Silencing of Phosphoinositide-specific Phospholipase C ε Remodulates the Expression of the Phosphoinositide Signal Transduction Pathway in Human Osteosarcoma Cell Lines

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Abstract. *Background: Ezrin, a member of the ezrin–radixin–moesin family, is involved in the metastatic spread of osteosarcoma. Ezrin binds phosphatydil inositol-4,5-bisphosphate (PIP2), a crucial molecule of the phosphoinositide signal transduction pathway. PIP2 levels are regulated by phosphoinositide-specific phospholipase C (PI-PLC) enzymes. PI-PLCε isoform, a well-characterized direct effector of rat sarcoma (RAS), is at a unique convergence point for the broad range of signaling pathways that promote RAS GTPase-mediated signalling. Materials and Methods. By using molecular biology methods and microscopic analyses, we analyzed the expression of ezrin and PLC genes after silencing of PLCE (OMIM *608414) in 143B and Hs888 cell lines. Results: The growth rate of the cells was slowed, and the expression of ezrin, PLCB1, PLCG2 and PLCD4 was significantly modified. Ezrin displacement from the plasma membrane was observed. Conclusion: The present results corroborate the hypothesis that ezrin and the PI signal transduction system are involved in a common network.*

Ezrin, a member of the ezrin–radixin–moesin (ERM) family involved in the rat sarcoma (RAS)-dependent signal transduction pathway, cross-links actin filaments (1-3), and has been suggested to play a central role in osteosarcoma metastasis (4). The protein 4.1, ezrin, radixin, moesin (FERM) domain (PBD ID:1GC6) recognizes plasma membrane phosphatydil inositol-4,5-*bis*phosphate (PIP2), a crucial molecule belonging to the phosphoinositide (PI)

signal transduction pathway (5). The reduction of PIP2 induces ezrin dissociation from the plasma membrane (6).

The levels of PIP2 are regulated by the PI-specific phospholipase C (PI-PLC) family (7), constituting thirteen enzymes divided into six sub-families on the basis of amino acid sequence, domain structure, mechanism of recruitment and tissue distribution (7-15). PI-PLCε, a direct effector of RAS (14-15), might be the point of convergence for the broad range of signalling pathways that promote the RASGTPase-mediated signalling (16).

In previous studies, we suggested a relationship between PI-PLC expression and ezrin (17-18). In the present study, we analyzed the expression of ezrin and *PLC* genes after silencing of *PLCE* (OMIM *608414), the gene which codifies for PI-PLCε enzyme.

Materials and Methods

Cell culture. 143B and Hs888 human osteosarcoma cell lines, obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%-15% foetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. Confluent monolayer cells were detached with 0.25% trypsin and counted using a Neubauer haemocytometer (Weber Scientific International Ltd., Middlesex, UK).

Cell survival trypan blue test. The number of viable cells was determined by adding 0.4% trypan blue staining (Sigma Aldrich, Dorset, UK) to an equal volume of cell suspension; a growth curve was designed counting the cells per square centimeter at different times. The following equation was used to calculate the number of viable cells in 1 ml suspension: number of viable cells in 1 ml (TC)=$\bar{x}$ 2×10^4, when $\bar{x}$ is the average of the cell counts from the squares of the haemocytometer grid, and 2 is the dilution factor (1:1). Student's *t*-test was used to evaluate the statistical significance between the survival rate of transfected cells *versus* metafectamine-transfected cells or *versus* non-transfected cells.

