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HUMAN PATHOLOGY Ph.D.

XXV Cycle

EZR-PLC and RhoGTPases interactions in osteosarcoma pathogenesis and metastatic process

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Ackowledgements

I would like to express my special gratitude and thanks to my advisor Professor Carlo Della Rocca for offering me this opportunity and leading me working on diverse exciting projects.
I would also like to thank my assistant advisor Dr. Rita Lo Vasco for the continuous support of my research, for his patience and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis.
My sincere thanks also goes to my labmates and my friends, in particular Micol for her fundamental support.

Last but not least, I would like to thank my family:
Lino and Caterina
My Parents, my Brother and my Grandparents
I love you

List of abbreviations

ANOVA Analysis of variance
ATCC American Type Culture Collection
C-terminus: Carboxyl-terminus
Ca2+: Calcium ions
cAMP Cyclic adenosine monophosphate
Cdc42: Cell division control protein 42 homolog
cDNA: Complementary DNA
CO2: Carbon dioxide
CPERMs: COOH-terminal threonine residue
Ct:Threshold cycleΔCt :Delta (difference) of threshold cycles
DAG: diacylglycerol
DAPI: 4',6'-dianidino-2-phenylindole
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Dimethyl sulfoxide
ECM: extracellular matrix
EGFR: epidermal growth factor receptor
ERK: Extracellular signal regulated protein kinase
ERM: ezrin-radixin-moesin proteins
EZR: ezrin
FBS Foetal bovine serum
FERM: Four point one, ERM domain
G proteins: Guanine nucleotide binding proteins
GAP: GTPase-activating protein
GDI: guanine nucleotide dissociation inhibitor
GDP guanosine diphospate
GEF guanine nucleotide exchange factor
GPCR: G protein coupled receptor
GTP guanosine triphosphate
Gα: Heterotrimeric G alpha protein
Gαβγ: Heterotrimeric G alpha, beta, gamma proteins (G protein complex)
Gβγ: Heterotrimeric G beta, gamma protein
IGF-1R: including insulin-like growth factor 1 receptor
IP3: inositol 1,4,5-trisphosphate
LPA: lysophosphatidic acid
mRNA: messenger RNA
N-terminus: amino-terminus
NHERF1 also named EBP50: ERM-binding phosphoprotein 50
NHERF2: Na+-H+ exchanger regulatory factor
OPG: Osteoprotegerin
OS: Osteosarcoma
PA: phosphatidic acid
PBS: Phosphate buffered saline
PCR: polymerase chain reaction
PDGFR: platelet-derived growth factor receptor
PDZ (Postsynaptic Density Protein) domains
PH: pleckstrin homology
PI: phosphatidyl inositol
PI3K: phosphatidylinosytol 3-kinase
PIP: phosphatidylinositol monophosphate
PIP2: phosphatidylinositol 4,5-bisphophate

PIP3: phosphatidylinositol trisphosphate
PIP5: kinase phosphatidylinosytol 4-phosphate 5-kinase
PIP5K-Iα: phosphatidylinositol 4-phospate 5-kinase type Iα
PKC: protein kinase C
PLA2: phospholipase A2
PI_PLC β1: Phospholipase C beta1
PI_PLC β2: Phospholipase C beta2
PI-PLC β3: Phospholipase C beta3
PI-PLC β4: Phospholipase C beta4
PI-PLC γ1: Phospholipase C gamma1
PI-PLC γ2: Phospholipase C gamma2
PI-PLC δ1: Phospholipase C delta1
PI-PLC δ3: Phospholipase C delta3
PI-PLC δ4: Phospholipase C delta4
PI-PLC ϵ: Phospholipase C epsilon
PI-PLC ζ: Phospholipase C zeta
PI-PLC η1: Phospholipase C eta1
PI-PLC η2: Phospholipase C eta2
PLC: phosphoinositide-specific phospholipase C
RAC1: Ras related C3 botulinum toxin substrate 1
RANK: receptor activator of NFkB
RANKL: receptor activator of nuclear factor kappa beta (NFkB ligand)
Ras: Rat sarcoma
Rho: Small G protein, a GTPase, a Ras superfamily
RhoA: Ras homolog gene family, member A
RIPA: Radio-Immunoprecipitation Assay Buffer
ROCK: Rho kinase
RT room temperature
RT – PCR: Real - time polymerase chain reaction
RT-PCR: Reverse transcription polymerase chain reaction
SDS sodium dodecyl sulfate
SDS-PAGE SDS polyacrylamide gel electrophoresis
SH: Src homology
SH2: proteins by binding to phosphorylated tyrosine residues
SH3: proteins by binding to proline-rich sequences
siRNA: Small interfering RNA
TIM: triose-phosphate-isomerase
Trypsin-EDTA: Trypsin Ethylene diamine tetracetic acid
U73122: 1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione
ΔΔCt: Comparative Ct method
U73343: 1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione
WB: Western-blot

Osteosarcoma

Overview of Osteosarcoma

Osteosarcoma is the most common primary malignancy of bone (Mirabello et al, 2009), characterized by the production of osteoid or immature bone from malignant cells. Osteosarcoma is a relatively uncommon cancer, accounting for 5% of childhood cancers and 8.9% of cancer-related deaths in children. The overall incidence is five cases per million persons per year (Ottaviani et al, 2009). The progression of osteosarcoma is marked by increased metastatic potential, with high tendency to disseminate to the lungs. Approximately 20% of patients present with lung metastases at diagnosis and, in 40% of non-metastatic patients, metastases occur at a later stage (Bacci et al, 2008).

Osteosarcoma has a bimodal age distribution, with the first peak during adolescence and the second peak in older adulthood. The first peak is in the 10–14-year-old age group, coinciding with the pubertal growth spurt. This suggests a close relationship between the adolescent growth spurt and osteosarcoma. The second osteosarcoma peak is observed in adults older than 65 years of age; it is more likely to represent a second malignancy, frequently related to Paget's disease. The incidence of osteosarcoma seems to be higher in males than in females, occurring at a rate of 5.4 per million persons per year in males vs. 4.0 per million in females, with a higher incidence in blacks (6.8 per million persons per year) and Hispanics (6.5 per million), than in whites (4.6 per million) (Ottaviani et al, 2009).

These differences may suggest that the underlying pathogenesis of two entities described is not the same; for instance, epidemiologic features such as Paget's disease and prior radiation exposure are risk factors unique to older patients (Mirabello et al, 2009).

Differences in tumour site and survival were also described according to age at presentation. The most frequent origin of the primary tumour are the metaphyseal (actively growing) regions of the distal femur, proximal tibia and proximal humerus. However, the tumour can develop in any bone (Marcove et al, 1970; Wittig et al, 2002).

Osteosarcoma arises from a mesenchymal cell that has or can acquire the capacity to produce osteoid (Janeway et al, 2009; Gorlick et al, 2010). Cells of this type exist in multiple compartments and include the adherent cell populations obtained in a bone marrow aspiration, hence the intramedullary space within bones, with cells of this type generally referred to as *mesenchymal stem cells* (Miura et al, 2004). They also include osteoblasts, concentrated along both the endosteal and periosteal surfaces of the bone and involved in

fracture repair and remodelling. Longitudinal growth of long bones is accomplished by proliferation of chondrocytes at the growth plates with subsequent bone mineralization as part of endochondral ossification, although the site of initial tumour formation (intramedullary or surface lesions) is a matter of speculation based on the radiographic appearance of the primary tumour. It helps explain why osteosarcomas develop more often in adolescents in the age range, which includes puberty, often in those regions where bone is actively growing.

Most osteosarcomas are classified as conventional, high-grade tumours (Marcove et al, 1970; Dorfman et al, 1998), tumour most often originating in the intramedullary space. However, low-grade intramedullary osteosarcomas are rarely observed (Schwab et al 2008). Similarly, surface osteosarcomas, presumably arising from cells in the periosteum, can occur in several variants, including parosteal lesions, which are low-grade osteosarcomas, high-grade surface osteosarcomas, and periosteal osteosarcomas that are intermediate in grade.

Historically, the prognosis for osteosarcoma was not good. Before 1970, amputation was the sole treatment for a high-grade osteosarcoma, and 80% of patients died of metastatic disease, most commonly in the lungs (Marcove et al, 1970). Over the past three decades, effective induction (neo-adjuvant/pre-operative) and adjuvant (post-operative) chemotherapy protocols have improved the ability to perform safe limb-sparing resections, and disease-free and overall survival rates have risen. Actually, 90 to 95% of patients with osteosarcoma can be treated with limb-sparing surgery, and 60 to 80% of patients with localized disease are long-term survivors (Bacci et al, 1993; Fuchs et al, 1998; Bickels, et al, 1999). Furthermore, limb salvage techniques were developed that help maintain function while not affecting the overall survival rate. Such techniques can be used in 90-95% of cases (Huth et al, 1989; Rubert et al, 1999; Malawer et al, 1995). Overall, those patients that present with primary disease only have a much better prognosis than those with metastatic disease.

Genomic Instability in Osteosarcoma

The majority of osteosarcomas origin as sporadic, however a small number of cases result from inherited predisposition (Wang et al, 2005). A number of recent reviews have focused on the genetic complexity of osteosarcoma, lamenting the inability to use the numerous abnormalities described in tumours either for diagnostic purposes or for prognostication of the clinical outcome (Chou et al, 2008; O'Day et al, 2008). They also have highlighted the inability to identify a precursor lesion. A unifying approach to bring genetic risk factors

together with epidemiologic and association data includes grouping gene or gene families into two categories: those which drive proliferation (growth) and those associated with inability to DNA repair associated to loss of cell cycle regulatory control.

Complex and largely inconsistent genetic alterations characterize conventional osteosarcoma. Overall, some frequent genetic alterations in conventional osteosarcoma are deletion of portions of chromosomes 3q, 6q, 9, 10, 13, 17p, and 18q and insertion of portions of chromosomes 1p, 1q, 6p, 8q, and 17p. In general, regions containing known tumour suppressor genes undergo deletion and mutation events, while those containing established oncogenes are added or amplified in cells (Martin et al, 2012)

The first genetic syndrome showing increased risk of developing osteosarcoma is hereditary retinoblastoma. Retinoblastoma is a malignant tumour of the retina involving mutations in the Rb (retinoblastoma tumour suppressor - RB1 - OMIM #180200) (Hurwitz et al, 2002). RB1 encodes the tumour suppressor protein pRB that is essential in preventing cell cycle progression through G1/S following DNA damage. Mechanistically, the protein inhibits members of the E2F transcription factor family, a process that requires strict regulation of the cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKNs), to promote stability of the genome (Manning et al, 2011).

The p53 pathway, extremely important in regulating the cell cycle and apoptosis, networks other cell cycle regulators, including RB1. Deregulation of TP53 is also thought to play a role in the development of osteosarcoma as it occurs due to mutations of the gene or gross changes to the gene locus at 17p13.1. Like pRB, p53 (TP53 - OMIM *191170) is a tumour suppressor protein activated upon DNA damage recognition and can induce cellular quiescence, senescence, or apoptosis. However, p53 is by far the more commonly inactivated protein in human cancer (Levine et al, 2009).

In individuals affected by the Li-Fraumeni syndrome (LFS1 - OMIM #151623), the manifestation of germline TP53 mutations, the incidence of osteosarcoma is increased (Fuchs et al, 2002; Malkin et al, 1990). Loss of heterozygosity (LOH) and deletions of the 17p13.1 locus have been detected in 29–42% of sporadic osteosarcomas (Patiño-García et al, 2003; Levine et al, 2009; Lau et al, 2004; Sztan, et al, 1997), and mutations of TP53 are present in 10–39% of cases (Overholtzer et al, 2003, Patiño-García et al, 2003; Tsuchiya et al, 2000).

Direct inactivation of TP53 expression is only one mechanism by which the p53 pathway can be disrupted. Functional inactivation of p53 at the post-translational level can also occur through regulation by tumorigenic proteins. The oncoprotein MDM2 is a well-described inhibitor of p53, functioning both in the promotion of p53 degradation and the downregulation

of its transcription. Amplification of the MDM2 gene (chromosome 12q15; MDM2 - OMIM *164782) is a relatively infrequent event in primary osteosarcoma, occurring in 3–25% of tumours (Duhamel et al, 2012; Lonardo, 1997) but appears to be considerably more frequent in metastases and recurrences (Miller et al, 1996; Lonardo, 1997).

There has always been an association between Paget's disease and increased risk of malignant transformation of the bone. Paget's disease is associated with development of osteosarcoma and is almost always the cause of osteosarcoma in patients over 40 years (Hansen et al, 2002). The overall rate of osteosarcoma in patients with Paget's disease is 2% (Link et al, 2002). Mutations in the SQSTM1 gene, which some patients with Paget's disease carry, are actually thought to play a role in osteosarcoma development, however no evidences confirm this relationship (Hansen et al, 2002).

In osteosarcomas a number of proto-oncogenes are overexpressed, however the exact role that such over-expression plays in the developing disease is still largely unknown (Ladanyi et al, 2000). Two oncogenes, c-myc and c-fos, both of which have been shown to be overexpressed in osteosarcomas, seem to have the most potential to be directly involved with osteosarcoma formation, especially c-fos, which plays a role in osteoclast differentiation and osteoblast transformation (Eferl et al, 2003).

A set of five inherited syndromes fall under the category of RECQ helicase disorders, and three of these are linked to cancer predispositions. Aberrations of the RECQL4 gene (8q24.4; RECQL4 - OMIM *603780) are also associated with osteosarcoma development. Loss of RECQL4 function following truncating mutation in individuals affected with the autosomal recessive familial Rothmund-Thomson syndrome (RTS #268400) results in significantly higher risk of osteosarcoma (Wang et al, 2003). However, in sporadic osteosarcoma the rate of RECQL4 mutation is less than 5% (Nishijo et al, 2004).

Bloom syndrome (BLM - OMIM #210900) and Werner syndrome (WRN - OMIM #277700) are two additional autosomal recessive syndromes that predispose affected individuals to osteosarcoma (Goto et al, 1996; German et al, 1997). Both syndromes result from genomic instability caused by hereditary mutation of a RECQL family DNA helicase gene (Mohaghegh and Hickson, 2001): Bloom syndrome due to mutation of BLM (RECQL3 - OMIM *604610) located at chromosome 15q26.1 and Werner syndrome due to mutation of WRN (RECQL2 - OMIM *604611) located at chromosome 8p12.

Both syndromes predispose the patient to developing cancer. However, BLM gene mutations predispose the patient to virtually all types of cancers at a very high rate and WRN gene mutations predispose the patient to developing osteosarcoma at a lower rate than

Rothmund-Thomson syndrome (RTS - OMIM #268400) (German et al, 1993; Goto et al, 1996).

Vascular endothelial growth factor A (VEGFA, 6p21.1 - OMIM +192240) is amplified in 25% of a cohort of osteosarcoma specimens (Yang et al, 2011), and its protein product promotes angiogenesis and blood vessel permeability in cancer (Harper et al, 2008). Also Cell Division Cycle 5 (CDC5L – OMIM *602868) encodes a cell cycle regulator, which may function in human osteosarcoma (Lu et al, 2008), and its overexpression may promote mitotic entry and shorten the G2 phase (Bernstein et al, 1998). Runt-related Transcription Factor 2 (RUNX2 – OMIM *600211) encodes a transcription factor important in osteogenesis (Lian et al, 2004) and was expressed in up to 87% of tumour specimens, including biopsy samples, implying that alteration of 6p12-p21 chromosome region may occur early. In another report, gain-related over-expression of RUNX2 was observed in 60% of the analysed osteosarcoma tumours (Sadikovic et al, 2009), and was correlated with poor response to chemotherapy (Sadikovic et al, 2010).

Clinical Presentation and Diagnosis of Osteosarcoma

While there is no definitive set of symptoms for osteosarcoma, generally pain and swelling or tenderness in the affected area are present (Bacci et al, OS FAQ). These symptoms are often attributed to stress or trauma, especially in active children where these might be considered “growing pains”. The increase in pain severity may correlate with tumour penetration of cortical bone and irritation of the periosteum, or with pathologic fracture. As the tumour invades the bone tissue, bones can weaken and occasionally fracture. Other symptoms may include lethargy, fever, weight loss, and anaemia (Bacci et al, OS FAQ).

When a bone tumour is suspected, the patient should undergo a complete medical examination, including blood test, as bone tumours can be associated with increased levels of Alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH).

Except for the slightly diaphyseal involvement, radiological findings are typical in osteosarcoma. On radiographic examination, conventional osteosarcoma present sclerotic and lytic lesion in the metaphyseal region of the involved bone (Wittig et al, 2005). The sclerotic appearance (small, irregular, cloud-like densities) is due to tumour osteoid production. Pathologic fractures might also be visible radiologically (Wittig et al, 2005). Most osteosarcomas present with destruction of the bony cortex and formation of a soft tissue

mass. The soft tissue component may ossify and thus may be detectable on radiographs. Pathologic fractures can also be identified (Wittig et al, 2005).

Magnetic resonance imaging (MRI) and computed tomographic (CT) scans are generally used in order to elucidate the involvement and location of the bone tumour. A biopsy is generally performed to identify the type of tumour.

Although it is not always possible to definitively diagnose osteosarcoma by radiography alone, the contemporary use of clinical symptoms, radiologic findings and histologic examination allow the diagnosis.

Surgery

Surgery for osteosarcoma includes both the biopsy to diagnose the cancer and the surgical treatment.

Biopsy is the key step in the diagnosis of osteosarcoma. Improperly performed biopsies are a frequent cause of misdiagnosis, and local recurrence, and the biopsy samples should be obtained by the orthopaedic oncologists. Staging studies are helpful in planning the surgical approach to the tumour and specifying the region of the tumour that will most likely yield representative pathologic material. In many instances, a percutaneous needle biopsy may be recommended, because it is minimally invasive, does not require wound healing and is associated with a lower risk of infection, contamination and post biopsy fracture (Wittig et al, 2005).

Surgery is an important part of treatment because the main goal is to remove all of the cancer. If even a few cancer cells are left, they can grow and multiply to make a new tumour. To avoid this, surgeons remove the tumour plus some of the surrounding normal tissue. This is known as wide excision. Removing some normal-looking tissue around the tumour improves the chance that all of the cancer is removed (American Cancer Society 2013).

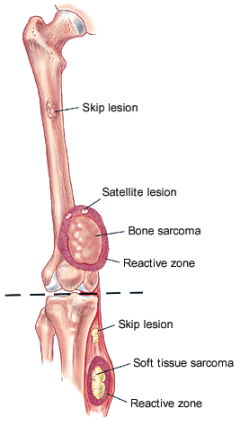
A pathologist will look at the removed tissue to see if the margins (outer edges) contain cancer cells. If cancer cells are seen at the edges of the tissue, the margins are called positive. Positive margins can mean that some cancer was left behind. When no cancer cells are seen at the edges of the tissue, the margins are said to be negative, clean, or clear. A wide excision with clean margins minimizes the risk that the cancer will grow back where it started.

The surgery approach depends on the location of the tumour. Although all operations to remove osteosarcomas are complex, tumours in the limbs (arms or legs) are generally not

as difficult to remove as those located at the base of the skull, in the spine, or pelvis (The American Cancer Society 2013).

Biologic Behaviour of Osteosarcoma

Osteosarcoma grows in a radial manner, forming a ball-like mass. When it penetrates the bony cortex, it compresses the surrounding muscles into a pseudocapsular layer referred to as the “reactive zone.” Tumour nodules representing micro-extensions of the primary mass invade the reactive zone. These nodules are termed “satellites.” The entire tumour mass, including the reactive zone (satellites), must be resected to ensure removal of all gross tumour. Thus, the surgical margin must be wide.



The tumour may metastasize regionally (within the same extremity) or systemically (to other organs, such as the lung). When metastases occur, the prognosis dramatically worsens. Tumour nodules growing outside the reactive rim but within the same bone or across a neighbouring joint are termed “skip lesions” and represent regional intra-osseous or trans-articular metastases, respectively (Bickels et al, 1999). Systemic metastases have a predilection for the lungs. The bones are the second most common site of metastasis, usually being involved only after pulmonary metastases have occurred. Distant bone metastases represent the latest stage of disease and are associated with the poorest prognosis (Bacci et al, 1992).

The vast majority of studies have been descriptions of osteosarcomas focused on the conventional, high-grade subtypes including the chondroblastic, fibroblastic, and osteoblastic variants. These are the most frequently occurring types of osteosarcoma. The rarer subtypes include telangiectatic, small cell, periosteal, high-grade surface, and low-grade osteosarcoma.

Approximately 75% of osteosarcomas arise in the intramedullary cavity and are referred to as conventional osteosarcoma.

The division into high-grade and low-grade variants depends on cellularity, pleomorphism, anaplasia and mitotic index of the tumour tissue. Tumour osteoid production is characteristic of each subtype. The neoplastic cells in high grade conventional osteosarcomas display marked nuclear pleomorphism, conspicuous chromatin abnormalities, prominent nucleoli and many mitotic figures, some of which are atypical.

In the fibrosarcomatous pattern of osteosarcoma, the stroma is composed of spindle cells. Osteosarcomas may contain large areas that resemble malignant fibrous histiocytoma.

Microscopically, conventional osteosarcomas are composed of malignant-appearing spindle cells that produce osteoid. The most common orthopaedic staging system is the Enneking system (Table) (Enneking et al, 1998), based on three key pieces of information:

- grade of the tumour (G)
- extent of the main (primary) tumour (T)
- metastasis (spread) to nearby lymph nodes or other organs (M)

The grade is a measure of how ithe tumour grow and spread, based on how it looks under the microscope examination. Tumours are either low grade (G1) or high grade (G2). The extent of the primary tumour is classified as either intra-compartmental (T1), meaning it is located only within the bone, or extra-compartmental (T2), meaning it has extended beyond the bone into other nearby structures. Tumours that have not spread to the lymph nodes or other organs are considered M0, while those that have spread are M1 (The American Cancer Society 2013).

Most conventional osteosarcomas present as stage IIB tumours (i.e. non-metastatic with an associated soft tissue mass).

Stage	*Grade*	*Site*	*Metastasis*
IA	G1(low)	T1(intracompartmental)	M0 (no metastasis)
IB	G1(low)	T2(extracompartmental)	M0
IIA	G2(high)	T1	M0
IIB	G2(high)	T2	M0
III	G1 or G2	T1 or T2	M1 (regional or distant)

G1: low-grade malignancy, with low likelihood of metastasis and frequent local recurrence, and G2: highgrade malignancy with frequent blood-borne metastases. (Adapted from Enneking WF, Spanier SS, Goodman MA. A system for the surgical staging of musculoskeletal sarcomas. Clin Orthop Rel Res 1980; 106-20.)

Low-grade, localized tumours are stage I.

High-grade, localized tumours are stage II.

Metastatic tumours (regardless of grade) are stage III.

Treatment Overview

Until the 1970s, osteosarcoma was treated by amputation in most cases or else by radiotherapy. In spite of local control, most osteosarcoma patients died within a short period

because of lung metastasis. The results of surgery alone as a treatment of osteosarcoma have not been satisfactory. The 5-year disease-free survival rate after treatment by surgery alone has been reported to be only 12% (Longhi et al, 2006). Neoadjuvant (preoperative) chemotherapy was introduced in 1978 (Rosen et al, 1982).

Once diagnosed, osteosarcoma treatment begins almost immediately. The treatment strategy for each individual is based on a number of factors, such as location, size and involvement of the primary tumour, as well as the presence of any secondary lesions, either regionally or systemically. The goal of treatment is to cure the cancer preserving as much function as possible. A general schedule of osteosarcoma treatment begins with chemotherapy followed by surgical resection of the gross lesion followed by chemotherapy for up to a year (www.mayoclinic.org). Radiation therapy is generally only used when surgery is not a possibility.

Chemotherapy is crucial in the treatment of osteosarcoma, in effort to kill cancer cells, control the spread of cancer and shrink the tumour to make surgery more manageable. Advances in chemotherapy over the past 30 years have been responsible for improved limb salvage and higher survival rates (Bacci et al, 1993; Bacci et al, 1998; Link et al, 1986; Eckardt et al, 1985; www.mayoclinic.org/chemiotherapy). After surgery, additional chemotherapy may improve the chances of killing any cancer cells that remain, but are not detected by imaging tests (www.mayoclinic.org).

Most osteosarcomas are treated with chemotherapy given before surgery (neoadjuvant chemotherapy) for about 10 weeks and again after surgery (adjuvant chemotherapy) for up to a year. People with high-grade osteosarcomas that responded well to chemotherapy before surgery usually get the same chemotherapy after surgery. People whose tumours responded poorly usually will get different chemotherapy after surgery.

Neoadjuvant chemotherapy induces tumour necrosis in the primary tumour and provides early treatment of micrometastatic disease. It helps to facilitate surgical resection with wide margins and therefore has been one of the main factors contributing to improved limb salvage rates.

Drugs that have been shown to be most effective against osteosarcoma include:

- doxorubicin (Adriamycin), that intercalates at points of local uncoiling of the DNA double helix; it also inhibits the synthesis of DNA and RNA;
- cisplatin (Platinol), that inhibits the synthesis of DNA through the formation of DNA cross-links; it binds directly to tumour DNA and denatures DNA double helix;
- ifosfamide (Ifex) with mesna (Mesnex), that causes cross-linking of DNA strands, inhibiting the synthesis of DNA and protein. Mesna is a prophylactic agent used to

prevent hemorrhagic cystitis; it has no intrinsic cytotoxicity. Mesna binds with acrolein (urotoxic metabolite of ifosfamide), inactivating it;

- high-dose methotrexate (Rheumatrex) with leucovorin calcium rescue, Methotrexate is a folate antimetabolite, with it inhibits the synthesis of purine and thymidylic acid by binding dihydrofolate reductase. Leucovorin is a tetrahydrofolic acid derivative that acts as a biochemical co-factor for carbon transfer reactions in the synthesis of purines and pyrimidines. Leucovorin does not require the enzyme dihydrofolate reductase; therefore, it is able to rescue normal cells and prevent severe myelosuppression and mucositis.

