Università degli Studi di Siena

Dipartimento Biotecnologie, Chimica e Farmacia

Dottorato di Ricerca in Biochimica e Biologia Molecolare

Ciclo XXIX

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A novel view on LMW-PTP involvement in tumorigenesis: from apoptosis resistance to metabolic reprogramming

Settore Disciplinare: BIO/10

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Anno Accademico: 2015-2016

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ABSTRACT

Protein tyrosine phosphorylation in eukaryotes is a key mechanism for cellular control, since it is involved in several processes, such as cellular metabolism, proliferation, differentiation and oncogenic transformation. A fine balancing of cellular protein tyrosine phosphorylation levels is determined by regulating the activities of protein-tyrosine kinases and/or protein-tyrosine phosphatases (PTPs) (Alonso et al., 2004). The PTP superfamily comprises almost 70 enzymes that, despite very limited sequence similarity, share a common CX5R active-site motif and an identical catalytic mechanism. LMW-PTPs are a group of cytosolic enzymes of 18 kDa that are widely expressed in different tissues. They are represented by two most abundant isoforms (arised from mutually exclusive alternative splicing), named fast and slow, according to their electrophoretic mobility (Tabernero et al., 2008; Wo et al., 1992; Dissing et al., 1991; Xing et al., 2007). LMW-PTP interacts with several receptor tyrosine kinases and docking proteins that may be involved in cancer progression, but the identification of functionally relevant interactors is not yet conclusive. Several works have shown how LMW-PTP overexpression is associated with human tumorigenesis, especially in breast, colon and kidney. In fact, results obtained with a wide array of human carcinomas indicate a significant increase in the expression of LMW-PTP in tumor tissue and a correlation between higher expression of LMW-PTP on one hand and worse prognosis and reduced survival on the other (Malentacchi et al., 2005). LMW-PTP acts also as a positive regulator of tumor onset and growth in in vivo animal models. (Chiarugi et al., 2004). Moreover there are many findings that LMW-PTP plays a key role in chemoresistance: Ferreira et al., demonstrated how the phosphatase overexpression induce resistance towards vincristine and imatinib, in a leukemia cell lines (Ferreira et al., 2012). Our studies were conducted on a Melanoma cell line, A375, to study in depth the role of LMW-PTP in skin tumor onset, in order to elucidate the molecular mechanism and pathways in which this enzyme is involved and also in the view of identifying new possible therapeutic targets. Using transient silencing technique, we tested the apoptotic response of A375 cells: citofluorimetric analysis showed that upon phosphatase silencing, treatment with 5FU increase cell mortality up to 50%. In agreement with these results, western blot analysis of apoptotic markers (Caspase3, Bcl2 and Bim) confirmed that LMW-PTP silencing sensitize cancer cells towards chemotherapic drugs. Looking for a link between LMW-PTP and apoptosis, we speculate that the phosphatase exerts its action through Cav1-Bcl2 pathways. In fact the phosphorylation on

Tyr14 of Cav-1 leads to Bcl2 degradation, increasing apoptosis. Our data demonstrate how LMW-PTP knocking-down lead to Cav-1 Tyr phosphorylation, and Bcl-2 down-regulation. Several studies have demonstrated how tumor cells can develop chemoresistance increasing the activity or the expression level of membrane transporters that actively expel chemotherapy drugs from inside. Our experiments suggest that LMW-PTP may confer resistance against anti-tumoral drugs, increasing the activity of some of these membrane transporters, thus limiting the toxic effect of chemotherapy. One of the most important treatment for Melanoma is Radiotherapy. Using therapeutic doses of radiation (2Gy), we demonstrated that silenced cells were more responsive to this treatment, with respect to control samples, confirming that LMW-PTP plays a key-role not only in chemio- but also in radio-resistance. Furthermore resistant cancer cells, usually, show common phenotypes: one of this features is self-renewal capability. Colony formation assay demonstrates that melanoma cells are able to reform new colonies, even when cells are exposed to a damage, such as 5FU treatment or 2Gy irradiation. When LMW-PTP is knocked-down and cells treated with 5FU or radiated, we observed no colony formation: we can assume that LMW-PTP silencing leads to the loss of some characteristics of self- renewal, fundamental for metastasis. LMW-PTP exerts its action also through some molecules implicated in cell migration and adhesion. Adhesion and Detachment assay showed that LMW-PTP silencing lead cells to be more adherent to a substrate. Accordingly Wound Healing Assay demonstrated that melanoma cells are able to migrate and refill the wound very quickly. We tested also the Invasiveness of A375 cells: silenced cells showed a decreased ability to invade, respect to untreated cells: in fact LMW-PTP down-regulation lead to a MMP-9 reduction.
Considering the importance of LMW-PTP in tumor onset we investigate the possibility to inhibit this enzyme as a new therapeutic approach.. Previous work demonstrated that a natural compound, Morin, is an enzymatic inhibitor of LMW-PTP. Morin is not toxic for Melanoma cells, but its combination with 5FU, causes a strong increase of apoptotic cells. Interestingly, this sensitization is not reproducible in non tumoral cell line, such as C2C12 myoblast: this co-treatment could be specific for cancer cells. Furthermore our studies demonstrate that Morin doesn't act only as an inhibitor of LMW-PTP: western blot analysis showed that the flavonol lead to phosphatase degradation, in a dose and time-dependent manner. This down-regulation may be due to different mechanism, but since this effect start 2h after Morin incubation, we hypothesized that a proteasome activation may be involved. Incubation with Morin together with a proteasome inhibitor (MG132), confirmed our hypothesis. The enhancement of chemotherapic action, obtained with Morin is reproducible even with Radiation therapy. A375 cells pre-treated with Morin and then radiated with 2Gy, decrease dramatically their viability. Moreover irradiation exposition didn't influence self-renewal capability of Melanoma cells: on the contrary, Morin treatment before irradiation is able to