Cell transfection for PLCE silencing. Cells were transiently transfected using METAFECTENE SI+ (Biontex, Munich,

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Table I. *Primer pairs used for Reverse Transcriptase Polymerase Chain Reaction.*

Protein (gene; ID)	Primers
PI-PLCβ1 (*PLCB1*; OMIM *607120)	Forward 5'-AGCTCTCAGAACAAGCCTCCAACA-3' Reverse 5'-ATCATCGTCGTCGTCACTTTCCGT-3'
PI-PLCβ2 (*PLCB2*; OMIM *604114)	Forward 5'-AAGGTGAAGGCCTATCTGAGCCAA-3' Reverse 5'-CTTGGCAAACTTCCCAAAGCGAGT-3'
PI-PLCβ3 (*PLCB3*; OMIM *600230)	Forward 5'-TATCTTCTTGGACCTGCTGACCGT-3' Reverse 5'-TGTGCCCTCATCTGTAGTTGGCTT-3'
PI-PLCβ4 (*PLCB4*; OMIM *600810)	Forward 5'-GCACAGCACACAAAGGAATGGTCA-3' Reverse 5'-CGCATTTCCTTGCTTTCCCTGTCA-3'
PI-PLCγ1 (*PLCG1*; OMIM *172420)	Forward 5'-TCTACCTGGAGGACCCTGTGAA-3' Reverse 5'-CCAGAAAGAGAG CGTGTAGTCG-3'
PI-PLCγ2 (*PLCG2*; OMIM *600220)	Forward 5'-AGTACATGCAGATGAATCACGC-3' Reverse 5'-ACCTGAATCCTGATTTGACTGC-3'
PI-PLCδ1 (*PLCD1*; OMIM *602142)	Forward 5'-CTGAGCGTGTGGTTCCAGC-3' Reverse 5'-CAGGCCCTCGGACTGGT-3'
PI-PLCδ3 (*PLCD3*; OMIM *608795)	Forward 5'-CCAGAACCACTCTCAGCATCCA-3' Reverse 5'-GCCA TTGTTGAGCACGTAGTCAG-3'
PI-PLCδ4 (*PLCD4*; OMIM *605939)	Forward 5'-AGACACGTCCCAGTCTGGAACC- 3' Reverse 5'-CTGCTTCCTCTTCCTCATATTC- 3'
PI-PLCε (*PLCE*; OMIM *608414)	Forward 5'-GGGGCCACGGTCATCCAC-3' Reverse 5'-GGGCCTTCATACCGTCCATCCTC-3'
PI-PLCη1 (*PLCH1*; OMIM *612835	Forward 5'-CTTTGGTTCGGTTCCTTGTGTGG-3' Reverse 5'-GGATGCTTCTGTCAGTCCTTCC-3'
PIPLCη2 (*PLCH2*; OMIM *612836)	Forward 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3' Reverse 5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3'
GAPDH	Forward 5' -CGAGATCCCTCCAAAATCAA-3' Reverse 5'-GTCTTCTGGGTGGCAGTGAT-3'

Germany). silencing RNA (siRNA) sequences targeting *PLCE* and negative control siRNA, obtained from Invitrogen (Life Technologies, Foster City, CA, USA), according to *PLCE* complementary DNA (cDNA) sequence (*PLCE* Gene ID: 51196). A suspension containing 1.5×10^5 cells/ml of 143B cells or 3×10^5 cells/ml of Hs888 cells was mixed with150 μl of 1× SI+ buffer, 72 μl of METAFECTENE® SI+ and 540 pMol of RNA stock solution within one hour from seeding. *PLCE* transcription was measured by reverse transcription–polymerase chain reaction (RT-PCR) and PI-PLC protein by western blot analysis 24, 48 and 72 h after transfection.

RNA extraction. RNA was extracted using SV Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells suspension was incubated with 175 μl of Lysis Buffer, then Dilution Buffer (350 μl) was added and centrifuged for 10 min at $14,000 \times g$. After the addition of 200 μl 95% ethanol, the mixture was transferred to a spin-column assembly, and centrifuged for one minute. The liquid was discarded, 600 μl of Wash Solution was added, centrifuged for one minute, and the collection tube was emptied. DNase incubation mixture (40 μl of Yellow Core Buffer, 5 μ1 of 0.09 M $MnCl_2$ and 5 μl of DNase I enzyme) was added. After 15 min incubation, 200 μl of Stop Solution was added, the mixture centrifuged for 1 min, and 600 μl of Wash Solution was added and centrifuged for one minute. The collection tube was emptied, 250 μl of Wash Solution was added, and the mixture centrifuged for 2 minutes. Nuclease-Free Water was added to an elution tube containing the spin basket, centrifuged for 1 min and RNA was eluted into a sterile tube with