Most standard protocols use doxorubicin and cisplatin, with or without high-dose methotrexate, for both neoadjuvant and adjuvant chemotherapy. The current standard protocol of a three-drug chemotherapy regimen using cispatin, doxorubicin and high-dose methotrexate provides about 70% long-term disease-free survival for osteosarcoma patients without metastatic (Chou et al, 2006; Sakamoto and Iwamoto, 2008).

The amount of tumour necrosis following preoperative chemotherapy has been known to be a reliable prognostic factor, enabling the effectiveness of the chemotherapy treatment to be accurately determined (Bielack et al, 2002; Glasser et al, 1992).

Tumour necrosis is usually assessed as follows (Rosen et al, 1982):

Grade I: no necrosis;

Grade II: necrosis of between 50 and 95%;

Grade III: necrosis greater than 95 but less than 100%;

Grade IV: total necrosis; 100%

Patients with tumour necrosis in excess of (>) 90% are classified as good responders to chemotherapy, whereas those whose tumour necrosis is less than (<) 90% are classified as poor responders.

Unfavourable prognosis is associated with tumour location, the number and location of the metastases and lack of adequate surgical resection. In particular, the prognosis of osteosarcoma patients with metastasis is still poor, with only 20% achieving a 5-year survival rate (Lamoureux et al, 2007).

New Treatment

In order to improve the survival of metastatic patients or patients classes as poor-responders following the standard protocol of a three-drug chemotherapy regimen using cispatin, doxorubicin and high-dose methotrexate, several protocols comprising intensive chemotherapy, such as high-dose or prolonged chemotherapy with/without peripheral blood stem cell rescue, have been considered, but the benefit of these therapies is not conclusive in long-term follow-up (Sakamoto and Yukihide, 2008).

A variety of agents, as monoclonal antibodies, heat-shock protein inhibitors or small molecule inhibitors, has been investigated for the treatment of osteosarcoma in clinical trials.

Identifying the genes and signal transduction pathways responsible for the development and malignant behavior in osteosarcoma will lead to the discovery of new drugs and therapy. It has been suggested that inhibition of the mammalian target of the rapamycin (mTOR) pathway may be effective in osteosarcoma (Wan, Khanna et al, 2005). Inhibitors of receptor tyrosine kinases, including insulin-like growth factor 1 receptor (IGF-1R) and a platelet-derived growth factor receptor (PDGFR).

Gene therapy is approaching readiness for clinical studies, and it now offers potentially promising results. The effectiveness of p53 gene therapy via a trans-ferring-liposome-p53 complex administered in animal models has also been reported (Nakase et al, 2005).

The receptor activator of nuclear factor-kß, its ligand and the osteoprotegerin (RANK/RANKL/OPG) system is important in bone remodelling and metabolism (Wuyts et al, 2001; Itoh et al, 2000). This RANK/RANKL/OPG system possibly plays a role in the pathogenesis of osteosarcoma (Miyamoto et al, 2002; Wittrant et al, 2006) and its interference may provide a new therapeutic strategy. Bisphosphonates inhibit osteoclast action and the resorption of bone, and it has been demonstrated that bisphosphonates inhibit tumour growth in vitro.

Differentiation therapy in osteosarcoma could be used as a new strategy.

Metastasis

The most significant negative prognostic factor in the treatment of cancer is the development of metastatic disease. In part this is the result of the differences in the biological behaviour of metastatic tumours from primary tumours (Krishnan et al, 2005; Kansara and Thomas, 2007; Bacci et al, 2008; De Nigris et al, 2008; Guo et al, 2008; Posthuma-DeBoer et al, 2011). As such, the therapeutic regimens that target primary tumours may not be successful in the treatment of metastatic disease (Osborne and Khanna, 2012).

The primary research need in the field is to understand the biology of metastasis in osteosarcoma and to use this understanding to develop therapeutics that target the metastatic phenotype. Dissemination of cancer may occur through haematogenous spread.

The metastatic cascade is an extremely complex multistage process. In order for a metastatic lesion to become clinically detectable, it must successfully complete a sequential series of steps of the metastatic cascade. Within this process, each step is subject to a wide variety of influences; thus, at any point in the sequence the tumour cells may not survive. Failure to complete any step results in failure to colonize a distant site.

The metastatic cascade can be divided into two phases:

- Invasion of the extracellular matrix (ECM);
- Vascular dissemination and arrest in a distant site (Kumar et al, 2005).

Prior to invasion of the ECM, clonal expansion, growth, diversification and angiogenesis must occur in order to produce metastatic subclones within the primary tumour (Krishnan et al, 2006). These 'transformed' tumour cells must then separate from the primary tumour and interact with the ECM at several stages in the metastatic cascade (Osborne and Khanna, 2012).

A number of research groups have tried to identify those genes and proteins that are important for metastasis by performing profiles of both primary and metastatic lesions. This has resulted in the finding of a number of differentially expressed genes, proteins or pathways, of which some appear they might play a key role in regulating metastasis.

In osteosarcoma, several associations with angiogenic phenotype, determinant of metastatic cells, have been demonstrated. This includes an association with metastatic risk and primary tumour microvessel density, expression of angiogenesis-associated growth factors, and the use of inhibitors of angiogenesis in osteosarcoma model systems (DuBois et al, 2007; Quan et al, 2006; Yin et al, 2008).

The integrins are a large family of membrane-associated receptors that interact primarily with matrix associated proteins (Ramsay et al, 2005). Integrin signalling has been suggested to be a primary mechanism whereby cancer cells interact with the cellular microenvironment. In osteosarcoma, the expression of specific integrin family members has been linked to metastasis (Wan et al, 2009). Similar to the integrin family of proteins, expression of chemokines and the chemokine receptors have been linked to osteosarcoma progression and metastasis in a number of preclinical and correlative studies (Laverdiere et al, 2005; Kim et al, 2008). Interactions between chemokines and integrin family members further contribute to the ability of metastatic cells to interact with their microenvironment and promote metastatic cell survival (Miura et al, 2008; Osborne and Khanna, 2012).

The IGF-1 pathway has been linked to the development and progression of many sarcomas, including osteosarcoma. The growth and development of adult mesenchymal tissues are largely the result of growth hormone–induced release of IGF-1 and its interaction with the IGF-1 receptors present on osteoblasts and other mesenchymal cells. Proliferation and survival of normal and malignant osteoblasts have been linked to activation of the IGF-1 pathway (Kappel et al, 1994; Osborne and Khanna, 2012).

Ezrin

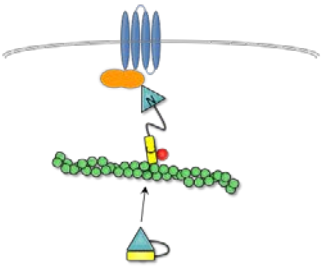
Tumour metastasis is a multi-gene and multi-step process, involving substantial interactions between neoplastic cells and adjacent non-neoplastic tissues (Li et al, 2012). Currently, increasing evidence suggests that a membrane-cytoskeleton linker, ezrin, plays a pivotal role in tumour invasion and metastasis (Neisch et al, 2011). Ezrin, which is encoded by the EZR gene (VIL2 – OMIM *123900) located at chromosome 6q25.2–q26, is a member of the ERM (ezrin-radixin-moesin) family of proteins, which provides a physical link from F-actin to cell membrane-associated proteins (Bretscher et al, 2002; Tsukita et al, 1994, Reczek et al, 1997), thus allowing the cell to interact directly with its microenvironment (Khanna et al, 2004). Ezrin plays a positive role in maintaining cell shape and polarity, and facilitates membrane-trafficking pathways, cell migration, growth regulation, and differentiation (Tsukita et al, 1997); it is also implicated in signal transduction networks both as a regulator and an effector interacting with various members of protein kinase A, Rho and phosphatidylinositol-3 kinase signalling pathways.

ERM proteins are three closely related proteins from the band 4.1 superfamily (Sato et al, 1992). Within this superfamily the members share a common domain, the FERM domain (Four point one, ERM), which in most cases mediates their association with the membrane. ERM proteins from evolutionarily diverse species, all share the same structural organization. They are composed of three distinct regions: an amino-terminal membrane-binding domain, FERM domain, followed by an α-helical midsection, and a carboxyl-terminal actin-binding domain containing the F-actin binding site (Turunen et al, 1994). ERM proteins are highly conserved in their primary structure throughout evolution with the highest level of identity observed in the FERM domains and the F-actin binding sites (Arpin et al, 2011).

Ezrin exerts its biological functions through protein–protein interactions generated upon its conformational change. Quiescent ezrin adopts an intramolecular head-to-tail amino–carboxyl termini complex, which can be modified by specific molecular interactions. The active sites in the protein are hidden by intramolecular interactions, so ezrin can neither bind to actin nor transmembrane proteins.

Two factors are involved in this conformational transition: binding of the amino-terminal domain to phosphatidylinositol 4,5-biphosphate (PIP2) and phosphorylation of a conserved threonine 567 (T567) in the actin-binding domain (Tsukita et al, 1994; Reczek et al, 1997; Makitie et al, 2001; Fehon et al, 2010; Nijhara et al, 2004). The resulting conformation perturbation creates new molecular interactions, the anchorage of the ezrin family to the plasma membrane occurs in different ways. Indirect binding to different proteins such as ion channels, transporters and receptors can bind indirectly through the scaffolding factors NHERF1 (also named EBP50 for ERM-binding phosphoprotein 50) or NHERF2 (Na+-H+ exchanger regulatory factor) (Reczek et al, 1997; Yun et al, 1997; Murthy et al, 1998). These proteins contain two PDZ (Postsynaptic Density Protein) domains known to mediate protein interaction followed by an ERM protein binding site or direct binding to cytoplasmic domains of several integral membrane proteins such as CD43, CD44, ICAM-1, -2 and -3, which mediate cell adhesion and migration (Algrain et al, 1993; Yonemura et al, 1993; Tsukita et al, 1994; Henry et al, 1995; Helander et al, 1996; Hirao et al, 1996; Serrador et al, 1997; Heiska et al, 1998; Yonemura et al, 1998).

The activation of ezrin requires separation of the N- and C-terminal domains. The FERM



domain consists of three lobes (F1, F2, and F3). Experimental evidence showing that the lysine pairs located in subdomains F1 and F3 of the ezrin FERM domain play an important role in the binding of ezrin to multilamellar vesicles containing PIP2 (Barret et al, 2000). Carvalho et al. demonstrated that a conformational change in ezrin is induced by the interaction of ezrin with the entire PIP2 molecule, while no conformational change is detected when PIP2 interacts solely with the FERM domain. Such conformational change does not occur when only the polar headgroup of the PIP2 molecule (IP3) is present, or when the length of the acyl chains of synthetic PIP2 analogues is too short (Carvalho et al, 2010).

As indicated above, phosphorylation of the conserved threonine residue present in the F-actin binding site has also been implicated in the activation of ERM proteins. In vivo, the sequence of events consisting of binding to PIP2 followed by phosphorylation at threonine

567 is critical for the proper activation of ezrin, since impairing one of these steps alters its correct localization and functions in epithelial cells.

In vertebrates, the three ERM proteins show tissue specific expression profiles (Niggli and Rossy, 2011). Ezrin, first isolated in gastric parietal cells, is expressed in a variety of normal cells including many types of epithelial, lymphoid and glial cells (Fehon et al, 2011; Mielgo et al, 2005; Wick et al, 2001; Crepaldi et al, 1997). Bretscher et al. first discovered ezrin in 1983 and found that it was present in very large amounts in membrane protrusions, such as intestinal microvilli (Bretscher et al, 1983). To investigate more precisely the involvement of ezrin in the morphogenesis of epithelial tissues, several studies using mutant mice have emerged. A lack of ezrin, the only isoform expressed in part of the polarized epithelia of the intestine, results in abnormal villus morphogenesis and a disrupted teminal web in these cells. As a consequence, neonate mice lacking ezrin fail to thrive and do not survive past weaning (Soatome et al, 2004). Using a knockdown approach in mice, a role of ezrin has been demonstrated in formation/expansion of canalicular apical membranes in gastric parietal cells, which express only ezrin, resulting in achlorhydria, suggesting ERM proteins play a role in vesicle trafficking (Tamura et al, 2005).

Among the ERM members, high ezrin expression has been identified as vital for metastatic behavior in a murine osteosarcoma model and its over-expression has been linked to a poor prognosis in murine and canine osteosarcoma (Khanna et al, 2001). Furthermore, ezrin was first identified as a crucial molecule in the dissemination of two paediatric tumours, rhabdomyosarcoma (Yu et al, 2004) and osteosarcoma (Khanna et al, 2004). In the two paediatric tumours, ezrin overexpression has been shown to correlate with clinical stage. Ezrin has been detected by high throughput screening of human OS, such as in GEM (Leonard et al, 2003) and proteomic analysis (Folio et al, 2009, Li et al, 2010). It was among the top deregulated genes in a GEM comparing metastatic and non-metastatic mouse OS cell lines (Khanna et al, 2001). The expression of ezrin and its association with metastasis was confirmed in several studies at the protein level by IHC (Boldrini et al, 2010, Kim et al, 2009, Kim et al, 2007, Park et al, 2006, Park et al, 2009, Salas et al, 2007) and at the mRNA level by qRT-PCR (Ogino et al, 2007, Salas et al, 2007, Wang et al, 2010, Xu-Dong et al, 2009). It is of note that exclusive cytoplasmic expression was associated with better disease free survival chances compared with both cytoplasmic and membranous expression (Ferrari et al, 2008). Nuclear expression of phosphorylated ezrin was reported in OS in one study (Di Cristofano et al, 2010), uncovering an unknown role of ezrin in OS.

Ezrin overexpression was demonstrated to increase migration in metastatic melanoma (Federici et al, 2009), pancreatic cancer (Meng et al, 2010) or hepatocellular carcinoma (Yeh et al, 2009). In melanoma cells, ezrin has been shown to be localized in phagocytic vacuoles, suggesting that its association with the actin cytoskeleton is crucial for the phagocytic activity (Lugini et al, 2003). Phagocytic behavior is usually considered to be an indicator of high-grade malignancy in melanomas. In pancreatic carcinomas, a high-level ezrin expression is associated with high metastatic potential; membrane translocation of ezrin might play a role in the progression from borderline tumour to malignant transformation (Yunxiao et al, 2010). In addition, immunohistochemical analysis has demonstrated a significant correlation between increased ezrin immunoreactivity and a high histological grade in astrocytoma (Geiger et al, 2000). Ezrin overexpression has also been considered as an independent predictor of adverse outcome of gastrointestinal stromal tumours (Wei et al,,2009).

Ezrin plays an important role in the metastatic progression of several tumours. However, the mechanisms by which ezrin contributes to the metastatic phenotype of cancer are largely unknown and will require further studies.

Suppression of ezrin protein expression and disruption of its function significantly reduced lung metastasis in a mouse osteosarcoma model (Khanna et al, 2001). Furthermore, high-level ezrin expression in canine osteosarcomas has been associated with early development of metastasis (Khanna et al, 2001).

Concomitantly, reduction of ezrin expression in rhabdomyosarcoma or osteosarcoma cell lines results in a decrease in pulmonary metastases in mice. In these cell lines, ezrin may contribute to metastasis by suppressing apoptosis, disturbing cell–cell adhesion and activating Rho. Depending on the type of adult human tumour, loss or upregulation of ezrin correlates with poor prognosis indicating cell-specific functions of ezrin in tumour progression (Hunter, 2004). Ezrin silencing by small hairpin RNA could reverse the metastatic behavior of human breast cancer cells (Li et al, 2001). In a complementary DNA (cDNA) microarray analysis of highly and poorly metastatic rhabdomyosarcomas, ezrin was indicated to be a key regulator of metastasis (Yu et al, 2004). These results imply that ezrin could be a therapeutic target in OS. Its inhibitor, sorafenib, was suggested for therapy in OS as it showed anti-tumour activity in vitro and in vivo in a pre-clinical study (Pignochino et al, 2009).

Paige et al. have recently identified that a small molecule, NSC 668394, is a potent inhibitor of ezrin function as determined by inhibiting migration in both in vitro and in vivo models. Moreover, inhibition of threonine 567 phosphorylation by NSC 668394 significantly

reduced the metastatic behavior in cellular and animal models and has thus emerged as an important lead inhibitor (Paige M et al, 2013).

Taken together, the observed effects of ezrin overexpression and ezrin silencing on the cell, indicate a role for ezrin in regulating tumour metastasis and progression (Curto an McClatchey, 2004), but little is known on how ezrin is regulated.

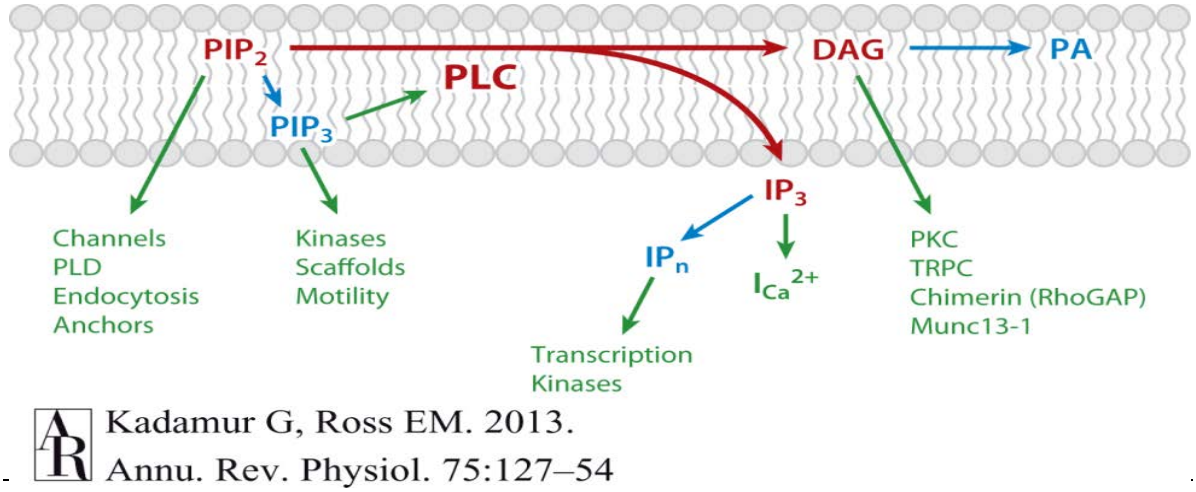
PI-PLC

A large number of extracellular signals stimulates hydrolysis of phosphatidylinositol 4,5-bisphophate (PIP2) by phosphoinositide-specific phospholipase C (PI-PLC) family of enzymes. These proteins, which function at key control points, respond to a variety of regulatory stimuli (Suh et al, 2008).

The first evidence of PI-PLC activity was suggested by Hokin et al in 1953, who reported specific hydrolysis of phospholipids in pigeon's pancreas slices after cholinergic stimulation (Hokin and Hokin, 1953). In 1983, Streb et al. demonstrated that IP3 generated from PIP2 hydrolysis is responsible for mobilization of intracellular calcium in the pancreatic acinar cells (Suh et al, 2008; Streb et al, 1983). Takenawa et al. purified the first PI-PLC with a molecular weight of 68 kDa in 1981 (Takenawa et al, 1981). Subsequently, a number of PI-PI-PLCs of different molecular masses, isoelectric points and calcium dependency have been identified in several tissues. In the late 80's, three PI-PLC isozymes, namely PI-PLC γ, -β, and -δ, were isolated and their cDNA sequences were obtained (Suh et al, 1988). Since then, multiple types of PI-PLC were found from various tissues using either an RT-PCR method by specifically designed primers or a screening method.

Basically, PI-PLCs cleave the polar head group from PIP2 (Suh et al, 2008; Katan, 2005). The best documented consequence of this reaction, like a major cell signalling response, is the generation of two second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG stimulates the activity of a variety of enzymes and structural proteins by binding to a conserved C1 domain and acts as substrate for synthesis of phosphatidic acid, a regulatory molecule. IP3 is a universal calcium-mobilizing second messenger, that gates a Ca2+ channel in the endoplasmic reticulum and is a major regulator of the cytoplasmic concentration of Ca2+, which itself is the centre of a major regulatory network. In addition, IP3 is the rate-limiting substrate for synthesis of inositol polyphosphates, which stimulate multiple protein kinases, transcription, and mRNA processing. These second messengers provide a common link from highly specific receptors for hormones, neurotransmitters, antigens, components of the extracellular matrix and growth factors. In this way, they contribute to the regulation of a variety of numerous biological functions as cell motility, fertilisation and sensory transduction. In addition to its role as a PI-PLC substrate, PIP2 has other functions. Changes in PIP2 concentration affect further cellular processes. One important role played by different species of PI is protein targeting to specific subcellular

compartments, with special regard to the control of membrane trafficking and cell motility (Di Paolo and De Camilli, 2006; Bunney and Katan, 2010).



Therefore, regulation of PI-PLC activity is crucial for cell survival and metabolism. The regulation and coordination of PI-PLC activity with further molecules of the same pathways are central to the control of cellular physiology (Kadamur and Ross, 2013). The precise mechanism of PI-PLC regulation is incompletely understood for all of the isoforms.

Structural and Regulation of PI-PLC enzymes

The family of phospholipase C enzymes includes 13 different isozymes grouped into 6 subfamilies, based on sequence homology, structure, regulation and tissue distribution.: four PI-PLC β, two PI-PLC γ, tre PI-PLC δ, one PI-PLC ϵ, one PI-PLC ζ and two PI-PLC η proteins.

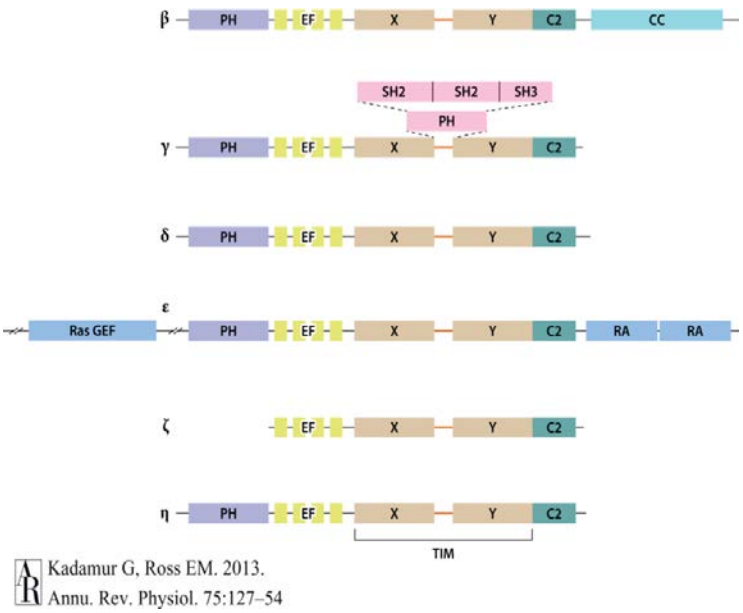
Studies of the catalytic properties of PI-PLCs revealed that all eukaryotic enzymes share common characteristics. All PI-PLC enzymes share a conserved core architecture containing an N-terminal pleckstrin homology (PH) domain, followed by a series of EF-hands motifs, a catalytic TIM barrel (formed by tightly associated X and Y domains) and a C-terminal 2 domain.

The catalytic TIM barrel is the most conserved domain in the PI-PLC isoforms, both structurally and functionally. The enzymatic reaction is catalysed by specific aminoacids in the catalytic domain, which are conserved in the different family members (Hondal et al, 1998). The catalytic domain binds the calcium ions. This domain contains two highly conserved regions, known as the X and Y domains, which form the catalytic core. The

aminoacid sequence similarity in the X and Y domains is about 60% in all isozymes, and it is substantially greater within the same PI-PLC subfamilies. Whereas PI-PLC β and PI-PLC δ isozymes contain a short sequence of 50–70 amino acids separating X and Y regions, PI-PLC γ isozymes owns a longer sequence of about 400 amino acids containing Src homology (SH) (two SH2 and one SH3) domains. The PH domains mediate the interaction with the membrane surface by binding to PIP2, while the SH domains mediate interactions with other proteins by binding to phosphorylated tyrosine residues (SH2) or proline-rich sequences (SH3).

Calcium binding has been reported to be essential for catalytic activity since single mutation of the calcium binding residue Glu-341 within the TIM barrel impairs enzymatic activity (Cheng et al, 1995; Raimondi and Falasca, 2012).

Further domains within the core structure contain highly conserved general folds. However, the ligand binding properties greatly differ. The C2 domain (calcium/lipid-binding domain, CaLB) is a protein module containing about 120 aminoacids present in different enzymes, such as Protein kinase C (PKCs), Phospholipase A2 (cPLA2) and PI-PLCs. C2 domains is formed by 8 antiparallel β-sheets, which define three loops, essential for calcium binding. It was suggested that the C2 domain of PI-PLCs could interact with phospholipids, allowing the enzymes to tether to the plasma membrane. Therefore, the C2 domain, together with the PH domain, when present, might represent a second site for PI binding that seems to be crucial in order to properly orientate the catalytic domain (Raimondi and Falasca, 2012; Bunney and Katan, 2010).