affect the formation of new colonies after the treatment. LMW-PTP is involved in cell-adhesion, migration and invasion: in fact Morin can affect this markers. After Morin treatment Melanoma cells are less able to migrate and invade; furthermore cells increase the number of focal adhesions.
To better understand the role of LMW-PTP in tumor onset, we looked for new substrates. To analyze this aspect we studied the phospho-proteomic profile of silenced Melanoma cells. Through these analysis we identified different proteins showing an higher levels of Tyr phosphorylation, upon LMW-PTP silencing. One of the new substrates identified with this experimental approach is Annexin-A1, a protein involved in apoptosis: this finding suggest a further possible link between LMW-PTP and apoptosis resistance, a link that will be further investigated. More interestingly, four of the proteins identified with the proteomic analysisbelong to glycolysis pathway, PKM2, α-Enolase, GAPDH and TIM. To confirm this results we performed immune and co-immunoprecipitations: these analyses confirmed that the mentioned enzymes get in contact with LMW-PTP and, presumably, are direct substrates of the phosphatase. It is well known that the "Warburg effect" causes alteration in cancer cell energetic metabolism, leading cells to consume large quantity of glucose, metabolizing it predominantly through glycolysis, and producing high level of lactate. Considering that four of these substrates are involved in glycolysis pathway, we tested some metabolic parameters. When LMW-PTP is silenced A375 cells consume less O2, and consequently their glucose up-take is higher, producing more lactate respect to controls. Pyruvate kinase controls the final and rate-limiting reaction of glycolysis: PKM2 undergoes conformational conversion between a tetrameric/full active and a dimeric/less active state. The conversion to a less active state, induced by Tyr phosphorylation, confers to PKM2 "non-metabolic" abilities. Indeed, PKM2 translocates into the nucleus and acts as a transcriptional co-activator of β-catenin and hypoxia-inducible factor 1α (HIF-1α), cooperating to control cell proliferation and glucose catabolism, respectively. Western Blot Analysis of the Glucose transporter GLUT-1 and Hesokinase II, confirmed our previous data. When LMW-PTP is down-regulated both proteins had an increased expression level, explaining, at least in part, the glycolytic metabolism showed by silenced cells. Moreover LMW-PTP influences not only the Tyr phosphorylation state of PKM2, but also its expression level: in fact when the phosphatase is down-regulated PKM2 protein level is higher.
In conclusion, our results demonstrated that LMW-PTP plays a key role in chemo and radio-resistance acquisition of Melanoma cells: in fact when the phosphatase is knocked-down cells are more responsive to therapy. Considering that gene silencing cannot be used in patients, the discovery that Morin is able to reproduce the same phenotype, open new possibilities for therapies. Moreover LMW-PTP seems to influence metabolism of Melanoma cells, a parameter often

deregulated in cancer cells. Further studies will be conducted to better characterize the role of this enzyme, and its role in the regulation of tumor metabolism.

INTRODUCTION

1 Protein Phosphorylation

Protein phosphorylation is a reversible event given by the balance of two classes of enzymes: kinases and phosphatases. It is a post-translational modification that occurs at the level of specific amino acid residues and it is a ubiquitous mechanism present in all organisms, both prokaryotic and eukaryotic. This mechanism represent the basis behind the phenomena of signal transduction cascade, and it's involved in many cellular processes, such as communication between cells, cell morphology, motility, decisions to proliferate versus differentiate, regulation of gene transcription, mRNA processing, molecules trafficking in or out the cells and even apoptosis (Alonso et al., 2004). In eukariotic cells kinases and phosphatases are classified depending on their substrate specificity. Protein kinases are divided into different groups: serine/threonine kinases and tyrosine kinases (PTKs); instead phosphoprotein phosphatase are divided into three groups: non specific phosphatases, phosphoserine/threonine protein phosphatases (PPases) and phosphotyrosine protein phosphatases (PTPases) (Kuba et al., 1992; Ohmori et al., 1993; Kim et al., 1993; Wong et al., 1993). Non-specific phosphatase comprise protein with broad specificity for phosphatase esters such as acid and alkaline phosphatases, but they don't show any amino acid sequence similarity to each other (Kim & Wyckoff, 1991; Coleman, 1992; Van Etten, 1982). Unlike from PPase, these proteins fulfill their principal functions in catabolic pathways.

Phosphoprotein phosphatase instead are composed of multiple classes structurally different from each other, but many of them have amino acid sequence similarities (Cohen et al, 1990; Ballou & Fischer, 1986). Moreover these enzymes use a mechanism of action catalysed by metals: two metal ions, Mn^{2+} and Fe^{2+} coordinated by conserved residues, bind and activate a water molecule that starts the nucleophic attack of phosphate (Zhang et al., 2002). Regarding protein tyrosine phosphatase, all of these class of enzymes that are known share the same structural features in the phosphate-binding active site and use a common catalytic mechanism. The binding to different

ligands and the substrate specificity are primarily due to the presence of regulatory domains or non-catalytic sub-pockets in the regions including the catalytic domains (Zhang et al.,2002; Alonso et al., 2004; Tonks et al., 2006; Puius et al., 1997; Sarmiento et al., 1998; Tabernero et al., 2008; Soulsby & Bennett, 2009). It is generally accepted by scientific community that tyrosine phosphorylation is regulated by equal and balanced action of the PTK and PTP but, nevertheless, most of the researches are focused on PTKs. This is partly because of the "history": the first PTP was purified and cloned ten years after the first PTK (Charbonneau et al., 1989; Guan et al., 1990; Czernilofsky et al., 1980). Moreover the discovery that several viral oncoproteins are constitutively active tyrosine kinases that transform cells through tyrosine phosphorylation, led to an immediate interest in the possibility of developing inhibitors that would block the action of such kinases; (Hunter T; 2007). But the most important reason is that the oncogenic characteristics of PTKs and the ability of PTP to hinder PTK actions through their antiproliferative effects conduct some authors to classify PTP's genes as tumor-suppressing or anti-oncogenes (LaForgia et al., 1991). In the last few years, new findings have led to the emerging view that PTPs play a dominant and specific role in controlling levels of tyrosine phosphorylation in the cells (Fischer et al., 1991; Walton & Dixon, 1993; Tonks & Neel, 1996; Mustelin et al., 2002a, 2002b; Muestelin & Tasken 2003). In fact, it has been confirmed that disorders in expression or activity of several PTPs are involved in the pathogenesis of several human diseases, such as diabetes, obesity, inflammation, autoimmune, cardiovascular disease and cancer (Zhang et al., 2001; Ostman et al., 2006; Sastry & Elferink, 2011).

