay medium (DMEM/F12 supplemented with 0.5% FBS and 5 μg/mL insulin). 1 × 10⁵ cells were plated, and cells numbers were counted on day 3 using a hemacytometer. 

Three-dimensional morphogenesis assay. MCF-10A stable cell lines (Vector, Par6α, K19A, and Par6β) were grown to confluency under normal growth conditions and then placed in assay medium (DMEM/F12 supplemented with 0.5% FBS and 5 μg/mL insulin). 1 × 10⁵ cells were plated, and cells numbers were counted on day 3 using a hemacytometer.

**Figure 1.** Overexpression of Par6 promotes proliferation without disrupting three-dimensional acini morphogenesis. A, phase contrast (top) and optical sections of acini stained with 4',6-diamidino-2-phenylindole (DAPI; bottom). Images of day 20 acini grown in 0.5 ng/mL EGF; insets show detail of hollow lumen. Scale bar, 50 μm. B, immunofluorescence staining of day 12 acini structures grown in 0.5 ng/mL EGF with the apical marker GM130 (green) and basal marker Laminin V (red) and costained with DAPI to monitor nuclei. Scale bar, 10 μm. C, distribution of acini size (circumferential area) of day 12 acini structures grown in 0, 0.1, and 0.5 ng/mL EGF. Area of each acini was measured using Zeiss AxioVision 4.5 software and plotted as box plots. Blue line, median value; spread, 1.5 times the interquartile range; circles, outliers. Each condition represents ~800 acini structures from three independent experiments. The P value between Vector and Par6 for each EGF concentration was <0.0001 calculated by Mann-Whitney test. D, quantitation of day 12 acini with at least one positive Ki-67–positive nuclei on day 12 acini structures grown in 0.5 ng/mL EGF. Data are represented as percentage of Ki-67-positive structures; bars, SD. Each bar represents ~700 acini from three independent experiments.
represents the median value, the spread represents 1.5 times the interquartile range, and outliers are shown as circles. Statistical analyses were performed using GraphPad Prism software and Mann-Whitney test. HC11 stable cell lines (Vector, Par6a) were grown on Matrigel with 0.5 ng/mL EGF, and day 12 structures were analyzed as done for MCF-10A cells.

Immunofluorescence. All immunofluorescence procedures were performed as previously described (26). Microscopy was performed on Zeiss Axiovert 200M using AxioVision 4.5 and ApoTome imaging system (Zeiss).

Biochemistry and immunoprecipitation. MCF-10A cells (Vector, Par6a, K19A, ΔPro136, M235W, and Par6h) were plated 2 x 10⁵ in growth media, and cells were stimulated on day 5 with 20 ng/mL EGF. Cells were lysed in TNE buffer and immunoprecipitated with Flag or Par6 antibodies as described previously (20).

DNA constructs. Carboxy or amino-terminal Flagepitope tag mouse par6a was generated by PCR amplification of mPar6a from pFlag-CMV-mpar6a (14) and cloned into MSCV-PURO-IRES-GFP (kindly provided by S. Lowe, Cold Spring Harbor Laboratory). Point mutations of mPar6a were generated using site-directed mutagenesis. Amino-terminal Flag epitope-tag human Par6h was generated by PCR amplification from plasmid(pClXhPar6) (American Type Culture Collection) and cloned into MSCV-PURO-RES-GFP. Two PKCα short hairpin vectors were obtained from Open Biosystems and subcloned into MSCV-LTR-PURO-RES-GFP (30); targeting sequence for PKCα1 CACAGACAGTATTTCCATATTAG and PKCα2 GATTATCTCTTCCAAGTTATTAG. Cdc42 short-hairpin vector number 3 was obtained from Open Biosystems and subcloned into MSCV-LTR-HYgro-RES-GFP (30). Cdc42 short-hairpin vector number 5 was generated by PCR and subcloned into MSCV-LTR-HYgro-RES-GFP: targeting