The PH domain, located in the NH2-terminal region, preceding the X domain, was first identified in the protein pleckstrin, the main substrate of PKC. Currently, several proteins were described to contain the PH domain (Musacchio et al, 1993), including PI-PLC δ1, PI-PLC γ1, GTPases, GTPase-activating proteins and Protein Kinase B (AKT). The PH domain is a protein module consisting of about 120 aminoacids with no catalytic activity (Lemmon et

al, 1995). Each PH domain presents a well-defined charge polarisation and defines the positively charged portion needed for ligand interaction and recognition. The side chains are responsible for further interactions, which define the identity of the ligand and the binding affinity (Lemmon and Ferguson, 2000). The majority of proteins hosting PH domains require membrane association to carry out their functions. The PI-PLC δ PH domain was identified to specifically interact with PIP2, leading to a plasma membrane recruitment and increase of the enzyme concentration (Lemmon et al, 1995). However, a few PH domains, such as PI-PLC δ1 PH domain, bind to PI with high specificity.

Most PH domains can bind several PI with different affinity and low specificity. For instance, the PH domain from PI-PLC δ1 and PI-PLC γ1 specifically bind to PIP2 and PIP3 with high affinity; whereas PH domain of PI-PLC β isozymes have low binding affinity to PI. PH domain from PI-PLC β2 mediates protein–protein interactions through binding the GTP-bound form of Rac and Cdc42. PI-PLC γ isozymes contain an additional PH domain that is split by the SH domains. Furthermore, β-type isozymes (with the exception of a splice variant of PI-PLC β4) contain a long COOH-terminal sequence about 450 residues long, located downstream of the Y domain, whereas this region lacks or is short in γ- and δ isozymes. The PI-PLC γ1 PH domain was demonstrated to be essential for PI-PLC γ1 plasma membrane recruitment and subsequent enzyme activation (Falasca, 1998), together with PI-PLC γ1 interaction with the phosphorylated epidermal growth factor receptor (EGFR)(Raimondi and Falasca, 2012).

PI-PLC ϵ does not contain a PH domain, and contains a NH2-terminal Ras guanine nucleotide exchange factor (RasGEF)–like domain and at least one, perhaps two, COOH-terminal Ras binding (RA) domains (Harden T. K. and John Sondek, 2006).

PI-PLCs differ for amino acid sequence, structure, regulation and tissue localisation. A great number of extracellular stimuli activate distinct PI-PLC isoenzymes involving different molecular mechanisms. However, some stimuli can simultaneously activate different PI-PLC isoenzymes, that makes it more difficult to distinguish the role of each single isoform.

PI-PLC β

Four mammalian PI-PLC β enzymes are stimulated by Gα subunits of the Gq family, Gβγ subunits and Ca2+ (Rebecchi and Pentyala, 2000). PI-PLC β isoforms may also be regulated by the small GTP-binding protein Rac (Snyder et al, 2003), phosphatidic acid (PA) and phosphorylation, catalysed by several protein kinases. PI-PLC β isoforms differ somewhat in

their tissue and cell distribution. PI-PLC β1 and PI-PLC β4 are preferentially, although not exclusively, expressed in the neural tissue. PI-PLC β2 is mainly expressed in immune cells, while PI-PLC β3 is more widely distributed. Two PI-PLC β1 proteins, known as 1a and 1b isoform, whose C-terminal are encoded by different exons, assume different subcellular distributions.

PI-PLC β subfamily members own a N-terminal PH domain, four EF-hand motifs, catalytic X and Y domains and a C2 domain. Compared to other PI-PLC subfamilies, PI-PLC β isoforms are unique in that they have a long α-helical C-terminal domain of about 450 aminoacids. The PH domain is involved in the interaction with Gβγ subunits of G-proteins and with phospholipids.

In the inactive GDP-binding state, the G-proteins form a heterotrimeric complex formed by Gα, Gβ and Gγ subunits. Upon stimulation of the receptor, the Gα subunit is activated through exchanging GDP with GTP. That induces the dissociation of Gα from Gβγ. The GTP-bound Gα subunit, together with the Gβγ heterodimer, activates PI-PLC β isoenzymes.

Each PI-PLC β enzyme shows variable responsiveness to the different G-subunits, and that suggests a subtle regulation of the activation (Rebecchi and Pentyala, 2000). PI-PLC β1 is the least sensitive to Gβγ activation, while PI-PLC β4 is completely insensitive to Gβγ. Recent works demonstrate that PI-PLC β can also be activated by the Rho-GTPase family member Rac, which interacts with the PH domain (Snyder et al., 2003). PI-PLC β2 is directly stimulated by the small GTP-binding proteins Rac1, Rac2, while Cdc42 and PI-PLC β3 respond weakly (Bahk et al, 1994). Rac binds the PH domain of PI-PLC β2 (Bahk et al, 1994), and the structure of the Rac1-PI-PLC β2 complex has been solved (Jezyk et al, 2003; Kadamur and Ross, 2013).

PI-PLC δ

Of six PI-PLC subfamilies, PI-PLC δ probably represents the most basal evolutive form in mammalian somatic cells. PI-PLC δ is the prototypical isoform of PI-PLC and its mechanism of interaction with the lipid bilayer and PI substrate is understood. Nevertheless, the activation mechanism and physiological function of the δ subfamily was not completely resolved.

Depending on the cell type, the PI-PLC δ isoforms are commonly distributed within the cytoplasm and in different membrane fractions. The mechanism which regulates the activation of PI-PLC δ isoenzymes differs from other PI-PLC subfamilies. PI-PLC δ isoforms are the most sensitive to variation of the intracellular calcium concentrations compared to

other PI-PLC isoenzymes. PI-PLC δ1 can be activated by calcium and in turn it can amplify the calcium-dependent signal cascade. Beside Ca2+ at the active site activation,Ca2+ in the 10–100 nM range is the second significant activator of PI-PLC δ (Allen et al, 1997; Lomasney et al, 2012). Three or four Ca2+ ions bind to the C2 domain of PI-PLC δ, in addition to Ca2+ at the active site in the TIM barrel domain (Grobler et al, 1998; Lomasney et al, 2012). Ca2+ binding moves of PI-PLC δ isoforms, particularly PI-PLC δ1, from cytosol to the plasma membrane, also increasing the catalytic in vitro activity. Moreover, the PH domains of the PI-PLC δ subfamily are unique in that tightly bind PIP2. They binding drives their membrane association, thus promoting PIP2 hydrolysis. Depletion of plasma membrane PIP2 in cells moves PI-PLC δ1 from the plasma membrane to the cytosol. Mutation of PIP2-interacting residues in the PH domain blocks membrane association and activity (Lomasney et al, 2012). PIP2-mediated membrane binding is not sufficient to activate PI-PLC δ (Kadamur and Ross, 2013). PI-PLC δ1 can also be activated by α1-adrenergic receptors through atypical G-protein, by Gi coupled receptor agonists (Murthy et al, 2004) and by RhoGAP, which stimulates PI-PLC δ1 activity up to 10-fold (Rebecchi and Pentyala, 2000).

PI-PLC γ

PI-PLC γs contains a unique region (PI-PLC γ-specific array, γSA) that comprises two tandem SH2 domains and an SH3 domain adjacent to a split PH (Katan and Williams, 1997). It is well known that the SH2 and SH3 domains facilitate interaction with diverse molecules, contributing to physiological functions of PI-PLC γ (Bunney and Katan, 2011). Whereas PI-PLC γ2 is mainly detected in hematopoietic cell lineages, PI-PLC γ1 is ubiquitously expressed. Thus, it has been suggested that PI-PLC γ1 plays pivotal roles in different tissues (Rebecchi and Pentyala, 2000; Suh et al, 2008).

The SH2 domain (Src homology domain 2) is responsible for protein–protein interaction, recognising specific phosphorylated tyrosine. Indeed, the SH2 domains are essential for interaction with phosphorylated tyrosine residues in activated tyrosine kinase receptors, which dimerize upon ligand engagement and get auto-phosphorylated in tyrosine residues. The SH3 domain recognises and interacts with proteins expressing polyproline sequence (Kadamur and Ross, 2013).

Prompted by suggestions of Rac-mediated PI-PLC γ2 activation in B lymphocytes,

Piechulek et al. showed that PI-PLC γ2, but not PI-PLC γ1, is directly stimulated by the small GTPases Rac1 and Rac2 (Piechulek et al, 2005). Stimulation is independent of tyrosine phosphorylation. Because only PI-PLC γ2 is sensitive to Rac, this path to PI-PLC activation exists only in immune cells. Rac2 regulates PI-PLC activity by binding to the split PH domain in the X-Y linker (Everett, 2011). However, the Rac binding surface is different from that on the N-terminal PH domain of PI-PLC β2. It is tempting to suggest that binding of Rac to the split PH domain moves the X-Y linker away from its autoinhibitory conformation, but this idea remains to be tested. Stimulation by Rac also involves recruitment of PI-PLC γ2 to sites of Rac activation on the plasma membrane (Kadamur and Ross, 2013).

PI-PLC ε

The PI-PLC ε sub-family was identified in 2001. PI-PLC ε isoenzymes are among most complex PI-PLC isoforms, as they present the highest number of different protein domains which regulate their activation (Kelley G.G., et al., 2001). PI-PLC ε is activated by Ras family members, such as Rap1, Rap2, TC21, Ral, Rho and Rac, as well as the heterotrimeric G protein subunits Gα12, Gα13. RA domains and the highly related Ras-binding domains (RBDs) are characteristically found in effectors of Ras family GTPases. Ras directly binds the RA domains to promote translocation of PI-PLC ε to membranes and increase inositol lipid hydrolysis by PI-PLC ε. In particular, PI-PLC ε has two RA domains that are associated with Ras and Rap1 wich are essential for PI-PLC ε activation (Song et al, 2002). Notably, Ras-mediated PI-PLC ε activation induces translocation of the enzyme to the plasma membrane, while Rap1 induced activation recruits the enzyme to the perinuclear region (Song et al, 2002). Furthermore, the PI-PLC ε sub-family presents a CDC25 homology domain and possesses guanine nucleotide exchange (GEF) factor properties for RAS proteins.

The CDC25 domain has GEF activity towards RAP1, but not towards any other Ras members. Therefore, CDC25 can stimulate Rap1 GTP binding and activation, which in turn activates PI-PLC ε activity and perinuclear translocation (Jin et al., 2001). This RasGEF domain directly binds Ras and Rap GTPases to catalyze the exchange of GTP for GDP, which leads to their activation. The combination of the RA domains and a RasGEF domain in PI-PLC ε adds considerable regulatory complexity to the signalling cascades controlled by PI-PLC ε, which is both a downstream effector and upstream regulator of various Ras GTPases.

PI-PLC ζ

PI-PLC ζ is a sperm-specific PI-PLC isoform. Recombinant protein injected in egg cells causes calcium oscillation. PI-PLC ζ isoenzyme is the shortest of the PI-PLC family and lacks the PH domain. The absence of the PH domain, important for PI binding, raised the question of how the enzyme can maintain the activity of substrate hydrolysis. It was shown that the C2 domain is sufficient for the membrane tethering and its deletion inhibits calcium oscillations upon PI-PLC ζ activation (Suh PG, et al., 2008; Kadamur G. and Ross E.M., 2013).

PI-PLC η

The activation of PI-PLC η sub-family is still not completely understood. The plasma membrane localization of the enzyme is independent from extracellular stimuli and depends on the PH domain. Indeed, deletion of the PH domain induces the enzyme to localise in the cytosol. Recent data show that PI-PLC η1 is involved in the signal amplification downstream to GPCR and that it is activated by intracellular calcium.LPA-induced calcium release is inhibited in PI-PLC η1 knock-down cell lines (Kim J.K., et al., 2011; Kadamur G. and Ross E.M., 2013).

PI-PLC in cancer and disease

Several studies suggested the existence of a link between PI signalling and cancer. Great attention was addressed to investigate the role of enzymes that regulate the levels of PIP2 and PIP3. Among those, phosphoinositide 3-kinase (PI3K) and PI-PLC have been involved in the generation and progression of tumours and, hence, considered as new potential targets for prognosis and therapeutic intervention.

Abnormal expression and activity of specific PI-specific PI-PLC isoforms are commonly detected in a variety of different human cancers and were associated with the appearance and progression of tumoral phenotypes. For instance, isozymes of the PI-PLC β sub-family were associated to hematopoietic malignancies and neuroendocrine tumours (Bertagnolo V., et al., 2006; Bertagnolo V., et al., 2007; Lo Vasco VR., et al, 2004). Members of the PI-PLC γ subfamily were described to be up-regulated in neoplastic leukocytes (Pozzobon M., et al.,

2004) and in colon carcinoma, where they might play a role in carcinogenesis (Nomoto K., et al., 1995). In addition, PI-PLC δ isozymes have been suggested to be involved in the neoplastic evolution of gastric mucosa and hepatic cells (Marchisio M., et al., 2001; Yang Y.R., et al., 2013).

Changes in the expression of certain PI-PLC β isoforms were observed since a long time during diverse physiological conditions. As an example, the expression of PI-PLC β1 is selectively increased during myoblast and adipocyte differentiation (Faenza et al., 2004 and O'Carroll et al., 2009). Likewise, PI-PLC β2 is highly up-regulated in breast tumours and correlates with poor clinical outcome, suggesting a role as a marker for breast cancer malignancy (Bertagnolo et al., 2006). In fact, PI-PLC β2 is crucial for migration of breast cancer-derived cell lines and mitosis in cultured breast-derived tumour cells (Bertagnolo et al., 2007). Also PI-PLC β3 was suggested to be a tumour suppressor in neuroendocrine tumours. In fact, PI-PLC β3 Knock-Out (KO) mice can develop myeloproliferative diseases, lymphoma and other tumours, resulting from an impaired Stat5-suppressive mechanism. Furthermore, PI-PLC β3 is down-regulated in leukocytes of patients affected with chronic lymphocytic leukaemia (Xiao et al., 2009) (Kawakami T. and Xiao W., 2013; Yang Y.R., et al., 2013).

Evidences indicated that PI-PLC γ signalling pathways might be important in cancer cells. PI-PLC γ expression evel is increased in cancers compared to the corresponding normal tissue counterpart (Arteaga et al., 1991; Noh et al., 1994). Many studies support the involvement of PI-PLC signalling in the migration of cellsfrom breast and prostate cancers, typically characterized by high levels of growth factor receptor expression (Kassis J., et al., 2001; Wells A. 2000). In most experiments, U73122, a widely used PI-PLC inhibitor, inhibited cell migration. PI-PLC γ is also important for growth-factor-induced gene expression (Cavallaro U., et al., 2001). Manos et al. have identified by microarray analyses some of the genes potentially involved in cell migration (Manos E.J., et al., 2001).

PI-PLC γ1 appears to be at the convergence point for different signalling pathways that activate cell spreading and migration mediated by integrins (Katan et al., 2005). Down-regulation of PI-PLC γ1 expression inhibits Rac1 activation and results in suppression of human breast cancer cell-derived lung metastasis in an *in vivo* mouse model (Falasca M., et al., 2008). In addition to mediating the effects of adhesion receptors on cell motility, PI-PLC γ1 was shown to mediate the cell motility effects of growth factors, including platelet-derived growth factor (PDGF) (Kundra et al., 1994), epithermal growth factor (EGF) (Chen et al., 1994; Xie et al., 2010), insulin-like growth factor (IGF) (Bornfeldt et al., 1994) and hepatocyte

growth factor (HGF) (Martin et al., 2008). Moreover, PI3K-mediated PI-PLC γ1 activation is required for EGF-induced migration of breast cancer cells (Piccolo M., et al., 2002). In fact, interactions between the SH3 domain of PI-PLC γ1 and Rac1 play a significant role in EGF-induced F-actin formation and cell migration (Li et al., 2009). *In vivo* mouse models showed the critical role of PI-PLC γ1 in metastasis. In transgenic mice carrying an inducible PI-PLC γ1 gene fragment, a fragment of dominant-negative PI-PLC γ1 limits the metastatic potential of carcinomas in oncogene-induced mammary and prostate cancer tissues (Shepard et al., 2007), strongly suggesting that PI-PLC γ1 might represent a potential therapeutic target for the treatment of tumour metastasis.

Given the importance of Ras and Rho-family GTPases in the generation and progression of tumours, several studies aimed to indentift a possible role for PI-PLC ϵ in tumour cells and cancer development (Bai et al, 2004, Campbell et al., 2006, Ikuta, 2008 and Sorli et al., 2005).

In fact, the observation that PI-PLC ε is a direct effector of the proto-oncogene Ras raised great interest about the potential role of PI-PLC ε in cancer. Ras activates multiple effector pathways. In many cancers, the Raf/ERK pathway, which drives cell division and transformation, is an important target of mutationally activated Ras.

Sorli S.C. et al. showed that PI-PLC ε plays a crucial role in skin papilloma formation and malignant progression induced by Ras activation followed by 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment. Furthermore, PI-PLC ε is shown to act down-stream of TPA to induce hyperproliferation of the basal layer cells and skin hyperplasia. They also showed that PI-PLC ε plays a crucial role in Ras oncogene-induced de novo carcinogenesis of skin epithelial cells. The authors also provided the evidence for the role of the PI-PLC signalling in carcinogenesis. This leads to the hypothesis that specific inhibitors of PI-PLC ε might be useful for treatment and prevention of certain types of cancer.

PLCE1 KO mice are resistant to intestinal tumour formation when crossed with APCmin/+ mice, which carry a non-sense mutation in the adenomatous polyposis coli (apc) gene (Li, M., et al., 2009).

Recent studies using genomic based approaches have identified association of the PI-PLC ε gene with various human cancers. Two separate genome-wide association (GWAS) studies identified PI-PLC ε1 single nucleotide polymorphisms (SNPs) associated with oesophageal squamous cell carcinoma (ESCC) (Abnet C.C., et al., 2009; Wang L.-D., et al., 2010). Those studies analysed DNA SNPs from over 1000 patients with ESCC and compared them with controls. Both studies identified polymorphisms in either the coding and non coding

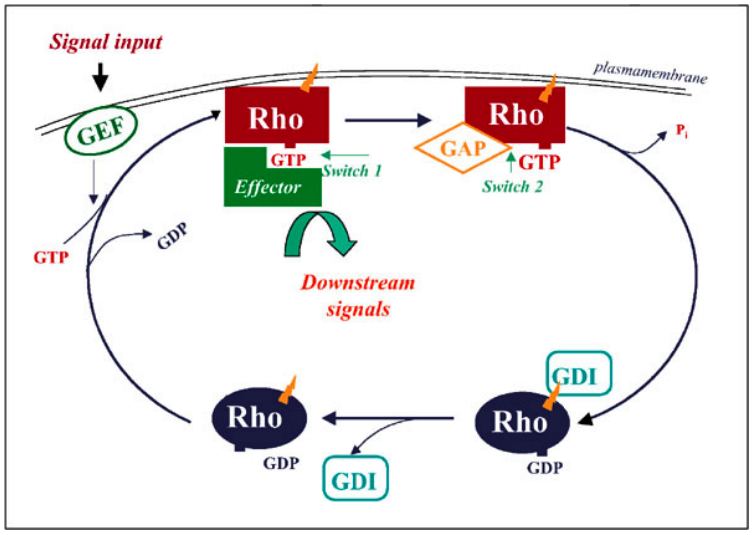
regions of the PI-PLCE gene, and demonstrated that those polymorphisms are associated with ESCC. One of these studies found that the same PI-PLC ε1 SNPs associated with ESCC are also associated with gastric adenocarcinoma (Abnet C.C., et al., 2009).

Several physiological functions indicate that PI-PLC ε may be a therapeutic target. In cardiac hypertrophy, PI-PLC ε appears to be a central player that integrates signals from multiple stimuli. The inhibition of PI-PLC ε function could be a strategy for treatment of heart failure. Similarly, inhibition of PI-PLC ε could prevent inflammatory reactions associated with tumour development in some cancers or in inflammation-related diseases (Wang H., et al., 2005). In pancreatic β-cells, PI-PLC ε can increase insulin secretion in response to changes in the levels of incretins, such as GLP1. Therefore. that role of PI-PLC ε might pave the way to new approaches to treatment of Type 2 diabetes (Dzhura I., et al., 2011). PI-PLC ε is highly expressed in the lung, suggesting that PI-PLC signalling plays a role in asthma, mediating contraction of bronchial smooth muscle. PI-PLC ε signalling may be associated with exchange protein directly activated by cAMP (Epac) in many cell types and might be a general regulator of calcium-induced calcium release (CICR) in response to cAMP in different excitable cells.

The general basis for receptor stimulated phosphoinositide hydrolysis is widely described, although probably incomplete and unexpected roles for phosphoinositide-specific PI-PLC isoforms still continue to emerge.

GTPases

Rho-family GTPases belong to the Ras superfamily of monomeric 20–30 kDa GTP-binding proteins; like RAS proteins, they have lipid modifications that target them to cell membranes, so that behave as molecular switches that couple signal transduction cascades of signaling pathways in the mammalian cell. Rho proteins are guanine nucleotide-binding proteins that switch between an active GTP-bound state and inactive GDP-bound state; these change controls their ability to activate their effector molecules.



Their activity is spatio-temporally regulated by positive regulator guanine nucleotide exchange factors (GEFs), negative regulators GTPases activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Ridley, 2001).

Once activated, RhoGTPases bind different effector molecules and trigger a signalling cascade to direct cellular responses; they are also involved in various cell functions, such as cell morphologic change, cell motility, and cytokinesis.

The small G proteins of the Rho family (Rho GTPase) include different subgroups: Rac (Rac1-3, RhoG), Rho (RhoA-C), Cdc42 (Cdc42, TC10, TCL, Chp/Wrch2, Wrch1), Rnd (Rnd1-2, Rnd3/RhoE), RhoD (RhoD, Rif/RhoF), RhoBTB (RhoBTB1-2) and RhoH (Etienne-Manneville et al, 2002). These proteins have been found as key regulators in one of the most important determinants for tumour cell migration and invasion, as actin cytoskeleton rearrangement (Hall, 1998). Rho GTPases have also been shown to be crucial for many aspects of tumorigenesis, including tumour cell adhesion and migration (Evers et al, 2000;

Ridley et al, 2001), stimulation of tumour cell invasion (Yoshioka et al, 1998), angiogenesis (Merajver et al, 2005) and anoikis (Cheng et al, 2004; Sahai et al, 1999). Rho GTPases have been reported the acquisition of unlimited proliferation potential, survival and evasion from apoptosis, tissue invasion and of metastasis. Also, they take part in control of other functions linked to actin cytoskeleton, including gene transcription, G1 cell cycle progression, microtubule dynamics, vesicle transport and a wide variety of enzymatic activities (Etienne-Manneville et al, 2002). In addition to their regulation of the cytoskeleton, Rho proteins could act as regulators of enzymatic activity; for example, Rac1 controls the enzyme complex responsible for superoxide formation (NADPH oxidase) (Sawada , 2010), whereas RhoA regulates the Rho-kinases.

Rho proteins are 190-250 residues long, composed only of GTPase domain and a short terminal C-terminal extension. Within their GTPase domain, they share approximately 30% amino acid identity with the Ras proteins and 40-95% identity within the family. All members contain the sequence motifs characteristic of all GTP-binding proteins, bind to GDP and GTP with high affinity. The majority of members undergoes C-terminal post-translational modification by isoprenoid lipids. This event, with other C-terminal modifications, facilitates their subcellular location and association with specific membranes or organelles. Lipid modification of Rho GTPases are also strategic for subcellular compartmentalization. The hydrolysis of GTP and the contact with GAPs allows a new association of the GTPases with GDI and their return to the cytosol (Pechlivanis and Kuhlmann, 2006).

GEFs act downstream of numerous growth factor receptors, integrins, cytokine receptors, and cadherins. Although still poorly characterized, over 30 GEFs have been identified that facilitate the exchange of GDP for GTP (Kjoller and Hall , 1999). All Rho GEFs contain a Dbl-homology (DH) domain which encodes the catalytic activity (Cherfils et al, 1999) and an adjacent pleckstrin homology (PH) domain. The PH domain is thought to mediate membrane localization through lipid binding but, in addition, structural and biochemical evidence suggests that it might also directly affect the activity of the DH domain.

Regulation of the cytoskeleton

GTPase effectors are a large group of proteins; two factors concur to determine specific Rho GTPase function: tissue specificity of GTPase effectors and distinct intracellular

localizations, due to different lipid modifications (Etienne-Manneville, 2002; Parri et al, 2010).

The best characterized Rho GTPAses are Rac1, Cdc42 and RhoA. They are ubiquitously expressed, and their major function is the regulation of the cytoskeleton. However, the impact of each member of Rho family on the cytoskeleton could be different.

RhoA activation leads to the formation of actin stress fibers and focal adhesion, as well as membrane protrusion through binding to mDia proteins and Rho-kinases (ROCK I and ROCK II), both of which are direct effectors of RhoA and RhoC. ROCK facilitates phosphorylation and activation of myosin light chain to induce myosin-based contractility and actin polymerization to produce long straight actin (Heasman and Ridley , 2010; Ridley, 2011; Loirand et al, 2013).

Stimulation of Rac1 induces actin polymerization at the cell periphery to generate protrusive actin-rich lamellipodia and membrane ruffling.