RNase-free water. The concentration and quality of the RNA was monitored using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

RT-PCR. RNA was reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Briefly, 2μg RNA were incubated with the master mix (2 μl of 10× Reverse Transcription Buffer, 0.8 μl of 25 x dNTPs (100 mM), 2 μl of 10× random primers, 1 μl of MultiScribe™ Reverse Transcriptase (50 U/μl) and 3.2 μl of DNase-free water). Ten microlitres of diluted RNA was added to 20 μl final volume and reverse transcribed for 10 min at 25˚C, 120 min at 37˚C and 5 minutes at 85˚C in a Gene Amp® PCR System 9700 (Applied Biosystems) thermocycler. Standard analytical PCR reaction was performed with GoTaq Master Mix (Promega) with 5× GoTaq buffer, 0.2 μM primers pairs (Bio Basic Inc, Amherst, NY, USA (Table I), 0.2 mMdNTPs, 0.5 mM $MgCl_2$, 1.25 U GoTaq and 3.5 μl (about 35 μg) of template cDNA to 50 μl final volume. Cycling was performed with a 95˚C initial denaturation step, followed by 40 cycles of denaturation at 95˚C (30 sec), annealing (30 seconds) at the appropriate temperature for each primer pair and 72˚C extension (1 min). PCR products were visualized by 1.5% ethidium bromide-stained agarose gel electrophoresis at 100 V using UV light transilluminator.

Real-time PCR. TaqMan® primers and probes for each gene, as well as the glyceraldehyde 3 phosphate dehydrogenase gene (*GAPDH*) reference gene, were obtained from Applied Biosystems. Reaction mixtures contained: 5 μl TaqMan® mastermix (2×; Applied

Biosystems), 0. 5 μl primer/probe mix specific for each analyzed gene, 1 μl PCR-grade water and cDNA (3.5 μl, 35 ng). After incubation for 2 min at 50°C and 10 min at 95°C, the reaction was carried out for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The reaction was carried out in triplicate on 96-well plate using a 7500 Real-Time PCR ABI PRISM (Applied Biosystems™) and products were evaluated using ABI PRISM 7500 software. The cycle threshold (Ct) values for each set of three reactions were averaged for calculations. The $2^{-\Delta\Delta IS}$ method was used to calculate relative changes in gene expression. PCR product concentrations of transfected and non-transfected cells were compared with student's one tailed *t*-test using Prism 5.0a software (GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered significant.

Western blot. Western blot analyses were conducted 24 and 48 hours from transfection and in non-transfected controls. Cells were processed in lysis buffer [50 mMTris-HCl, 150 mMNaCl, 2 mM EDTA, 1% NP-40, 2 mM sodium fluoride, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) containing protease inhibitors]. Fifty micrograms of protein was separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Invitrogen), blocked with 5% skimmed milk for 1 hour and incubated overnight with primary antibodies directed against PI-PLC enzymes. Membranes were stained adding appropriate secondary antibodies (Jackson Immunoresearch Laboratories, Newmarket, Suffolk, UK) withchemiluminescence. Expression of β-actin was used as an internal control to normalize results. The densities of the bands on the membrane were scanned and analysed with ImageJ software (NIH Image J version 1.38 software;http://rsb.info.nih.gov/ij/).

Immunofluorescence analysis of subcellular distribution of target molecules. Immunofluorescence detection of PI-PLCε, PI-PLCβ1, PI-PLCγ2, PI-PLCδ4 and ezrin was performed on coverslip-cultured transfected and non-trasfected cells, fixed with 4% paraformaldehyde, incubated with primary antibodies directed against each PI-PLC isoform (Santa Cruz Biotechnologies Inc, Santa Cruz, CA, USA) for 1 h and then with the specific secondary antibody Texas Red or fluorescein-conjugated for 1 hour. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and visualized using an inverted microscope.

