Phosphoinositides and lipid kinases in oxidative stress signalling and cancer
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CHAPTER 7

Low PIP4K2B expression in human breast tumours correlates with reduced patient survival: a role for PIP4K2B in the regulation of E-cadherin expression

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Abstract
Phosphatidylinositol-5-phosphate 4-kinase beta (PIP4K2B) directly regulates the levels of two important phosphoinositide second messengers PtdIns5P and Phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P₂). PtdIns5P has been linked to the regulation of gene transcription, TP53 and AKT activation and to the regulation of cellular reactive oxygen accumulation. PtdIns(4,5)P₂ is at the heart of PI signalling as it is the preferred substrate for both PI-3-kinase and phospholipase C. PtdIns(4,5)P₂ also acts as a second messenger regulating ion channel and integrin function, endocytosis, membrane trafficking and transcription in the nucleus. Although PIP4K2B can regulate cancer relevant functions in cells it is not known if PIP4K2B expression impinges on human tumour development and on patient survival. Here we have developed a specific antibody recognising PIP4K2B and have characterised its use to interrogate the expression of PIP4K2B in a large cohort (489) of human breast tumour samples with associated clinical outcome data. PIP4K2B is expressed in the ducts of normal breast tissue and in tumours we found a strong variability in its expression. Surprisingly, low PIP4K2B expression was associated with increased tumour size, high histological grade (NHG) and Ki67 expression and an increase in distant metastasis. Kaplan Meier curves showed that event free survival of patients was negatively correlated with PIP4K2B staining. In breast tumour (MCF7) and normal (MCF10A) breast epithelial cell lines, PIP4K2B knockdown led to a decrease in the transcription and expression of the tumour suppressor protein E-cadherin (CDH1). PIP4K2B knockdown also primed MCF10A cells to undergo an epithelial to mesenchymal transition (EMT) in response to TGFβ treatment. Data mining of two independent expression arrays using Oncomine confirmed an associated decrease in CDH1 expression with low PIP4K2B expression. The deregulation of CDH1 expression and priming of TGFβ-induced EMT by reduced PIP4K2B expression might in part explain the negative correlation between PIP4K2B expression and patient survival.

Key words: Phosphoinositides, E-cadherin, Breast Cancer, Phosphatidylinositol-5-phosphate, Phosphatidylinositol-5-phosphate-4-kinase, PIP4K2B
Introduction

Neoplasms derived from breast epithelium often originate as a consequence of alterations in various signalling pathways. For example amplifications of the ERBB2 gene, mutations in BRCA1, BRCA2 and RAS genes and modulation of the estrogen/progesterone receptor status have all been shown to play a role in the development of breast neoplasms. However, most cancer deaths occur as a consequence of the spread of therapy resistant cells throughout the body. Metastatic cells acquire somatic mutations and epigenetic changes that increase their ability to migrate away from the original tumour site, survive within lymphatics and the blood stream and to invade and colonise new tissues. The acquisition of these characteristics is often associated with a decrease in the levels of CDH1 and a subsequent decrease in cell-cell adhesion and increase in cell motility and degradation of the stromal matrix. CDH1 loss is also associated with an increased in epithelial to mesenchymal transition (EMT). CDH1 is an epithelial Ca\(^{2+}\)-dependent cell adhesion molecule which facilitates cell-cell adhesion through homophilic interactions with adjacent CDH1 molecules expressed on neighbouring cells (1-2). Loss of CDH1 has been observed in several tumours and has been shown to contribute to invasiveness and the development of metastasis (3). EMT is an important process in normal development enabling cell migration and the consequent differentiation and generation of new organs and tissues. EMT is controlled by a number of master regulators of gene transcription such as the Snail family, Goosecoid and members of the ZFH family (zinc-finger homeodomain, ZEB1 and ZEB2) (4) and has been suggested to be required for the acquisition of metastatic stem cell-like characteristics of tumour cells (5-6). For example TGF\(\beta\) which is normally tumour suppressive also induces EMT-like characteristics during late stage tumour development enhancing cell metastasis. The loss of CDH1 is considered to be one of the hallmarks of the EMT programme (7-8), and recent studies have shown that loss of CDH1 can also facilitate the induction of the EMT programme (9). Loss of CDH1 function can occur through mutation, gene deletion, silencing of the CDH1 promoter, regulation of its transcription and by proteolytic degradation. Understanding how various signalling pathways impinge on CDH1 levels is of utmost importance in understanding how the EMT programme and metastasis might be facilitated.