Figure 2. Par6-induced cell proliferation requires interaction with aPKC and Cdc42. A, table summary of the Par6 mutants and their different binding partners. B, immunoprecipitation of cell extracts with Par6 (anti-flag antibodies) and immunoblotting with anti-aPKCα, anti-Flag, anti-Par3, anti-Cdc42, and anti-Lgl antibodies. C, growth curve of vector control cells compared with Par6 wt and mutant Par6 cells over a period of 11 d in EGF-free media. Points, means of estimated cell numbers from three independent experiments. D, distribution of acini size (circumferential area) of day 12 structures grown in 0.5 ng/mL EGF. Each condition represents ~800 acini structures from three dependent experiments. The P value between Vector and Par6wt or Par6wt and K19A is <0.0001 calculated by Mann-Whitney test.
sequence for Cdc42-3 CGGCTTAAAGAATGTATTGTAT and Cdc42-5 CAAAATGTATTTGACAGA.

Quantitative PCR. Twenty-five breast tumor samples were obtained from the Wigler laboratory (Cold Spring Harbor Laboratory). RNA was isolated using a Versagene RNA tissue kit (Genta Systems). Normal breast tissue RNA was a gift from David Mu (Cold Spring Harbor Laboratory). Breast cancer cell line RNA was a gift from Adrian Krainer (Cold Spring Harbor Laboratory). MCF-10A control and Par6 over-expressing RNA were obtained from trizol lysis (Invitrogen) according to the manufacturer’s protocol. cDNA was generated using a Taqman reverse transcriptase kit (Roche). Quantitative real-time PCR was performed using SYBR green master mix (Applied Biosystems). The following primer sequences were used for Par6, 5'-GTGAGAAGCAATGGACG-3' and 5'-GATGGCTGTA- TAGATCCTAA-3' and GAPDH, 5'-CCGACAGTCGCCCATGC-3' and 5'-CGGTGACTCTGCTCCA-3'. Samples were run on a Peltier thermalcycler (PTC-200) from MJ Research, and data were collected using an Opticon monitor chromo4 continuous fluorescence detector. Cycles were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared with MCF-10A or normal breast tissue Par6 levels.

Antibodies. Antibodies used in this study were against Ki67 (Zymed); PKC, GM130, Cdc42, ERK2 (BD Biosciences); HphaR antibody (previously described; ref. 20); mPar3 (Upstate Biotechnology); PKC (Santa Cruz Biotechnology); cleaved caspase-3, p-ERK, p-AKT (Cell Signaling Technology); Flag M2, α-actin (Sigma); laminin (Chemicon); p-ERK1 and p-ERK2, p-PKC, Alexa-Fluor–conjugated secondary antibodies (Invitrogen).

Analysis of human breast cancers. Gene expression was performed on 112 breast tumors samples, and data were generously provided by Therese Sorlei and colleagues. The expression values are median-polished log transformation of breast tumors samples, and data were obtained using an Opticon monitor chromo4 continuous fluorescence detector. Cycles were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared with MCF-10A or normal breast tissue Par6 levels.

Results

Overexpression of Par6 does not disrupt three-dimensional acini morphogenesis. Although Pard6b is amplified in breast cancer, it is not known what role, if any, overexpression of Par6 plays during transformation on breast epithelial cells. To investigate if overexpression of Par6 affects polarization and morphogenesis of mammary epithelial cells in three-dimensional culture, we expressed Pard6a (protein called Par6α) and Pard6b (protein called Par6β) in an EGF-dependent, nontransformed human mammary epithelial cell line, MCF-10A. When plated on Matrigel in the presence of EGF, MCF-10A cells form three-dimensional acini-like structures comprised of a single layer of polarized, proliferation-arrested epithelial cells surrounding a hollow central lumen (31). Overexpression of either Par6 isoform did not affect the ability of MCF-10A cells to form acini with hollow lumens (Fig. 3A). In addition, these acini had no detectable loss of apical-basal polarity as determined by using the apical polarity marker GM130, a basal polarity marker Laminin V (Fig. 1B and Supplementary Fig. S1), and a basolateral marker E-cadherin (data not shown). Thus, overexpression of Par6 did not affect polarization and morphogenesis of MCF-10A cells on Matrigel.