1.1 Protein Phosphatases

Differently from PTKs that originate from a common ancestor, PTPs evolution led to the development of a number of separate families that are structurally different. Inside human genome the number of genes (107) that encode enzymes belonging to the PTPs family is higher than the number of genes (90) encoding PTKs. Among these 107 PTP's genes, 11 are catalytically inactive, 2 dephosphorylate mRNA and 13 remove phosphate from inositol phospholipids. Therefore only 81 PTPs are enzymes with the capability to dephosphorylate phosphotyrosine (Manning et al., 2002). Among the 90 PTKs, only 85 are catalytically active: so the numbers of active PTKs and PTPs are similar, and we can assessed that they have equal substrate specificity. Moreover both types of enzymes show similar tissue distribution, from ubiquitous to very cell type restricted: in this way every single cells express 30-60% of the entire amount of PTKs and PTPs. The main feature of the

superfamily of protein tyrosine phosphatase is the conserved CX_5R domain in the active site, which forms the phosphate binding loop rich in cysteine (called P-loop or PTP-loop), responsible for the catalytic mechanism of these molecules. (Tabernero et al., 2008). Considering the amino acid sequences of their catalytic domains, the PTPs can be classified in four families:

- ***Class I Cysteine-based PTPs***
- ***Class II Cysteine-based PTPs***
- ***Class III Cysteine-based PTPs***
- ***Aspartate-based PTPs***

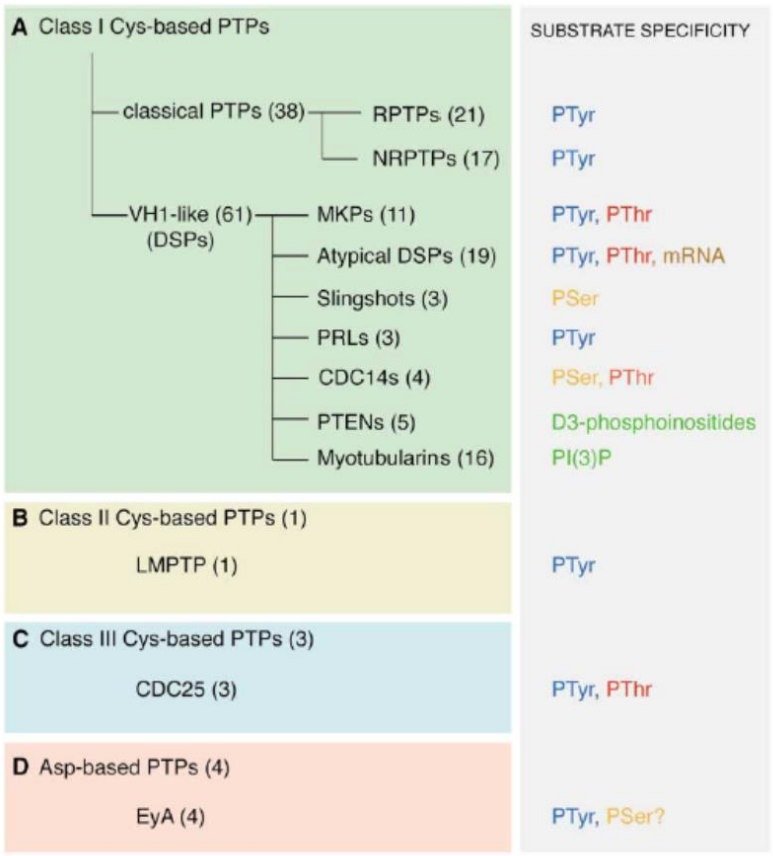


Figure 1: Classification and Substrate Specificity of PTPs. The PTP families are color coded: class I Cysbased PTPs (green), class II Cys-based PTPs (pale yellow), class III Cys-based PTPs (pale blue), and Asp-based PTPs (pink). The substrate specificity of each group or class of PTPs is listed (Alonso et al., 2004).

The **Class I Cysteine-based PTPs** can be divided in several subclasses basing on the homology and structure between catalytic domains. The 38 strictly tyrosine specific *"classical PTPs"* include transmembrane receptor-like proteins (RPTPs) and the intracellular non-receptor PTPs (NRPTPs). RPTPs are able to control signaling through ligand-controlled protein tyrosine dephosphorylation and many of them are involved in cell-cell and cell-matrix contact (Andersen et al., 2004). Moreover some RPTPs contain extracellular regions with differing composition characterized by immunoglobulin-like, carbonic anhydrase-like or fibronectin type III-like domains (Streuli & Saito, 1993). NRPTPs instead can be localized both in cytoplasm or into nucleus: they are similar to RPTPs, but, in addition to the catalytic domain, they have amino- and carboxy-terminal sequences maybe with regulatory functions (Mauro & Dixon, 1994). The *Dual Specificity Phosphatase (DSP)* have little sequence similarity between each other and possess smaller catalytic domains than the classical PTPs. They work through the same catalytic mechanism, but the structure of the active site allows them to interact both with phosphoserine (pSer)/phosphothreonine (pThr) and pTyr residues. One of the most studied sub-classes of DSP catalyzes the dephosphorylation of both Tyr and Thr sites in the activation loop of MAPK (Alonso et al 2003a; Keyse 1998; Saxena & Mustelin, 2000; Tonks, 2006). The **Class II** is represented in the human genome by a single gene, called ACP1, which encodes the Low M_r PTPase (LMW-PTP): they are widely expressed in humans and have been identified in most tissues and organs, such as erythrocytes, endothelial and muscle cells, epithelial, connective and nervous tissue, liver, placenta and lens (Wo et al., 1992; Dissing et al., 1991; Xing et al., 2007). These proteins are structurally unrelated to the other PTPs and don't share any sequence homology with transmembrane and non-transmembrane PTPs, except for the signature motif common to all PTPs (Ramponi, 1994; Ramponi & Stefani 1997; Heinrikson, 1969; Rehkop & Van Etten 1975; Chernoff & Li, 1985; Oakada et al., 1986; Wahed et al., 1988; Ferreira et al., 2006). The conservation of this class of PTP through evolution and the interconnection with many human pathologies such as rheumatoid arthritis, asthma, diabetes, cardiomyopathy, Alzheimer's disease and cancer, suggest that LMW-PTP may be involved in the control of several crucial processes inside cells (Bottini et al., 2002). The **Class III** is composed, in humans, by three cell cycle regulators: cdc25a, cdc25b and CDC25C. The role of these enzymes is to dephosphorylate Cdks (Ciclin-dependent Kinase) at the inhibitory N-terminal motifs: the reaction is fundamental to obtain kinase activation and to lead progression of cells through the cell cycle (Honda et al, 1993).

