


RESEARCH ARTICLE

Ectopic expression of PLC- β 2 in non-invasive breast tumor cells plays a protective role against malignant progression and is correlated with the deregulation of miR-146a

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Cells in non-invasive breast lesions are widely believed to possess molecular alterations that render them either susceptible or refractory to the acquisition of invasive capability. One such alteration could be the ectopic expression of the β 2 isoform of phosphoinositide-dependent phospholipase C (PLC- β 2), known to counteract the effects of hypoxia in low-invasive breast tumor-derived cells. Here, we studied the correlation between PLC- β 2 levels and the propensity of non-invasive breast tumor cells to acquire malignant features. Using archival FFPE samples and DCIS-derived cells, we demonstrate that PLC- β 2 is up-regulated in DCIS and that its forced down-modulation induces an epithelial-to-mesenchymal shift, expression of the cancer stem cell marker CD133, and the acquisition of invasive properties. The ectopic expression of PLC- β 2 in non-transformed and DCIS-derived cells is, to some extent, dependent on the de-regulation of miR-146a, a tumor suppressor miRNA in invasive breast cancer. Interestingly, an inverse relationship between the two molecules, indicative of a role of miR-146a in targeting PLC- β 2, was not detected in

Abbreviations: DABCO, 1,4-diazabicyclo [2.2.2] octane; DAPI, 4',6-diamidino-2-phenylindole; DCIS, Ductal carcinoma in situ; DMEM F-12, Dulbecco's modified Eagle's and Ham's F-12 media; EMT, Epithelial-to-mesenchymal transition; FFPE, Formalin fixed paraffin-embedded; IDC, Invasive ductal carcinoma; NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; PBS, Phosphate buffered-saline; PLC- β 2, Phosphoinositide-dependent phospholipase C- β 2; siRNAs, Small interfering RNAs; TNBC, Triple-negative breast cancer.

Valeria Bertagnolo and Silvia Grassilli contributed equally to this work.

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primary DCIS from patients who developed a second invasive breast neoplasia. This suggests that alterations of the PLC- β 2/miR-146a relationship in DCIS may constitute a molecular risk factor for the appearance of new breast lesions. Since neither traditional classification systems nor molecular characterizations are able to predict the malignant potential of DCIS, as is possible for invasive ductal carcinoma (IDC), we propose that the assessment of the PLC- β 2/miR-146a levels at diagnosis could be beneficial for identifying whether DCIS patients may have either a low or high propensity for invasive recurrence.

KEYWORDS

breast tumor recurrence, ductal carcinoma in situ (DCIS), epithelial-to-mesenchymal transition (EMT), miR-146a, phosphoinositide-dependent phospholipase C beta 2 (PLC- β 2)

1 | INTRODUCTION

Since the introduction of mammography screening, ductal carcinoma in situ (DCIS) constitutes about 20-25% of newly diagnosed cases of breast cancer in industrialized countries^{1,2} and, although DCIS is a non-obligate precursor, it may constitute a crucial step in the progression toward invasive ductal carcinoma (IDC).^{3,4} The progression of DCIS to IDC is a very complex biological phenomenon and recent studies indicate that cells in DCIS lesions likely harbor molecular alterations that render them prone to the acquisition of invasive capability.⁵

Among the signaling molecules deregulated in breast cancers is the beta2 isoform of phosphoinositide-dependent phospholipase C (PLC- β 2). Physiologically expressed in hematopoietic cells only, PLC- β 2 is up-modulated in the large majority of primary invasive mammary tumors independent of their histological subtypes, in which positively correlates with malignancy and poor prognosis.⁶ In highly invasive breast tumor-derived cells, PLC- β 2 sustains invasive capability⁷ and is inversely correlated with the cancer stem cell marker CD133,⁸ involved in cell invasion and lymph node metastases.^{8,9} In low-invasive breast tumor-derived cells, PLC- β 2 is down-modulated by hypoxia, a crucial condition for the malignant progression of solid tumors, including breast cancer.¹⁰ The forced up-regulation of PLC- β 2 in pre-EMT breast tumor-derived cells prevents E-cadherin levels from decreasing and blocks the up-regulation of the stem cell marker CD133 induced by low oxygen,¹⁰ suggesting that this PLC isozyme has a role in preventing the malignant progression of early breast neoplasia.

The primary aim of this work was to assess the relationship between PLC- β 2 expression in non-invasive breast tumors and their propensity to progress into invasive lesions. In addition, since the regulation of gene expression during malignant breast cancer evolution is mainly attributed to miRNAs,^{11,12} the miRNA responsible for modulating PLC- β 2 levels in breast tumors was investigated. Using both archival formalin-fixed breast tumor samples and breast

tumor-derived cells, we demonstrate that primary DCIS express PLC- β 2 at varying levels and that PLC- β 2 amounts are inversely correlated to those of miR-146a, which is known to reduce the metastatic potential of invasive breast cancer cells. The involvement of miR-146a in modulating PLC- β 2 in DCIS-derived cells is addressed, as is the potential prognostic relevance of the PLC- β 2/miR-146a ratio in primary DCIS.

2 | MATERIALS AND METHODS

All reagents were from Sigma (St Louis, MO) unless otherwise indicated.

2.1 | Breast tumor tissues

Cohort 1 included 70 formalin fixed paraffin-embedded (FFPE) samples of primary pure DCIS selected from a collection of tumors archived by the Pathological Anatomy Division of the Verona and Rovigo Hospitals and from the Surgical Pathology and Cytopathology Unit of the Padua University. The age of patients at diagnosis ranged between 32 and 84 yr (median: 51). A total of 9% of DCIS were comedo, 64% non comedo, and 27% mixed histotype. Concerning histological grade, 20% were low, 33% intermediate, and 47% high grade tumors.

Cohort 2 included 20 FFPE samples of consecutive primary IDC collected at the Surgical Pathology and Cytopathology Unit of Padua University. Nine showed Luminal A, 4 Luminal B, 2 HER2, and 5 TNBC molecular phenotype. Three were low, 7 intermediate, and 10 high grade tumors.

Cohort 3 included 24 archived FFPE samples of primary unilateral pure DCIS from patients with at least 5 years available follow-up (Verona site). The Cohort included 12 patients who later developed a contralateral IDC and 12 control patients who did not recur, selected on the basis of patients' homogeneity in terms of age (median: 53, range: 51-55), adjuvant therapy, and radiotherapy administration. A total of 12% displayed comedo, 53% non

comedo, and 35% mixed histotype. A total of 29% were low grade, 12% intermediate grade, and 59% high grade tumors. The median follow-up for Cohort 2 was of 94 months (range: 61–120 months).

Patients were required to give written informed consent for their tissue samples to be archived and used for research purposes.

2.2 | Immunohistochemical analysis of PLC- β 2

Five micrometer thick tissue sections were subjected to immunohistochemical analysis with an anti-PLC β 2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) following a previously described procedure.¹³ Each stained tissue sample was analyzed by an optical microscope (Carl Zeiss Axiophot 100, Zeiss, Göttingen, D) equipped with a Nikon Digital Sight DS Vi1 camera (Nikon, Lijnden, NL) and the stain intensity was estimated by analysis of acquired images with ImageScope software (Aperio, Vista, CA). Each tumor was categorized according to arbitrarily defined criteria into “weak,” “moderate,” and “strong” staining, as previously described.⁶

2.3 | Cell culture and transfection

The non-transformed MCF10A cells were purchased from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1, v/v) (DMEM F-12, Gibco Laboratories, Grand Island, NY) containing 10 μ g/mL bovine insulin, 100 ng/mL cholera toxin, 0.5 μ g/mL hydrocortisone, 20 ng/mL recombinant human epidermal growth factor, and 10% horse serum. The breast cancer-derived cell line MCF10DCIS, kindly provided by Dr. Macpherson (Beatson Institute for Cancer Research, Glasgow, UK), was grown in DMEM F-12 supplemented with 5% horse serum and 1% Penicillin-streptomycin solution (Gibco Laboratories). Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air, monthly tested for mycoplasma and other contaminations and quarterly subjected to cell identification by single-nucleotide polymorphism.