Overexpression of Par6 promotes cell proliferation. Although we did not detect any difference in the organization of the acinar structures, we did observe that Par6 expressing acini were larger than the control acini (Fig. 3A). To test the hypothesis that Par6 overexpression promotes acinar growth, acini size was quantitated by measuring the area occupied by each acinus. Par6α and Par6β overexpressing cells formed acini that were significantly larger than those formed by control cells, irrespective of the dose of EGF (Fig. 1C and Supplementary Fig. S2B). To determine if the increase in size was due to an increase in cell number or cell size, we counted the number of cells per structure. Acini derived from Par6 over-expressing cells had significantly more cells than those derived from control cells (Supplementary Fig. S3).

To determine if the increase in cell number is due to changes in cell proliferation rates, we monitored the presence of the proliferation marker Ki-67 during three-dimensional morphogenesis. Consistent with our previous results (31), acini derived from control cells had low proliferation rates on day 12 (Fig. 1D). However, acini derived from Par6-expressing cells had higher proliferation rates until day 20 compared with acini derived from control cells (Supplementary Fig. S4A). Furthermore, we did not observe any evidence for inhibition of cell death pathways in acini derived from Par6-overexpressing cells (Supplementary Fig. S4B and C). Thus, Par6 overexpression induces development of hyperplastic acini by inhibiting proliferation arrest during three-dimensional morphogenesis without disrupting polarity.

The ability of Par6 to promote proliferation was observed in multiple independent populations of MCF-10A cells and in a mouse mammary epithelial cell line, demonstrating that there was no clonal or cell line bias to the phenotype (Supplementary Fig. S5).

Par6-induced cell proliferation requires interaction with aPKC and Cdc42. To gain insight into the mechanisms by which Par6 induces cell proliferation, we generated Par6 mutants that are defective in binding to members of the Par complex, such as Par3, Cdc42, Lgl, and aPKC. We generated a lysine-to-alanine mutation in the PB1 (Phox/Bem1p) domain (K19A) to abolish binding to aPKC, deleted Proline 136 in the semi-CRIB binding domain (ΔPro136) to disrupt binding to Cdc42, and substituted a methionine with a tryptophan in the PDZ domain (M235W) to disrupt binding to Lgl (refs. 14, 32–35; Fig. 2A). Each mutant was expressed in MCF-10A cells, and the ability of the mutants to interact with components of the Par complex was determined by coimmunoprecipitation analysis (Fig. 2B–D). As expected, K19A failed to associate with aPKC and retained its ability to associate with Cdc42 (Fig. 2B). In addition, we found that this mutant also lost its ability to interact with Par3 and Lgl (Fig. 2B). The ΔPro136 mutant was defective in its ability to bind Cdc42 but associated with Par3, aPKC, and Lgl. The M235W mutant did not bind Cdc42, Lgl and was defective in binding Par3 but still associated with aPKC (Fig. 2B).

Stable populations of MCF-10A cells expressing comparable levels of the Par6 mutants were generated and analyzed for EGF-independent cell proliferation. None of the mutants induced EGF-independent cell proliferation in monolayer cultures (Fig. 2C) or enhanced cell proliferation during acini formation (Fig. 2D and data not shown). The inability of Par6K19A to promote proliferation showed that aPKC binding was necessary and that Cdc42 binding is not sufficient. Conversely, the Par6ΔPro136 mutant showed that interaction with Cdc42 was necessary whereas aPKC, Par3, and Lgl were not sufficient to promote proliferation. The inability of Par6K19A to promote proliferation confirmed our conclusion that interaction with Cdc42 was necessary and that an interaction with aPKC was not sufficient to induce EGF-independent proliferation. Because the binding of Cdc42 to Par6 induces aPKC kinase activity in the Par6 complex (3, 7), it is likely that Par6-aPKC-Cdc42 forms a core complex to promote EGF-independent cell proliferation of mammary epithelial cells.