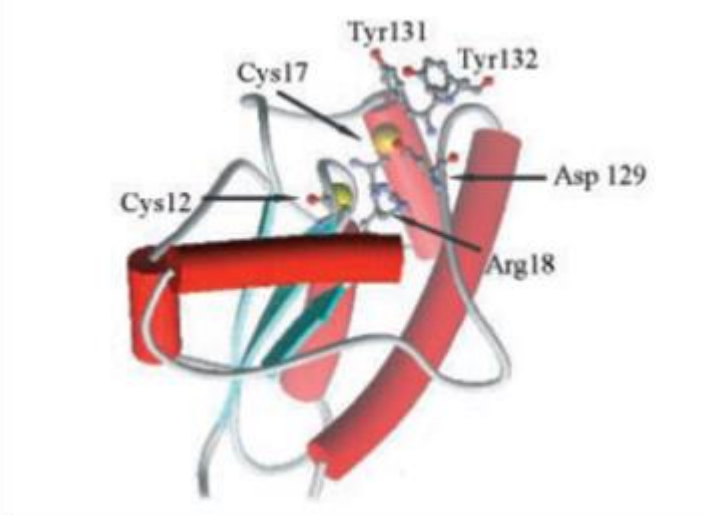


Fig. 2: The tridimensional structure of LMW-PTP, with features of the catalytic site residues. The sulfur atoms of both Cys12 and Cys17 are shown in yellow (Raugei et al., 2002)

1.2 LMW-PTP

The *ACP1* human gene, which expresses LMW-PTP, is located on 2p25.3 and composed of seven exons and six introns, spanning approximately 18kD of genomic DNA. This gene is genetically polymorphic having three alleles, called A, B and C, that produce six different genotypes: AA, BB, CC, AB, AC, BC (Hopkinson et al., 1963; Dissing et al., 1991). The three alleles (A, B and C) show an homology close to 100%, since they differ from each other only for the presence of three single nucleotide polymorphisms (SNPs), that affect the enzymatic activity (Dissing, 1987). In A allele, the amino acid 105 is a residue of Arginin, while in B and C there is a Glutamin. The other two SNPs instead do not change the encoded amino acid residues: the B allele differs from A for a CT transition in codon 41 (located in exon 4), while the C allele differs from both A and B for a silent CT transition in codon 43 (located in exon 3). This polymorphism strongly affects alternative splicing of mRNA. The *ACP1* gene produces 13 primary transcripts from which originate only 5 proteins. Of the remaining 8 transcripts: 4 undergo to a nonsense mediated decay, a process that prevents the expression of truncated or invalid proteins; two do not contain an Open Reading Frame (ORF) and two contain only intronic sequences. Two of the five proteins produced correspond to the most active isoforms of the gene:

- ACP1_001 (NM_004300.3; *fast*)
- ACP1_002 (NM_007099.3; *slow*)

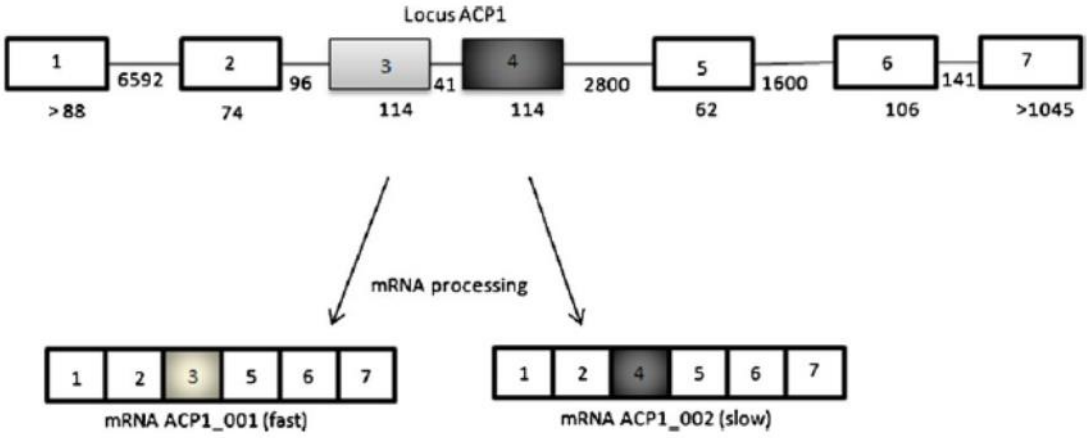


Fig. 3: Gene structure of the human *ACP1* locus. Mechanism for the generation of fast and slow isoforms by mutually exclusive RNA splicing (Alho et al., 2013).

These two isoforms are electrophoretically, kinetically and immunologically distinct, and are defined *Fast* and *Slow* according to their electrophoretic mobility (Bryson et al., 1995): they are the product of a mutually alternative splicing of exon 3 or 4 of the primary transcript. In each one of the two isoforms exon 3 and 4 encode sequences for the amino acid residues in position 39-76 for both isoforms. The remaining exons, 1-2 and 5-7, are identical for *Fast* and *Slow*, encoding the amino acid residues 1-38 and 77-157 respectively (Bryson et al., 1995; Rudbeck et al., 2000).