Phosphoinositides are signalling lipids that regulate many cellular processes (10). Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)\(P_2\)) is at the centre of phosphoinositide signalling providing the substrate for both receptor regulation of PLC (phospholipase C) and of PtdIns-3-kinase family members. PtdIns(4,5)\(P_2\) also acts as a signalling moiety itself by recruiting and regulating the activity of specific interacting proteins (11). Regulation of CDH1 signalling by phosphoinositides is complex. The concentration of CDH1 mRNA and protein can be regulated by AKT signalling, one of the major downstream targets for PtdIns-3-kinase signalling. CDH1 activation also leads to increased PtdIns(3,4,5)\(P_3\) signalling required for the regulation of cell-cell adhesions.
suggested the existence of complex signalling feedback loops (12). The localisation of CDH1 can also be regulated by PtdIns(4,5)_{2} signalling (13). Two families of lipid kinases are known to generate PtdIns(4,5)_{2}; PIP5Ks regulate the bulk of PtdIns(4,5)_{2} by phosphorylation of PtdIns4P and the gamma isoform of PIP5K regulates CDH1 trafficking and localisation (13). PIP4Ks phosphorylate PtdIns5P on the 4-position to generate PtdIns(4,5)_{2} and their likely role in vivo is to regulate cellular pools of both PtdIns5P and PtdIns(4,5)_{2}. There are three isoforms of PIP4K, 2A, 2B and 2C, (14-17) which appear to have distinct subcellular localisations, although PIP4K can homo and heterodimerise. PIP4K2B localises in the plasma membrane, the cytoplasm and in nuclear speckles. Overexpression of PIP4K2B can suppress AKT activation induced by insulin and PIP4K2B knockout mice show enhanced insulin sensitivity in muscle and increased AKT activation (18-19). In the nucleus PIP4K2B controls nuclear PtdIns5P levels (20) and through its interaction with PHD finger containing proteins PtdIns5P can regulate gene transcription (21). Furthermore in response to H_{2}O_{2} PtdIns5P acts as a signalling intermediate to regulate the expression of genes required for the management of oxidative stress responses (22).

In normal tissues PIP4K2B is highly expressed in brain and in muscle and its levels in tumour cells vary markedly. High expression levels of PIP4K2B can be found in several breast cancer derived cell-lines such as UACC-812, BT474 and T47D cells and is particularly associated with ERBB2 positive breast cell lines. The gene encoding PIP4K2B is located at 17q21.2 and can be amplified as part of the ERBB2 amplicon (23).

Using a specific antibody against PIP4K2B (24) we interrogated PIP4K2B expression in 489 human breast tumour samples for which extensive associated clinical outcome data was available. An inverse correlation between PIP4K2B expression and tumour size, the proliferation marker Ki67 and observation of distant metastasis during follow up was found. Kaplan Meier survival curves showed a decrease in event free survival in patients that expressed low levels of PIP4K2B leading to worse patient outcome. Loss of PIP4K2B in breast tumour cells led to decreased transcription of the cell-cell adhesion protein CDH1 and to enhanced EMT in response to TGF{\beta} signalling. Reduced CDH1 expression in breast tumours might in part underlie the poorer survival observed in patients with low PIP4K2B expression.

Materials and methods

Cell lines and reagents

Cell lines (HEK293, MCF7 and MCF10A) were obtained from the American Type Culture Collection. HEK293 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FCS whilst MCF7 cells were grown in RPMI medium (Invitrogen) supplemented with 10% FBS. MCF10A cells were grown in DMEM/F12 medium (Invitrogen) supplemented with horse serum (Invitrogen), 20ng/ml EGF (R&D), 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma) and 10 μg/ml insulin
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(TGFβ (R&D systems). The following plasmids were used: pRetro and pLKO.1 (lentiviral vector). pGL3-E-cadherin-luciferase was a gift from J.A. Martignetti (Mount Sinai School of Medicine, New York, USA). RNAi constructs were generated by cloning the sequence below into pRetroSuper. PIP4K2B 5′-AGATCAAGGTGGACAATCA-3′. Retroviral particles were generated in Phoenix ecotropic cells to transduce HEK293 cells expressing the ecotropic receptor. Cell populations were selected with puromycin (2μg/ml).

The lentiviral vector pLKO.1 containing shRNA targeting sequences were used to knock down PIP4K2B in MCF7 and MCF10A cells. Viral particles were generated in HEK293FT cells in 6 well plates using 2 μg of total DNA consisting of pLKO.1 vector and plasmids encoding GAGPol and VSVG (the ratio of being 4:2:1, respectively). Cells were transduced in the presence of polybrene (5μg/ml) and populations of cells were selected using puromycin (2μg/ml).