The down-modulation of PLC- β 2 was performed with specific siRNAs (Santa Cruz Biotechnology) and PLC- β 2 over-expression was achieved by transient transfection with a plasmid expressing a full-length human PLC- β 2, following previously described procedures.¹⁰ As control of PLC- β 2 down-modulation, cells were transfected with a further siRNAs mixture (PLC- β 2 siRNAs#, QIAGEN S.p.A., Milan, I), as previously reported.⁷ The transfected cells were incubated at 37°C in a 5% CO₂ atmosphere for 48 h prior to RNA extraction and cellular assays.

For specific modulation of miR-146a, transient transfections were carried out with synthetic inhibitor or mimic (miRVana miRNA, Life Technologies, Monza, I), and Lipofectamine 2000 in Opti-MEM I medium (Gibco Laboratories) without serum, according to standard procedures. Random sequences were used as negative controls.

2.4 | Isolation of nuclei and immunochemical analysis

Nuclei from MCF10DCIS cells were isolated essentially as previously described.¹³

Total lysates from cells and nuclei (25 μ g protein) and PLC- β 2 immunoprecipitates, obtained following a previously reported procedure,⁷ were separated on 8.5% polyacrylamide /SDS gels and blotted to nitrocellulose membranes (GE Healthcare Life Science, Little Chalfont, UK). The membranes were incubated with antibodies directed against Vimentin and β -Tubulin (Sigma), E-cadherin, Ikb α , p65 and Lamin B (Santa Cruz Biotechnology), as previously reported,¹⁰ and revealed with ECL (PerkinElmer, Boston, MA). The chemiluminescent bands were detected with ImageQuant™ LAS 4000 imager and densitometry was performed with Image Quant TL (GE Healthcare).

2.5 | Cytofluorimetric evaluation of cancer stem cell markers

CD133 surface expression was evaluated in both MCF10A and MCF10DCIS cells by means of flow cytometry after direct staining with phycoerythrin (PE)-conjugated anti-CD133/2 monoclonal antibody (293C3, Miltenyi Biotec, Bologna, I), as previously reported.⁸

The expression of CD44 and CD24 surface antigens were evaluated following a previously described procedure.¹⁴ Briefly, in a one-tube assay, cells were stained with allophycocyanin (APC)-conjugated anti-CD44 and fluorescein isothiocyanate (FITC)-conjugated anti-CD24 monoclonal antibodies (Becton Dickinson, San José, CA).

All samples were analyzed by a FACSCalibur flow cytometer (Becton-Dickinson) with CellQuest Pro 6.0 software (Becton-Dickinson). Data collected from 10 000 cells are shown as percentage of positive cells or as mean fluorescence intensity (MFI) values.

2.6 | Real-time cell invasion assays

Cell invasion of MCF10A and MCF10DCIS cells was evaluated with the xCELLigence Real-Time Cell Analyzer System (Acea Biosciences Inc., San Diego, CA) that monitors cell events by measuring electrical impedance, as previously reported.^{8,10} In particular, 40 000 cells/well were seeded onto the top chambers of CIM-16 plates (Acea Biosciences) covered with a layer of Matrigel (BD Biosciences, Milan, I) diluted 1:20. The bottom chambers were filled with medium containing 10% serum and the signal was detected every 15 min for a total of 24 h. Impedance values were expressed as a dimensionless parameter (cell index, CI). Data were also subjected to slope analysis that describes the steepness, incline, gradient, and changing rate of the CI curves over time.

2.7 | miRNA-Seq analysis of formalin fixed DCIS tissues

Four consecutive 10 μ m thick sections of 30 DCIS samples selected from Cohort 1, where the tumor constituted over 70% of the tissue area and infiltrating blood cells were virtually absent, were subjected to manual macro-dissection. Dissected areas were processed for RNA extraction using AllPrep DNA/RNA FFPE Kit (QIAGEN), following the manufacturer's instructions. The short RNA was profiled using an RNAseq approach on SOLiD (Thermo Fisher Scientific, Paisley, UK),

following a previously described procedure.¹⁵ Each sample was barcoded and the RNAseq data were quality checked, pre-processed, mapped, and annotated on miRBase v21 using NovoAlign (Novocraft Technologies, Selangor, Malaysia).

2.8 | Laser-capture micro-dissection and miR-146a analysis

Five micrometer tissue sections on frame slides (Leica Microsystems, Wetzlar, D), were deparaffinized with xylene, rehydrated in RNase-free 100% ethanol, rinsed in RNase-free water, lightly stained with RNase-free hematoxylin (Bio-Optica, Milan, I) and dehydrated. For each sample, 5–10 000 cells were micro-dissected using the Leica LMD6500 laser capture microscope (Leica Microsystems). Micro-dissected tissues were subjected to RNA extraction with miRNeasy FFPE Kit (QIAGEN), following the manufacturer's protocol. RNA was subjected to real-time PCR for quantitative measurement of miR-146a using the TaqMan MicroRNA assay (Life Technologies) and a Bio-Rad CFX96™ system, as previously reported.¹⁶ Fold-changes were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the expression of U6 snRNA. Cycle threshold >35 were excluded. Control PCR samples were run without cDNA.

2.9 | Quantitative analysis of PLC-β2 mRNA and miR-146a in cell lines

High-quality total RNA from MCF10A and MCF10DCIS cells was extracted with mirVana™ miRNA Isolation Kit (Life Technologies) according to the manufacturer's instruction. RNA quantity and quality was determined by using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The first-strand cDNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), following the manufacturer's instruction. The cDNAs were employed as template for real-time RT-PCR for PLC-β2 quantitative measurement using the TaqMan® Gene Expression Assay (Life Technologies), as previously reported.¹⁰ Fold-changes were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the expression RPL13A (Ribosomal Protein L13a) mRNA levels.

MicroRNAs from MCF10A and MCF10DCIS cells were extracted with miRNeasy Micro kit (QIAGEN), following the manufacturer's instructions. For miR-146a analysis, RNA was subjected to single stranded cDNA synthesis using a TaqMan MicroRNA Reverse Transcription Kit (Life Technologies), according to the manufacturer's instructions. The cDNAs obtained were employed as templates for quantitative Real-Time PCR, following a previously described procedure.¹⁶

2.10 | Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) as previously reported.¹⁶ The samples were subjected to immunoprecipitation at 4°C overnight with antibodies directed against p65 or with a non-specific IgG, used as a negative control (Santa Cruz Biotechnology). DNA was recovered

using a PCR purification kit (Promega, Madison, WI) in 50 μL elution buffer. PCR was performed to assess for the presence of DNA precipitated by the antibodies using specific primers (Forward: 5'-GCCTCCACTGGGAGTTTAT-3', Reverse: 5'-GCATACCCCAA-CAACGGTA-3') to amplify a 137 bp region encompassing the putative NF-κB binding site located at -1645/-1633 bp from the transcriptional start in the human PLC-β2 promoter. Input corresponding to 1% of the total sonicated DNA was used as a positive control. PCR products were separated on tris-acetate 2% agarose gels, stained with ethidium bromide and visualized by UV light apparatus.