Par6-induced cell proliferation requires both aPKC and Cdc42. To determine if aPKC and Cdc42 play a role during Par6-induced cell proliferation, we knocked down expression of aPKC and Cdc42 in both control and Par6-expressing cell lines using two independent short hairpin RNAs for each aPKC and

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Cdc42 (Fig. 3A). The aPKC RNA interference (RNAi) vectors significantly down-regulated the expression level of PKC\(_{L}\) and had a modest effect on the levels of PKC\(_{\sim}\) (Fig. 3A). Decreased expression of PKC\(_{L}\) significantly impaired the ability of Par6-overexpressing cells to proliferate in the absence of EGF while having no significant effect on the basal proliferation observed in vector control cells (Fig. 3B).

We identified two independent short hairpins that target Cdc42 (Fig. 3C). Both RNAi vectors significantly down-regulated Cdc42 in both control and Par6-overexpressing MCF-10A cells (Fig. 3C). As observed for aPKC, down-regulation of Cdc42 inhibited the ability of Par6 to induce proliferation in the absence of EGF, whereas loss of Cdc42 did not have a significant effect on the basal proliferation observed in vector control cells (Fig. 3D).

We therefore investigated cell autonomous mechanisms by which Par6 promotes cell proliferation. Activation of the EGF receptor (EGFR) pathway is an attractive possibility, because our data showed that Par6 induces EGF-independent proliferation of MCF-10A cells (Fig. 2D and Supplementary Figs. S2C and S5A). However, we did not observe EGF stimulation–independent phosphorylation of EGFR in Par6-overexpressing cells (data not shown), suggesting that Par6 overexpression does not directly induce EGFR phosphorylation.

To determine if Par6 activates pathways downstream of EGFR, we investigated activation of PLC\(_{\gamma}\), phosphatidylinositol 3-kinase (PI3K), Ras/MAPK, and c-Jun NH\(_2\) kinase (JNK), both in the presence and in the absence of EGF stimulation. Par6 overexpression had no effect on activation of PLC\(_{\gamma}\), PI3K, or JNK pathways, either in the presence or in the absence of EGF (Fig. 4A and data not shown). However, Par6-overexpressing cells had a significant increase in extracellular signal-regulated kinase (ERK) phosphorylation in the absence of EGF. In the presence of EGF stimulation, ERK phosphorylation returned to basal levels by 30 minutes in vector control cells, whereas in Par6-overexpressing cells, it was prolonged for 60 (Fig. 4B) and 120 minutes (Supplementary Fig. S2D).

In contrast to Par6, the Par6 mutants neither induced phosphorylation of ERK in the absence of EGF nor promoted sustained ERK phosphorylation in the presence of EGF (Fig. 4B and Supplementary Fig. S6).
To confirm that the effect of Par6 on proliferation is directly due to its effect on ERK activation, we inhibited ERK activation using the MAPK/ERK kinase (MEK) kinase inhibitor U0126. Treatment of Par6-overexpressing cells with U0126 blocked both basal and EGF-induced sustained ERK phosphorylation (data not shown). In addition, the inhibitor also blocked EGF-independent cell proliferation (Fig. 4D), suggesting a role for MEK kinase in Par6-induced cell proliferation. Thus, we conclude that Par6 overexpression promotes EGF-independent cell proliferation by activating the MEK/ERK signaling pathway.

**Par6b is overexpressed in ER-positive human breast tumors.** Next, we investigated if the genes encoding for the Par6 family of proteins (Par6) are targeted for overexpression in overexpression in breast cancer. Interestingly, Par6b (Chr. 20q13.13), but not Par6a (Chr. 16q22.1) or Par6g (Chr. 18q23), is located in a region of the genome that is frequently amplified in breast cancer (22–25), and this amplification correlated with increased Par6b gene expression (23, 24). To directly test if Par6b is overexpressed in primary human breast cancers, we performed quantitative PCR analysis from 25 primary breast tumors, 2 normal breast tissue samples, 6 breast cancer cell lines, and MCF-10A cells. We found that 6 of 25 primary tumors and 2 of 6 breast cancer cells express mRNA at least 2-fold more than the levels observed in their respective controls (Fig. 5A and B). Thus, among the Par6 family members, Par6b is genomically amplified and transcriptionally up-regulated in breast cancer.