Patients and tumour samples
The tissue microarray used included tumour cores from 489 consecutive breast cancer cases diagnosed at the Department of Pathology, Malmö University Hospital, Sweden between 1988 and 1992. (25)

Immunocytochemistry
For immunocytochemistry HEK293 cells were harvested, washed in PBS and fixed for 4 hours in 4% paraformaldehyde. Cell pellets were dehydrated in a graded ethanol series and embedded in paraffin. The TMA slides were deparaffinised, rehydrated and microwave-treated in target retrieval solution citrate buffer (10mM, pH 6.0), Sections were incubated with the indicated antibodies and were visualised was visualized using DAB. The PIP4K2BP6 antibody was used at 1:500, while the Anti-ERBB2 antibody was used according to manufactures instructions (Pathway CB-USA, 760-2694)

PIP4K2B staining intensities were subdivided into six categories (groups 0, 1, 2, 3, 4 and 5) and for further statistical analysis we combined groups 0 and 1, groups 2 and 3 and groups 4 and 5 to generate three intensity groupings. ERBB2 staining was scored semi-quantitatively by the intensity and percentage of staining as 0 and 1+ negative, 2+ equivocal and 3+ as positive. Evaluation was performed by two independent observers (one a pathologist), with the pathologist’s score superseding the other observer’s at consolidation. Conflicting observations were low (< 5%) for all evaluations made. All immunohistochemical evaluations were performed without prior knowledge of tumour characteristics.

Cell viability/proliferation assay
MCF7 cells were plated (3000 cells/well) in clear 96-well plates and allowed to adhere overnight. Cell viability/proliferation was monitored at day 1, 3 and 5 post plating. Alamar Blue reagent (Invitrogen) was added to the plate, incubated for 2 hours at
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37°C and fluorescence was measured on a POLARstar Omega plate reader (BMG) using 530nm excitation and 590nm emission.

**Anchorage-dependent and -independent clonogenic growth assays**
MCF7 cells were plated at low density (1000 cells/10cm plate) and allowed to grow for 10 days. Colonies were stained with crystal violet (0.5% w/v in PBS-0.5% formaldehyde v/v). After extensive washing to remove non-specifically bound dye the plates were dried, scanned and colonies were quantified with Image J software. For colony density analysis, colonies were stained with ethidium bromide (Sigma) and fluorescence and phase-contrast images were taken with an Axiocamera and quantified with Image J software. Data were presented as number of colonies divided by colony area. Anchorage-independent growth was monitored in soft agar. Cells were embedded in low-melting agarose (Sigma) and allowed to grow for 10 days. Images were taken using an Axiocamera and colonies were quantified with Image J software.

**Western immunoblot analysis**
Protein expression was analysed by standard Western blotting procedures employing the following antibodies: PIP4K2BP6 was generated in house by immunising New Zealand white rabbits with a specific PIP4K2B peptide (CNLLSFPRFFGP) coupled to keyhole limpet haemocyanin). Control antibody supernatant was obtained by immunopurification of PIP4K2BP6 serum using a PIP4K2B epitope containing column. ERBB2 (Labvision Neomarkers) CDH1 (BD), Actin (Chemicon International), Fibronectin (BD), Vimentin (Novo Castro), B-catenin (Cell Signalling Technology) and Tubulin (Sigma).

**Luciferase assay**
Reporter assays were performed using the Dual-GLO® Luciferase System (Promega). Cells transfected with a renilla vector and pGL3-E-cadherin-luciferase were lysed in passive lysis buffer and transferred to a white polystyrene 96-well plate (Corning). Luminescence was measured using a POLARstar Omega plate reader (BMG).

**RT-PCR analysis**
RNA was isolated with an RNeasy® Plus Mini kit and QIAshredder spin columns (Qiagen). cDNA was generated using the high capacity reverse transcription kit (Applied Biosystems). qPCR assays were performed in MicroAmp® optical 384-well reaction plate (Applied Biosystems) and analysed using an Applied Biosystems 7900HT Sequence Detection System (SDS). Ribosomal protein L32 was used as a housekeeping gene loading control. Primers were designed and probes selected using the universal probe library. SDS 2.1 software provided with the 7900HT system was used to interpret the raw qPCR data to produce Ct values. Once normalised for housekeeping gene expression and control samples these values represented quantitation of mRNA transcript levels.
Results