2.11 | Luciferase reporter assay

HeLa and MCF10DCIS cells were co-transfected with 50 or 250 ng pMIR-report luciferase reporter vector containing the wild-type 3'-UTR sequence of human PLC-β2 (Promega) and with 10 or 30 nM miR-146a mimic, using Lipofectamine 2000 (Life Technologies) diluted in Opti-MEM I medium (Gibco Laboratories), according to the manufacturer's instructions. After 5 h of incubation at 37°C in a 5% CO₂ atmosphere, transfected cells were cultured in growing medium for 24 h and then subjected to luciferase reporter assay using the Luciferase Assay System (Promega) in a GloMax 20/20n single tube Luminometer (Promega) according to the manufacturers' protocol. Non-targeting CONmiR mimic (Riboxx Life Sciences, Radebeul, D) was used as negative control.

2.12 | Statistical analysis

The relationships between PLC-β2 expression and clinic-pathological parameters were assessed by χ^2 test. The correlations of PLC-β2 with miRNAs were determined by using Spearman Rho. Statistical analysis of data from densitometry of WB bands was performed using the two-sided Mann-Whitney *U*-test for unpaired data (SPSS Inc., Chicago, IL). The relationship between PLC-β2 levels and patients outcome was assessed by Fisher exact test. *P* values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | PLC-β2 is present in DCIS tissues and affects EMT markers, CD133 level, and invasion capability in DCIS-derived cells

Immunohistochemical analysis performed on FFPE tissue sections from 70 pure DCIS (Cohort 1) with different histopathological features demonstrated that PLC-β2 is expressed, to a variable extent (Figure 1A), in all breast tumor samples. The analysis of PLC-β2 staining, arbitrarily quantified as weak, moderate or strong following a previously established criterion,⁶ showed that protein levels are significantly lower in DCIS with respect to unrelated IDC (Cohort 2), even though this PLC isozyme was expressed at moderate or high levels in 26% and 20% of DCIS samples, respectively (Figure 1B).

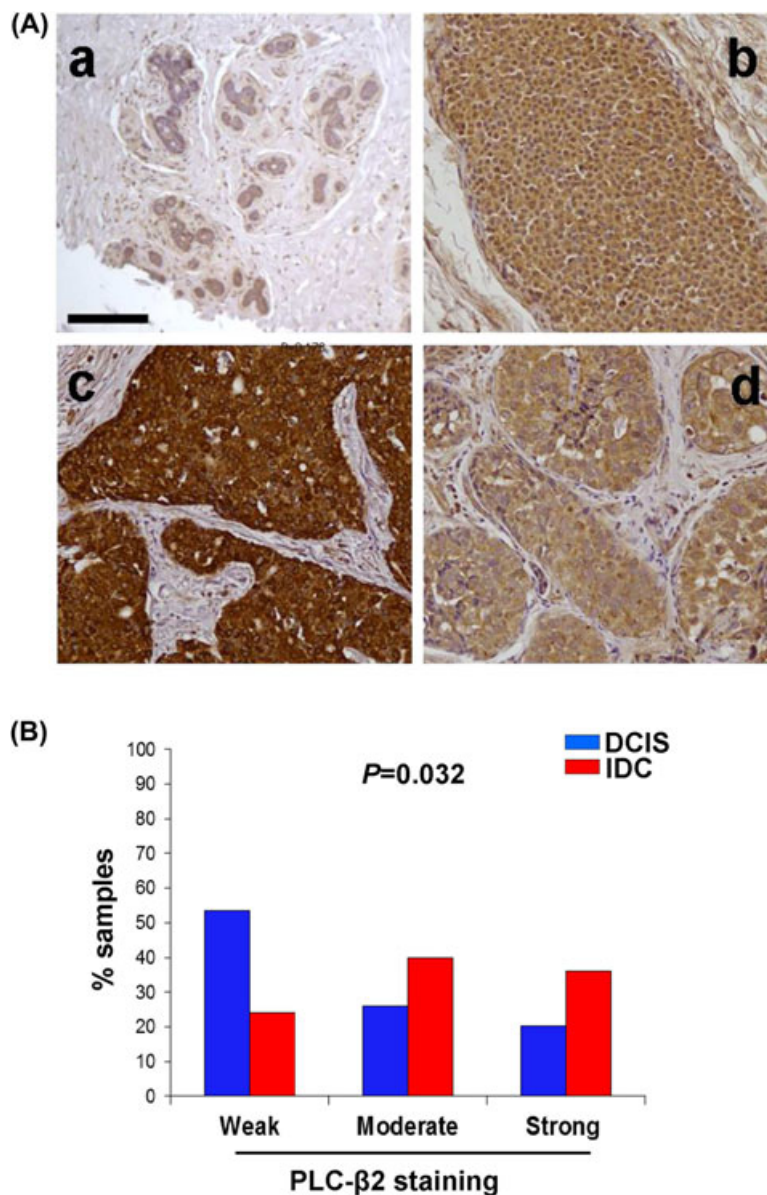


FIGURE 1 PLC-β2 is expressed in primary DCIS. In (A) immunohistochemical analysis of PLC-β2 expression of FFPE sections from healthy breast tissue (a) and DCISs with different histological features (b-d) derived from Cohort 1. b: low-grade ductal non comedo; c: intermediate-grade ductal non comedo; d: high grade ductal comedo. Bar = 100 μm. In (B) graphical representation of levels of PLC-β2 staining in primary DCIS (Cohort 1) and unrelated IDC (Cohort 2). [Color figure can be viewed at wileyonlinelibrary.com]

The amount of PLC-β2 found in DCIS did not significantly correlate with any of the main clinic-pathological factors and biological markers for breast tumors (Table 1). Conversely, a significant correlation was observed between PLC-β2 staining and the age at diagnosis, since the majority of patients ≤ 50 developed primary DCIS with low levels of the protein and none of the tumors from patients over 65 showed strong PLC-β2 staining (Table 1).

The effects of different levels of PLC-β2 on malignant features of breast cells were investigated *in vitro* in the non-transformed MCF10A cells and in the non-invasive MCF10DCIS cell line, a well-established model of high grade, comedo-like DCIS tumor.¹⁷ Stemming from our previous data indicating that PLC-β2 may regulate the levels of EMT markers in low invasive breast

tumor-derived cells,¹⁰ E-cadherin, and Vimentin were measured in cells in which the PLC isozyme was forcedly modulated. As reported in Figures 2A and 2B, in MCF10DCIS but not in MCF10A the epithelial marker E-cadherin increased and the mesenchymal marker Vimentin decreased when PLC-β2 was over-expressed. Only in DCIS derived cells a substantial increase of Vimentin was observed as a consequence of specific silencing of PLC-β2 (Figures 2A and 2B).

The analysis of invasion capability performed with the xCELLigence real-time system indicated that down-modulation of PLC-β2 is sufficient to increase the ability of MCF10DCIS to pass through Matrigel, while it was completely ineffective on motility of untransformed MCF10A (Figures 2C and 2D).