The differences in the expression ratios of these isoforms may be linked to the different genotypes. The slow isoform produced by the C allele is much more widespread than the fast form (ratio 1/4), otherwise the fast B isoform shows an higher activity than the slow B (ratio 4/1). The fast A and slow A occur in the ratio 2/1 (Bottini et al. 1995).

1.3 Structure of the two isoenzyme, localization and catalytic mechanism.

Both human isoforms IF1 and IF2 are formed from a single peptide chain consisting of 157 amino acid residues. The sequence of amino acids is identical in the two isoforms, with the exception of the sequence 40-73, called *variable loop*, which provides only 41% homology (Wo et al., 1992; Dissing et al., 1991). This *loop* flanks the catalytic site and determines the isoform specificity for the substrates and modulators binding, suggesting the different roles of the two isoforms (Cirri et al., 1998; Zabell et al., 2006). The three-dimensional structure of the two iso-enzymes is composed of a nucleus formed by four-stranded β sheet surrounded on both sides by α-helices. The P-loop, consisting of 12-19 residues, forms the conserved sequence CLGNICRS and connects the helix α1 to the β1 sheet. This phosphatase active site is located at the bottom of a crevice formed by the C-terminal side of the parallel β sheet This crevice, as other phosphotyrosine-specific protein phosphatases, is deep enough to specifically catalyze the dephosphorylation of phosphotyrosine residues, avoiding the action of phosphoserine and phosphothreonine substrates, whose side chain is shorter (Maccari & Ottanà, 2012; Ramponi & Stefani, 1997). The side of the crevice is ranged by hydrophobic residues (Leu13, Ile 16, Trp49, Asn50, Tyr131 and Tyr132) which probable supply an hydrophobic interaction surface for both the aromatic moiety of the pTyr substrate and the neighbouring residues. In IF1 isoform the residues Trp49 and Asn50 are substituted by a Tyr and a Glu residue, respectively. These substitutions are responsible for the differing catalytic features of the two iso-enzymes (Mondesert et al., 1994; Zhang et al., 1995; Modesti et al., 1995; Li & Strhol, 1996). The Backbone of the P-loop assumes a rigid conformation, which is stabilized by the formation of multiple hydrogen bonds between the Asn15 of the active site and the conserved residues Ser19, Ser43, His72 and is optimal for binding to the phosphate substrate. In this conformation, a system of hydrogen bonds between different nitrogen atoms of the backbone and the oxygen atoms of the phosphate group of the substrate is set during the formation of the enzyme-substrate complex. In addition, an interaction between the negatively charged phosphate group and the guanidine dell'Arg18 positively charged group is crucial for the recognition and binding of phosphorylated substrate. Moreover an interaction between the negatively charged phosphate group and the positively charged guanidium group of Arg18 is crucial for the binding and recognition of phosphorylated substrate. The ionized residue Cys12 of the P-loop acts as the catalytic nucleophile that attacks the phosphorus atom of the substrate to form a phospho-enzyme intermediate. A proximal residue, Ser19, seems to play a key role in this step because it can form a hydrogen bond with the thiolate anion of the Cys12 which allows it to adopt an efficient positioning for the

nucleophilic attack (Evans et al., 1996; Chiarugi et al., 1992; Cirri et al., 1993). In addition, Asp19 appears involved in both phosphointermediate formation and breakdown: this residue seems to act as a general acid by protonating the leaving-group phenolic oxygen and as a base by activating the catalytic water molecule accountable of the nucleophilic attack at the cysteinylphosphate intermediate leading to the release of inorganic phosphate and restoring the enzyme (Taddei et al., 1994; denu et al., 1996; Wu & Zhang, 1996). The Cys17 residue of the active site contributes to the hydrolysis mechanism coordinating the water molecule in a suitable orientation for the hydrolysis, which is analogous to the role acted by a glutamine residue in high molecular weight PTPs (Kolmodin & Aqvist, 2001).

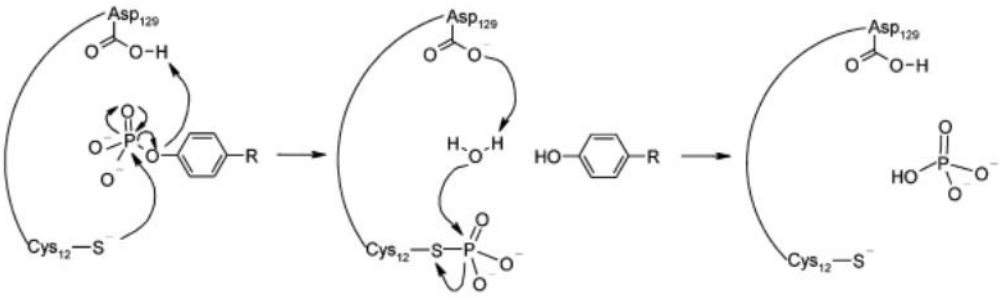


Fig. 4: Mechanism of the dephosphorylation reaction catalyzed by LMW-PTP (Maccari & Ottanà, 2011).

1.4 LMW-PTP activity: phosphorylation and redox regulation.

PTPs activity is controlled at different levels, such as expression, alternative splicing and limited proteolysis, subcellular localization, oxidation phosphorylation, dimerization and interactions with ligands (den Hertog et al., 2008). Regarding LMW-PTP, the modulation of its activity is based on phosphorylation/dephosphorylation and reversible oxidation of cysteine residues.