**PIP4K2B antibody characterisation**

In order to study PIP4K2B signalling we developed a PIP4K2B-specific antibody (24) generated to the peptide sequence NLLSFPRFFGP which is present in 2B but absent in both the 2A and 2C isoforms (Figure 1A). The antibody, PIP4K2BP6, specifically recognised purified GST-PIP4K2B compared to an equivalent amount of GST-PIP4K2A by both ELISA and by western blotting (Figure 1B and C). We first assessed if PIP4K2BP6 could be used to stain cells that had been fixed and embedded in paraffin in which we had suppressed or increased the expression of PIP4K2B. As an additional control, we also used a pre-immune serum from the rabbit used to generate the PIP4K2BP6 antibody. The PIP4K2BP6 antibody specifically stained wild type HEK293 cells compared to the pre-immune serum (Figure 1D panel 1 and 2). The specific staining observed with PIP4K2BP6 was diminished when endogenous PIP4K2B expression was suppressed by shRNA (Figure 1D panel 3 and insert) and the staining intensity was increased in cells that overexpressed PIP4K2B (Figure 1D panel 4). We next stained sections derived from several normal breast and tumour samples. To control for antibody staining, the PIP4K2BP6 serum was immuno-depleted for PIP4K2B antibodies by passing the serum through a column to which the P6 peptide had been coupled. Immunoblotting demonstrated that the PIP4K2BP6 antibody no longer recognised GST-PIP4K2B after passing through the P6 column (Supplementary Figure 1). In normal breast tissue, PIP4K2B was strongly expressed in the luminal epithelial cells of ducts and acini where it was predominantly localised at the plasma membrane. Little staining was observed in the myoepithelial cell layer (Figure 1E panel 1). In tumours heterogeneous staining was observed in which plasma membrane staining was often lost and cytosolic and nuclear staining became apparent (Figure 1F panel 1). The specificity of PIP4K2B staining was observed in which plasma membrane staining was often lost and cytosolic and nuclear staining became apparent (Figure 1F panel 1). The specificity of PIP4K2B staining was demonstrated by using the P6-peptide depleted antiserum which showed decreased staining of both normal and tumour tissue (Figure 1E and F panel 2). The heterogeneous cellular localisation of PIP4K2B observed in normal and tumour tissue was also observed in several breast cancer cell-lines fixed and embedded in paraffin, which showed both nuclear, cytosolic and membrane staining. Notably we observed strong nuclear staining of PIP4K2B in MCF7 cells (Supplementary Figure 2).

**PIP4K2B staining in breast tumours correlates with several clinico-pathologic tumour parameters**

To study PIP4K2B levels in breast tumours a tissue micro array (TMA) containing 489 advanced breast tumour samples was stained with the PIP4K2BP6 antibody. PIP4K2B expression varied dramatically between tumour cores across the array (Figure 2A panel 1-3). For statistical analysis we categorised PIP4K2B expression in to three groups with 1 representing the lowest intensity and 3 the highest (Figure 2B). As the PIP4K2B gene can be co-amplified with the ERBB2 gene (23), we also assessed ERBB2
Figure 1. Characterisation of the PIP4K2BP6 antibody. A. Sequence alignment of PIP4K2A, PIP4K2B and PIP42C of the region around the epitope (highlighted in red) to which the P6 antibody was raised. B. Serial dilutions of P6 serum were applied to a 96-well Elisa plate containing recombinant PIP4K2B and PIP4K2A protein. C. Recombinant PIP4K2B and PIP4K2A were analysed by Western blotting with the PIP4K2BP6 antibody. D. HEK293 cells were transduced with a control vector (pRetro panel 1 and 2), or a shRNA construct targeting PIP4K2B (panel 3). The shRNA vector targeting PIP4K2B decreased PIP4K2B protein levels compared to control.
Figure 2. Expression of PIP4K2B and ERBB2 in breast tumour samples. A. Examples of typical staining patterns observed in breast tumour tissue stained with PIP4K2BP6. Low (1), intermediate staining (2) and high intensity (3) staining are shown. Scale bar represents 50 μM (1) 100 μM (2 and 3). B. After staining with the PIP4K2BP6 antibody the intensity was scored and examples of the scoring for the three different categories of PIP4K2B staining intensities are shown. Group 1, weak or no staining, group 2, intermediate staining and group 3, high staining. C. Cores derived from the same breast tumour sample were stained for PIP4K2B and for ERBB2. The images in the red boxes show higher magnifications as indicated by the scale bars.

Vector (inset next to panel 3). Cells were also transduced with a construct to overexpress PIP4K2B (panel 4). Cells were fixed and embedded in paraffin and stained with a control pre-immune antibody (1) or the PIP4K2BP6 antibody (2, 3 and 4). The images show that PIP4K2BP6 specifically recognises PIP4K2B. E. Normal breast tissue was stained with PIP4K2BP6 before (panel 1) or after (panel 2) specific depletion of PIP4K2BP6 with the P6 peptide. F. Breast tumour tissue was stained with PIP4K2BP6 before (panel 1) or after (panel 2) specific depletion of PIP4K2BP6 with the P6 peptide. Scale bar represents 50 μm (E) or 100 μm (F).
expression in this TMA. ERBB2 staining was scored semi-quantitatively by the intensity and percentage of staining as 0 and 1+ negative, 2+ equivocal and 3+ as positive. Figure 2C shows a sample core that strongly stained positive for both PIP4K2B and ERBB2. Strong membrane staining of ERBB2 could be seen, while PIP4K2B staining again appeared more cytosolic. Analysis of the whole TMA clearly demonstrated a strong significant correlation between high ERBB2 expression and high PIP4K2B expression (Figure 3A) confirming and extending the studies of Luoh et al (23).