TABLE 1 Correlation of PLC-β2 staining with clinico-pathological factors and biological markers in DCIS

Features	Weak staining n (%)	Moderate staining n (%)	Strong staining n (%)	P
Age at diagnosis (n = 70)				0.0489 ^a
≤50	22 (64.7)	7 (20.6)	5 (14.7)	
50-65	10 (40)	6 (24)	9 (36)	
≥65	6 (54.5)	5 (45.5)	0 (0)	
Histotypes (n = 70)				0.348
Comedo	3 (50)	3 (50)	0 (0)	
Non comedo	26 (57.78)	11 (24.44)	8 (17.78)	
Mixed	9 (47.37)	4 (21.05)	6 (31.58)	
Size (n = 56)				0.616
≤2 cm	23 (53.49)	11 (25.59)	9 (20.92)	
>2 cm	5 (38.46)	4 (30.77)	4 (30.77)	
Histological grade (n = 70)				0.830
Low	8 (57.14)	3 (21.43)	3 (21.43)	
Intermediate	11 (47.83)	8 (34.78)	4 (17.39)	
High	19 (57.58)	7 (21.21)	7 (21.21)	
ER (n = 69)				0.648
≤10	3 (50)	1 (16.67)	2 (33.33)	
>10	34 (53.97)	17 (26.98)	12 (19.05)	
PR (n = 69)				0.340
≤10	9 (40.91)	7 (31.82)	6 (27.27)	
>10	28 (59.57)	11 (23.4)	8 (17.03)	
HER-2 (n = 15)				0.254
0	2 (50)	1 (25)	1 (25)	
1	0 (0)	4 (66.67)	2 (33.33)	
2	0 (0)	3 (100)	0 (0)	
3	1 (50)	1 (50)	0 (0)	
Ki-67 (n = 59)				0.838
≤13	18 (48.65)	10 (27.03)	9 (24.32)	
>13	11 (50)	7 (31.82)	4 (18.18)	

P, Pearson's χ^2 test.

^aStatistically significant.

Since we have previously demonstrated that, in low and highly invasive breast tumor-derived cells, PLC-β2 regulates the expression of CD133,^{8,10} this surface antigen was measured in MCF10A and MCF10DCIS subjected to forced modulation of the protein. As revealed by flow cytometry (Figure 2E), only in MCF10DCIS the number of cells expressing CD133 at surface level significantly increased as a consequence of the silencing of PLC-β2. The analysis of the surface expression of CD44 and/or CD24 in the same experimental conditions failed to reveal, in both MCF10A and MCF10DCIS cells, effects of the forced modulation of PLC-β2 (Supplementary Figure S1).

The effects of the down-modulation of PLC-β2 on EMT markers, on invasion capability and on CD133 expression in MCF10DCIS cells were confirmed by transfection with a different siRNA mixture (Supplementary Figure S2).

3.2 | miR-146a influences PLC-β2 expression in non-transformed and DCIS-derived cells

To establish whether the ectopic expression of PLC-β2 in DCIS correlates with miRNA/s de-regulated in breast tumors, 30 DCIS selected from Cohort 1 were macro-dissected and profiled by short RNA NGS. For each DCIS sample, the level of each miRNA was compared with the amount of PLC-β2 determined by immunohistochemical analysis. Of the 1222 different mature miRNAs expressed across all samples, none of those predicted to target the PLC-β2 gene (TargetScan, <http://www.targetscan.org>) were found to significantly correlate with the protein amount (data not shown). However, when the inverse relationship was considered, miR-146a emerged as the miRNA that displayed the most significant ($P < 0.001$) negative correlation with PLC-β2 staining (Table 2). The inverse relationship

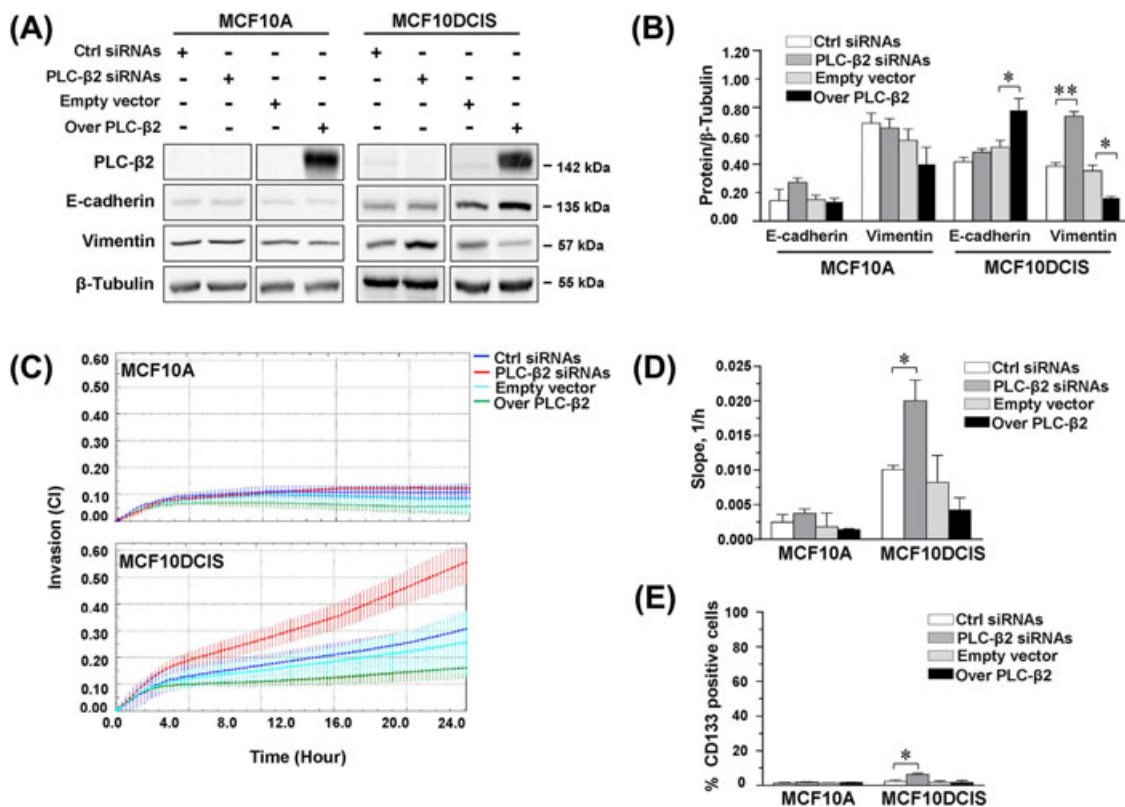


FIGURE 2 PLC-β2 modulates EMT markers, invasion capability, and CD133 expression in MCF10DCIS cells. In (A) representative Western blot analysis with the indicated antibodies of MCF10A and MCF10DCIS cells transfected with siRNAs specific for PLC-β2 (PLC-β2 siRNAs) or with a construct expressing the full-length human PLC-β2 (Over PLC-β2). Scramble siRNAs (Ctrl siRNAs) and an empty vector were used as controls. In (B) relative amounts of the indicated proteins as deduced from the densitometry of Western blot bands normalized with β-Tubulin, used as internal control for equivalence of loaded proteins. The mean expression level of three separate experiments ± SD is shown. The asterisks indicate statistically significant differences compared to transfection with respective controls (Ctrl) taken as 1. In (C) dynamic monitoring of invasion through Matrigel using the xCELLigence system RTCA system. Cell Index (CI) is reported and error bars indicate ±SD. Slope analysis that describes the steepness, incline, gradient, and changing rate of the CI curves over time, is shown in (D). In (E) cytofluorimetric evaluation of CD133 surface expression in MCF10A and MCF10DCIS cells under the above reported experimental conditions, after labelling with a phycoerythrin (PE)-conjugated anti-CD133 antibody. The number of cells expressing high levels of CD133 is reported as percentage. All the data are the mean of 3 separate experiments ± SD. (* $P < 0.05$; ** $P < 0.01$). [Color figure can be viewed at wileyonlinelibrary.com]

between PLC-β2 staining and the miR-146a expression resulting by the NGS analysis (Figure 3A) was confirmed by RT-PCR of micro-dissected areas from some selected samples showing high and low PLC-β2 staining (Figure 3B).