- **Phosphorylation/dephosphorylation**

LMW-PTP contains two conserved adjacent tyrosine, Tyr131 and Tyr 132, which are preferential sites for phosphorylation by PTKs, mainly Src kinases. The phosphorylation of these residues is independent and lead to different outcomes (Julien et al., 2011; Bucciantini et al., 1999; Tailor et al., 1997). Although Src strongly and specifically phosphorylates both Tyr residues, Tyr 131 phosphorylation induces 25-fold increase in LMW-PTP activity, whereas phosphorylation at Tyr132 does not affect the enzyme activity. Despite not having an obvious effect on LMW-PTP enzymatic activity, phosphorylation of Tyr132 by Src appears to be important for this enzyme to interact with other proteins via SH2 domains. Buccianti et al. demonstrated that SH2 domain-containing protein Grb2 (growth factor receptor bound protein 2) can bind LMW-PTP only when this phosphatase is phosphorylated at Tyr132. As Tyr132 is positioned close to the active site, the interaction of Grb2-like proteins with phosphorylated LMW-PTP could limit the access of the ligand binding surface and active site entrance, leading to selection of substrates by size or enzyme inactivation (Bucciantini et al., 1999). The result is an increase of the strength of cell adhesion through down-regulation of MMP expression, probably through the inhibition of the Grb2/MAPK pathway. On the other and phosphorylation of Tyr131 leads to regulation of Rho-mediated cell adhesion rate through dephosphorylation of p190RhoGAP. Therefore Chiarugi et al. proposed that LMW-PTP is a bifunctional phosphatase that regulates both the strength and the rate of cell adhesion through alternative phosphorylation of Tyr131 or Tyr132 (Chiarugi et al., 2000).

- **Redox modulation**

Reactive oxygen species (ROS) such as O_2 or H_2O_2 are transiently generated intracellularly in many physiological and pathological conditions, such as lymphocytic and macrophage oxidative burst, cell stimulation with cytokines and growth factors. The generation of these reactive molecules induces an increase in tyrosine phosphorylated proteins, which can be obtained by the activation of PTKs and/or inactivation of PTPs (Chiarugi & Buricchi, 2007; Monteiro et al., 2008; Chiarugi et al., 2005). In the case of LMW-PTP the oxidation of an essential cysteine (Cys12) present at the active site, which forms a disulfide bond with a proximal cysteine (Cys17), leads to its inactivation. In fact the phosphatase is not able to form a cystenyl-phosphate intermediate during the first step of the catalysis (Chiarugi et al., 2001; Caselli et al., 1998). LMW-PTP oxidation can be reversed by the glutaredoxin/glutathione/glutathione reductase/NADPH system, leading to the availability of the reduced catalytic cysteines (Chiarugi et al., 2001). Souza et al., proposed that the redox regulation of LMW-PTP may be useful for surviving and proliferation of cancer cells. They suggested that the two forms of the phosphatase, active (reduced) or inactive (oxidized), play crucial roles in cancer

cells signaling: the oxidized can support survival pathways through activation of JAK2 and STAT5; the reduced form can be involved in transformation through EphA2 dephosphorylation, and loss of adhesion by inhibition of p190RhoGAP (Souza et al., 2009).

There is also a specific regulation of each one of the two iso-enzymes by several molecules, such as purine derivatives. In particular isoform IF1 is inhibited by adenine and activated by hypoxanthine. In contrast, adenine activates IF2, whereas hypoxanthine does not affect this isoform. The explanation for the effect of hypoxanthine activating exclusively the IF1 iso-enzyme was that the only difference between the structures of these purines is the presence of an amine group at the C6 position of adenine which is replaced by a carbonyl group in hypoxanthine. This may significantly influence the interactions of the protonated N1 atom of hypoxanthine with the amino acid residues present in

the various LMW-PTPs (Wang et al., 2000; Maccari & Ottanà, 2011). Modulating effects are also exerted by purine derivatives toward electrophoretically fast and slow isoenzymes of different origins. cGMP induced a strong increase in the activity of the slow isoform AcP2 from rat liver, whereas the fast isoenzyme AcP1 was only moderately activated, suggesting that variations in the cellular cGMP concentration could result in the modulation of the LMW-PTP activity in vivo. In addition, uric acid was shown to inhibit bovine LMW-PTP at concentrations in the low millimolar range (Zabell et al., 2004; Cirri et al., 1995; Granjeiro et al., 2002).

Moreover LMW-PTP shuttles between two different compartments, giving levels of control. Indeed, while LMW-PTP is constitutively carried from both cytosolic and cytoskeleton subcellular fractions, Src-phosphorylated-LMW-PTP has been found only in the cytoskeleton fraction, whereas only the cytosolic fraction is able to interact with activated PDGF receptor. LMW-PTP membrane-associated component bind and dephosphorylates activated PDGF receptor, while the cytoskeleton-associated pool specifically acts on substrates that become tyrosine phosphorylated upon PDGFR treatment. These findings show the importance of these differences in localization on LMW-PTP molecular functioning (den Hertog et al, 2008; Cirri et al., 1998).