We next analysed correlations between PIP4K2B expression and clinical pathological parameters (Figure 3A). There was a statistically significant correlation between PIP4K2B expression and the Nottingham histological grade (NHG) \( (P = 0.007, \text{Pearson Chi Square test}) \); low PIP4K2B expression was associated with a high NHG. PIP4K2B expression also negatively correlated with both tumour size \( (P = 0.021, \text{Pearson Chi Square test}) \) and expression of the proliferation marker Ki67 \( (P = 0.011, \text{Pearson Chi Square test}) \) indicating that tumours with low expression of PIP4K2B appear to be larger in size, more proliferative and associated with more malignant grade. ER and PR status did not correlate with PIP4K2B expression nor was there a statistically significant correlation with age or lymph node positivity. The majority of cores included in the TMA originated from ductal and lobular tumours with no statistically significant difference between PIP4K2B expression and histological type. Interestingly, observation of distant metastasis during follow up correlated with PIP4K2B expression \( (P = 0.040, \text{Pearson Chi Square test}) \); more patients with low PIP4K2B expression exhibited metastasis during follow up compared to high PIP4K2B expression. Together these data correlate low PIP4K2B expression with worse clinico-pathological parameters.

To investigate if PIP4K2B expression correlated with breast cancer outcome, a Kaplan Meier curve was generated. PIP4K2B expression correlated with patient outcome (Log Rank chi-squared = 6.753, \( P = 0.034 \)) as defined by time from diagnosis until local, regional or distant metastasis or breast cancer-specific death (Figure 3B). Low PIP4K2B expression correlated with poorer patient outcome indicating that loss of PIP4K2B may facilitate a more malignant tumourigenic phenotype. Intermediate or high PIP4K2B expression (group 2 and 3, respectively) correlated with better cumulative survival and less observation of cancer specific events.

**PIP4K2B knock down in MCF7 cells affects colony formation in anchorage-dependent clonogenicity.**

To begin to deconstruct the correlation between poorer patient survival with tumours that have low PIP4K2B expression we examined the role of PIP4K2B in the growth of the MCF7 breast tumour cell line. PIP4K2B expression was targeted using two separate lentiviral-driven shRNAs against PIP4K2B. Western blotting demonstrated that both of these constructs strongly reduced the level of PIP4K2B protein by more than 90% compared to vector alone (pLKO1) transduced cells (Figure 4A). Although
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Consecutive array PIP4K2B

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**Figure 3.** Clinico-pathological parameters of the TMA correlated with expression of PIP4K2B. A. Correlation between PIP4K2B staining and clinico-pathological and molecular parameters of advanced breast cancer. B. Kaplan-Meier curve showing the effect of PIP4K2B expression levels on event free survival as a function of time from diagnosis until local, regional or distant metastasis or breast cancer-specific death. Low PIP4K2B expression group 1 correlated with poor patient outcome in contrast to the intermediate and high expression of PIP4K2B (group 2 and 3), \( \text{Log Rank chi-squared} = 6.753, P = 0.034 \)
PIP4K2B expression plays a role in priming breast cells for an EMT-like phenotype

A. MCF7 cells were transduced with a control lentiviral vector or two different shRNA vectors targeting PIP4K2B (#1 and #2). Cell lysates were subjected to western blotting to detect PIP4K2B and actin.

B. MCF7 cells as described in A were plated and viability/proliferation was measured on day 1, 3 and 5 post plating using alamar blue. Data are representative of three experiments and represent the mean ± SD. No statistically significant changes were observed between samples.

C. Cells were fixed and labelled with propidium iodide and analysed by FACS.

D. MCF7 cells were plated in soft agarose to assess anchorage-independent growth. Images were taken with an Axiocamera and quantified with Image J software. Images are representative of 3 separate biological replicates. The graph presents the

Figure 4. PIP4K2B knock down in MCF7 cells decreases colony formation in anchorage-dependent clonogenic growth assays. A. MCF7 cells were transduced with a control lentiviral vector or two different shRNA vectors targeting PIP4K2B (#1 and #2). Cell lysates were subjected to western blotting to detect PIP4K2B and actin. B. MCF7 cells as described in A were plated and viability/proliferation was measured on day 1, 3 and 5 post plating using alamar blue. Data are representative of three experiments and represent the mean ± SD. No statistically significant changes were observed between samples. C. Cells were fixed and labelled with propidium iodide and analysed by FACS. D. MCF7 cells were plated in soft agarose to assess anchorage-independent growth. Images were taken with an Axiocamera and quantified with Image J software. Images are representative of 3 separate biological replicates. The graph presents the
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numbers of three different sizes of colonies (Image J Arbitrary Units, from small 0.002 to big 0.05) divided by total number of colonies. Data are representative of two experiments performed in triplicate and the data represent the mean ± SD. E. 1000 cells from pLKO1 control and PIP4K2B knock-down cell lines were plated in a 10 cm plate and grown for 10 days to assess anchorage dependent clonogenic growth. Typical images of the colonies are shown and quantitation showed significant differences (** P <0.01, *** P <0.001 (student t-test)) between pLKO and PIP4K2B knockdown constructs (#1 and #2). F. Colony morphology of cells from E, was evaluated by staining with ethidium bromide (EtBr) to assess cell number per colony and phase contrast images were used to determine the colony area. The data are presented as the number of cells per colony surface area (Image J, arbitrary units). ** P <0.01, *** P <0.001 (student t-test).
PIPK2B expression plays a role in priming breast cells for an EMT-like phenotype.