The role of miR-146a in modulating PLC-β2 levels was investigated in the MCF10A and MCF10DCIS cell lines, which express the two molecules at different levels. In particular, PLC-β2 was almost undetectable in MCF10A cells, coherently with their non-transformed status, while a higher expression was found in MCF10DCIS, in terms of both mRNA (Figure 4A) and protein (Figures 2A and 4C). Concerning miR-146a, a significantly lower expression was observed in the DCIS derived cells in comparison with the non-transformed MCF10A (Figure 4A). The use of synthetic mimic and inhibitors showed that silencing of miR-146a induces the expression of PLC-β2 in both cell lines and that its up-regulation partially down-modulates PLC-β2 expression only in MCF10DCIS (Figures 4B and 4C).

The possible role of PLC-β2 in regulating the levels of miR-146a was also investigated, failing to show significant modifications of

the miRNA expression as a consequence of the forced modulation of the protein in both MCF10A and MCF10DCIS cell line (Figure 4D).

3.3 | miR-146a inactivates NF-κB and targets PLC-β2

The NF-κB pathway is the main intracellular signaling known to be linked to miR-146a^{18,19} and p65 subunit was demonstrated to regulate PLC-β2 expression in platelets.²⁰ Once established that miR-146a may modulate the expression of PLC-β2 in both MCF10A and MCF10DCIS cells, the possible involvement of NF-κB was investigated in cells in which the miRNA was forcedly modulated. A strong increase of the IκBα kinase, indicative of its reduced degradation, was observed only in MCF10DCIS cultured in the presence of the miR-146a mimic (Figures 5A and 5B). In the same experimental condition, a significant decrease of p65 inside the nuclear compartment was revealed (Figure 5C), indicative of a reduced activity of NF-κB at the nuclear level. Accordingly, the use

TABLE 2 Inverse relationship between PLC- β 2 and microRNAs levels ($n = 30$)

Sample	Correlation coefficient	Parametric <i>P</i> -value	FDR	UniqueID
1	-0.46	0.0002521	0.0531	hsa-miR-146a
2	-0.534	0.0002802	0.0531	hsa-miR-3147
3	-0.448	0.0003808	0.0531	hsa-miR-320b
4	-0.417	0.001365	0.103	hsa-miR-1246
5	-0.413	0.0018919	0.103	hsa-miR-3195
6	-0.404	0.001961	0.103	hsa-miR-1291
7	-0.394	0.0019766	0.103	hsa-miR-1296
8	-0.388	0.0023279	0.108	hsa-miR-148a
9	-0.377	0.0031934	0.12	hsa-miR-146b-5p
10	-0.392	0.0035889	0.12	hsa-miR-3714
11	-0.372	0.0038837	0.12	hsa-miR-3609
12	-0.356	0.0054383	0.142	hsa-let-7f
13	-0.347	0.0068014	0.163	hsa-miR-769-5p
14	-0.344	0.0085625	0.188	hsa-miR-187
15	-0.33	0.0118621	0.222	hsa-miR-188-5p
16	-0.327	0.0124876	0.222	hsa-miR-3687
17	-0.343	0.013265	0.222	hsa-miR-320d
18	-0.319	0.0151405	0.229	hsa-miR-363
19	-0.313	0.0153457	0.229	hsa-miR-19b
20	-0.305	0.0182477	0.235	hsa-let-7e
21	-0.304	0.0185355	0.235	hsa-miR-15a
22	-0.358	0.0206028	0.247	hsa-miR-2115
23	-0.315	0.0206831	0.247	hsa-miR-3607-3p
24	-0.296	0.0217612	0.253	hsa-miR-29astar
25	-0.319	0.0228089	0.258	hsa-miR-4295
26	-0.288	0.025747	0.264	hsa-miR-362-3p
27	-0.287	0.0263124	0.264	hsa-miR-3607-5p
28	-0.287	0.0265537	0.264	hsa-miR-340
29	-0.286	0.0272886	0.265	hsa-miR-127-3p
30	-0.282	0.0294648	0.28	hsa-miR-32
31	-0.273	0.03536	0.308	hsa-miR-100
32	-0.291	0.0408292	0.325	hsa-miR-2110
33	-0.265	0.040889	0.325	hsa-miR-194
34	-0.264	0.0418104	0.325	hsa-miR-1274b
35	-0.315	0.042441	0.325	hsa-miR-519a
36	-0.263	0.0427038	0.325	hsa-miR-19a
37	-0.262	0.0435214	0.325	hsa-miR-339-3p
38	-0.258	0.0465823	0.334	hsa-miR-22
39	-0.258	0.0466792	0.334	hsa-miR-142-3p

of the miR-146a inhibitor induced a significant increase of nuclear p65 (Figure 5C).

ChIP experiments were then performed to explore the association of the p65 subunit of NF- κ B with the PLC- β 2 promoter in vivo. Primers able to amplify a 137 bp region encompassing

the putative NF- κ B binding site located in the human PLC- β 2 promoter (Supplementary Figure S3) were used, failing to detect significant amount of p65 associated DNA in control conditions as well as in cells in which miR-146a was forcedly modulated (Figure 5D).

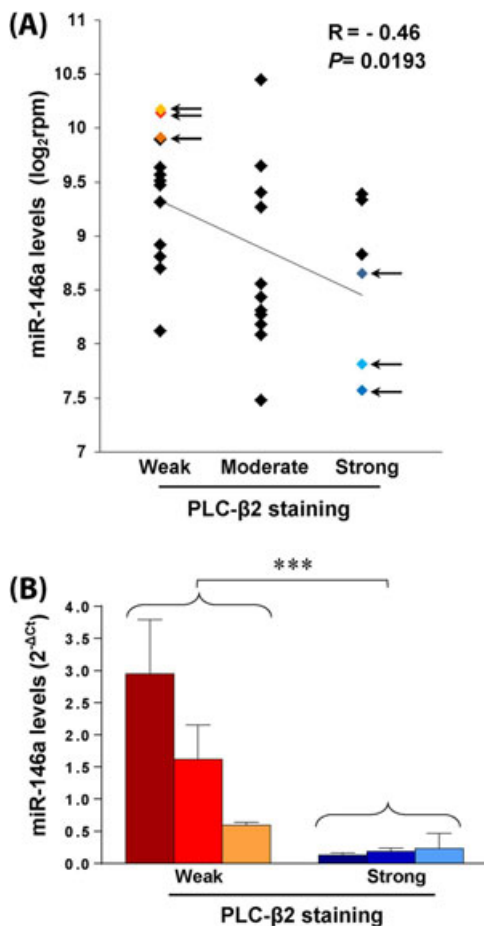


FIGURE 3 Relationship between the levels of PLC-β2 and miR-146a in primary DCIS. In (A) the expression levels of miR-146a (reads per million, rpm) in DCISs from Cohort 1 were log₂ transformed and correlated with the PLC-β2 immunohistochemical staining of the same tissue samples. The regression line with the Spearman correlation coefficient and the *P*-value was reported. The arrows indicate samples subjected to the RT-PCR analysis of miR-146a levels, whose relationship with PLC-β2 is shown in B. Relative transcript levels were determined using the 2^{-ΔCt} method. ****P* < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]

Even if the use of the TargetScan system did not predict PLC-β2 as a target of miR-146a, the use of EMBOSS Needle algorithm allowed to identify putative binding regions for miR-146a inside the 3'UTR of PLC-β2 (Supplementary Figure S4A), suggesting a direct regulatory role of this miRNA. The entire 3'-UTR of PLC-β2 was then cloned into a pMIR vector which was transfected into cells with miR-146a mimic or miR-control. The luciferase activity, first evaluated in HeLa cells, significantly decreased as a consequence of transfection with miR-146a mimic (Supplementary Figure S4B). The inhibitory effect of miR-146a on the luciferase activity in the presence of the PLC-β2 3'UTR was also revealed in MCF10DCIS cells (Figure 5E), suggestive of a direct role of the miRNA in modulating PLC-β2 expression.