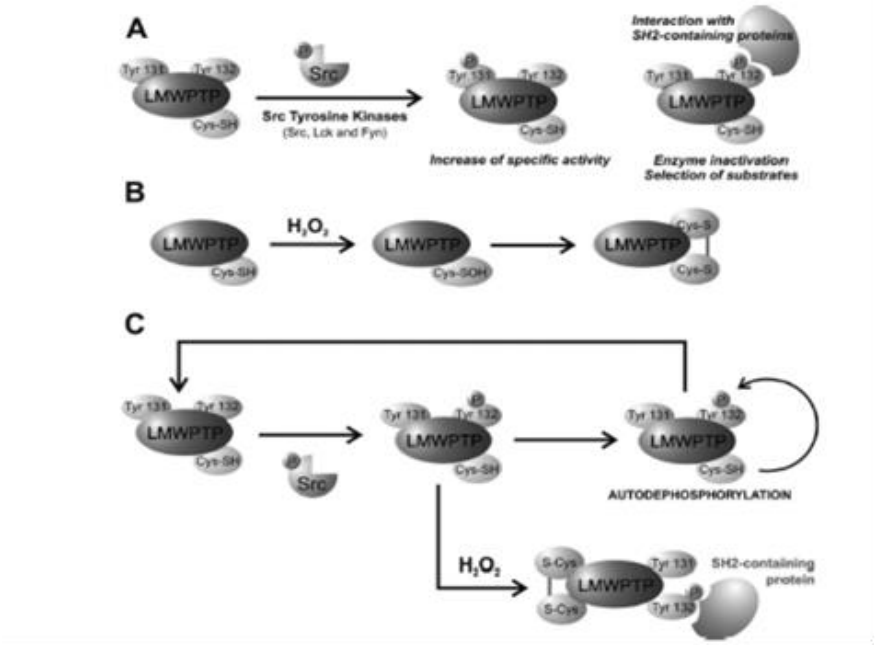


Fig. 5: Modulation of LMWPTP activity. A) Phosphorylation of Tyr131 by the Src kinases induces an increase in LMWPTP specific activity, while phosphorylation at Tyr132 enables LMWPTP interaction with SH2-containing proteins**. B)** In the presence of ROS or other oxidants, the oxidation of the cysteine residue in the catalytic site leads to the formation of a S-S disulfide, and its inactivation. **C)** LMWPTP oxidation not only inhibits the catalytic activity of the enzyme, but also potentiates the action of PTKs, such as Src. Once phosphorylated by a PTK, oxidized/inhibited LMWPTP cannot dephosphorylate itself and therefore has no ability to counteract the action of PTK (Souza et al., 2008)

2. Biological hallmarks of cancer.

It is well known that tumors develop through a succession of genetic and epigenetic alterations that confer neoplastic characteristics (Hanahan & Weinberg, 2002). "The non-lethal genetic damage" is the centerpiece of carcinogenesis; such damage (or mutations) may be caused by the action of environmental agents, such as chemicals, radiation, viruses, defined with the generic term of *"carcinògens"*; or they can be inherited through the germ line. Carcinogens damage DNA causing breaks in the filaments or by interfering with the replication. When a normal cell is damaged on the nucleic acids , it can react in two different ways: die or try to repair the damages. If this attempt failed because the extent of damage exceeds the capacity of the cell to repair the DNA, this starts apoptosis. However, sometimes the damaged cell can survive, repaired and dysplastic, and sometimes has a replicative advantage. Then carcinogenesis is a multiphasic and progressive process both at the phenotypic and genetic level, which allows the cell to acquire different characteristics such as uncontrolled growth, invasiveness and the ability to form metastasis.

All these phenomena takes place gradually, through a mechanism called *Tumor Progression* (Price et al., 1997; Yokota, 2000; Price & Thompson, 2002).
There are three classes of regulatory genes most commonly affected by mutations and therefore related to the neoplastic progression:

- *Proto-oncogenes*, such as c-myc, c-erb-B2, ras;
- *Onco-suppressor*, such as Rb, p53, PTEN;
- Genes involved in DNA repair.

Proto-oncogenes are highly conserved during evolution and encode proteins essential for survival, proliferation and cell differentiation. The conversion of proto-oncogenes to oncogenes occurs by in heterozygous mutations, that are able to facilitate the transformation and tumor progression. The process that leads to the activation of oncogenes can take place through two mechanisms:

- qualitative alterations caused, for example, by point mutations, deletions or insertions of the gene and subsequent creation of chimeric genes
- quantitative alterations due to gene amplification events or alteration of transcriptional control

The Onco-suppressor are genes that, when inactivated, contribute to the process of carcinogenesis. Usually these genes encode proteins which arrest cell growth, promote apoptosis or are antagonists of oncogenes. Loss of function can be linked to multiple factors such as deletions, inactivating mutations or reduced expression; it is also common that different mechanisms can co-exist on the two alleles of the same onco-suppressor. In fact in most cases, mutations in these genes determine a phenotypic alteration only if both alleles are modified. The malfunctioning of genes involved in DNA repair mechanism causes genomic instability that favors the accumulation of mutations in proto-oncogenes and tumor suppressor genes that lead to neoplastic transformation. Thanks to this genomic instability, cells acquire the ability to survive, proliferate and spread, even in an unfavorable microenvironment or different from the one where the primary tumor developed (Yokota, 2000). Then the accumulation of various mutations cause the acquisition of the six fundamental alterations in the physiology of the cell, which together define the malignant phenotype:

- **Sustaining Proliferative Signaling:** Normal tissues control the production and release of growth-promoting signals that instruct entry into and progression through the cell growth-and-division cycle, thereby ensuring a homeostasis of cell number and thus maintenance of normal tissue architecture and function. Cancer cells, by deregulating these signals, have the ability to proliferate without the presence of external stimuli. This characteristic is acquired through different mechanisms: they may produce growth factor ligands themselves, to which they can respond via the expression of cognate receptors, resulting in autocrine proliferative stimulation. Otherwise, cancer cells may send signals to stimulate normal cells within the supporting tumor-associated stroma, which reciprocate by supplying the cancer cells with various growth factors (Cheng et al., 2008; Bhowmick et al., 2004). Growth factor independence may also derive from the constitutive activation of components of signaling pathways operating downstream of these receptors, obviating the need to stimulate these pathway by external stimuli. Receptor signaling can also be deregulated by increasing the levels of receptor proteins present at the cancer cell membrane, rendering such cells hyper responsive to otherwise-limiting amounts of growth factor ligand; the same result can be obtained from structural alterations in the receptor molecules that facilitate ligand-independent firing. Usually all of these modifications derived from oncogene activation (Mendelsohn et al., 2001).

- **Evading Growth Suppressors:** cancer cells may not be sensitive to molecules that inhibit the proliferation of normal cells; many of this molecules belong to the action of onco-suppressor genes. . For example, the products of tumor suppressor genes such as RB (retinoblastoma-associated) and TP53 proteins operate to control a cellular regulatory circuits that govern the decisions of cells to proliferate or, alternatively, activate senescence and apoptotic programs. Frequently in tumor cells there is a RB aberrant protein, leading to uncontrolled cell proliferation (Burkhart & Sage, 2008; Deshpande et al., 2005).