Cdc42 initiates long parallel bundles of actin filaments to induce the formation of filopodia. These reorganizations of the actin cytoskeleton induced by Rho proteins have consequences on cell morphology, polarity, motility, and adhesion. RhoA and RAC1 can regulate the function of ezrin, moesin and radixin: these related proteins promote cell motility by linking the actin cytoskeleton to the plasma membrane through the membrane-spanning ECM receptor CD44. RhoA can promote phosphorylation of ezrin by ROCK, leading to its increased association with the cytoskeleton (Matsuiet al, 1998). Direct inhibition of RhoA function, using the Clostridium botulinum toxin C3 transferase, can have a dramatic effect on ERM protein activity. Treatment of most cell types with C3 transferase results in microvillar collapse and concomitant inactivation of ERM proteins (Nobes and Hall, 1995; Ivetic and Ridley, 2004).

Rac and ERM proteins are both required for membrane ruffling and lamellipodium extension and are important for cell migration, as well as for the production of reactive oxygen species in phagocytes (Ridley, 2001). Although molecular links between Rac and ERM proteins have not been studied extensively, different studies suggest that Rac might regulate positively or negatively ERM proteins. On the one hand, Rac can regulate PIP2 production and could thereby increase ERM activity; ERM proteins can be activated by PIP2 independently of Rho and it is possible that Rac is involved here. On the other hand, Rac has recently been proposed to down-regulate ERM activity by stimulating dephosphorylation (Ivetic and Ridley, 2004).

Rac and Rho proteins in cancer

As a consequence of the large number of key functions assigned to Rho proteins, like proliferation, apoptosis/survival, cell polarity, cell adhesion and plasticity of cell migration, it is not surprising that they play important roles in tumour biology (Boettner et al, 2002). A clear connection can be established between Rho proteins overexpression and cancer initiation and progression of a large variety of human tumours including the acquisition of unlimited proliferation potential, survival and evasion from apoptosis, angiogenesis, tissue invasion and establishment of metastases (Aznar et al, 2004; Sahai et al, 2002). Some Rho GTPases stimulate cell cycle progression and regulate gene transcription; this could in part explain their pro-oncogenic properties, for example in promoting Ras-induced transformation (Benitah et al, 2004). Some Rho GTPases are thought to be able to regulate the release of pro-angiogenic factors to promote neovascularisation (Merajver et al, 2005). RhoA and RhoC expression and/or activity is frequently increased in human tumours, whereas RhoB is often downregulated (Gomez del Pulgar et al, 2005). Increased RhoA expression was described in various human tumors including liver (Li et al, 2006), skin (Abraham et al, 2001) and colon cancer (Fritz et al, 1999). RhoA has been implicated in virtually all stages of cancer progression: for example, in vitro, constitutively active RhoA can stimulate transformation (Jaffe et al, 2005).

The Rac subfamily of Rho GTPases includes Rac1, Rac2, Rac3. Rac1 is over-expressed in various tumours and accumulating evidence indicate that Rac1-dependent cell signalling is important for malignant transformation (Gomez del Pulgar et al, 2005). Overexpression of Rac1 correlates with progression of testicular (Kamai et al, 2004), gastric (Pan et al, 2004), and breast cancer (Schnelzer et al, 2000). Rac1 is also overexpressed in oral squamous cell carcinoma (Liu et al., 2004). Rac1 could contribute to cancer cell proliferation by regulation of the cell cycle: for example, it stimulates expression of cyclin D1 and induces cell transformation in vitro (Benitah et al, 2004). The contribution of different Rac isoforms to migration is likely to depend on the cell type and their relative expression levels. Rac2 is required for neutrophil migration but whether it acts similarly in tumors is not known (Roberts et al, 1999). In contrast, Rac1 and Rac2 are dispensable for cell migration in macrophages, although Rac1 is required for invasion (Wheeler et al, 2006). However, in fibroblasts, Rac1 but not Rac3 suppression by RNAi affects lamellipodium formation although cell invasion is reduced in both cases (Chan et al, 2005).

The discovery of Ras GTPases as important proteins in oncogenesis led to necessity assigning effectors and understanding signal transduction pathways downstream.

GTPase-PI-PLC EZR

The small GTPase RhoA, an organizator of stress fibers (Tapon and Hall, 1997), was shown to be the principal regulator of ERM proteins activation. Interestingly, ERM proteins may regulate the Rho activity and vice versa (Bretscher, 1999; Mangeat et al, 1999; Tsukita and Yonemura, 1999). Literature evidences reported that ERM associates to plasma membranes following RhoA activation (Hirao et al, 1996). It was also suggested that ERM proteins regulate the Rho function by binding RhoGDI, a negative regulator of Rho activity, thus releasing the inactive Rho, allowing the GDP/GTP exchange, thus resulting in Rho activation (Hunter, 2004). Over-expression of another direct effector of Rho, phosphatidylinositol 4-phospate 5-kinase type Iα (PIP5K-Iα), which produces PIP2, increases the level of COOH-terminal threonine residue (CPERMs) and induce microvillar formation (Matsui et al,1999). Furthermore, Barret et al. showed that mutagenesis of the PIP2-binding site in the N-terminal domain of ezrin alters its membrane localization, suggesting that PIP2 might be important in ERM protein function at the plasma membrane (Barret et al, 2000).

As ERM proteins were implicated in Ras signaling (Orian-Rousseau et al, 2007), the uncontrolled Ras activation, via ERM proteins, was thought to promote tumour progression.

PLC isoforms were preferential site of link Ras to inositol phosphates. One of the first examples of PI-PLC activation by small GTPases was the isolation of Rac proteins, which act as activators of PI-PLC β2 isoform in neutrophils (Moon et al, 2003). Further studies showed that several Rac proteins (but not RhoA) can directly bind and activate the PI-PLC β2 isoform in vitro. Rac proteins, and not other members of Rho GTPases, regulate PI-PLC γ2 (Piechulek et al, 2005). By contrast, PI-PLC ε is activated by RhoA, RhoB and RhoC, whereas Rac and Cdc42 have no effect (Wing et al, 2003). To explain the difference between these two sets of data, authors postulated that the interaction sites of each PI-PLC isoform for Rho GTPases are unique. Studies of the interactions of respectively PI-PLC β2 and PI-PLCε with Rac and Rho access to different structural domains. Two studies about PI-PLC β2 identified the PH domain as an interaction site; by contrast, a site in PI-PLC ε necessary for Rho-mediated activation was mapped to a unique region (designated the Y-insert) specifically present in the catalytic domain of PI-PLC ε. The different potential binding

interfaces is consistent with studies about the interaction sites of different effectors with Rho family GTPases, showing that no particular domain or structural motif accomunates them all (Dvorsky and Ahmadian, 2004)

Studies of PI-PLC β2 and PI-PLC γ2 and activated Rac in transfected cells suggest that Rac could mediate membrane translocation of PI-PLC β and PI-PLC γ2 (Illenberger et al, 2003; Wing et al, 2003; Jezyk et al, 2008). Binding of Rac to the PI-PLC β2 PH domain is likely to provide a bridge to the membrane surface. Given that Rac makes no contacts with the catalytic core, it is difficult to predict whether it might activate the PI-PLC through a conformational change in more complex cellular settings. The observation that a conformational change and the subsequent translocation to the membrane is necessary for PI-PLC activation, was suggested for further PI-PLC isoforms. This event might be required to release intra-molecular inhibitory interactions that maintain the PI-PLC enzymes inactive in the absence of stimulus.

Aim of the study

Ezrin a member of the ERM family of proteins, has been associated with osteosarcoma metastasis to the lungs and its over-expression has been linked to a poor prognosis in murine, canine, and human OS cases.

A relatively rare phospholipid, the phosphatidylinositol 4,5-bisphosphate (PIP2) plays an essential role in the regulation of cytoskeleton remodelling by binding to ezrin protein, inducing its activation and ultimately association to the plasma membrane. PIP2 belongs to the phosphoinositide (PI) signalling system, an intricate network of enzymes and phospholipids which regulates a number of cell and tissue activities and interacts with RHO-GTPases.

Aim of this study was to investigate the existence of a possible relationship between ezrin activity and the PI signal transduction pathway. In particular, we intended to study the involvement of phosphoinositide-specific phospholipase C (PI-PLC) enzymes that selectively catalyze the hydrolysis of PIP2. Due to the relationship between ezrin and PIP2, we investigated whether the expression of PI-PLC enzymes might be related to ezrin activity in different osteosarcoma cell lines.

Materials and Methods

Materials

Cell culture

We first investigated the PI-PLC expression panel in MG-63, 143B, SaOS-2 and Hs888 human osteosarcoma cell lines to obtain a preliminary tool in order to analyse the role of PI signal transduction pathway in the pathogenesis of osteosarcoma.

The cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

MG-63 cells are used as experimental models for human osteoblasts, have low levels of alkaline phosphatase activity, and PTH unresponsive adenylate cyclase (Fukayama and Tashjian, 1990).

143B, thymidine kinase negative cells, are known to develop osteolytic tumours (Kaminski et al, 2003).

SaOS-2 is an established epithelial-like human osteosarcoma cell line (Fogh et al, 1975) used as experimental model for human osteoblasts (McQuillan et al, 1995; Rodan et al, 1987).

Hs888 cells were derived from lung metastasis of osteosarcoma.

Antibodies

antibody	clone		diluition	
PI-PLC β1	G-12	rabbit polyclonl IgG	1:50	**Santa Cruz Biotechnology, Santa Cruz, CA**
PI-PLC γ2	K-18	goat polyclonal IgG	1:100	**Santa Cruz Biotechnology, Santa Cruz, CA**
PI-PLC δ4	Q-15	goat polyclonal IgG	1:100	**Santa Cruz Biotechnology, Santa Cruz, CA**
PI-PLC ε	C-18	goat polyclonal IgG	1:100	**Santa Cruz Biotechnology, Santa Cruz, CA**
EZRIN	3C12	mouse monoclonal IgG	1:100	**Santa Cruz Biotechnology, Santa Cruz, CA**
Rac-1	Clone 23A8	mouse monoclonal IgG	1:150	**Millipore, CA, USA**
RhoA	Clone 119	rabbit polyclonl IgG	1:300	**Santa Cruz Biotechnology, Santa Cruz, CA**
β-actin		Rabbit polyclonal IgG	1:20000	**aBcam, Cambridge, UK**
anti-mouse		AlexaFluor Donkey 488	1:300	**Invitrogen, Carlsbad, CA, USA**
anti-rabbit		AlexaFluor Donkey 594	1:100	**Invitrogen, Carlsbad, CA, USA**
anti-goat		AlexaFluor Donkey 595	1:300	**Invitrogen, Carlsbad, CA, USA**
anti-mouse HRP		immunoglobulin G	1:1000	**Jackson Immunoresearch, UK**
anti-rabbit HRP		immunoglobulin G	1:1000	**Jackson Immunoresearch, UK**

Methods

Expression of PI-phospholipase C in osteosarcoma cell

Cell culture

MG-63, 143B, SaOS-2 and Hs888 were separately cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%-15% foetal bovine serum (FBS), 1mM sodium piruvate, 100 U/mL of penicillin, and 100 mg/mL of streptomycin, at 37°C and 5% CO2 according to ATCC recommendations.

Cells were grown at 37ºC in a humidified atmosphere containing 5% CO2 in a Forma Scientific incubator (USA). Growth medium was replaced with fresh medium at one to three day intervals. All cell cultures were propagated in large tissue culture plates until a confluent monolayer had formed. When confluent, the medium was discarded and the cells rinsed with phosphate-buffered saline (PBS). Cells were detached by adding 3 ml of 0.25% Trypsin/EDTA (disodium ethylene diaminetetraacetate) for 3-5 minutes at 37ºC and gently shaking the flask. One milliliters growth medium (see below) was then added to neutralise the trypsin. Cells were stored frozen in FBS supplemented with 10% DMSO at a density of 1 to 3 million per ml. They were counted using a Neubauer haemocytometer (Weber Scientific International Ltd., Middlesex, UK) diluted 1:1 in trypan blue (Sigma Aldrich, Dorset, UK) for dead cell identification before freezing or prior to any assay.

Trypan blue stain

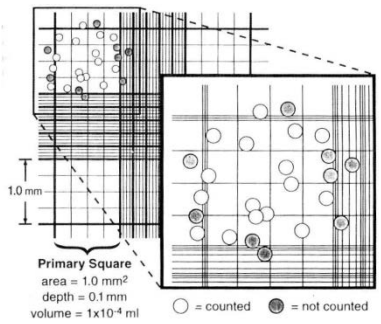
A growth curve was established counting the quantity of cells by cm2 at different times. At each time, cultured cells were trypsinised using 1ml of 0.25% Trypsin/EDTA for each well. After cell detachment the trypsin was neutralised with 1 ml of culture medium. The number of viable cells was determined by 0.4% Trypan blue staining added to an equal volume of cell suspension. Viable cells were counted using a Neubauer haemocytometer and a phase contrast microscope. Approximately 10 µl of cell suspension/trypan blue stain was pipetted at one edge of the cover slip, so that it ran under the cover-slip. The haemocytometer grid was then visualised with a phase contrast microscope where the viable cells appeared bright

and colourless, whereas the dead cells appeared blue. Both viable and dead cells were counted in the 4 large, corner squares of the grid and an average taken.

Using the following equation the total number of viable cells in 1 ml was calculated:

TC= $\bar{x}$ *2*10^4

Where TC was the number of total viable cells in 1 ml; $\bar{x}$ was the average of the cell counts from the squares of the haemocytometer grid, 2 was the dilution factor (1:1 dilution of cell suspension and trypan blue stain) and represented the conversion factor. The number of live cells was used to determine the growth rate and experiments were repeated three times.



U73122 treatment

MG-63, 143B, SaOS-2 and Hs888 cell lines were treated with 30 µmol U-73122 (Sigma–Aldrich) in dimethylsulfoxide (DMSO) for respectively 3h, 6h and 24 h. Cells were detached by adding 1 ml of 0.25% Trypsin/EDTA, and counted using a Neubauer haemocytometer. RNA and proteins were extracted for molecular biology analysis. Experiments were independently repeated at least 3 times for each line.

RNA Isolation

After a confluent monolayer was obtained, the MG-63, 143B, SaOS-2 and Hs888 cell lines were harvested in trypsin-EDTA, washed in PBS twice and stored as a pellet at -20°C until processing.

Total RNA was extracted with a SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

The cells were transferred to a microcentrifuge tube containing 175 µL of SV RNA Lysis Buffer and were passed through a 20-gauge needle to shear the genomic DNA for 4 to 5 times. 350 µL of SV Dilution Buffer was then added, mixed by inverting 4 times, and placed in a heating block at 70°C for 3 minutes. The sample was centrifuged for 10 minutes at 14,000 x g. The lysate solution was transferred to a new microcentrifuge tube, 200 µL of 95% ethanol were added, and the mixture was transferred to a spin column assembly, and centrifuged at

14,000 x g for one minute. The liquid was discarded from the collection tube, 600 µL of SV RNA Wash Solution was added to the column, centrifuged at 14,000 x g for one minute, and the collection tube was emptied. A DNase incubation mixture was prepared per sample by combining 40 µL Yellow Core Buffer (Promega), 5 µL 0.09M MnCl2 and 5 µL of DNase I enzyme. The DNase incubation mixture was added directly to the membrane of the spin basket. The mixture was incubated for 15 minutes at room temperature, 200 µL of SV DNase Stop Solution was added to the spin basket, and centrifuged at 14,000 x g for one minute. Next, 600 µL of SV RNA Wash Solution was added and centrifuged at 14,000 x g for one minute. The collection tube was emptied, 250 µL of SV RNA Wash Solution was added, and centrifuged at 14,000 x g for two minutes. The spin basket was transferred from the collection tube to an elution tube, 100 µL of Nuclease-Free Water was added to the membrane and centrifuged at 14,000 x g for one minute. Finally, RNA was eluted into a sterile collection tube with RNase-free water. The procedure was carried out for each sample.

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

RT-PCR

RNA was reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA). Briefly, 2µg RNA, from cells cultured, were incubated with the master mix for reverse transcription. The mix consisted of 2µl of 10 x Reverse Transcription Buffer, 0.8µl of 25 x dNTPs (100mM), 2µl of 10 x random primers, 1µl of MultiScribeTM Reverse Transcriptase (50 U/µl) and 3.2µl of DNase-free water. 10µl of diluted sample RNA was then added to make a final volume of 20µl. for 10 min at 25°C, 120 min at 37°C and 5 min at 85°C in a thermocycler Gene Amp® PCR System 9700 (Applied Biosystems).

Polymerase Chain Reaction (PCR)

PCR was used to study the panel of expression of PI-PLC enzymes in MG-63, 143B, SaOS-2 and Hs888 human osteosarcoma cell lines.

The primer pairs (Bio Basic Inc, Amherst, New York, USA) are listed in TableX. To amplify glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Bio Basic Inc, Amherst, New York, USA) the following primer pair was used: forward 5′ -CGAGATCCCTCCAAAATCAA-3′ reverse 5′ -GTCTTCTGGGTGGCAGTGAT-3′ . The specificity of the primers was verified by searching in the NCBI database for possible homology to cDNAs of unrelated proteins.

Standard analytical PCR reaction were performed with GoTaq Master Mix (Promega) and each PCR tube contained the following reagents: 5X GoTaq buffer, 0.2 µM forward primer , 0.2 µM reverse primer, 0.2 mM dNTPs, 0.5 mM MgCl2, 1.25 U GoTaq and 3,5µl of (about 35µg) template cDNA. The final volume was 50 ml.

Cycling conditions were as follows. The amplification was started with an initial denaturation step at 95°C for 1 min and was followed by 40 cycles consisting of denaturation (30 s) at 95°C, annealing (30 s) at the appropriate temperature for each primer pair and extension (1 min) at 72°C in a thermocycler Gene Amp® PCR System 9700 (Applied Biosystems).

GENE		Primer sequence	conditions
PI-PLC β1	Foward	5′ -AGCTCTCAGAACAAGCCTCCAACA-3′	
OMIM*607120	Reverse	5′ -ATCATCGTCGTCGTCACTTTCCGT-3′	64°C
PI-PLC β2	Foward	5′ -AAGGTGAAGGCCTATCTGAGCCAA-3′	
OMIM *604114	Reverse	5′ -CTTGGCAAACTTCCCAAAGCGAGT-3′	64°C
PI-PLC β3	Foward	5′ -TATCTTCTTGGACCTGCTGACCGT-3′	
OMIM *600230	Reverse	5′ -TGTGCCCTCATCTGTAGTTGGCTT-3′	60°C
PI-PLC β4	Foward	5′ -GCACAGCACACAAAGGAATGGTCA-3′	
OMIM *600810	Reverse	5′ -CGCATTTCCTTGCTTTCCCTGTCA-3′	60°C
PI-PLC γ1	Foward	5′ -TCTACCTGGAGGACCCTGTGAA-3′	
OMIM *172420	Reverse	5′ -CCAGAAAGAGAG CGTGTAGTCG-3′	60°C
PI-PLC γ2	Foward	5′ -AGTACATGCAGATGAATCACGC-3′	
OMIM *600220	Reverse	5′ -ACCTGAATCCTGATTTGACTGC-3′	60°C
PI-PLC δ1	Foward	5′ -CTGAGCGTGTGGTTCCAGC-3′	
OMIM *602142	Reverse	5′ -CAGGCCCTCGGACTGGT-3′	60°C
PI-PLC δ3	Foward	5′ -CCAGAACCACTCTCAGCATCCA-3′	
OMIM *608795	Reverse	5′ -GCCA TTGTTGAGCACGTAGTCAG-3′	60°C
PI-PLC δ4	Foward	5′ -AGACACGTCCCAGTCTGGAACC- 3′	
OMIM *605939	Reverse	5′ -CTGCTTCCTCTTCCTCATATTC- 3′	64°C
PI-PLC E	Foward	5′ -GGGGCCACGGTCATCCAC-3′	
OMIM *608414	Reverse	5′ -GGGCCTTCATACCGTCCATCCTC-3′	64°C
PI-PLC H1	Foward	5′ -CTTTGGTTCGGTTCCTTGTGTGG-3′	
OMIM *612835	Reverse	5′ -GGATGCTTCTGTCAGTCCTTCC-3′	64°C
PI-PLC H2	Foward	5′ -GAAACTGGCCTCCAAACACTGCCCGCCG-3′	
OMIM *612836	Reverse	5′ -GTCTTGTTGGAGATGCACGTGCCCCTTGC-3′	64°C
EZRIN	Foward	5'-GCTGTGCAGG CCAAGTTT-3'	
OMIM*123900	Reverse	5'-TCCCACTGGT CCCTGGTA-3'	60°C
GAPDH	Foward	5'-CCCACTCCTC CACCTTTGAC-3'	
	Reverse	5'-CATACCAGGA AATGAGCTTG ACAA-3'	60°C

Following thermocycling, PCR products were analysed by 1.5% TAE ethidium bromide-stained agarose gel and electrophoresed for 1 h at 100 V. Results were visualized and photographed using UV light transillumination.

Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for each primer pair. RNA samples were also amplified by PCR without RT. No band was observed, excluding DNA contamination during the procedure. Experiments were independently repeated at least 3 times for each line.

Agilent 2100 bioanalyzer

The reaction products were further quantified with the Agilent 2100 bioanalyzer using the DNA 1000 LabChip kit (Agilent Technologies, Deutschland GmbH), according to the manufacturer's instructions. Briefly, the gel-dye mix was prepared by equilibrating the DNA dye concentrate and DNA gel matrix to room temperate for 30 min. After equilibration, 25 µl of the DNA dye concentrate was added to the vial DNA gel matrix. The gel-dye mix was vortexed and briefly centrifuged. After the gel-dye mix was transferred to the spin filter, it was centrifuged for 15 min at 2240g in a Fisherbrand Vortex Genie-2 (Fisher Scientific, France). Between uses, the gel-dye mix was stored at 4°C. For each analysis, the gel-dye mix was equilibrated to room temperature for at least 30 min. The DNA 1000 chip was primed by adding 9 µl DNA gel matrix to the assigned well and applying pressure for 1 min, with the plunger of the chip priming station at 1 ml. Another 9 µl gel-dye mix was added to each of the next two assigned wells. Then 5 µl marker was added to each of the 12 sample wells and the ladder well. Of each sample, 1 µl was added to a separate well, and 1 µl DNA ladder was added to the ladder well. The marker mixture contained lower and upper molecular size markers of 15 and 1500 bp, respectively. The DNA 1000 chip was subsequently vortexed for 1 min at 2400 rpm. Within 5 min the DNA 1000 chip was run on the Agilent 2100 Bioanalyzer. All of the data was analyzed using Agilent 2100 Expert Software (Agilent Technologies, Waldbronn, Germany).

Targeting of Ezrin and PI-PLC ε with RNA Interference

In order to analyse the interaction between ezrin and PI-PLC ε, we silenced ezrin and PI-PLC ε expression by small interfering RNA. Silencing was validated by real time PCR (RT-PCR) analysis of mRNA extracts from 143B and Hs888 cells transfected with ezrin siRNA and PI-PLC ε siRNA and compared to a non-targeting control siRNA.

Transient transfections

Transient transfections of Ezrin and PI-PLC ε were carried out using METAFECTENE SI+ (Biontex Laboratories GmbH, Munich, Germany) on 143B and Hs888 cell line. siRNA sequences targeting Ezrin and PI-PLC ε, as well as a negative control siRNA, were designed and synthesized by Invitrogen (Life Technologies, Foster City, CA, USA). The siRNAs were designed according to the Ezrin and PI-PLC ε complementary DNA (cDNA) sequence respectively (EZR Gene ID: 7430; PI-PLCE Gene ID: 51196).

Briefly, 2.2 ml cell suspension were prepared in complete cell culture medium with a concentration of 1,5·10^5 cells/ml of 143B cells and 3·10^5 cells/ml of Hs888. Cells were seeded, in 6-well plates, shortly before the addition of the lipoplex, according to the manufacturer's instructions. The 143B and Hs888 cells were incubated under normal culture conditions (37°C in CO2–containing atmosphere) until the lipoplex is added. Before transfection, 150 µl of 1× SI+ buffer were mixed with 72 µl of METAFECTENE® SI+ and 540 pMol of RNA stock solution. The mixture was incubated for 15 min at room temperature and subsequently added to the cells during the first hour after seeding. The cells were incubated an additional 24 to 72 hours after which functional siRNA was measured by reverse transcription–polymerase chain reaction (RT-PCR) and western blot analysis. A growth curve was established counting cells using a Neubauer haemocytometer at each times.

Real-Time PCR

Gene expression of Ezrin, PI-PLC ε, PI-PLC β1, PI-PLC γ2, PI-PLC δ4, Rac-1 and RhoA were determined by real-time PCR using the 7500 Real-Time PCR instrument from Applied Biosystems™. TaqMan® primers and probes for each gene, as well as the GAPDH reference gene, were obtained from Applied Biosystems™.

Transfected 143B and Hs888 cells and normal controls were harvested 24, 48 and 72 hours after transfection. The messenger RNA (mRNA) expression of Ezrin, PI-PLC ε, PI-PLC β1, PI-PLC γ2, PI-PLC δ4, Rac-1 and RhoA was determined by real time-PCR. Total RNA was extracted with a SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. We confirmed purity and quantity of RNA by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc. USA). The RNA was reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA).