3.4 | The relationship between PLC-β2 and miR-146a in primary DCIS has a clinical significance

To assess the potential prognostic relevance of the PLC-β2 levels in primary pure DCIS, their correlation with the risk of the appearance of an IDC in the other breast was investigated. PLC-β2 levels were then estimated in primary unilateral DCISs from patients without recurrence and from patients who developed a subsequent contralateral IDC after at least 5 years from surgery (Cohort 3). As reported in Figure 6A, PLC-β2 staining of tissue sections did not show significant relationship with recurrence, even though primary DCIS from patients who developed a contralateral recurrence expressed levels of PLC-β2 lower than primary DCIS from patients who did not recur.

By comparing the levels of PLC-β2 and miR-146a in the same primary DCIS, we found that the strong inverse PLC-β2/miR-146a relationship that characterized samples from patients who did not recur (Figure 6B) was not shown by DCIS from patients who developed a contralateral IDC (Figure 6C). In particular, the majority of DCIS from patients who developed a second invasive breast neoplasia displayed low levels of miR-146a not accompanied by increase of PLC-β2 expression (Figure 6C).

When the expression of miR-146a was allocated in two levels on the basis of an arbitrarily selected cut-off, allowing to identify "high" or "low" samples (Figures 6B and 6C), we found that all patients bearing primary DCIS with weak PLC-β2 and high miR-146a or strong PLC-β2 and low miR-146a did not recur. On the other hand, all patients whose DCIS expressed low PLC-β2 and low miR-146a developed a contralateral IDC. Finally, the patients with DCIS showing an intermediate PLC-β2 staining had more than 50% probability to develop a second breast neoplasia provided that miR-146a in their primary tumor was expressed at low levels (Figure 6D).

4 | DISCUSSION

DCIS is extremely heterogeneous and different degrees of aggressiveness may characterize lesions showing the same phenotype,^{5,21} suggesting that each DCIS intrinsically possesses features that can prevent or promote the acquisition of invasive properties. We previously demonstrated that PLC-β2 is ectopically expressed in invasive breast tumors in which it is positively correlated with malignancy in highly invasive tumors⁶ while it plays a protective role against hypoxia in low-invasive cells.¹⁰ Here, we investigated whether PLC-β2 is de-regulated and involved in determining the malignant potential of non-invasive breast tumors. We found that PLC-β2 is expressed in DCIS showing different phenotypes and histological features. Even if the majority of DCIS displayed levels of PLC-β2 lower than those in IDC, high levels of the protein were expressed in some lesions, opening up the issue of its functional significance in non-invasive breast tumor cells.

Using the non-transformed MCF10A and the MCF10DCIS cells, a well-established model of DCIS-derived cells that spontaneously progress into IDC in immune-deficient mice,¹⁷ we demonstrate that the forced modulation of PLC-β2 is unable to modify the phenotype of

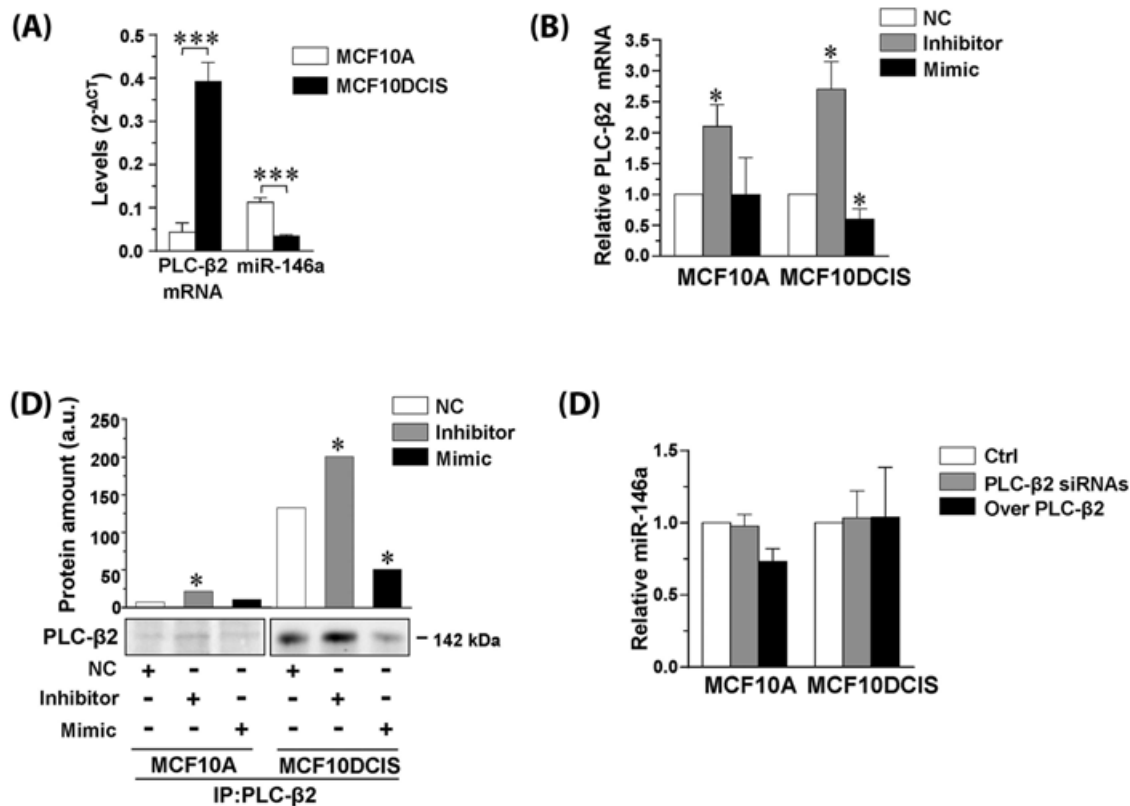


FIGURE 4 MiR-146a modulates PLC-β2 expression in non-transformed and DCIS-derived cells. In (A) total RNA isolated from MCF10A and MCF10DCIS cells was subjected to quantitative RT-PCR analysis of PLC-β2 mRNA and of miR-146a. Transcripts levels were calculated by the $2^{-\Delta CT}$ method. In B, total RNA isolated from MCF10A and MCF10DCIS cells transfected with miR-146a inhibitor or mimic was subjected to quantitative RT-PCR analysis of PLC-β2 mRNA. Relative transcript levels were determined using the $2^{-\Delta\Delta CT}$ method. Values obtained from cells transfected with a scramble sequence were taken as 1. In (C) representative Western blot analysis of PLC-β2 immunoprecipitated from MCF10A and MCF10DCIS cells transfected with miR-146a inhibitor or mimic and revealed with the anti PLC-β2 antibody. Histograms report data from densitometrical analysis of Western blot bands. In D, RNA isolated from MCF10A and MCF10DCIS cells in which PLC-β2 was silenced (PLC-β2 siRNAs) or over-expressed (Over PLC-β2) was subjected to quantitative RT-PCR analysis of miR-146a. Relative transcript levels were determined using the $2^{-\Delta\Delta CT}$ method. Values obtained from cells transfected with the scramble sequences were taken as 1. The data are the mean of three separate experiments \pm SD. * $P < 0.05$; *** $P < 0.001$

MCF10A. On the other hand, up-modulation of PLC-β2 promotes an epithelial-like phenotype in non-invasive DCIS, as we previously demonstrated in pre-EMT BT-474 cells.¹⁰ Accordingly, its silencing in MCF10DCIS cells is sufficient to up-regulate the mesenchymal marker Vimentin. Since EMT may be involved in the progression from in situ to invasive phenotype, at least in basal-like breast cancer,^{22,23} our data suggest that different levels of PLC-β2 may influence the malignant potential of non-invasive breast tumor cells. The increased ability of PLC-β2-silenced MCF10DCIS cells to pass through matrigel definitively confirms that low levels of the protein predispose DCIS-derived cells to acquire invasive properties.