A. pLKO1 shPIP4K2B #1 shPIP4K2B #2

B. Day 1 Day 2 Day 3 Day 4

CDH1
P-473 AKT
AKT1S1
PIP4K2B
ACTA

C. Day 1 Day 4

MG132 10nM/6hrs

CDH1
PIP4K2B
ACTA

D. E.
a small reduction in cell growth was apparent in the first two days using shPIP4K2B #2 this was not observed using shPIP4K2B #1, and after day two the growth rate was comparable with the growth rate of the pLKO1 transduced control cells (Figure 4B). No statistically significant differences in cell cycle distribution between PIP4K2B knockdown cells compared to control cells were found (Figure 4C). We next studied anchorage-dependent and independent clonogenic growth. Cells were plated in soft agar and anchorage independent growth was analysed after ten days. No significant changes were observed in colony numbers between control and PIP4K2B knockdown cells. Although, there was a small reduction in colony numbers when PIP4K2B was knocked down with shPIP4K2B #2, this was not observed with shPIP4K2B #1 (Figure 4D). In anchorage-dependent clonogenic assays, both PIP4K2B knockdown constructs led to a decrease in the number of colonies observed compared to pLKO1 control (Figure 4E). Microscopic analysis of the colonies suggested that knockdown of PIP4K2B led to a change in the morphology of the colonies. More specifically colonies from cells in which PIP4K2B was knockdown were less tightly packed than the pLKO1 transduced control cells.

To quantify this we stained the colonies with ethidium bromide to visualise nuclei and quantify cell number and measured colony area. The data are presented as the number of cells/colony area and quantitation showed a statistically significant decrease when PIP4K2B was knocked down (Figure 4F). The reason for the decrease in cell number per colony area is likely to be complex and might be explained by changes in contact growth inhibition, surface area/cell number maintenance mechanisms or may reflect a decrease in cell/cell contact together with increased cell migratory behaviour.

Lentiviral-mediated knock down of PIP4K2B leads to a decrease in CDH1 expression

As colonies from PIP4K2B knockdown cells appeared less tightly packed we investigated if there were any changes in the levels of CDH1. Previous studies have
shown that loss of cell-cell contacts are often associated with loss of CDH1 in cancer cells which can contribute to a malignant transition (3, 9). MCF7 cells transduced with PIP4K2B knockdown constructs showed less total CDH1 expression as assessed by immunofluorescence (Figure 5A). We observed less staining at cell/cell junctions and less overall staining (Figure 5A). We also assessed CDH1 levels in the non-tumourigenic breast cell line MCF10A. PIP4K2B was knocked down using two separate shRNAs (#1 and #2) and CDH1 levels were assessed over 4 days. In control cells CDH1 levels steadily increased. However in PIP4K2B knockdown cells the levels of CDH1 remained low. PIP4K2B expression was reduced by approximately 80% and this was maintained throughout the time course, although shPIP4K2B #2 appeared to be slightly less efficient than shPIP4K2B #1 at day 1 perhaps explaining the difference in relative inhibition of CDH1 expression observed (Figure 5B). AKT activation decreases CDH1 expression and increases CDH1 sequestration in intracellular vesicles. As PIP4K2B has been suggested to modulate AKT activation (18), we monitored AKT activation (serine 473 phosphorylation) and the phosphorylation status of its downstream target AKT1S1. No significant differences in either AKT or AKT1S1 were observed between control and PIP4K2B knockdown cells although AKT activation was down regulated during the time course of the experiment.

CDH1 expression can be regulated transcriptionally and post transcriptionally and we determined if PIP4K2B might regulate proteasome-mediated degradation of CDH1. PIP4K2B knockdown decreased CDH1 at day 1 and day 4 but the decrease was not prevented by pre-incubation with the proteosome inhibitor MG132 (Figure 5C). To determine if PIP4K2B knock down might affect the transcription of CDH1, we measured the levels of CDH1 mRNA. PIP4K2B expression was reduced by both knockdown constructs and correlated with a significant decrease in CDH1 mRNA (Figure 5D). Furthermore, to establish if transcription of the CDH1 gene might be decreased, we assessed CDH1 promoter activity in MCF10A cells after knock down of PIP4K2B using the CDH1 gene promoter coupled to luciferase transcription. Both shPIP4K2B #1 and shPIP4K2B #2 constructs decreased luciferase activity driven by the CDH1 promoter (Figure 5E). These data indicate that PIP4K2B regulates CDH1 transcription.