PCR products were detected using gene-specific primers and probes labeled with reporter day FAM which yielded a predicted amplicons of 82, 84, 61, 78, 64, 93 and 62 base pairs respectively; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard, which yielded a predicted amplicon of 58 base pairs. Reaction mixtures for all gene expression assays contained: 5 µl TaqMan® mastermix (2X; Applied Biosystems™), 0,5 µl gene of interest primer/probe mix and 1 µl PCR grade water. To each reaction, 3,5 µl of the diluted cDNA (35 ng) were added. All samples were assayed in triplicate. PCR reaction was carried out in triplicate on 96-well plate with 10 uL per well using 1X TaqMan Master Mix. After an incubation for 2 minutes at 50°C and 10 minutes at 95°C, the reaction continue for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. At the end of the reaction, the results were evaluated using the ABI PRISM 7500 software. For each sample, ΔCt value was calculated as Ct of the target gene minus Ct of the endogenous gene. Subsequently, for each sample, ΔΔCt value was calculated as ΔCt of the sample minus ΔCt of the control sample. Relative quantification was obtained as the mathematical function $2^{-\Delta\Delta Ct}$. Based on these calculations, the control sample has a value of 1, taken as 100%.

Immunofluorescence

In order to analyse the interaction between ezrin and PI-PLC proteins in osteosarcoma tumorigenesis, we localized the protein in the cells by immunofluorescence.

Immunofluorescence detection of Ezrin, PI-PLC ε, PI-PLC β1, PI-PLC γ2, PI-PLC δ4 expression was performed on coverslips cultured cells, both transfected and normal controls. Cells were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 10 min at 4°C, followed by three washes with PBS. Cells

were incubated with primary antibodies diluted in PBS for 1 hr at room temperature. After washing with 1X PBS, the cover-slips were incubated with the specific secondary antibody Texas Red or fluorescein-conjugated for 1h at room temperature. Cells were washed twice for 5 min each with 1X PBS. Finally, the cells were counterstained with DAPI to visualize nuclei before mounting. The slides were visualized using an inverted microscope.

Western Blot

To verify the effectiveness of silencing, was carried the Western Blotting analysis. After 24 and 48 hours of transfection, the cells were washed twice with cold PBS before lysis in a cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 2mM sodium fluoride, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. 50ug of protein was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Invitrogen, Life Technologies, Foster City, CA, USA). The membranes were blocked in PBS with 5% skim milk for 1 hour and incubated overnight with the primary antibodies. Finally, membranes were visualized by the addition of anti-mouse immunoglobulin G (Jackson Immunoresearch, UK) and anti-rabbit immunoglobulin G (Jackson Immunoresearch, UK) enhanced chemiluminescence. Expression of β-actin was used as an internal control to normalize for loading and to allow comparisons of target protein expression to be made among all groups of cells. The densities of the bands on the membrane were scanned and analyzed with ImageJ software. The ratios of Ezrin and PI-PLC ε, protein expression were calculated as follows: ratio = [1 − (treated group Target Protein density/treated group β-actin density)/(control group Target Protein density/control group β-actin density)] × 100%.

Statistical analysis

For in vitro studies, differences were determined either with two-way repeated measures analysis of variance (ANOVA) with Bonferroni's multiple comparison test, or by student's t test, using Prism 5.0a software (GraphPad Software, San Diego, CA, USA). A p value <0.05 was considered significant.

Results

Expression of PI-Phospholipase C in osteosarcoma cell

In MG-63 cell line, PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1 and PI-PLC δ3 resulted expressed (Table 1; Fig, 1A). Specific bands were obtained at the gel electrophoresis analysis. Analysis with the bioanalyzer confirmed the data obtained with the gel electrophoresis. The following concentrations were obtained: PI-PLC β1= 10.18 ng/ml, PI-PLC β3 = 17.49 ng/ml, PI-PLC β4 = 17.79 ng/ml, PI-PLC γ1, = 8.02 ng/ml, PI-PLC γ2 = 21.4 ng/ml, PI-PLC δ1 = 15.42 ng/ml, PI-PLC δ3 = 15.13 ng/ml. Weak signals for PI-PLC β2 and PI-PLC ε were detected with gel electrophoresis analysis. Analysis with the bioanalyzer detected a small peak for PI-PLC β2, although the quantification was not possible. A small peak was also detected for PI-PLC ε isoform and the measured concentration was 0.77 ng/ml (Table 1).

In 143B cell line, PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1, PI-PLC δ3 and PI-PLC ε, were expressed (Table 2; Fig. 1A). Specific bands were obtained at the gel electrophoresis analysis. Analysis with the bioanalyzer confirmed the expression and the following concentrations were obtained: PI-PLC β1= 4.54 ng/ml, PI-PLC β3= 25.06 ng/ml, PI-PLC β4= 46.4 ng/ml, PI-PLC γ1= 10.38 ng/ml, PI-PLC γ2= 43.23 ng/ml, PI-PLC δ1= 9.0 ng/ml, PI-PLC δ3= 15.56 ng/ml, PI-PLC ε = 15.55 ng/ml (Table 1). A weak signal for PI-PLC β2 was detected with gel electrophoresis analysis. Using the bioanalyzer, a small peak was detected, although the quantification was not possible.

SaOS-2 is an established epithelial-like human osteosarcoma cell line (Fogh et al, 1975) used as experimental model for human osteoblasts (McQuillan et al, 1995; Rodan et al, 1987).

In SaOS-2 cell line, PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1, PI-PLC δ3, PI-PLC ε and PI-PLC η1 were expressed (Table 1; Fig. 1B). Specific bands were obtained at the gel electrophoresis analysis. Analysis with the bioanalyzer confirmed the expression and the following concentrations were obtained: PI-PLC β1= 5.84 ng/ml, PI-PLC β3= 33.83 ng/ml, PI-PLC β4= 30.07 ng/ml, PI-PLC γ1= 11.87 ng/ml, PI-PLC γ2= 47.59 ng/ml, PI-PLC δ1= 12.67 ng/ml, PI-PLC δ3= 32.64 ng/ml, PI-PLC ε = 37.99 ng/ml, PI-PLC η1 = 8.18 ng/ml (Table 1).

Hs888 cells were derived from lung metastasis of osteosarcoma.

In Hs888 cell line, PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC δ1, PI-PLC δ3, PI-PLC δ4, PI-PLC ε and PI-PLC η1 were expressed (Table 1; Fig. 1B). Specific bands were obtained at the gel electrophoresis analysis. Analysis with the bioanalyzer confirmed the expression and the following concentrations were obtained: PI-PLC β1= 2.5 ng/ml, PI-PLC β3= 27.52 ng/ml, PI-PLC β4= 38.47 ng/ml, PI-PLC γ1= 11.91 ng/ml, PI-PLC δ1= 54.51 ng/ml, PI-PLC δ3= 13.63 ng/ml, PI-PLC δ4= 11.59 ng/ml, PI-PLC ε = 4.43 ng/ml, PI-PLC η1 = 8.29 ng/ml (Table 1).

Statistical analysis

The ANOVA test results were significant ($p < 0.01$) comparing the concentrations of each isoform in the 4 cell lines. The t-test used to compare the panels of expression of the cell lines did not identify statistical significant results.

Comparison of cell lines	One tailed *t*-test
MG-63/143B	$p=0.11784$
MG-63/SAOS	$p=0.09538$
MG-63/Hs888	$p=0.16348$
143b/SAOS	$p=0.39689$
143B/Hs888	$p=0.48501$
SAOS/Hs888	$p=0.41439$

Study of ezrin and PI-PLC ε expression in molecular pathway of osteosarcoma cells

In 143 cell line, ezrin was localized in cytoplasm and showed moderate signal intensity; PI-PLC ε showed the same localization, but with weak signal intensity; there was only a focal cytoplasmatic colocalization of Ezrin and PI-PLC ε (Fig. 2A).

Quantitative Real-Time PCR assay revealed that Ezrin mRNA levels were significantly higher compared to PI-PLC ε mRNA levels in the 143B ($p<0,0005$) and Hs888 ($p<0,05$) cell lines (Fig. 2B). PI-PLC ε expression in 143B cells was significantly higher compared to PI-PLC ε expression in Hs888 ($p<0,05$), while the levels of expression of the gene ezrin were comparable in the 2 cell lines (Fig. 2B).

Effective Targeting of Ezrin and PI-PLC ε with RNA Interference

In order to analyse the interaction between ezrin and PI-PLC ε, we silenced ezrin and PI-PLC ε expression by small interfering RNA. Silencing was validated by real time RT-PCR analysis of mRNA extracts from 143B and Hs888 cells transfected with ezrin siRNA and PI-PLC ε siRNA and compared to a non-targeting control siRNA.

Growth curve

The growth rate of the ezrin siRNA-treated cells decreased in a time-dependent manner, both in 143B (Fig. 3A) and in Hs888 (Fig. 3B) cell lines. In 143B cells line, the growth of silenced cells is significantly slowed with respect to untreated cells ($p<0,5$).

The growth rate of the PI-PLC ε siRNA-treated cells decreased in a time-dependent manner both in 143B (Fig. 4A) both in Hs888 (Fig. 4B) cells line. The effect of PI-PLC ε silencing in 143B was higher with respect to Hs888 cells line.

Effects of siRNA on cell morphology

Ezrin silencing has higher effect on 143B cell respect Hs888 (Fig. 5B, 5D). In 143B, silencing resulted in an irregular outline of the plasma membrane and was associated with a reduced intercellular adhesion. Microvacuolization of the cytoplasm was also observed. In Hs888 we observed a quantitative reduction of cellular elements, which showed a substantially well-conserved structure.

PI-PLC ε silencing has higher effect upon Hs888 cell respect 143B (Fig. 6B, 6D).

PI-PLC ε silencing in Hs888 induces the cytoplasmic maro/micro-vacuolisation with slight reduction of the number of cells and without alteration of morphology (Fig. 6 D). In the 143B induces an important change of conformation of the cells, cell rounding, interpreted as cytotoxic effect (Fig. 6B).

siRNA Transfection efficiency evaluation

24 and 48 hours after transfection, RT-PCR and quantitative Real-Time PCR assay were performed to analyse the expression of ezrin and PI-PLC ε. The transcription of ezrin and PI-PLC ε mRNA was suppressed in the 143B and Hs888 cell lines which were transfected with siRNA compared to the control group (not transfected) (t=0) (Fig. 7).

The mRNA expression level of ezrin was significantly reduced with respect to untreated cells ($p<0,001$), both in 143B and Hs888 cell lines (Fig. 7A).

In 143B cell line, the mRNA expression level of PI-PLC ε was significantly slowed with respect to untreated cells ($p<0,001$); in Hs888 cell line, the expression is significantly slowed only at t=24h ($p<0,05$) (Fig. 7B).

The expression of GAPDH mRNA showed no change as an internal control.

24 and 48 hours after transfection, western blot assay was performed to analyse the expression of ezrin and PI-PLC ε protein. There was no change in the expression of b-actin as internal control. While the expression of ezrin protein was decreased in the cell lines which were transfected with ezrin siRNA comparing to the group without transfection (control group) (Fig. 7C).

Real-Time PCR after ezrin silencing

In 143B cell line the ezrin silencing had different results (Fig. 8 - green):

- there was no differences between the mRNA expression levels of PI-PLC ε in treated and untreated cells;
- the expression of PI-PLC γ2 increased about 40%;
- the expression of PI-PLC β1 decreased about 55%;
- the expression of RhoA increased about 10% after 24 and increased about 3 times after 48 hours;
- the expression of Rac1 increased 50% after 24 hours.

In Hs888 cell line the ezrin silencing also had different results (Fig. 8 - blue):

- the expression of PI-PLC ε was similar to the untreated cells after 24 hour, and increased about twice after 48 hours;
- the expression of PI-PLC γ2 was not significantly modified;
- the expression of PI-PLC β1 significantly increased in the 24-48 hours interval;
- the expression of PI-PLC δ4 was double after 24 hours;
- the expression of RhoA significantly decreased about 75% after 24 and 50% after 48 hours;
- the expression of Rac1 significantly decreased about 95% after 24 and 50% after 48 hours.

There was a statistically significant difference in the mRNA expression levels of PI-PLC ε ($p<0,005$) in 143B and Hs888 after 48 hours; there was a statistically significant difference of mRNA expression levels of PI-PLC β1 both after 24 ($p<0,0005$) and 48 ($p<0,0125$) hours in 143B and Hs888; after 24 hours, there was a statistically significant difference in the mRNA expression levels of RhoA ($p<0,0005$) and RAC1 ($p<0,005$).

Real-Time PCR after PI-PLC ε silencing

In 143B cell line the PI-PLC ε silencing had different results (Fig. 9 - green):

- the expression of ezrin decreased about 75 in the interval 24-48 hours;
- the expression of PI-PLC γ2 increased about 50%;
- the expression of PI-PLC β1 decreased about 80% after 24 hours;
- the expression of PI-PLC δ4 appeared after 24 hours, but was extremely little;
- the expression of RhoA decreased about 50%;

- the expression of Rac1 decreased about 40% after 24 hours.

In Hs888 cell line the PI-PLC ε silencing also had different results (Fig. 9 - blue):

- the expression of ezrin increased about 40% after 24 hours;
- the expression of PI-PLC β1 significantly increased in the interval 24-48 hours;
- the expression of PI-PLC δ4 was not significantly modified;
- the expression of RhoA significantly decreased about 90% in the interval 24-48 hours;
- the expression of Rac1 significantly decreased about 95% in the interval 24-48 hours.

There was a statistically significant difference of mRNA expression levels of ezrin ($p<0,00025$), PI-PLC β1 ($p<0,0025$ - $p<0,0005$), RhoA ($p<0,00125$ - $p<0,0125$) and RAC1 ($p<0,05$ - $p<0,025$) in 143B and Hs888 in the interval 24-48 hour.

Immunofluorescence

In order to analyse the interaction between ezrin and PI-PLC proteins in osteosarcoma tumorigenesis, we localized the protein in the cells by immunofluorescence after ezrin and PI-PLC ε silencing.

In 143B cell line:

- Ezrin showed a moderate cytoplasmatic staining, with signal enhancement on the membrane, while PI-PLC ε showed strong cytoplasmatic and perinuclear staining. Ezrin and PI-PLC ε co-localized in the cytoplasm (Fig. 10);
- When Ezrin was silenced, a slight reduction of PI-PLC ε staining was observed. However, the cytoplasmatic localization persisted (Fig. 10);
- When PI-PLC ε was silenced, intensity of ezrin staining slightly decreased, and move from the plasma membrane to perinuclear localization. (Fig. 10).

In 143B cell line PI-PLC γ2 showed a weak cytoplasmatic staining. After ezrin silencing, a stronger cytoplasmic signal and a new membrane staining were observed. After PI-PLC ε silencing there was slight intensity increase in the cytoplasm (Fig. 11A)

In 143B cell line PI-PLC β1 showed a strong cytoplasmatic staining. Both ezrin and PI-PLC ε silencing induced a significant reduction of the signal intensity of PI-PLC β1 in cytoplasm, more marked after ezrin absence (Fig. 11B).

In 143B cell line RhoA showed a perinuclear and cytoplasmatic staining with strong signal intensity. After ezrin silencing the signal intensity did not change, and caused a displacement

of the staining in the nucleus, while PI-PLC ε silencing caused a moderate reduction of signal intensity, persistently localized in cytoplasm (Fig. 11C).

In 143B cell line RAC1 showed weak signal intensity localized in the perinuclear zone. After ezrin was silenced, there was a strong increase of signal intensity of RAC1 was observed, persistently localized in the same zone. Silencing of PI-PLC ε induced marked reduction of the perinuclear signal intensity (Fig. 11D).

In Hs888 cell line:

- Ezrin was localized in cytoplasm, showing mild signal intensity. PI-PLC ε was also localized in cytoplasm, although showing weak signal intensity. A focal cytoplasmatic colocalization of Ezrin and PI-PLC ε was observed (Fig. 12).
- After Ezrin silencing, there was a slight increase of PI-PLC ε signal intensity, localized in cytoplasm (Fig. 12);
- The PI-PLC ε silencing caused a moderate increase of Ezrin signal intensity that maintained cytoplasmatic localization (Fig. 12).

In Hs888 cell line PI-PLC γ2 was not detected. Ezrin silencing induced the expression of PI-PLC γ2 showing a moderate signal intensity in the cytoplasm, with strong perinuclear enhancement. PI-PLC ε silencing also induced moderate cytoplasmatic signal intensity (Fig. 13A). In Hs888 cell line PI-PLC β1 showed a moderate cytoplasmatic staining. Both Ezrin and PI-PLC ε silencing induced a significant increase of the signal intensity of PI-PLC β1 in cytoplasm (Fig. 13B).

In Hs888 cell line RhoA showed a perinuclear and cytoplasmatic staining with strong signal intensity; after Ezrin silencing the signal intensity was weaker, while PI-PLC ε silencing induced marked reduction of the signal, always localized in cytoplasm (Fig. 13C).

In Hs888 cell line RAC1 showed strong signal intensity localized in the cytoplasm and perinuclear zone; both in Ezrin and PI-PLC ε silencing, the signal of RAC1 moderately decrease, although in the same cytoplasmic localization (Fig. 13D).

Effect of U73122 and U73343 treatment

Growth curve

The growth rate of the U73122-treated cells decreased in a time-dependent manner both in 143B (Fig. 15A) both in Hs888 (Fig. 15B) cell lines. In 143B cells line, the administration of U-73122 reduced the viable cells within the 1-3 hours interval; but after 6 hours, the effect of the treatment was finished (Fig. 15A). In HS888 cells, the administration of U-73122 reduced the viable cells of more than 80% within the 1-3 hours interval (Fig. 15B).

In 143B and hs888 cells lines, the administration of U-73343 reduced slowly the viable cells within the 1-6 hours interval. After 6 hours, the effect of the treatment seemed to be lethal for all the cells (Fig. 15 A, 15B).

Effects of treatment on cell morphology

U73122 treatment had a greater effect on cell morphology (Fig. 16), while U73343 treatment had the greatest effect on cell death (Fig. 16). At the beginnig of U73122 treatment, the cells reduced intercellular adhesion. After 6 hours microvacuolization of the cytoplasm appeared both in 143B and Hs888.

After 3 hours of U73343 treatment, the cells started to detach (Fig. 16).

Real-Time PCR after U73122 treatment

In 143B cells the U73122 treatment had different results (Fig. 17 green):

- there was no differences between the mRNA expression levels of ezrin between cells treated and untreated;
- the expression of PI-PLC ε decreased about 55% after 1 hour;
- the expression of PI-PLC γ2 decreased about 10% after 1 hour;
- the expression of PI-PLC β1 decreased about 85% after 1 hour;
- the expression of PI-PLC δ4 appeared after 1 hour, while remaining very low;
- the expression of RhoA increased about 50%;
- there was no differences between the mRNA expression levels of RAC1 between cells treated and untreated.

In Hs888 cells the U73122 treatment had different results (Fig. 17- blue):

- there was no differences between the mRNA expression levels of ezrin between cells treated and untreated, except at 3 hours of treatment where the expression decreased about 40%;
- the expression of PI-PLC ε decreased about 50%;
- the expression of PI-PLC γ2 decreased about 25%, but at 6 hour increased about 35%;
- the expression of PI-PLC β1 decreased about 70%;
- the expression of PI-PLC δ4 decreased about 20% after 1 hours and then 50%;
- the expression of RhoA decreased about 20% only at 3 hour of treatment;
- the expression of Rac1 decreased about 60%.

There was a difference statistically significant between the mRNA expression levels of PI-PLC ε ($p<0,05$), of PI-PLC γ2 ($p<0,05$), of PI-PLC β1 ($p<0,05$), of PI-PLC δ4 ($p<0,0125$), of RhoA ($p<0,005$) and RAC1 ($p<0,005$) between 143B and Hs888 at 3 hours of treatment.

Real-Time PCR after U73343 treatment

In 143B cells the U73343 treatment had different results (Fig. 18 green):

- the expression of ezrin was double after 1 hour of treatment and then normalize;
- the expression of PI-PLC ε was double to 1 hour after;
- the expression of PI-PLC γ2 increased about 20% after 1 hour and then normalize;
- the expression of PI-PLC β1 decreased about 60%;
- the expression of PI-PLC δ4 appeared normalize;
- the expression of RhoA increased about 150%;
- the expression of Rac1 increased about 10% after 1 hour and then decreased linearly to 40%.

In Hs888 cells the U73343 treatment had different results (Fig. 18 blue):

- the expression of ezrin decreased about 60% after 1 hour and then decreased linearly to 75%;
- the expression of PI-PLC ε decreased about 50%;
- the expression of PI-PLC γ2 decreased about 40%;
- the expression of PI-PLC β1 decreased about 80%;

- the expression of PI-PLC δ4 increased about 10%;
- the expression of RhoA decreased linearly to 60%;
- the expression of Rac1 decreased about 70%.

There was a difference statistically significant between the mRNA expression levels of ezrin ($p<0,00025$), of PI-PLC ε ($p<0,125$), of PI-PLC γ2 ($p<0,025$), of PI-PLC β1 ($p<0,00025$), of RhoA ($p<0,00125$) and RAC1 ($p<0,0005$) between 143B and Hs888 at 3 hours of treatment.

Discussion

Expression of PI-Phospholipase C in osteosarcoma cell

Little is known about osteosarcoma carcinogenesis, especially in regard to the complex molecular pathway network that regulates the survival and metabolism of the cells.

Ezrin, which is a member of the ERM family of proteins, is involved in the signal transduction networks, both as regulator and effector, by interacting with different signalling pathways. Currently, increasing evidence suggests that ezrin plays a pivotal role in tumour invasion and metastasis (Neisch et al, 2011). However, the regulation of ezrin is largely unknown.

The phosphatidylinositol 4,5-bisphosphate (PIP2), a relatively rare phospholipid, has a key role in the regulation of cytoskeleton remodelling. The bond between PIP2 to ezrin protein induces its activation and ultimately association to the plasma membrane. PIP2 belongs to the phosphoinositide (PI) signalling system, an intricate network of enzymes and phospholipids which control a number of cell and tissue activities and interacts with RHO-GTPases.

Several studies have documented the existence of a well-established link between phosphoinositides signalling and cancer. Particular attention has been focused on the enzymes that regulate the levels of PIP2 and PIP3. Among these, PI3K and PI-PLC have been implicated in the generation and progression of tumours and hence considered new potential targets for therapeutic intervention.

Expression of Phospholipase C in osteosarcoma cell

Emerging evidence regarding the close connection between Ca^{2+} signalling and cytoskeletal activation in multiple cell systems lead us to investigate the role of PI-PLC enzymes in osteosarcoma cell lines.

The expression of PI-PLC enzymes, PI-PLC β1, PI-PLC β2, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1, PI-PLC δ2, PI-PLC δ3, PI-PLC ε, PI-PLC η1 and PI-PLC η2, was analysed in four cultured human osteosarcoma cell lines: MG-63, 143B, SaOS-2 and Hs888.

PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1 and PI-PLC δ3 resulted expressed in MG-63 cell line as shown by Agilent bioanalyzer (Table 1; Fig. 1A). In agreement with previous studies (Hoberg et al, 2005), high expression of PI-PLC β1, PI-PLC β3, PI-PLC γ1, PI-PLC γ2 and PI-PLC δ1 was observed.

On the contrary, PI-PLC β2 resulted weakly expressed in MG-63 partially contrasting the results of Hoberg et al., that demonstrated a specific role of PI-PLC β2 in the

mechanotransduction and cell attachment (Hoberg et al, 2005). However, the basal concentration of the enzyme in unstimulated cells was not reported (Hoberg et al, 2005) making difficult the comparison with our results. In osteoblasts, PI-PLC β2 transduces signals from prostaglandin E2 and other prostanoids (Kondo and Togari, 2004). The expression of PI-PLC β2 is generally correlated to specific stimuli and probably the observed low basal expression of PI-PLC β2 in osteoblast-like cells is justified by the use of unstimulated MG-63 cells. Different and specific stimuli, both mechanic and biochemical, might have increased the signal and it will be taken into consideration in future experiments.

Surprisingly, weak expression of PI-PLC ε was also observed. PI-PLC ε is known to be a downstream effector of Ras superfamily GTPases and an upstream activator of small GTPases, both Ras and Rap (Seifert et al, 2008). Therefore, further and deeper studies are needed to explain our findings in order to elucidate the role of this enzyme in MG-63 cells.

SaOS-2 is an established epithelial-like human osteosarcoma cell line (Fogh et al, 1975), which is conventionally used as model for human osteoblasts (McQuillan et al, 1995; Rodan et al, 1987). In SaOS-2 cell line, PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1, PI-PLC δ3, PI-PLC ε and PI-PLC η1 were expressed (Table 1; Fig. 1B). Our results confirm previous data on the expression of PI-PLC β1, PI-PLC β3, PI-PLC γ1, PI-PLC γ2 and PI-PLC δ1 (Hoberg et al, 2005) and provide for the first time evidence of the role of PI-PLC β4, PI-PLC δ3 and PI-PLC η1 in the induction of calcium osteosynthesis even in osteosarcoma.

143B, thymidine kinase negative cells, are known to develop osteolytic tumours (Kaminski et al, 2003). Our results demonstrated that PI-PLC β1, PI-PLC β2, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1, PI-PLC δ3 and PI-PLC ε, are expressed also in 143B, though the signal from PI-PLC β2 was quite weak (Fig. 1A). To the author knowledge, this was not investigated and observed before.

In our study PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC δ1, PI-PLC δ3, PI-PLC δ4, PI-PLC ε and PI-PLC η1 in Hs888 cell line were expressed (Table 1; Fig. 1B). No literature data were available for PI-PLC expression in Hs888 cell line. Surprisingly, PI-PLC γ2 was unexpressed in Hs888, whereas it was detected in other three cell lines. Previous research suggests that PI-PLC γ2 isoform is required for early phase osteoclast differentiation (Kertèsz et al, 2012). PI-PLC δ4 was expressed exclusively in Hs888. Noteworthy, studies of breast cancer MCF-7 cells suggested that abnormal expression of PI-PLC δ4 contributes to carcinogenesis through upregulation of ErbB expression and activation of ERK pathway (Leung

et al, 2004). The peculiar expression of these two isoforms in Hs888 cells might be related to the metastasizing nature of the lineage and suggest that they could be involved in differentiation, carcinogenesis and metastatic process of osteosarcoma.