Similarly to what we have previously demonstrated in invasive breast tumor-derived cells,^{8,10} in the MCF10DCIS cell line silencing PLC-β2 induces a significant increase in the number of cells expressing CD133, a breast cancer stem cell marker²⁴ which positively correlates with the occurrence of lymph node metastases in breast tumors with a triple-negative phenotype (TNBC).^{9,25} Since the number of cells with a CD44+/CD24- phenotype, associated with enhanced invasive potential,²⁶ was not affected by the forced

modulation of PLC-β2, we propose that in non-invasive breast lesions high levels of this protein, rather than limiting the number of cells with a stem-like phenotype, play a protective role by limiting the expression of CD133, directly involved in invasion of MDA-MB-231 cells.⁸

Having established that different expression levels of PLC-β2 may influence the malignant potential of non-invasive breast tumor cells, we investigated the possible mechanism underlying its de-regulation. Stemming from the notion that the levels of signaling molecules involved in tumor formation, progression, and metastasis may arise from the down-modulation of tumor suppressor miRNAs and/or the overexpression of oncogenic miRNAs,¹¹ we searched in DCIS tissues for miRNAs, with a known role in breast cancer, involved in the de-regulation of PLC-β2. Considering that no miRNA able to target PLC-β2 directly has been identified to date, the miRNoma of a selected number of DCIS was analyzed by applying an NGS strategy, that has allowed our understanding of breast cancer genomics to be significantly advanced in recent years.²⁷ We found that miR-146a, known to have a tumor suppressor role in solid tumors including breast

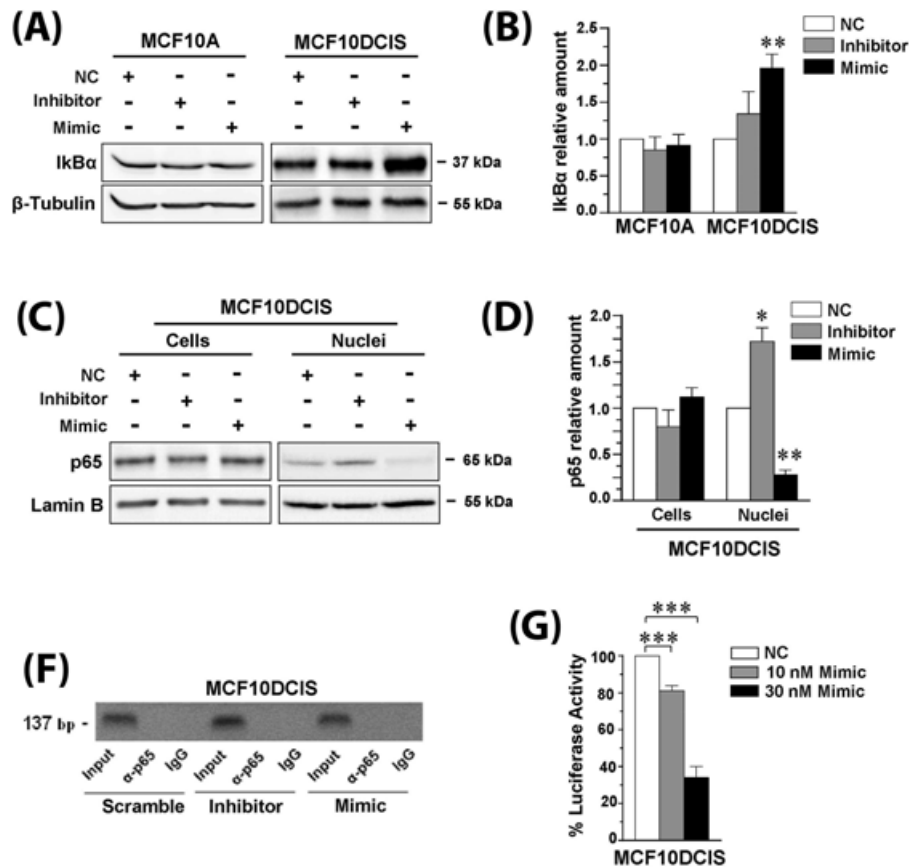


FIGURE 5 miR-146a targets PLC-β2 in MCF10DCIS cells. In (A) representative Western blot analysis with the indicated antibodies of MCF10A and MCF10DCIS cells transfected with miR-146a inhibitor or mimic. In (B) amounts of IκBα as deduced from the densitometry of Western blot bands normalized with β-Tubulin, used as internal control for equivalence of loaded proteins. The mean expression level of three separate experiments ± SD is shown. ***P* < 0.01 compared to transfection with scramble oligonucleotides. In (C) representative Western blot analysis with the anti-p65 antibody of total cells and nuclear lysates from MCF10DCIS cells transfected with miR-146a inhibitor or mimic. In (D) amounts of p65 as deduced from the densitometry of Western blot bands normalized with Lamin B, used as internal control for equivalence of loaded proteins. The mean expression level of three separate experiments ± SD is shown. **P* < 0.05; ***P* < 0.01 compared to transfection with scramble oligonucleotides. In (E) representative analysis of *in vivo* recruitment of the p65 subunit of NF-κB to human PLC-β2 promoter by chromatin immunoprecipitation in MCF10DCIS cells transfected with miR-146a inhibitor or mimic. The bands correspond to PCR products obtained amplifying a 137 bp DNA fragment encompassing a consensus-binding site for NF-κB. Input: genomic DNA not subjected to immunoprecipitation (positive control); IgG: samples immunoprecipitated with a non-specific antibody (negative control). All experiments were performed in triplicate. In (F) percentage of luciferase activity in MCF10DCIS cells co-transfected for 24 h with 250 ng of PLC-β2 3'-UTR luciferase reporter vector and with different concentrations of miR-146a mimic. Values obtained from cells transfected with scramble miRNA sequences (NC: negative control) were taken as 100. The data are the mean of three separate experiments ± SD. ****P* < 0.001

tumors,^{28–30} had the highest significant inverse correlation with PLC-β2 in DCIS, suggestive of its role in the regulation of PLC-β2 in breast tissue. This was addressed in MCF10-derived cells that, despite their high content of miR-146a, typical of triple-negative breast tumors,³¹ are suitable for studying the PLC-β2/miR-146a relationship since a lower miR-146a level and higher PLC-β2 level characterizes MCF10DCIS when compared with non-transformed MCF10A. In this breast cancer progression model³² we demonstrate that inhibition of miR-146a significantly increases the PLC-β2 level in both non-transformed and DCIS-derived cells, suggesting that the down-modulation of this miRNA may underlie the ectopic appearance of PLC-β2 in mammary tumor cells. To address the mechanism involved, we first considered NF-κB signaling since this transcription factor has

been described to be strongly interconnected to miR-146a in a number of tumor cells, including breast cancer cells,^{18,19} and to directly target PLC-β2 in platelets.²⁰ Only in MCF10DCIS we observed that restoration or inhibition of miR-146a inactivates or activates NF-κB, respectively, but we did not find evidence of any interaction between NF-κB and the PLC-β2 promoter. Although PLC-β2 mRNA is not a predicted target of miR-146a, we explored the possibility of a direct role of this miRNA in modulating PLC-β2 expression. Even if the exact binding sequence remains to be identified, our experiments revealed an interaction between miR-146a and the 3'-UTR of PLC-β2, and thus point toward the direct involvement of this miRNA in the regulation of the PLC. On the other hand, since miR-146a induces incomplete response, it is very likely that other miRNAs are involved in the