Results

Efficacy of transfection. *PLCE* mRNA was not detected in the transfected 143B and Hs888 cell lines (Figure 1). Western blot analyses confirmed marked reduction of PI-PLCε protein (Figure 1).

Cell growth. The growth rate of *PLCE*siRNA-transfected cells decreased in a time-dependent manner in both 143B and Hs888 cell lines (Figure 2) with respect to non-transfected cells and to cells transfected exclusively with metafectamine vector.

Microscopy analyses. In Hs888 cells, *PLCE* silencing in induced cytoplasmic macro/microvacuolisation, and reduction of cells numbers (Figure 2). Ezrin was slightly reduced, localized in the cytoplasm with evident peri-nuclear
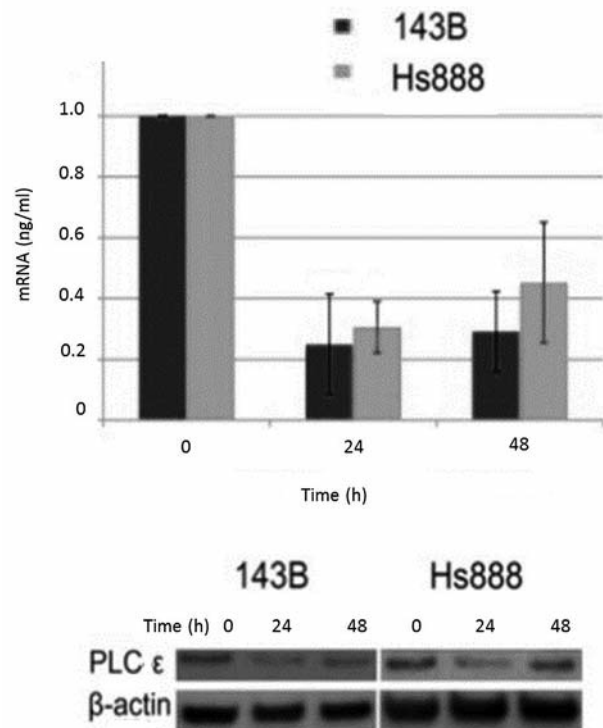

Figure 1. *Efficacy of Phospholipase C E gene (PLCE) silencing. Transcript concentrations after 0, 24 and 48 h from PLCE silencing in 143B (gray) and Hs888 (black) cell lines; standard deviation bars are indicated. In 143B cells, the expression of PLCE was significantly slowed in siRNA-transfected cells with respect to the untreated counterpart (p<0.001); in Hs888 cells line, the expression of PLCE was significantly slowed in siRNA-transfected cells at t=24h (p<0.05). Lower line: western blot of PI-PLε protein in 143B and Hs888 cell lines compared to actin protein loading control.*

staining, and lack of membranous staining (Figure 3). PI-PLCβ1 in the cytoplasm was significantly increased, PI-PLCγ2 was weakly detected (Figure 3). In 143B cells, *PLCE* silencing induced cell rounding. Cytoplasmic ezrin slightly decreased, with marked peri-nuclear staining, and less marked membranous staining (Figure 3). PI-PLCβ1 in the cytoplasm was significantly reduced, and PI-PLCγ2 was slightly increased (Figure 3).