Remarkably, we detected PI-PLC η1 in SaOS-2 epithelial-like osteosarcoma lineage and Hs888 metastatic cells. PI-PLC η1 is considered a sensor activated by very small calcium increases, and, therefore, involved in the finest regulation of signalling, as is essential for downstream ERK1/2 phosphorylation (Kim et al, 2011).

This pathway is frequently altered in tumours. The PI-PLC η1 expression directly or through the release of intracellular Ca2+ could change the protein phosphorylation process. Although the role of PI-PLC η1 is not fully elucidated, the activation of PI-PLC η1 is known to enhance the GPCR mediated calcium pathway (Kim et al, 2011).

Given the high tissue specificity of PI-PLC enzymes, the panel of expression we delineated in the analysed osteosarcoma cell lines slightly differed. Some enzymes, such as PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC δ1 and PI-PLC δ3, were commonly expressed, although at variable concentrations, in all four cell lines. PI-PLC η2 was also commonly unexpressed in all the analysed cell lines. Beside the results regarding PI-PLC β2 and PI-PLC ε, already commented, the remaining enzymes were differently expressed. Summarizing, the PI-PLC γ2 was expressed in MG-63, 143B and SaOS-2, whereas it was absent in Hs888 cells. The concentration measured in 143B and SaOS-2 cells was about 50% higher than in MG-63 cells. Only Hs888 metastatic cell lines did not express PI-PLC γ2 and express PI-PLC δ4.

Study of ezrin and PI-PLC ε expression in molecular pathway of osteosarcoma cells

Ezrin over expression seems to be crucial for metastatic behaviour in a murine osteosarcoma model (Khanna et al, 2001) and correlated with poor prognosis in murine and canine osteosarcoma (Khanna et al, 2001). Ezrin was also suggested to have a key role in the dissemination of two paediatric tumours, rhabdomyosarcoma (Yu et al, 2004) and osteosarcoma (Khanna et al, 2004). On the contrary, reduction of ezrin expression in rhabdomyosarcoma or osteosarcoma cell lines decreased pulmonary metastases in mice (Yu et al, 2004; Wan et al, 2005). Given that, it was hypothesized that ezrin might contribute to metastasis by suppressing apoptosis, disturbing cell–cell adhesion and activating Rho (Chen et

al, 2011).

In this study ezrin silencing decreased significantly in a time-dependent manner the growth rate of 143B (Fig. 3A) and Hs888 (Fig. 3B) silenced cell lines (p-value < 0,5). The effect of ezrin silencing on the actin cytoskeleton, cell morphology and cell adhesion resulted stronger on 143B than Hs888 cell lines. In 143B cells, ezrin silencing resulted in irregular outline of the plasma membrane and was associated with a reduced intercellular adhesion and microvacuolization of the cytoplasm (Fig. 5B, 5D). In Hs888, we observed a quantitative reduction of cellular elements, though with a substantially well-preserved structure. These results corroborate previous research that suggests that the solely modification of the interaction plasma membrane-actin cytoskeleton is sufficient to interfere with the morphological features (Do Choi et al, 2012). These changes in cytoskeleton might be a key factor in regulating neoplastic progression and tumour growth (Curto, 2004; Turunen et al, 1994; Park et al, 2006; Meng et al, 2010).

Immunofluorescence analysis on 143B cell line showed that ezrin has a moderate cytoplasmatic staining, with signal enhancement on the plasma membrane (Fig. 10), while in Hs888 it is localized in the cytoplasm, showing moderate signal intensity (Fig. 12). Probably, this is related to the different nature of the two cell lines: 143B originates from highly aggressive primary tumor, while Hs888 derives from lung metastasis. Ezrin can be found in the cytoplasm in two forms, active or dormant. After leaving the Golgi complex, it remains dormant in the perinuclear area of the cytoplasm until it is activated (Do Choi et al, 2012). The conformational change of ezrin in its activated form is fundamental to act as a membrane-cytoskeleton linker. After changing into its active form, it moves promptly to the membranous portion to bind to specific molecules that transduce cellular signalling and maintain cellular scaffolding. Do Choi hypothesized that when ezrin is in its dormant form, cancer cells can detach from primary sites more easily (Do Choi, 2012). Therefore, depending on the type of the adult human tumour, loss or over-expression of ezrin correlates with poor prognosis, indicating cell-specific functions of ezrin in tumour progression (Hunter, 2004).

The PI-PLC ε sub-family was identified in 2001. PI-PLC ε isoenzymes are among most complex PI-PLC isoforms, as they present the highest number of different protein domains which regulate their activation (Kelley G.G., et al., 2001). PI-PLC ε plays an important role in carcinogenesis and increased expression of PI-PLC ε seems to be correlated with a more aggressive phenotype and higher capacity to develop cancer, though the mechanism is still unclear. PI-PLC ε1 expression was found to be correlated with human bladder cancer, as there is evidence that the knockdown of PI-PLC ε1 in vitro and in vivo inhibits the growth of bladder tumoral cells (Cheng et al, 2011; Ou et al, 2010). Over expression of PI-PLC ε1 was also

reported in murine skin cancer (Bai et al, 2004). Oka et al. observed that PI-PLC ε gene-knockout (PI-PLC ε (-/-)) mice exhibits marked resistance to tumour formation in two-stage skin chemical carcinogenesis (Oka et al, 2010). Li et al. showed that Apc(Min/+) mice lacking PI-PLC ε (PI-PLC ε (-/-)) exhibit marked resistance to spontaneous intestinal tumorigenesis compared with those with the PI-PLC ε (+/+) background (Li et al, 2009). Bourguignon et al. proposed that PI-PLC ε1 promote the progression in head and neck cancer. Hao et al., in a meta-analysis, showed that the PI-PLC ε1 rs2274223 polymorphism was significantly associated to increased risk of squamous cell carcinoma and gastric cancer (Hao et al, 2013). These data suggest that PI-PLC ε plays an important role in carcinogenesis and that increased expression of PI-PLC ε can be correlated with a more aggressive phenotype and higher capacity to develop cancer. In this study we observed a higher expression of PI-PLC ε in 143B cell lines, known to develop osteolytic tumours (Kaminski et al, 2003), rather than in Hs888 cell line, already metastatic cells, that probably lost their aggressiveness.

It is also known that PI-PLC ε is activated by Ras family members (Wing et al, 2003; Bunney and Katan, 2006). Given the importance of Ras and Rho-family GTPases in the generation and progression of tumours, several studies aimed at establishing a possible role of PI-PLC ε in the development of tumour cells and cancer (Bai et al, 2004; Campbell et al, 2006, Ikuta, 2008; Sorli et al, 2005). By silencing the cells using PI-PLC ε siRNA, the growth rate linearly decreased over time in both 143B (Fig. 3A) and Hs888 (Fig. 3B) cell lines. However, morphological analysis revealed that PI-PLC ε silencing had different effects the two cell lines (Fig. 6B, 6D). In Hs888, the silencing promoted a cytoplasm macro-microvacuolization with minimal quantitative reduction of the number of cell and no conformational alteration (Fig. 6D). In 143B, the morphology change drastically, including cell rounding (Fig. 6B). This is probably a consequence of the delocalization of ezrin from plasma membrane to cytoplasm as shown by parallel immunofluorescence analyses (Fig. 10). Exclusive cytoplasmic expression of ezrin is often associated with better disease free survival chances compared with both cytoplasmic and membranous expression (Ferrari et al, 2008).

In this study, the expression of PI-PLC ε decreases concurrently with the decrease of the expression of RhoA and Rac1. It is widely known that actin reorganization and cytoskeleton dynamics are regulated by the Rho family of small GTPases (Hall, 1998; Parri et al. 2010). Rho regulates stress fiber and focal adhesion assembly, while Rac controls the formation of lamellipodia protrusions and membrane ruffles (Ridley, 2001). PI-PLC ε is a bifunctional enzyme that also regulates small GTPases of the Ras superfamily through the activity of its Ras guanine nucleotide exchange factor (RasGEF). As an effector of heterotrimeric and small G-protein, it might play a role in actin organisation.

In 143B cell line, the PI-PLC ε silencing caused the displacement of ezrin from the plasma membrane to the perinuclear (Fig. 10), probably in an inactivated form. In addition to its PI-PLC catalytic activity, this enzyme has an N-terminal domain with guanine nucleotide exchange (GEF) activity. These data could suggest that being PI-PLC ε linked to GEF protein, if PI-PLC ε decreases, GEF will be available to form complex with GDI and, subsequently, to detach itself from ezrin. Detaching of GDI from ezrin will lead to inactivating ezrin, changing its localization (perinuclear, Fig. 10) but not its expression (mRNA, Fig. 9). It has been suggested that ERM proteins regulate Rho function by binding RhoGDI, a negative regulator of Rho activity (Hunter et al, 2004). Our observations suggest that PI-PLC ε suppression, leading to ezrin inactivation, might result in producing negative regulators involved in the RhoGTPase pathway and thus lead to interfere in metastatic signalling. Seifert et al. confirmed that PI-PLC ε is a unique G protein-regulated effector that is stimulated by both Ras and Rho GTPases (Seifert et al, 2008). All these data suggest a pivotal role of PI-PLC ε in cancer.

The expression of PI-PLC in the two cell lines is different and peculiar, and possibly related to a different crosstalk. In Hs888, decrease of PI-PLC ε expression resulted in a low expression of RhoA and Rac1 mRNA, both after PI-PLC ε silencing and treatment with the PI-PLC inhibitor, U73122. Moreover, in ezrin silencing PI-PLC ε decreased and, subsequently, RhoA and Rac1 were also reduced. In 143B, PI-PLC ε silencing decreased the expression of RhoA and Rac1 with consequent decrease of the expression ezrin. Therefore, further and deeper studies are needed to explain these findings in order to elucidate the role of this enzyme in RhoGTPases pathway.

There is evidence that PI-PLC γ signalling pathways might be important in cancer cells. Cancers have higher level of PI-PLC γ than normal tissues (Arteaga et al, 1991; Noh et al, 1994). PI-PLC γ contains a unique region that comprises two tandem SH2 domains and an SH3 domain adjacent to a split PH (Katan and Williams, 1997). The SH2 and SH3 domains facilitate the interaction with diverse molecules, contributing to physiological functions of PI-PLC γ (Bunney and Katan, 2011). Ezrin interacts with the SH2 domain of different proteins. Normally PI-PLC γ2 is expressed only in 143B cells (Fig. 1A, 1B) and this is quite surprising because 143B cell line are known to develop osteolytic tumours (Kaminski et al, 2003). It is known that PI-PLC γ2 has a role in osteoclast formation and function (Mao et al, 2006) and in the modulating of bone loss (Cremasco et al, 2010). In this study, reduced ezrin expression is concomitant with increased levels of PI-PLC γ2 mRNA both in 143B (40%) and in Hs888 (30-90%) cell lines (Fig. 8). In Hs888 cells line, PI-PLC γ2 appeared after ezrin silencing (Fig. 8). Immunofluorescence analysis

revealed that PI-PLC γ2 has a weak cytoplasmatic staining in 143B cell line. When ezrin decreased, both in ezrin silencing and PI-PLC ε silencing, there was a stronger signal intensity in cytoplasm and a new membrane staining (Fig. 11A). PI-PLC γ2 is involved in actin cytoskeleton reorganization and Rac-activation in dendritic cells (DCs) (Cremasco et al, 2010), in the integrin-mediated processes of adhesion, migration and bone resorption in osteoclast. (Epple et al, 2008). Mao et al. showed that PI-PLC γ2 regulates Proto-oncogene tyrosine-protein kinase Src (c-Src) activation and membrane localization in osteoclasts, while c-Src null osteoclasts lack podosomes. Absence of these structures can affect the capacity of the cell to rapidly rearrange the actin cytoskeleton during cell migration or intercellular contacts (Cremasco et al, 2010). The actin binding proteins seem to act as negative regulators of PI-PLC γ, because binding PIP2, they could subtract it to PI-PLC γ. Then, PIP3 is a high specific positive regulator, because it binds to PH domain, leading PI-PLC γ on the membrane surface and increasing the PIP2 hydrolysis. Therefore, one might speculate that EZR silencing leads to PI-PLC γ2 overexpression.

Fluctuations in the expression of certain PI-PLC β subtypes during diverse physiological conditions have been observed. For example, expression of PI-PLC β1 is selectively increased during myoblast and adipocyte differentiation (Faenza et al, 2004; O'Carroll et al, 2009). Molinari et al. reported that PI-PLC β1 might be altered in breast cancer, especially in the most aggressive phenotypes; however, they did not find a significant correlation between the PI-PLCB gene and lymph node status. In this study, in 143B cell line, PI-PLC β1 shows a strong cytoplasmatic signal, while the ezrin silencing determines a significant reduction of the intensity of PI-PLC β1 in cytoplasm (55%) (Fig.11B). In Hs888 cell line, PI-PLC β1 was found to have a moderate cytoplasmatic staining; ezrin silencing determines a significant increase of mRNA expression during the 24-48 hours interval (Fig. 8) and of the cytoplasm signal intensity of PI-PLC β1 (Fig. 13B). These observations suggest that high ezrin expression might correlate to PI-PLC β1 expression in the primary tumour and vice-versa. Therefore, when ezrin expression decreases in 143B, PI-PLC β1 is reduced too. By contrast, in metastatic line, PI-PLC β1 expression increases only after ezrin silencing, that leads to the lack of RhoA and Rac1. The activation of PI-PLC β1 probably interferes with alternative pathways. Recent works demonstrated that PI-PLC β can also be activated by the Rho-GTPase family member Rac, which interacts with the PH domain (Snyder et al, 2003). The PH domain is involved in the interaction with Gβγ subunits of G-proteins and with phospholipids. In the inactive GDP-binding state, the G-proteins form a heterotrimeric complex formed by Gα, Gβ and Gγ subunits. Upon stimulation of the receptor, the Gα subunit is activated through exchanging GDP with GTP that

induces the dissociation of Gα from Gβγ. The GTP-bound Gα subunit, together with the Gβγ heterodimer, activates PI-PLC β isoenzymes. Chikumi et al. indicated that receptors, transmitting signals through Gq, can promote Rho activation, thereby initiating the activity of intracellular pathways controlled by Rho. Similarly, they observed that Gαq and Gq-coupled receptors can potently stimulate the Rho-related GTPase Rac1 (Chikumi et al, 2012). These data, together with our findings, suggest that if the PI-PLC β1 decreases, Gαq could be more available for activating RhoA and Rac1.

Stimulation of Rac1 induces actin polymerization in the cell periphery to generate protrusive actin-rich lamellipodia and membrane ruffling. In particular, Rac has been reported to be a critical player in cell motility in various cell types (Benvenuti et al, 2004; Minobe et al, 2009). These data suggest that suppression of ezrin expression might modulate mainly Rac1 expression. Piechulek T et al. reported that Rac proteins, but not other members of Rho GTPases (Cdc42 or RhoA), regulate PI-PLC γ2 (Piechuleket al, 2005). Interestingly, ERM proteins might regulate Rho activity and vice-versa (Bretscher, 1999; Mangeat et al, 1999; Tsukita and Yonemura, 1999). After ezrin silencing, in 143B, RAC1 was increased about 50%. This high expression could activate PI-PLC γ2, that increased by 40%. The contribution of different Rac isoforms to migration is likely to depend on the cell type and on their relative expression levels. Rac2 is required for neutrophil migration, but whether it acts similarly in tumours is not known (Roberts et al, 1999). In contrast, Rac1 and Rac2 are dispensable for cell migration in macrophages, although Rac1 is required for invasion (Wheeler et al, 2006). However, in fibroblasts, Rac1, but not Rac3 suppression by siRNA, affects lamellipodium formation, although cell invasion is reduced in both cases (Chan et al, 2005).

The GTPases Rac1 and Cdc42 promote polymerization at the leading edge, whereas RhoA antagonizes this effect, promoting retraction of the leading edge and the assembly of stress fibers (Schmitz et al, 2000). Rho is also thought to be involved in ERM protein activation (Tapon and Hall, 1997). In vitro association of ERM proteins with the plasma membranes of cultured cells is reported to be dependent on Rho activation (Hirao et al, 1996). In several lines of cultured cells, activation of RhoA, but not Rac1 or Cdc42, produced CPERMs, forming microvilli with a concomitant accumulation of ERM proteins in microvilli (Shaw et al, 1998; Matsui et al, 1998; Matsui et al, 1999). In this study, both in ezrin and PI-PLC ε silencing, mRNA RhoA expression decreased in Hs888 cell line, but the protein localization changed from the cytoplasm to actin filaments. Microscope visualization confirmed their co-localization with actin antibody (Fig. 16). In 143B cell line, after ezrin silencing, RhoA moved from cytoplasm to nucleus. Further studies are needed to understand these data.

PI-PLC δ4 plays a key role in cell proliferation, though the regulation of its activity is less understood. It is reported that certain isoforms, such as PI-PLC δ1, might be regulated through the interaction with a dual function G-protein (Gh) (Murthy et al, 1999). In the regenerating liver, PI-PLC δ4 mRNA is expressed at higher levels than in normal resting liver, as well as in hepatoma cells, and in src-transformed cells than in non-transformed cells (Santi et al, 2003). Leung et al. examined the expression of PI-PLC δ4 in normal vs. tumour cells. They found that normal kidneys tissue showed the highest expression, while a moderate expression was detected in normal gastro-intestinal tissues. PI-PLC δ4 expression is highly down-regulated in different tumours as prostate, vulva, thyroid, skin, and pancreatic tumour. By contrast, it is up-regulated at a high percentage (>25%) in breast and testicular tumour tissues and at low percentage (<5%) in lung and uterine tumour tissues, compared to the corresponding normal counterpart (Leung et al, 2004). In our study, in Hs888 cells, after ezrin silencing, the expression of PI-PLC δ4 is significantly increased (100% - Fig. 8). PI-PLC δ4 is mainly expressed by metastatic cell line and not by primitive tumour, suggesting that PI-PLC δ4 over-expression or dys-regulation might contribute to transform the phenotype and participate to metastatic progression. PI-PLC δ4 decreases also after PI-PLC ε silencing, suggesting a possible role of PI-PLC ε in inducing PI-PLC δ4.

Conclusions and future perspectives

PLC mRNA and protein expression varies after ezrin silencing or PI-PLC ε silencing. We observed an overall complex re-organization of the expression profile of PI-PLC genes with respect to unsilenced controls. These data suggest a possible role of PI-PLC enzymes in osteosarcoma progression. Future studies are required in order to further investigate the nature and the meaning of this relationship.

Our results also corroborate the hypothesis that a complex, and still unclear relationship exists among GTPases, RhoA, and RAC1, selected PI-PLC enzymes and ezrin.

Based on the previous studies, here we confirm the complexity of the pathways, and that differences exist in the expression of PI-PLC genes in the different osteosarcoma cell lines we analysed. Unfortunately, no literature data are available.

The panel of expression of PI-PLC enzymes in Hs888 cell line is comparable to the corresponding panel of fibroblast cells we had analysed.

Future studies should be designed to confirm these observations.

- complete the preliminary study performed with PI-PLC inhibitor U73122 and the inactive analog U73343 further investigate the transduction pathway conducting simultaneus silencing of ezrin and further selected PI-PLC isoforms further investigate the relationship between ezrin and PI-PLC γ2
- further investigate whether a relationship exists between RhoA and PI-PLC β1 and the possible role in osteosarcoma tumor and metastasis
- analyze the two different PI-PLC β1 splicing isoforms, taking into account the localization (nuclear versus cytoplasmic)
- culture 143B and Hs888 cell lines in micromass, in order to analyze the ezrin related PI-PLC expression in a live model
- extend the study to other osteosarcoma cell lines, including primary cell lines
- ultimately, analyze the expression of PI-PLC enzymes in osteosarcoma biopsies from patients in order to investigate the role of one of the signal transduction systems acting in osteosarcoma and the possible influence upon the natural history of the disease and the clinical outcome of the patients.

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Tables and Figures

Table 1

PI-PLC		MG-63	143B	SAOS	HS888	*p*
β1	EXP1	10.18	4.54	5.84	2.50	<0.01
	EXP2	10	4.44	5.88	2.5	
	EXP3	10.6	4.6	5.8	2.51	
	EXP4	10.02	4.55	5.85	2.5	
β2	EXPS1-2–3	0	0	0	0	
β3	EXP1	17.49	25.06	33.83	27.52	<0.01
	EXP2	17.58	25.06	33.9	27.51	
	EXP3	17.48	25.05	33.99	27.5	
	EXP4	17.5	25.05	33.2	27.52	
β4	EXP1	17.79	46.40	30.07	38.47	<0.01
	EXP2	17.8	46.66	30.06	38.48	
	EXP3	18.01	46.29	30.07	38.48	
	EXP4	18.01	46.49	30.07	38.46	
γ1	EXP1	8.02	10.38	11.87	11.91	<0.01
	EXP2	7.98	10.39	11.77	11.9	
	EXP3	8.2	10.38	11.9	11.9	
	EXP4	7.98	10.38	11.77	11.92	
γ2	EXP1	21.40	43.23	47.59	0	<0.01
	EXP2	22	43.02	47.55	0	
	EXP3	21.1	43.33	47.58	0	
	EXP4	21.40	43.2	47.59	0	
δ1	EXP1	15.42	9.00	12.67	54.51	<0.01
	EXP2	15.1	9	12.66	54.5	
	EXP3	15.5	9.01	12.67	54.5	
	EXP4	15.4	9.01	12.68	54.51	
δ3	EXP1	15.13	15.56	32.64	13.63	<0.01
	EXP2	15.2	15.55	32.64	13.62	
	EXP3	15.1	15.6	32.6	13.65	
	EXP4	15.13	15.56	32.65	13.64	
δ4	EXP1	0	0	0	11.59	<0.01
	EXP2	0	0	0	11.6	
	EXP3	0	0	0	11.6	
	EXP4	0	0	0	11.58	
ε	EXP1	0.77	15.55	8.0	4.43	<0.01
	EXP2	0.79	15.6	8	4.42	
	EXP3	0.77	15.46	7.9	4.41	
	EXP4	1	15.55	8	4.44	
η1	EXP1	0	0	8.18	8.29	<0.01
	EXP2	0	0	8.18	8.3	
	EXP3	0	0	8.2	8.26	
	EXP4	0	0	8.15	8.29	
η2	EXPS1-2–3	0	0	0	0	

Table 1: Results of RT-PCR.
Quantification of the expressed PI-PLC isoforms in osteosarcoma cell lines with Agilent Bioanalyzer. The concentrations were measured in 4 independent experiments, listed as EXP1, 2, 3 and 4. Concentrations are intended in nanograms per microliter .