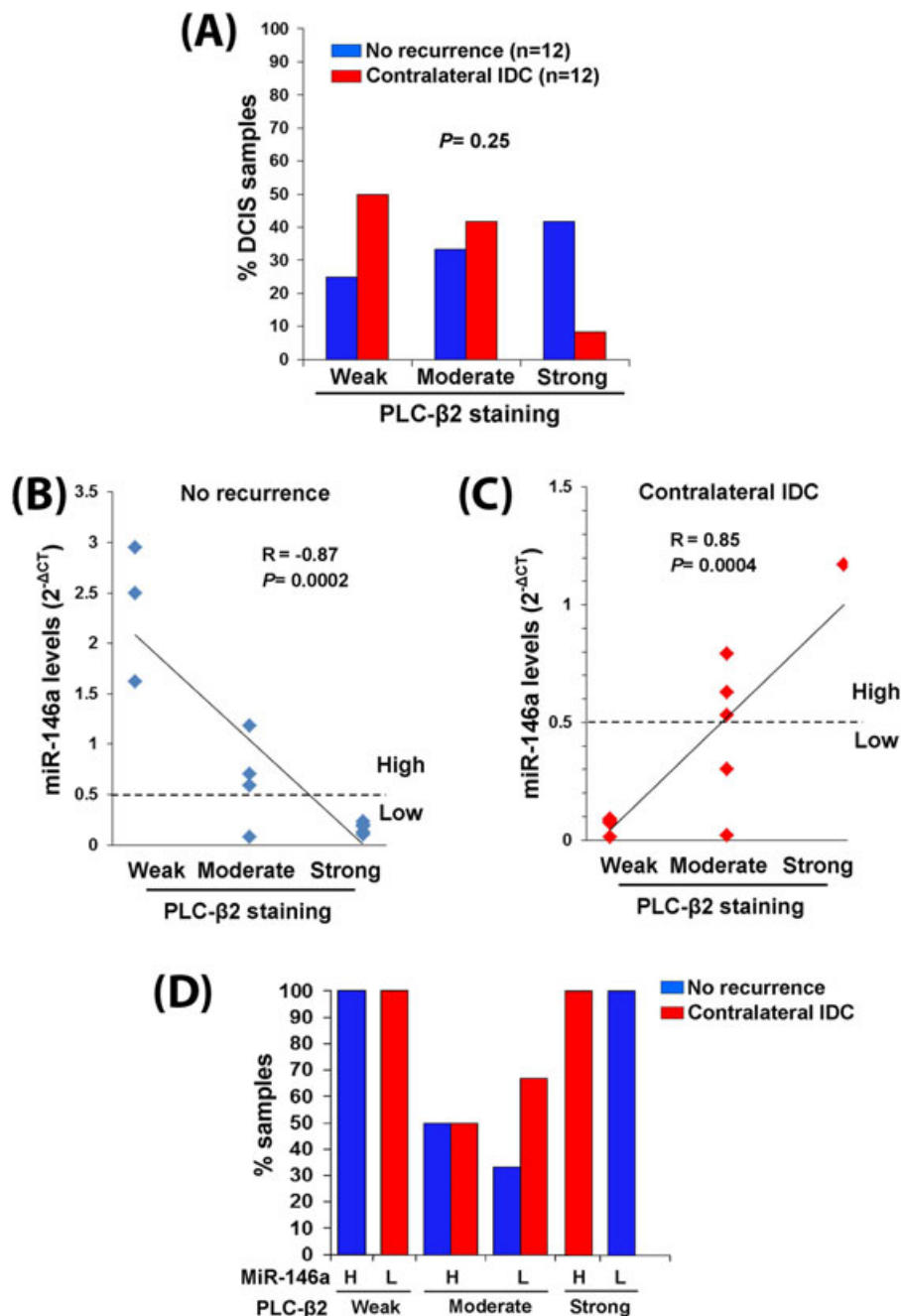


FIGURE 6 Correlation of PLC-β2 and miR-146a in DCIS with invasive recurrence. In (A) relationship between PLC-β2 staining intensity and the outcome of DCIS patients (No recurrence or appearance of a further contralateral IDC). PLC-β2 staining intensity was correlated to miR-146a levels in DCIS from patients who did not recur (B) or who developed a further contralateral IDC (C). The dotted horizontal line defines the arbitrary cut-off between high and low levels of miR-146a expression. In (D) the percentages of patients with primary DCIS showing the different combinations of PLC-β2 (weak, moderate, strong) and miR-146a (H: high, L: low) expression levels are reported. [Color figure can be viewed at wileyonlinelibrary.com]

regulation of PLC-β2 in this cell model, leaving open the question of the significance of its ectopic expression in breast cancer.

This bulk of our data allow us to conclude that the expression of PLC-β2 in DCIS-derived cells due, at least in part, to the down-regulation of miR-146a, promotes an epithelial-like phenotype, and may have a protective role against the acquisition of invasive properties. We also found that high levels of miR-146a in DCIS-derived cells promote the

accumulation of the p65 subunit of NF-κB at the cytoplasmic level, an event reported to have a positive prognostic significance in TNBC.³³ This suggests that different PLC-β2 to miR-146a ratios might characterize DCIS with extremely different malignant potentials. Establishing the PLC-β2 to miR-146a ratio could also, therefore, have significant prognostic importance in early breast lesions, the actual risk of which is extremely difficult to predict for individual patients.^{34,35}

To assess the potential prognostic relevance of PLC- β 2 and/or miR-146a in primary DCIS, we selected a specific cohort of DCIS patients, including those who developed a second IDC in the other breast at least 5 years after surgery. Considering the low frequency of relapse in patients with DCIS,² we investigated a relatively high number of samples selected on the basis of comparable age at diagnosis and therapeutic approach. In contrast with invasive breast tumors, in which high PLC- β 2 levels correlate with poor prognosis,⁶ we found low levels of protein staining in DCIS from patients who developed a contralateral IDC. Interestingly, in this cohort the majority of samples showed low levels of both miR-146a and PLC- β 2, suggesting that the as yet unknown transcription mechanism regulating the expression of PLC- β 2 in this cell model is relatively inefficient. Although we cannot correlate the second neoplasia with the primary DCIS, we can conclude that the down-modulation of miR-146a in breast lesions not accompanied by an increase of PLC- β 2 may constitute a risk for malignant progression as well as for the appearance of new lesions.

Since neither traditional classification systems nor molecular characterizations can reliably predict the malignant potential of DCIS, at variance with those for IDC, we propose that assessment of the PLC- β 2 to miR-146a ratio in primary DCIS at diagnosis could help identify patients with either a low or high propensity for the development of invasive recurrence; this could, in turn, assist physicians select the most appropriate intervention strategy.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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