**Lentiviral mediated knock down of PIP4K2B induces EMT characteristics in MCF10A cells**

Deregulation and loss of CDH1 expression is considered to be one of the hallmarks of EMT, although CDH1 loss can also induce EMT. We considered that PIP4K2B knock down and subsequent down-regulation of CDH1 might prime cells to undergo EMT more readily. TGFβ is a well characterised inducer of EMT in MCF10A cells and was therefore used to stimulate cells to acquire mesenchymal features (26). PIP4K2B was knocked down in MCF10A cells and the cells were maintained untreated or treated with TGFβ for 72 hours. Cell lysates were prepared and probed with antibodies
against various markers of EMT (Figure 6A). Treatment of control cells with TGFβ led to the expected decrease in CDH1 and increase in the mesenchymal markers fibronectin (FN1) and vimentin (VIM). PIP4K2B knock down decreased the basal level of CDH1 to a similar extent as that observed after treatment of control cells with TGFβ (Figure 6A). Treatment with TGFβ further decreased CDH1 levels. Reduced PIP4K2B expression also augmented the increase in the mesenchymal markers, fibronectin and vimentin, upon TGFβ treatment. SNAI1 is a master transcriptional regulator of the EMT programme and can repress CDH1 transcription. In control cells the level of SNAI1 was increased in response to TGFβ treatment and this was augmented by PIP4K2B knockdown (Figure 6A). TGFβ controls EMT through regulating gene transcription and therefore we assessed if the changes in EMT markers observed in PIP4K2B knockdown cells might be regulated transcriptionally. MCF10A cells were transduced with the pLKO1 empty vector or two separate shRNAs (#1 and #2) targeting PIP4K2B. Cells were stimulated with TGFβ for 48 hours or left as control and the levels of PIP4K2B, CDH1, VIM, FN1 and SNAI1 mRNA were assessed by qRT-PCR (Figure 6B). Both shRNAs against PIP4K2B knocked down PIP4K2B mRNA to approximately 50% compared to PLKO in both control cells and those treated with TGFβ. CDH1 mRNA levels decreased in control cells and VIM, FN1 and SNAI1 mRNA increased, as expected, when stimulated with TGFβ. PIP4K2B knockdown decreased basal levels of CDH1 mRNA to a level similar to control cells treated with TGFβ. The basal levels of VIM, FN1 and SNAI1 mRNA were not significantly increased by knockdown of PIP4K2B but their expressions were increased compared to control cells after TGFβ treatment. PIP4K2B knock down did not significantly change the expression of Slug (SNAI2) before or after treatment with TGFβ. These data suggest that PIP4K2B-mediated regulation of CDH1 expression levels primes cells to undergo EMT in response to TGFβ.

As reduced CDH1 expression is associated with a more aggressive tumour type showing increased tendency to metastasise, the poorer patient prognosis observed in tumours that have low PIP4K2B expression might in part be explained by a reduction in CDH1 expression. To determine if low CDH1 expression correlates with decreased PIP4K2B expression in human breast tumours, we interrogated their expressions in a publically available database using Oncomine. The expression data sets for PIP4K2B and CDH1 were downloaded from the unpublished Bittner breast tumour array and from the published Curtis array (27). The data were sorted with respect to PIP4K2B expression and the CDH1 levels were determined in the top and bottom 10% of the PIP4K2B expressing population. We observed a significant decrease in the expression levels of CDH1 in the low PIP4K2B expressing tumours compared to the high PIP4K2B expressing populations in both arrays (P = 0.0039 in the Bittner array and P = 0.000437 in the Curtis array, Figure 6F). No significant changes were observed in SNAI1 or TWIST1, two upstream repressors of CDH1.
PIP4K2B expression plays a role in priming breast cells for an EMT-like phenotype

A. 

B. 

C. 