Figure 1

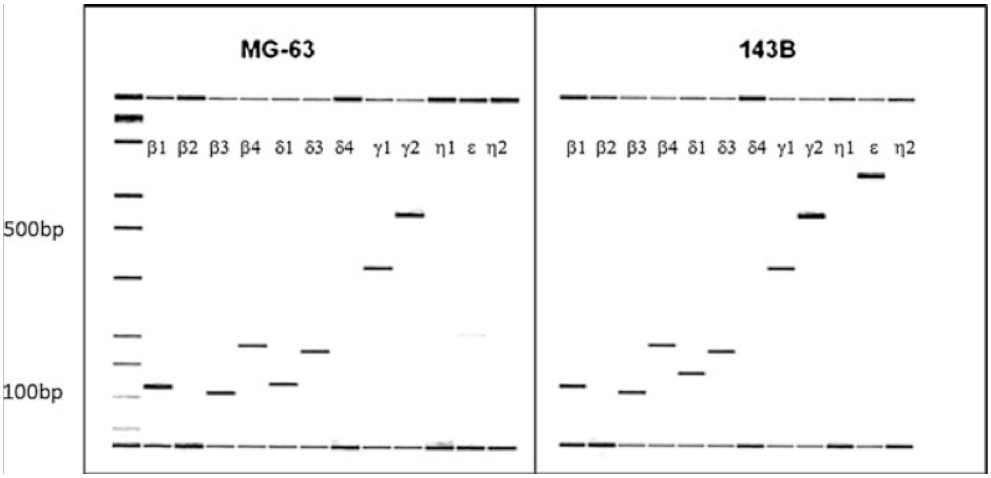

Fig. 1A: RT-PCR results in MG-63 and 143B.
Left: MG-63 cell line. Visualization of specific bands for the transcripts of PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1 and PI-PLC δ3
Right: 143B cell line. Visualization of specific bands for the transcripts of the genes codifying for PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1, PI-PLC δ3 and PI-PLC ε

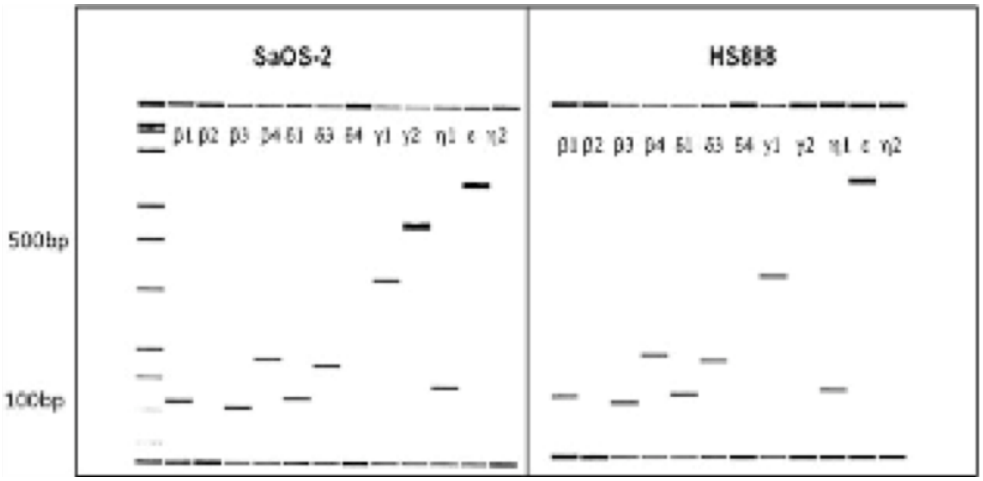

Fig. 1B: RT-PCR results in SaOS and Hs888.
Left: SaOS-2 cell line. Visualization of specific bands for the transcripts of the genes codifying for PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC γ2, PI-PLC δ1, PI-PLC δ3, PI-PLC ε and PI-PLC η1
Right: Hs888 cell line. Visualization of specific bands for the transcripts of the genes
codifying for PI-PLC β1, PI-PLC β3, PI-PLC β4, PI-PLC γ1, PI-PLC δ1, PI-PLC δ3, PI-PLC δ4, PI-PLC ε and PI-PLC η1

Figure 2

A

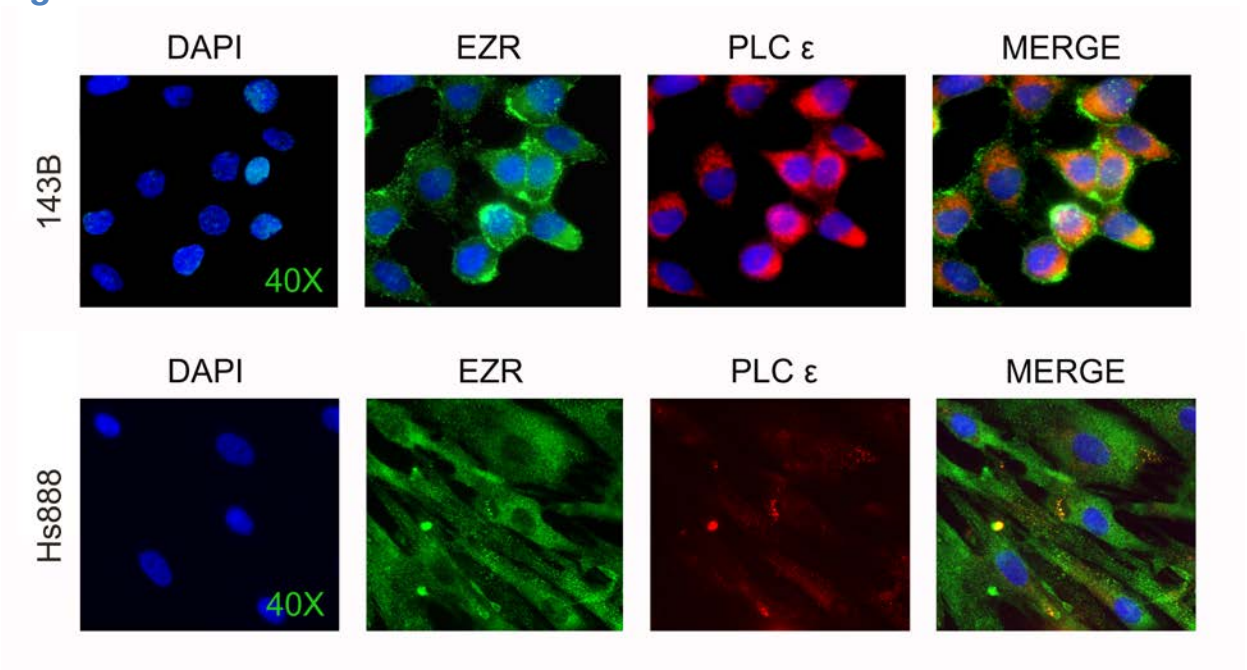

Fig. 2A: Immunofluorescence in143B and Hs888 cells.
Ezrin localized at cytoplasmic and membranous level of all the cell type.
PI-PLC ε localizated at cytoplasmic and peri-nucleus level.
Ezrin and PI-PLC ε partially colocalized in the citoplasm in 143B, while in hs888 results in differt localization

B

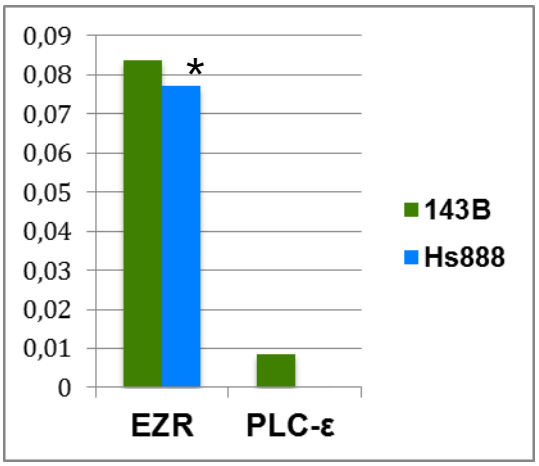

Fig. 2B: The mRNA expression levels of Ezrin, PI-PLC εin143B and Hs888 cells.

Figure 3

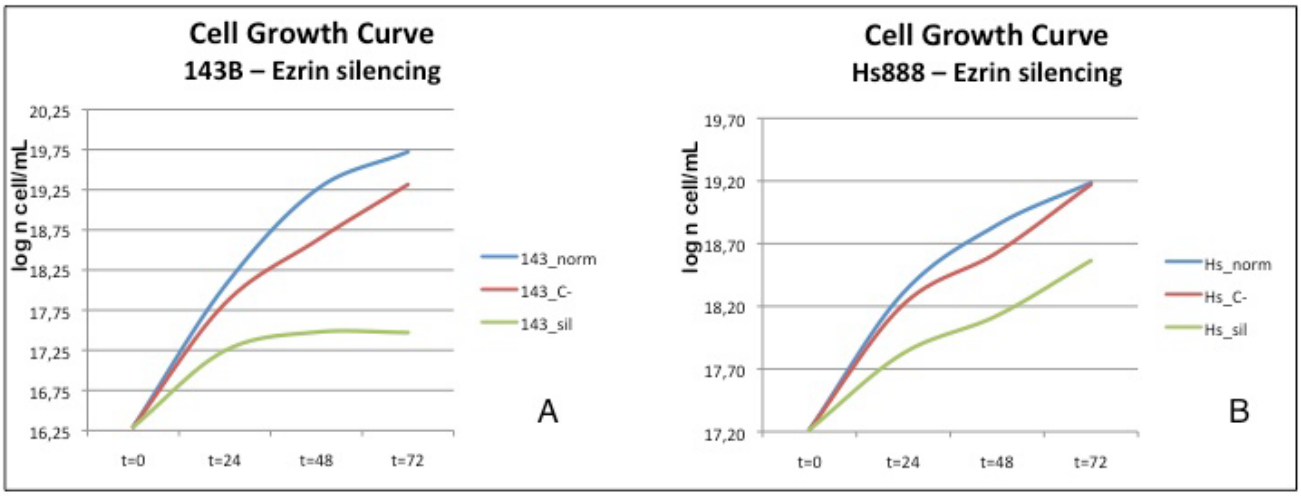

Fig 3:
A - in 143B cells line, the growth of silenced cells is significantly slowed with respect to untreated cells ($p<0,5$).
B - in Hs888 cells line, the growth of silenced cells is slowed with respect to untreated cells.

Figure 4

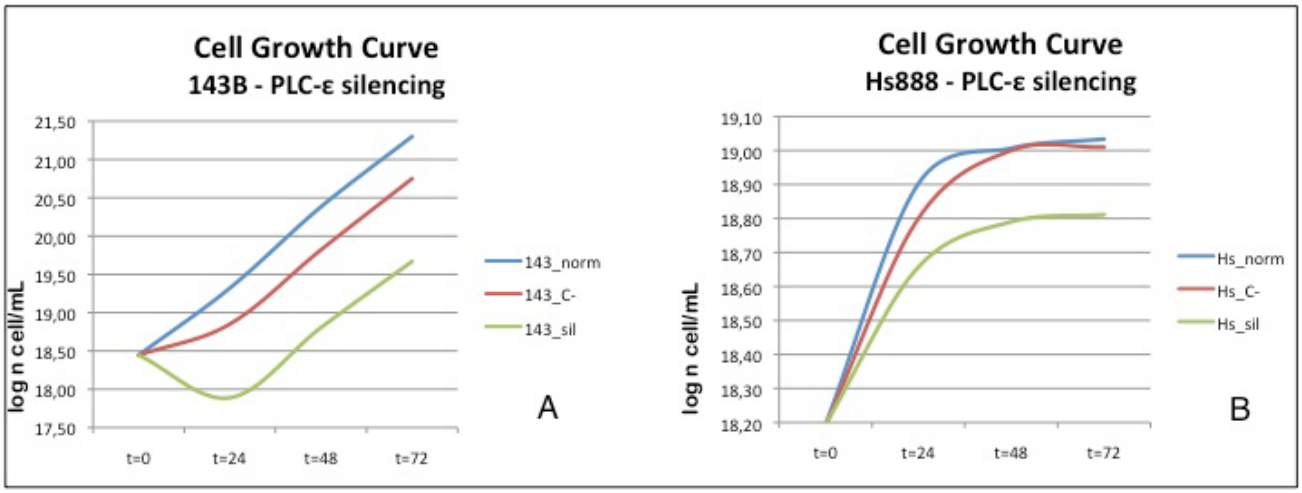

Fig. 4:
A - in 143B cells line, the growth of silenced cells is significantly slowed with respect to untreated cells ($p<0,5$).
B - in Hs888 cells line, the growth of silenced cells is slowed with respect to untreated cells.

Figure 5

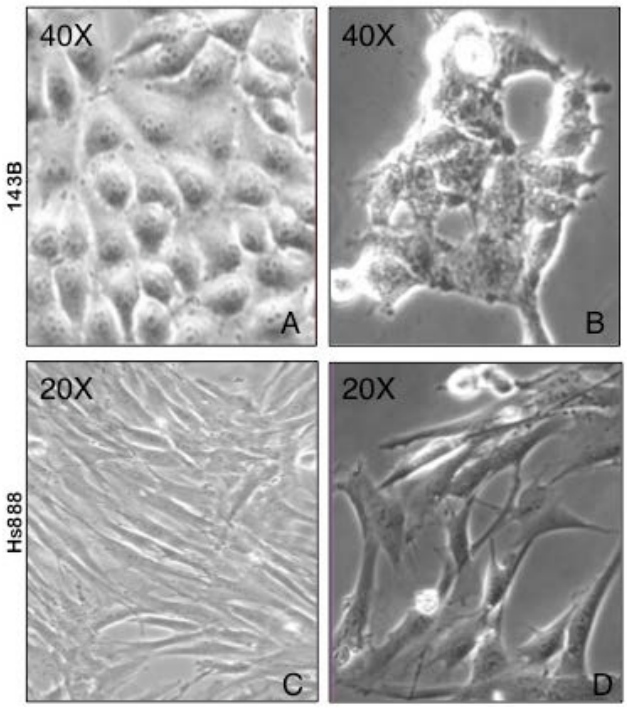

Fig. 5: effect of Ezrin siRNA on cell morfology.

In 143B, silencing resulted in an irregular outline of the plasma membrane and was associated with a reduced intercellular adhesio (B). Microvacuolization of the cytoplasm was also observed.

In Hs888 we observed a quantitative reduction of cellular elements, which showed a substantially well-conserved structure (D).

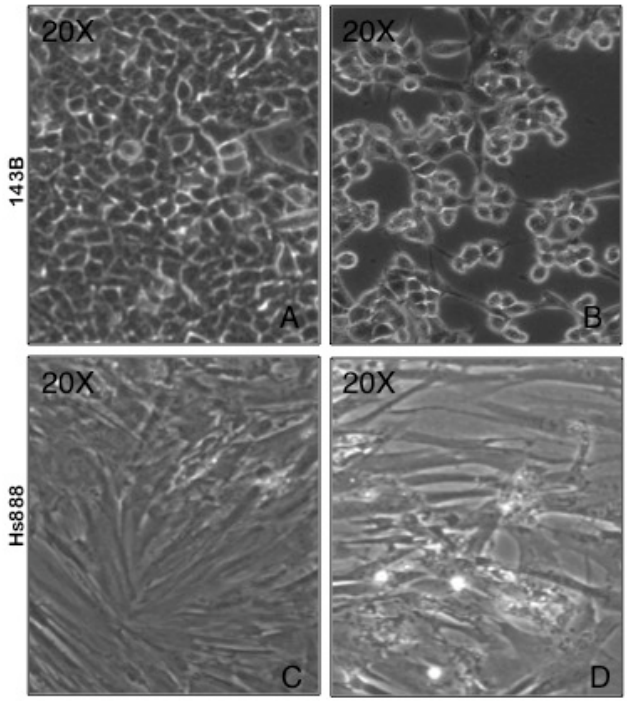

Figure 6

Fig. 6: effect of PI-PLC ε siRNA on cell morfology.
In the 143B induces an important change of conformation of the cells and cell rounding (Fig. 6B).

in Hs888 induces the cytoplasmic maro/micro-vacuolisation with slight reduction of the number of cells and without alteration of morphology (Fig. 6 D).

Figure 7

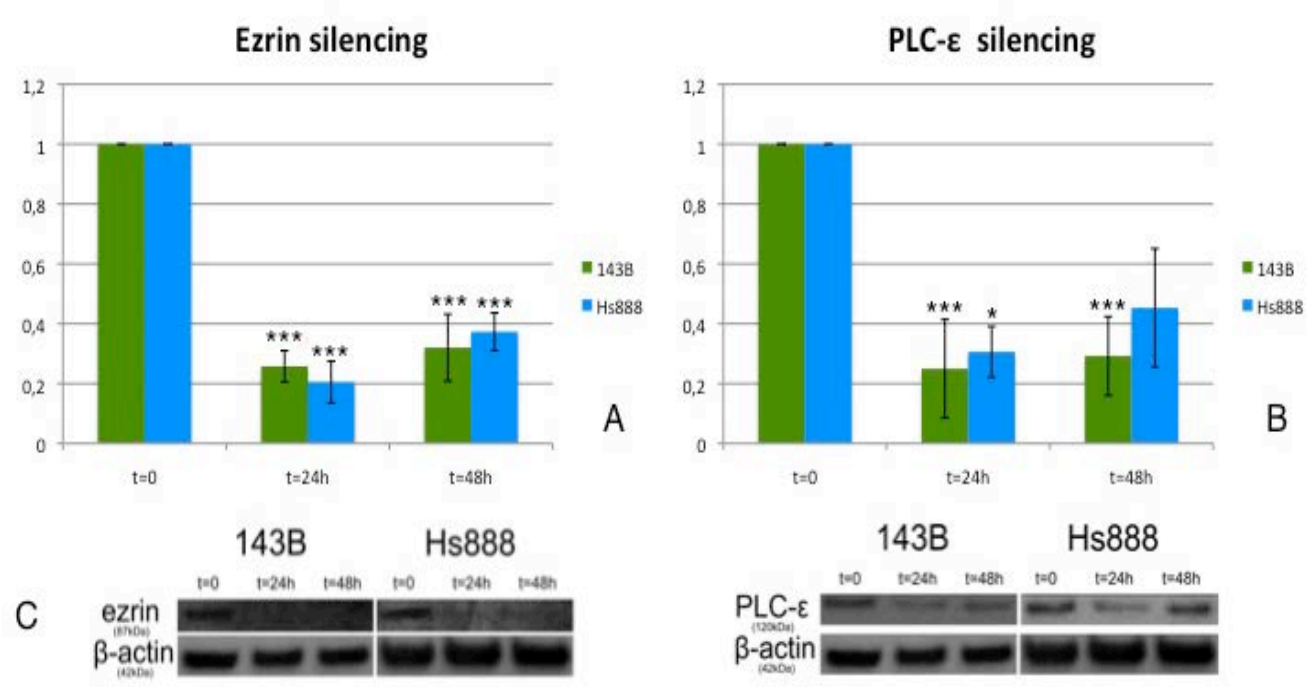

Fig. 7:
A - The mRNA expression level of ezrin detected by RealTime-PCR 24 and 48 hours after ezrin siRNA transfection in 143B and Hs888 cell lines. Both, in 143B and Hs888 cells line, the expression of ezrin in silenced cells is significantly slowed with respect to untreated cells ($p<0,001$).

B - The mRNA expression level of PI-PLC ε detected by RealTime-PCR 24 and 48 hours after ezrin siRNA transfection in 143B and Hs888 cell lines. In 143B cells line, the expression of PI-PLC ε in silenced cells is significantly slowed with respect to untreated cells ($p<0,001$); in Hs888 cells line, the expression is significantly slowed only at t=24h ($p<0,05$).

C – 24 and 48h hours after transfection, western blot assay was performed to analyse the expression of ezrin and PI-PLC ε protein. There was no change in the expression of b-actin as internal control. While the expression of ezrin protein was decreased in the cell lines which were transfected with ezrin siRNA comparing to the group without transfection

Figure 8

Fig. 8: The mRNA expression levels in 143B (green) and Hs888 (blue) cell lines trasfected with ezrin siRNA.

Figure 9

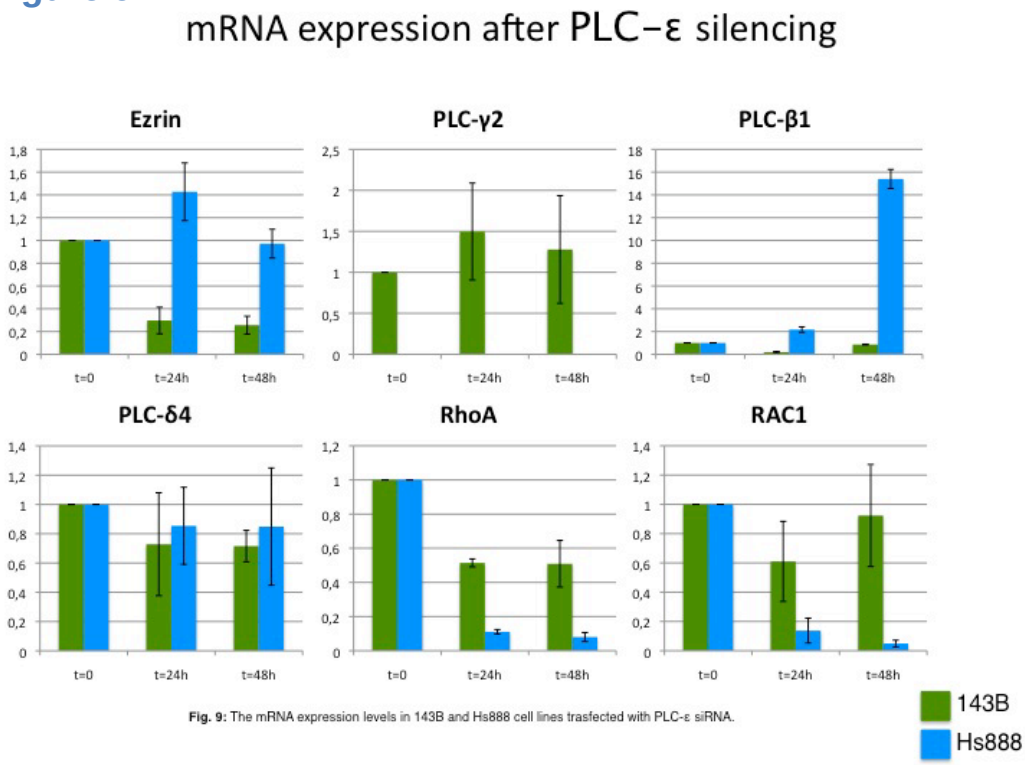

Fig. 9: The mRNA expression levels in 143B and Hs888 cell lines trasfected with PLC-ε siRNA.

Figure 10

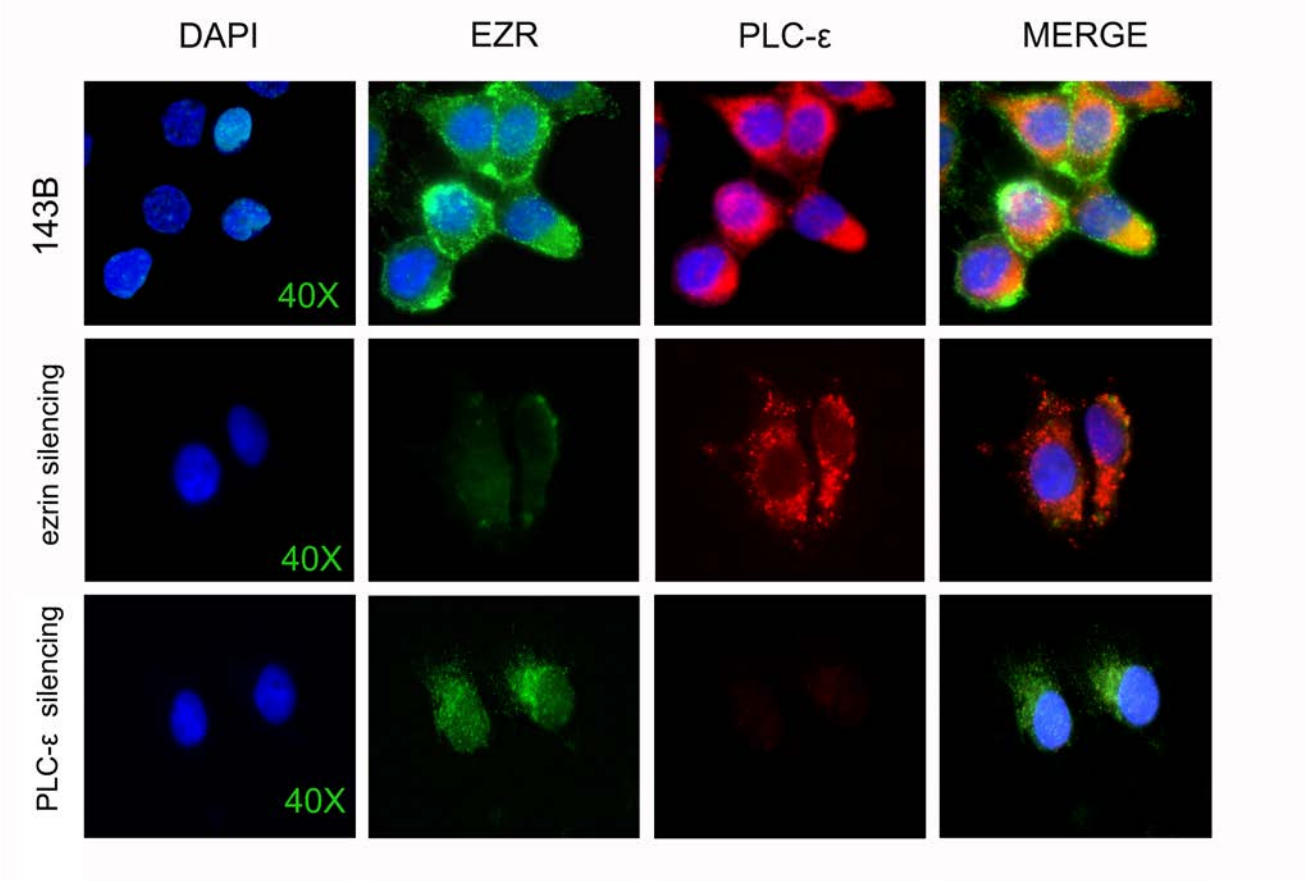

Fig.10: **Immunofluorescence anti-ezrin (green) and PI-PLC ε (red) in 143B cell line.**

• 143B (t=0): Ezrin showed a moderate cytoplasmatic staining, with signal enhancement on the membrane, white PI-PLC ε showed strong cytoplasmatic and perinuclear staining. Ezrin and PI-PLC ε colocalized in the cytoplasm;

• ezrin silencing: ezrin disappaired, while there was a slight reduction of PI-PLC ε stain, but the cytoplasmatic localization remained;

• PI-PLC ε silencing: PI-PLC ε disappaired, ezrin showed slight intensity decrease, the same cytoplasmatic localization but a more evident perinuclear staining, and losed the membrane staining.