Among the breast cancer cell lines tested, the two cell lines (MCF-7 and T47D) that overexpressed Par6b are also ER-positive, suggesting a relationship between Par6b overexpression and ER status. To determine if there is a relationship between ER status and Par6 expression, we compared Par6b mRNA levels in 68 of ER-positive and 44 of ER-negative breast tumors. Overexpression of Par6b showed a significant positive correlation with ER+ status (Fig. 5C). We did not observe any relationship between Par6 overexpression and ErbB2 amplification in human breast tumors (data not shown), demonstrating that Par6b overexpression is specific to the ER-positive subtype of breast cancer. In addition, analysis of a public gene expression database Oncomine (36) showed that Par6b expression positively correlated with ER in four independent studies (Supplementary Fig. S5; refs. 23, 37–39). Inhibition of ER signaling in MCF-7 cells by treatment with tamoxifen did not result in a significant decrease in the levels of Par6b mRNA (Supplementary Fig. S8), suggesting that Par6b levels may not be directly regulated by estrogen. Thus, it is likely that overexpression of Par6 cooperates with EGF to provide a proliferative advantage to ER-positive breast cancers.

Almost all precancerous breast lesions are ER-positive (40). Furthermore, we recently showed that hyperplastic enlarged lobular units (HELU), the earliest histologically identifiable precursor of breast cancer, show high proliferation rates and an increased expression of members of both EGF and ER signaling modules (40, 41). Interestingly, like the three-dimensional acini derived from Par6 overexpressing MCF-10A cells, HELUs are hyperplastic structures wherein the acini are enlarged in size due to increased in cell number without apparent loss of acinar architecture. Given the architectural similarity, we tested if Par6 was overexpressed in HELUs. RNA isolated from microdissected HELU and adjacent TDLSU were analyzed by microarray (41). Par6b, but not Par6a or Par6g, was overexpressed by 2.5-fold (P = 0.002) in HELUs compared with adjacent TDLSUs. The gene encoding for PKCa (PRKCZ), a component of Par6 complex, was also up-regulated by 1.46-fold (P = 0.029) compared with TDLSUs (ref. 41; Fig. 5D). Thus, our results show that overexpression of Par6/aPKC is observed early in precancerous lesions and retained during development of ER-positive cancers.

It is possible that the Par6/aPKC/Cdc42 complex is a novel target for therapeutic intervention for both early and late stage of breast cancers. To test this possibility, we down-regulated expression of aPKC in a breast cancer–derived cell line, MCF7. Down-regulation of PKCa decreased cell proliferation (Fig. 6A and B), suggesting that inhibiting aPKC is likely to have important therapeutic relevance. Taken together, our results show that Par6/aPKC/Cdc42 is a regulator of cell proliferation and suggests that the Par6 complex is a novel therapeutic target for breast cancer.

**Discussion**

We show that overexpression of Par6 in mammary epithelial cells promotes activation of MAPK and induces cell proliferation. In addition, we show that Par6 is overexpressed in both precancerous human breast lesions and in advanced breast cancers.
cancers. Thus, our results identify a novel role for the polarity protein Par6 as an inducer of cell proliferation, which is likely to play an important role during initiation and progression of breast cancer.

Our results show that overexpression of Par6α and Par6β in nontransformed mammary epithelial cells does not affect establishment of apical-basal polarity during acinar morphogenesis. Whereas several studies have reported that altered expression of both Par6α and Par6β delays establishment of apical polarity (2, 3), they also find that the cells recover from the effects of Par6 overexpression and eventually develop cell polarity. This suggests that compensatory mechanisms act redundantly to ensure apical polarity formation in the presence of altered Par6 expression. Because MCF-10A acinar morphogenesis takes several days, it is likely that Par6-overexpressing cells use these compensatory mechanisms to establish proper apical polarity. We found that ectopic expression of Par6 promotes proliferation of multiple mammary epithelial cell lines, demonstrating that, in addition to its known role as a polarity regulator, Par6 can also induce cell proliferation.