**Log2 array median centered expression intensity**

low PIP4K2B

High PIP4K2B
Discussion

In this study we describe the characterisation and use of a PIP4K2B specific antibody to interrogate its expression in paraffin embedded cells and tissues. We have used this antibody to probe a TMA of breast tumours and show that the expression of PIP4K2B correlates with several clinico-pathological parameters. To our knowledge this is the first time expression of PIP4K2B has been studied in a large cohort of patient tumour samples. We found that high expression of PIP4K2B correlates with ERRB2 expression, which concurs with the findings of Luoh et al. (23). Interestingly in their study they found one tumour that showed amplification of the PIP4K2B gene in the absence of amplification of ERRB2. Although we did not study gene amplification, we observed 58 tumours that showed high expression of PIP4K2B which were scored negative for ERRB2 expression, suggesting that PIP4K2B can be overexpressed in the absence of increased ERRB2 expression. The number of tumours that expressed ERRB2 with or without PIP4K2B expression was too low to derive significant clinical outcome data relating the role of PIP4K2B in clinico-pathological parameters of ERRB2 positive tumours. Analysis of the whole array showed that tumours with low expression of PIP4K2B had significantly greater size, NHG grade, Ki67 staining and distant metastasis during follow up. Kaplan Meier survival curves indicated that decreased expression of PIP4K2B was associated with poorer patient outcome related to a greater incidence of metastases.

In order to define plausible mechanisms for poorer patient outcome in PIP4K2B low expressors, we investigated how PIP4K2B knockdown might impinge on a number of different tumour specific parameters in breast tumour and normal breast cell lines. PIP4K2B knockdown had little effect on the growth rate of MCF7 cells in soft agar but did decrease their growth in anchorage dependent clonogenic assays. Furthermore, we observed a significant change in the morphology of the colonies during anchorage dependent colony growth when PIP4K2B was depleted. PIP4K2B knockdown significantly decreased the number of cells observed per colony area. The change in colony morphology could occur as a consequence of changes in many different parameters of tumour cell growth, however we observed that PIP4K2B knockdown significantly...
decreased the expression of CDH1, a tumour suppressor and a critical regulator of cell/cell adhesion. CDH1 expression regulates cell/cell contact and its loss is associated with an increased malignant phenotype and can drive invasive carcinoma formation in a mouse model (3, 9, 28-29). In order to correlate PIP4K2B expression with CDH1 expression in human tumours, we interrogated public databases of gene transcription arrays of primary human tumours and found that decreased expression of PIP4K2B was associated with decreased CDH1 expression confirming our studies in human breast tumour and normal cell lines. Previous studies have demonstrated that low tumour CDH1 is associated with high grade tumours and poor prognosis (30-32). Finally we observed that down regulation of PIP4K2B primes MCF10A cells for TGFβ induced EMT. The priming may be a consequence of the decrease in CDH1 expression or might be a consequence of a direct effect of PIP4K2B in TGFβ signalling. SNAI1 expression can be directly regulated by TGFβ-mediated activation of Smad2/3 and we observed an increase in Snail expression after TGFβ treatment in PIP4K2B knockdown cells compared to control cells.

How PIP4K2B regulates CDH1 transcription is not clear. PIP4K2B can negatively regulate AKT activity and increased AKT has been associated with decreased CDH1 expression through the activation of the transcriptional regulator SNAI1. However, during the 4-day time course of PIP4K2B knock down in MCF10A cells we did not observe PIP4K2B dependent changes in AKT activity suggesting that AKT is unlikely to mediate the effect of PIP4K2B knockdown on CDH1 expression. CDH1 expression is regulated by a number of transcriptional repressors including ZEB1, ZEB2, SNAI1, SNAI2 and TWIST family members, although PIP4K2B knockdown in MCF10A cells did not change the basal expression levels of SNAI1 or SNAI2. PIP4K2B can regulate the nuclear levels of both PtdIns(4,5)P$_2$ and PtdIns5P. Changes in nuclear phosphoinositides have been associated with changes in gene transcription although the direct mechanisms are still unclear. Interestingly Vitamin-D3 induced CDH1 transcription, which may constitute a major mechanism for the tumour suppressive effects of Vitamin D3 in colon cancer, requires the expression of PIP4K2B (33). Although the mechanism was not defined, the authors suggest that synthesis of nuclear PtdIns(4,5)P$_2$ might be important for PIP4K2B mediated regulation of CDH1 expression. We have previously demonstrated that PIP4K2B is important in regulating the levels of its substrate PtdIns5P within the nucleus. PtdIns5P can regulate chromatin remodelling and transcription through its ability to interact with nuclear proteins that contain a PHD zinc finger motif. We suggest that a decrease in CDH1 expression in tumours that express low levels of PIP4K2B ultimately might lead to a decrease in patient event free survival. Targeting other tumour arrays will be important to determine if PIP4K2B expression also correlates with poor patient prognosis.

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References


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Supplementary materials

Supplementary Figure 1. The PIP4K2BP6 antibody was immunodepleted by passing it through a column to which the P6 antigenic peptide was coupled. The antibody before or after, as indicated, was used to probe western blots of increasing levels of PIP4K2B.

Supplementary Figure 2. Cells as indicated were fixed and embedded in paraffin and sections were cut and mounted. The sections were stained with the PIP4K2BP6 antibody after antigen retrieval.