Real-time PCR. In the Hs888 cell line, *PLCE* silencing increased the expression of ezrin by approximately 40% after 24 h; the expression of PI-PLCβ1 significantly increased; the expression of PI-PLCδ4 was not significantly modified (Figure 4). There was a statistically significant difference of mRNA expression of ezrin (*p*<0.00025) and PI-PLCβ1 (*p*<0.0005) between transfected and non-transfected cells. With respect to their untreated counterpart, in 143B cells, *PLCE* silencing reduced the expression of ezrin by
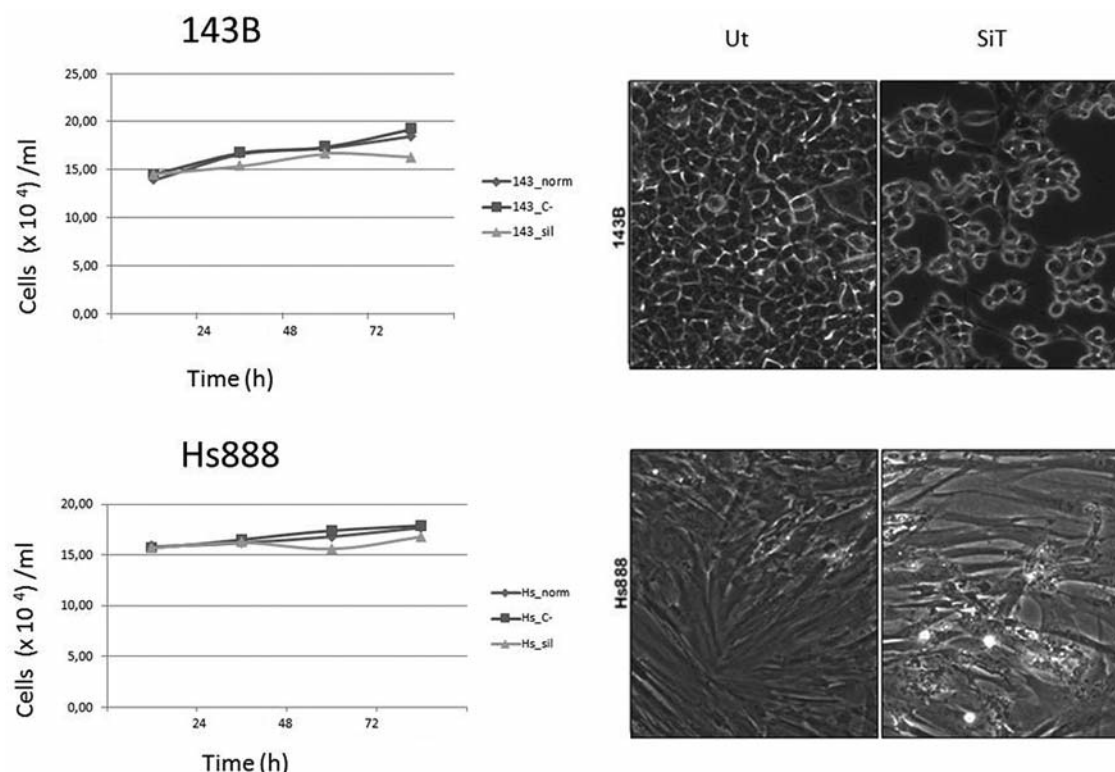
Figure 2. *Growth curve after silencing of the gene which codifies for Phospholipase C ε (PLCE) and effect of PLCE siRNA on cell morphology. A: The growth of silenced cells was slowed with respect to untreated 143B cells (p<0.05) (upper) and in Hs888 cells (p<0.05) (lower). B: Morphological changes, phase-contrast microscopy in 143B (×20; upper) and Hs888 cells (×20; lower). Ut: Untransfected cells, Mt: metafectamine-transfected cells, SiT: PLCE siRNA-transfected cells.*

approximately 75% in the interval 24-48 h; the expression of PI-PLCγ2 increased about 50%, of PI-PLCβ1 decreased about 80% after 24 h and PI-PLCδ4 was detected after 24 h, although at a low concentration. A statistically significant difference of mRNA expression of ezrin ($p<0.00025$) and of PI-PLCβ1 ($p<0.0025$) was found comparing transfected cells and non-transfected cells (Figure 4).