Both αPKC and Cdc42 are required for Par6 to stimulate cell proliferation. This is consistent with the established role of αPKC and Cdc42 in Par6-mediated regulation of tight junction biogenesis, polarized cell migration, and cell death (3, 6, 19, 42). Although previous studies have shown that Par6-induced modulation of GSK3β activity is required for directed cell migration and apoptosis, GSK3β is unlikely to play a role in Par6-induced cell proliferation, because we did not see any differential phosphorylation of GSK3β in Par6-overexpressing cells (data not shown). Instead, we found that Par6 overexpression induces ERK

Figure 5. Pard6b is overexpressed in ER-positive human breast tumors. A, quantitative PCR analysis of Par6β gene expression using cDNA from breast cancer cell lines and normalized to GAPDH gene expression. Data are represented as fold increase over MCF-10A control cells. B, quantitative PCR analysis of Par6β gene expression using cDNA generated from primary breast tumors and normalized to GAPDH gene expression. Data are represented as fold increase over the average levels expressed in normal breast tissue. C, a box plot of the microarray data comparing Pard6b gene expression in ER− versus ER+ tumor samples. A Kolmogorov-Smirnov null hypothesis test was used to calculate the P value (P = 2.9 × 10−7). D, hierarchical clustering from a supervised comparison between paired samples of normal TDLUs and HELUs. Pard6b is expressed 2.5-fold (P = 0.02) and PKCZ 1.46-fold (P = 0.029) in HELU compared with TDLU (45).
phosphorylation in the absence of EGF, identifying MAPK pathway as an effector of the Par6-aPKC-Cdc42 complex. MAPK signaling can be activated by ligand-independent phosphorylation of EGFR (43). However, we did not observe phosphorylation of EGFR in the absence of EGF, ruling out a role for EGFR phosphorylation as a mechanism to activate MAPK pathway in Par6-overexpressing cells. Previous studies have shown that PKC is not only sufficient but is also required for activation of ERK in a MEK-dependent manner in response to serum stimulation (44–47). Similarly, we find that Par6 uses aPKC to activate ERK in a MEK-dependent manner. The precise mechanism by which aPKC activates MEK remains to be understood. Thus, in addition to its known effectors that regulate cell polarity, the Par6-aPKC-Cdc42 complex also regulates molecules that induce cell proliferation.

Our results show that Par6β is overexpressed both in HELUs and ER-positive advanced carcinomas. HELUs are characterized by high rates of cell proliferation, ER positivity, and increased expression of EGF family of growth factors. It is possible that HELUs arise from cooperation between overexpressed Par6 and EGF ligands, similar to how Par6 overexpression induced hyperplastic acini in our MCF-10A three-dimensional cultures. Considering that Par6 overexpression promoted hyperplastic acini in MCF-10A three-dimensional culture in cooperation with EGF, it is possible that these two factors could cooperate during development of premalignant lesions in the breast. Our data combined with other studies that show that Par6 is amplified genomically and overexpressed in ER-positive advanced carcinoma suggest that there is a genetic advantage associated with increased Par6 expression during breast cancer progression.

Par6 is also known to cooperate with oncogenes associated with breast cancer progression. For example, Par6 plays a critical role during ErbB2-induced transformation of organized epithelia (20) and ErbB2 amplification is associated with the premalignant breast disease DCIS (48). We did not observe either a positive or negative correlation between Par6 overexpression and ErbB2 status. Therefore, ErbB2 requires expression of Par6, but not overexpression. In addition, Par6 is required for TGFβ-induced epithelial-to-mesenchymal transition, a cellular process that is associated with invasion. The ability of Par6 to interact with oncogenes in distinct cellular processes suggests that Par6 has multiple functions. The results presented in this manuscript, taken together with those above, suggest that the polarity signaling pathways regulated by Par6 plays a cooperative role during the initiation and progression to breast cancer. Thus, a further understanding of the alterations in the Par6 signaling pathways could identify both novel drug targets and predictive biomarkers for breast cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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