Figure 11

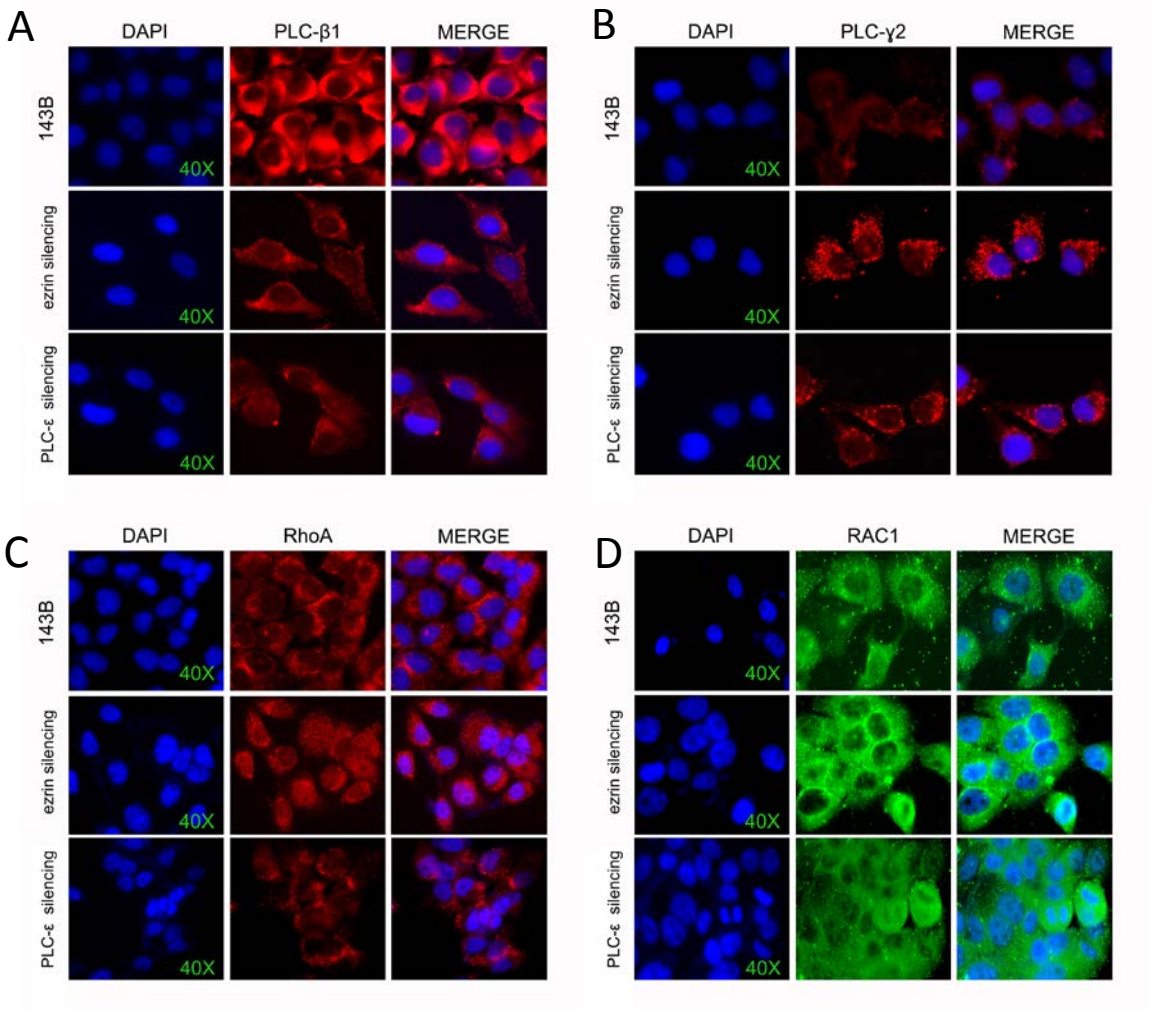

Fig.11
A: Immunofluorescence anti- PI-PLC γ2 (red):
• 143B (t=0) PI-PLC γ2 showed a weak cytoplasmatic staining;
• Ezrin silencing, there was a stronger signal intensity in cytoplasm and a new membrane staining;
• PI-PLC ε silencing there was slight intensity increase in the cytoplasm .
B: Immunofluorescence anti- PI-PLC β1 (red):
• 143B (t=0) PI-PLC β1 showed a strong cytoplasmatic staining;
• Ezrin silencing determined a significant reduction of the signal intensity in cytoplasm;
• PI-PLC ε silencing determined a significant reduction of the signal intensity in cytoplasm.
C: Immunofluorescence anti-RhoA (red):
• 143B (t=0) RhoA showed a perinuclear and cytoplasmatic staining with strong signal intensity;
• Ezrin silencing, the signal intensity did not change, but caused a displacement of the staining in the nucleus;
• PI-PLC ε silencing caused a moderate reduction of signal intensity, always localized in cytoplasm.
D: Immunofluorescence anti- RAC1 (green):
• 143B (t=0) RAC1 showed weak signal intensity localized in perinuclear zone;;
• Ezrin silencing there was a strong increase of signal intensity of RAC1, that remained localized in perinuclear zone;
• PI-PLC ε silencing the signal intensity decreased.

Figure 12

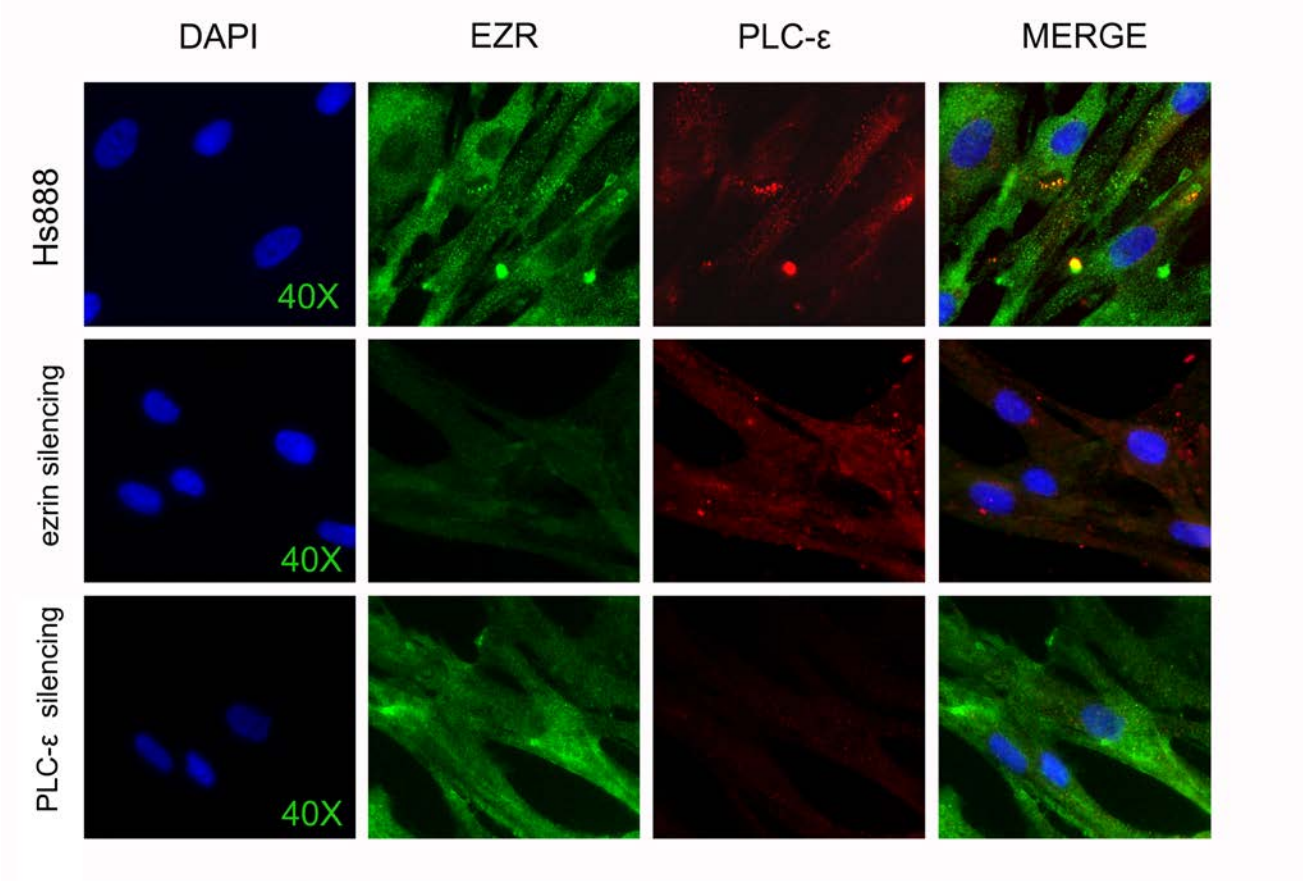

Fig.12: Immunofluorescence anti-ezrin (green) and PI-PLC ε (red) in Hs888 cell line.

Hs888 (t=0): Ezrin showed a moderate cytoplasmatic staining, with signal enhancement on the membrane, white PI-PLC ε showed strong cytoplasmatic and perinuclear staining. Ezrin and PI-PLC ε colocalized in the cytoplasm;

ezrin silencing:ezrin disappaired, while there was a slight reduction of PI-PLC ε stain, but the cytoplasmatic localization remained;

PI-PLC ε silencing: ezrin showed slight intensity decrease, the same cytoplasmatic localization but a more evident perinuclear staining, losing the membrane staining

Figure 13

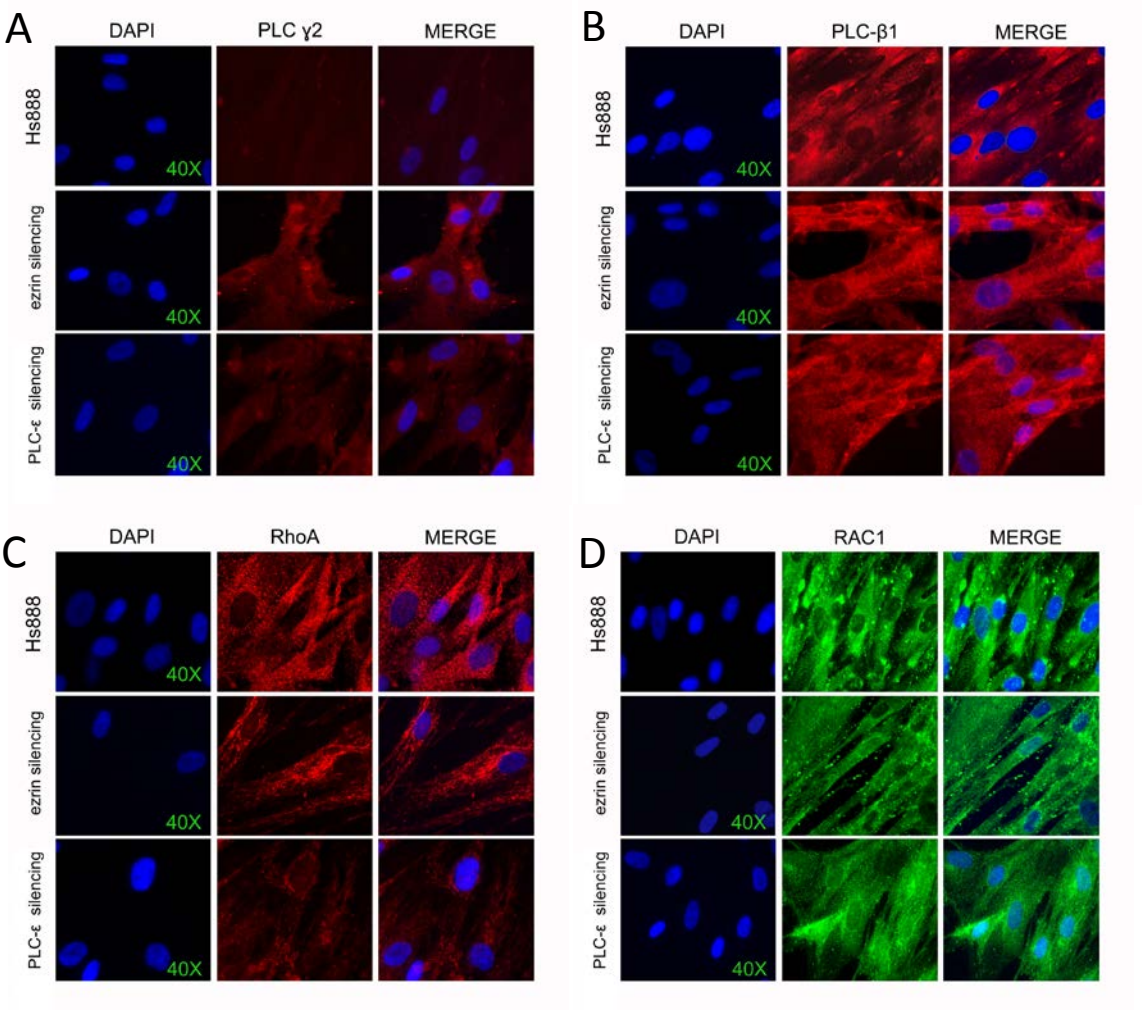

Fig. 13
A: Immunofluorescence anti-PI-PLC γ2 (red):
• Hs888 (t=0) PI-PLC γ2 did not show signa;
• Ezrin silencing determined a moderate signal intensity of PI-PLC γ2 in cytoplasm, with strong perinuclear enhancemen;
• PI-PLC ε silencing determined moderate cytoplasmatic signal intensity of PI-PLC γ2 .
B: Immunofluorescence anti- PI-PLC β1 (red):
• Hs888 (t=0) PI-PLC β1 showed a moderate cytoplasmatic staining;
• Ezrin silencing determined a significant increase of the signal intensity of PI-PLC β1 in cytoplasm;
• PI-PLC ε silencing determined a significant increase of the signal intensity of PI-PLC β1 in cytoplasm.
C: Immunofluorescence anti-RhoA (red):
• Hs888 (t=0) RhoA showed a perinuclear and cytoplasmatic staining with strong signal intensity;
• Ezrin silencing the signal intensity became weaker;
• PI-PLC ε silencing caused a notable reduction of signal intensity, always localized in cytoplasm .
D: Immunofluorescence anti- RAC1 (green):
• Hs888 (t=0) RAC1 showed strong signal intensity localized in cytoplasm and perinuclear zone;
• Ezrin silencing there was a moderate decrease of signal intensity of RAC1, that remained localized in cytoplasm;
• PI-PLC ε silencing there was a moderate decrease of signal intensity in cytoplasm.

Figure 14

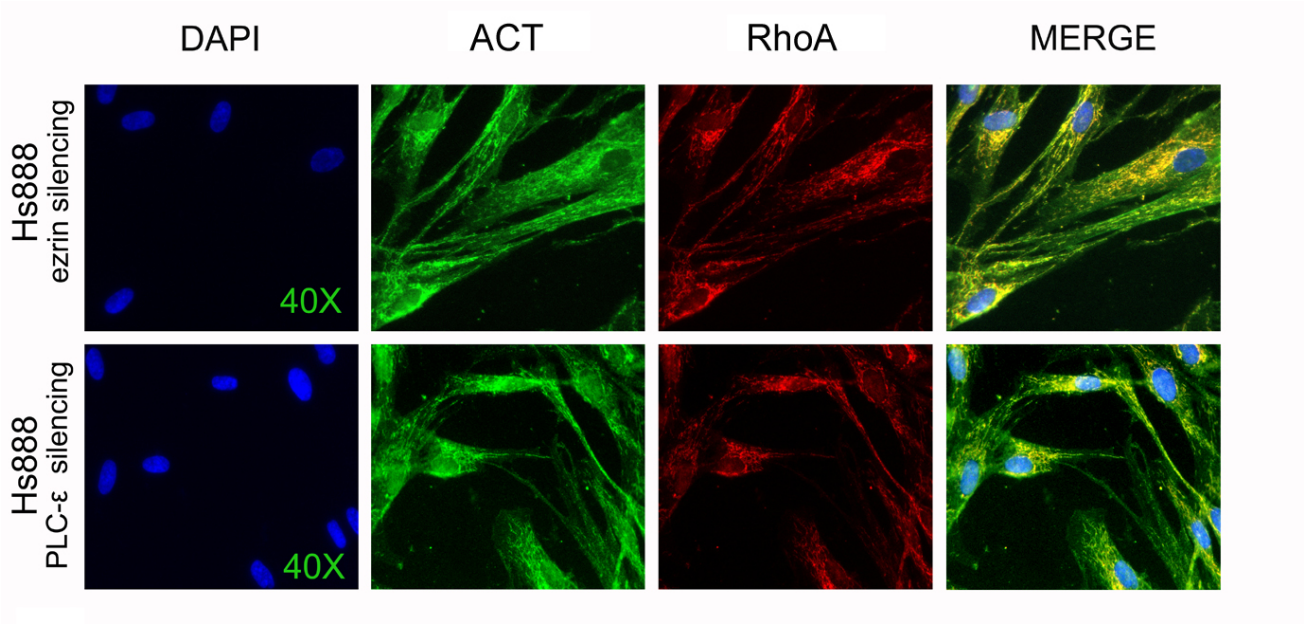

Fig.14: Immunofluorescence anti-actin (green) and RhoA (red) in Hs888 cell line

ezrin silencing: RhoA and actin colocalized in the cytoplasm (merge);

PI-PLC ε silencing: ezrin showed slight intensity decrease, the same cytoplasmatic localization but a more evident perinuclear staining, losing the membrane staining

Figure 15

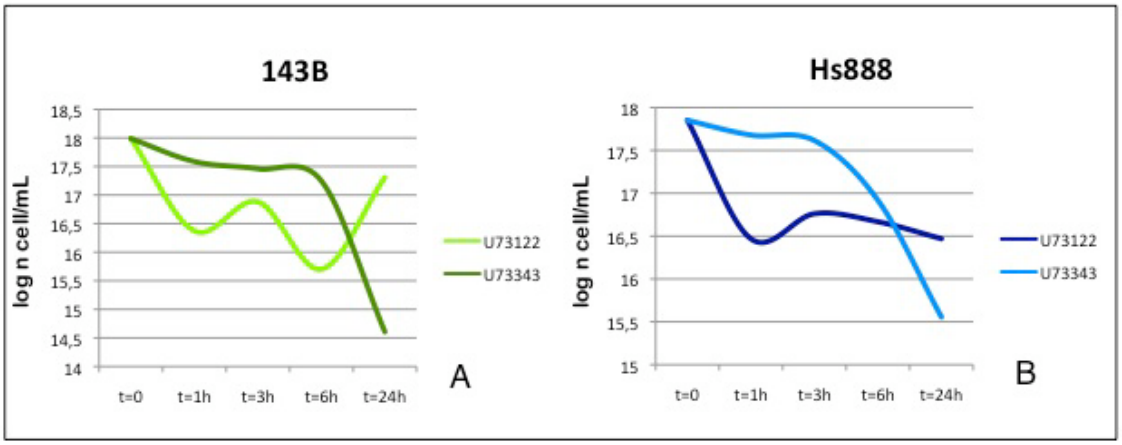

Fig. 15: U73122 and U73343 growth curve

Figure 16

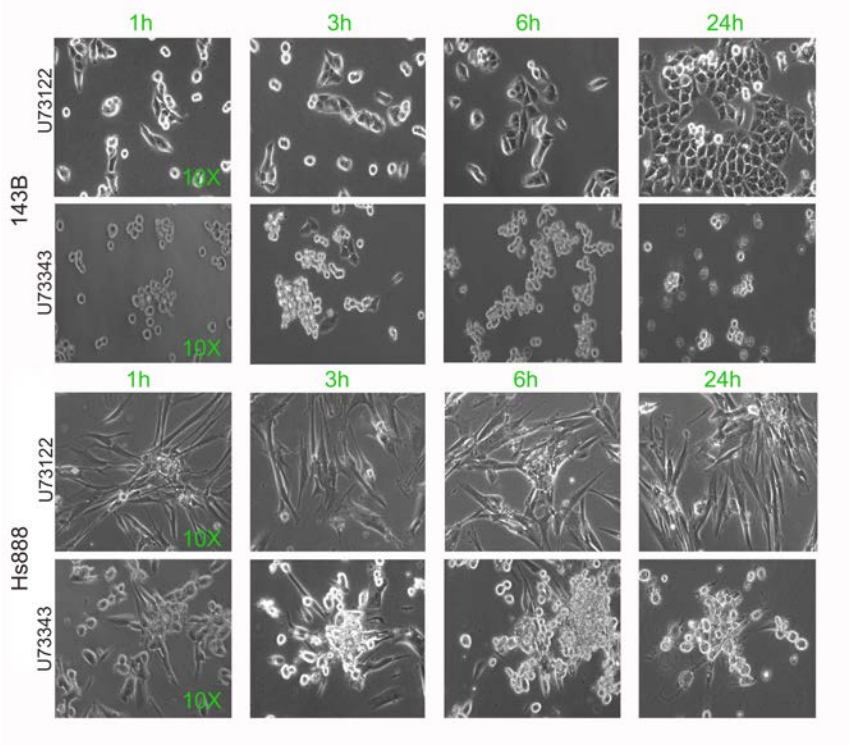

Fig.16: effect of U73122 and U73343 on cell morfology.
U73122 treatment had a greater effect on cell morphology while U73343 treatment had the greatest effect on cell death.
At the beginnig of U73122 treatment, the cells reduced intercellular adhesion. After 6 hours microvacuolization of the cytoplasm appeared both in 143B and Hs888.
After 3 hours of U73343 treatment, the cells started to detach.

Figure 17

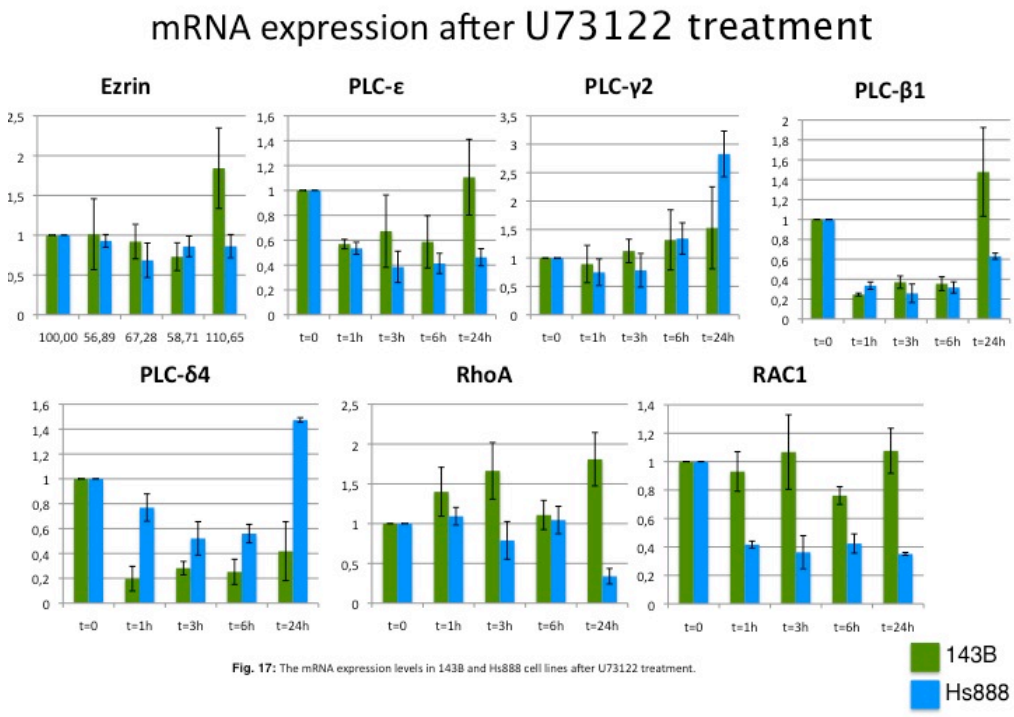

Fig. 17: The mRNA expression levels in 143B and Hs888 cell lines after U73122 treatment.

Figure 18

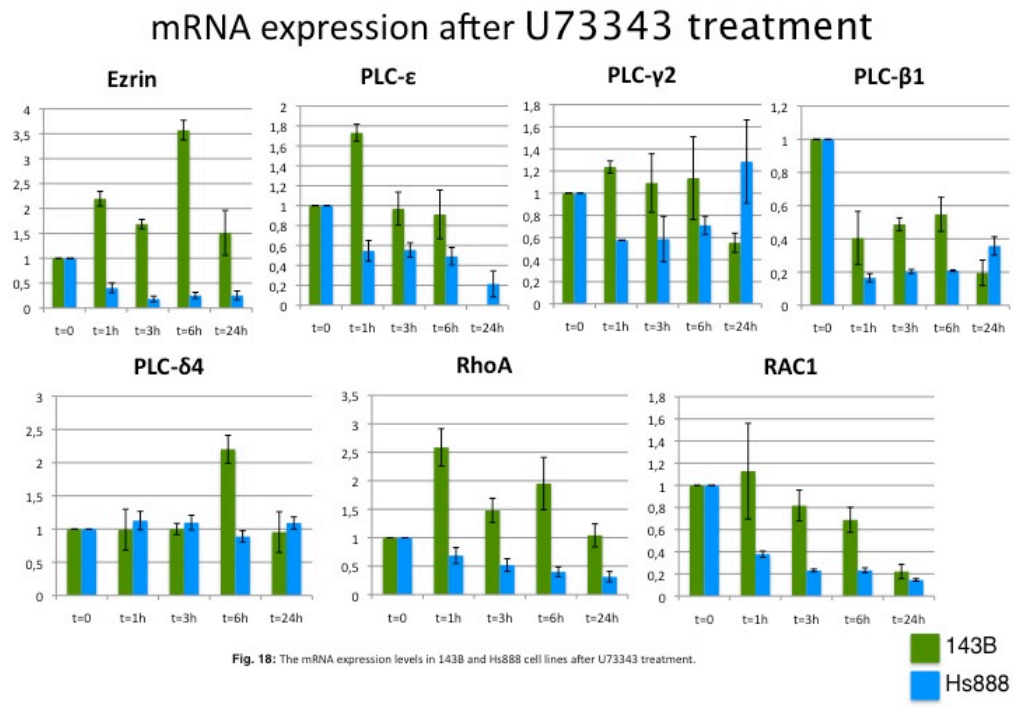

Fig. 18: The mRNA expression levels in 143B and Hs888 cell lines after U73343 treatment.