Discussion

PI-PLC ε enzyme or the expression of *PLCE* has been frequently described to be involved in carcinogenesis. A favourable activity of PI-PLC ε was observed in bladder and intestinal tumour, oesophageal squamous cell carcinoma, gastric adenocarcinoma, murine skin cancer, head and neck cancer (19-23). However, controversial observations were reported and recently, a tumour-suppressive role for PI-PLC ε was suggested in RAS-triggered cancer (24). In the present experiments, the modifications induced by *PLCE* silencing upon the transcription of other PI-PLC enzymes, such as PI-PLCβ1, PI-PLCγ2, and PI-PLCδ4, confirms the hypothesis that an extensive crosstalk among the PI-PLC enzymes occurs

in cells (12). *PLCE* silencing had different effects upon 143B and Hs888 cell lines, probably due to the different features of the cells, confirming data from previous reports (17, 18). *PLCE* silencing linearly reduced the cell growth rate in both 143B and Hs888 cell lines, although with different effects upon cell morphology. 143B Cell rounding was observed, and quantitatively reduced ezrin translocated from the plasma membrane to the perinuclear area. Cell rounding, usually considered a secondary cytotoxic effect, might be due the displacement of ezrin. Notably, literature data indicate that ezrin displacement might influence its activity and exclusive cytoplasmic expression of ezrin was associated with better disease-free survival likelihood compared with both cytoplasmic and membranous expression (1-3). One might speculate that *PLCE* silencing might play a role upon the activity of ezrin, influencing its displacement from the plasma membrane to the perinuclear area, probably secondarily modifying its activation.

In both 143B and Hs888 cell lines, PI-PLCβ1 is expressed at low concentrations. In Hs888, a significant increase of PI-PLCβ1 was observed after ezrin reduction (18). The present results partially confirm this hypothesis. In fact, after 48 h
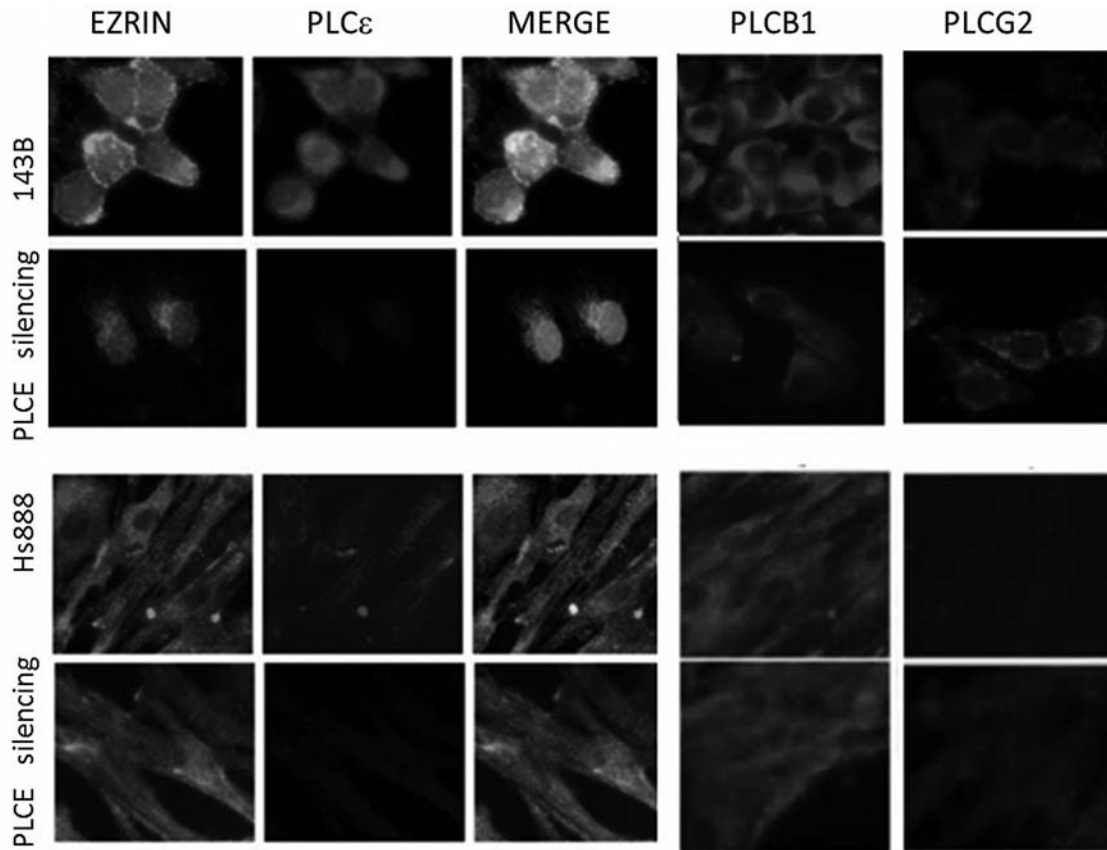
Figure 3. *Localization of ezrin and Phospholipase C (PI-PLC) enzymes. Fluorescence immunocytochemistry in 143B (upper panels) and Hs888 (lower panels) cell lines; standard deviation bars are indicated. Localization of ezrin, PI-PLCε, co-localization (merge) of ezrin and PI-PLCε, and localization of PI-PLCβ1 and of PI-PLCγ2 in 143B and in 143B cells after PLCE silencing (×40) and in Hs888 and in Hs888 after PLCE silencing (×40) are shown.*
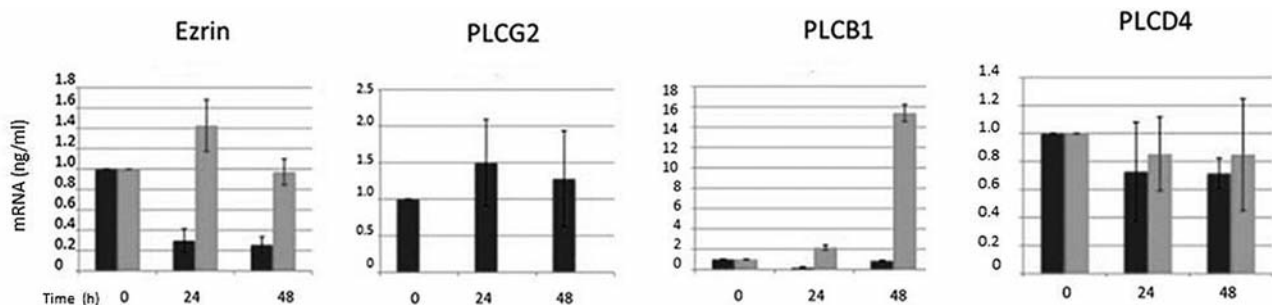

Figure 4. *Real-time Polymerase Chain Reaction results after silencing of the gene which codifies for Phospholipase C ε (PLCE) silencing. Transcript concentrations of PLC genes after 0, 24 and 48 hours from PLCE silencing in 143B (gray) and Hs888 (black) cell lines.*

from *PLCE* silencing, ezrin slightly decreased and PI-PLCβ1 significantly increased. Partial or total lack of PI-PLCβ1 might promote cancer progression in myeloid tissue (26, 27). One might speculate that, on the other hand, increased *PLCB1* expression might oppose cancer progression. However, this observation requires further investigation, in order to address the cross-talk among the PI-PLC enzymes and the role of possible alternative pathways involving the PI molecules, including RAS signaling.

PI-PLCγ2, absent in Hs888 cells, is up-regulated in 143B cells after *PLCE* silencing, similarly to that observed after ezrin silencing (18). The PI-PLCγ2 isoform is required for

osteoclast differentiation (28-30). The overexpression of PI-PLCγ2 observed in the case of ezrin reduction might be related to the features of 143B cell line, which can develop osteolytic tumours (30).

In conclusion, in the analyzed human osteosarcoma cell lines, PI-PLC expression varies after *PLCE* silencing with respect to the corresponding non-transfected control cells. The overall reorganization of expression of the PLC genes seems to be complex, as already observed after ezrin silencing (18). Other PI-PLC enzymes, such as PI-PLCβ1 and PI-PLCγ2 might play a role in osteosarcoma-related signal transduction pathways. Further studies are required to elucidate the relationship between ezrin and selected PI-PLC enzymes in order to highlight the crosstalk and the extensive network of interactions with G proteins, which are related both to ezrin and to the PI signal transduction system.

Acknowledgements

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