- **Evading Cell Death**: studies conducted in the last decades have shown that programmed cell death represents a natural barrier to cancer development (Adams & Cory, 2007; Lowe et al., 2004; Evan & Littlewood, 1998). The apoptotic system is composed of both upstream regulators and downstream effector components (Adams & Cory, 2007). The *regulators*,can be divided into two main groups, one receiving and processing extracellular death-inducing signals (*the extrinsic apoptotic program*, involving the Fas ligand/Fas receptor), and the other sensing and integrating a many signals of intracellular origin (*the intrinsic program*).

Each one culminates in activation of a normally latent protease (pro-caspases 8 and 9, respectively), which start a cascade of proteolysis. This process leads to the execution phase of apoptosis, in which the cell is progressively disassembled and then destroyed, both by the other neighbor cells and by professional phagocytic cells. Currently, the intrinsic apoptotic program is more widely implicated as a barrier to cancer pathogenesis. Tumor cells develop several abnormalities in those molecules that play a key role in apoptosis regulation: most common is the increase expression of anti-apototic regulators (Bcl-2, Bclxl) or the down-regulation of pro-apoptotic factors (Bax, Bim, Puma). Alternatively cancer cells loose the function of one of the most important tumor suppressor: TP53.

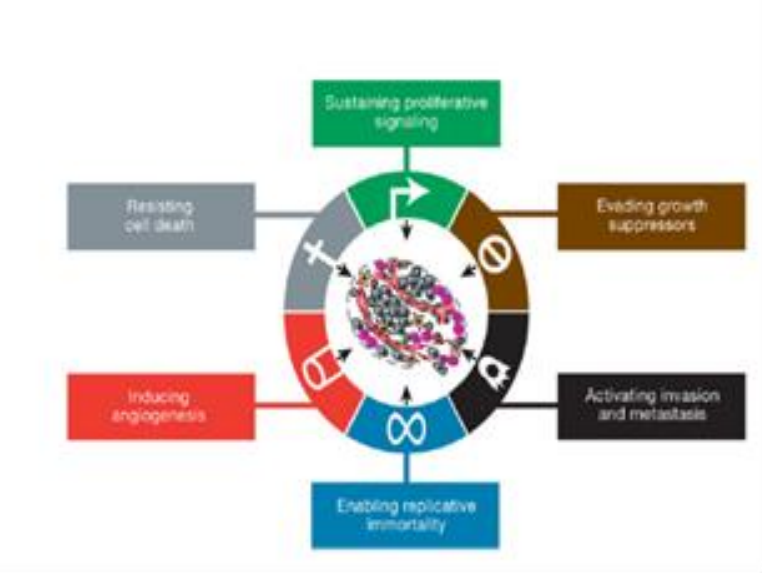


Fig. 6: Schematic representation of the six fundamental alterations in the physiology of the cell, which together define the malignant phenotype (Hanahan & Weinberg, 2011).

- **Enabling Replicative Immortality:** at every cell division occurs a shortening of specialized structures, called telomeres, placed at the ends of chromosomes. When these structure are shortened to the limit, the loss of telomeric function leads to activation of the cell cycle check-point, causing the proliferative arrest or apoptosis. In germinal cells this process is prevented by Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA: this enzyme is not present in most somatic cells. Immortalization of some cells, which leads to neoplasia, seems to occur in about 90% of human tumors. This ability of cancer cells is due to the maintenance of telomeric DNA at

a length that allows to avoid the onset of senescence and apoptosis through the increased expression of the enzyme telomerase (Blasco 2005; Shay & Wright, 2000).

- **Inducing Angiogenesis:** as well as the normal cells, even tumors need vascularization for the supply of nutrients and oxygen, and the removal of waste substances and carbon dioxide (CO_2). During embryogenesis, the development of the vasculature involves the birth of new endothelial cells and their assembly into tubes (vasculogenesis) in addition to the sprouting (angiogenesis) of new vessels from existing ones. The normal vasculature becomes largely quiescent and in the adult, as part of physiologic processes such as wound healing and female reproductive cycling, angiogenesis is turned on, but only transiently. In contrast, during tumor progression, angiogenesis is always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths. The well-known tumor-associated angiogenetic factor are: Vascular Endothelial Growth Factor (VEGF) and Basic Fibroblast Growth Factor (BFGF) (Hanahan & Folkman, 1996; Ferrara et al., 2003). The modification of tumor phenotype from Angiostatic to Angiogenetic is called the *"angiogenic switch"* and is a prerequisite for the development to metastasis (Dirkx et al., 2006). Tumor angiogenesis can occur by recruitment of endothelial cell precursors or by budding from existing capillaries, such as physiological angiogenesis. However, tumor blood vessels differ from normal vascular system for their winding and irregular form, and for the higher permeability. Angiogenesis is induced early in the development of invasive tumors, thus it contributes not only to the macroscopic phase of tumor progression but also to the microscopic and pre-malignant (Jain, 2003).
- **Activating Invasion and Metastasis:** invasiveness derives from an increased motility of tumor cells. These cells typically developed alterations in their shape as well as in their attachment to other cells and to the extracellular matrix (ECM). The best characterized alteration involved the loss by carcinoma cells of E-cadherin, a key cell-to-cell adhesion molecule. By forming adherens junctions with adjacent epithelial cells, E-cadherin helps to assemble epithelial cell sheets and maintain the quiescence of the cells within these sheets. Increased expression of E-cadherin was well established as an antagonist of invasion and metastasis, whereas reduction of its expression was known to potentiate these phenotypes (Berx & van Roy, 2009; Cavallaro & Christofori, 2004). Another alteration is the up-regulation of molecules associated with cell migration: for example N-cadherin, which is normally expressed in migrating neurons and mesenchymal cells during

organogenesis, is upregulated in many invasive carcinoma cells. Metastatization is the process by the malignant cells migrates from the place of origin (primary tumor) to form other tumors (secondary tumors) at distant sites. However cancer cells must successfully complete a cascade of events before forming a metastasis (invasion-metastasis cascade) and only a part of them may have the set of properties necessary for the completion of this process. The sequential steps begin with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed "colonization" (Fidler, 2003; Talmadge & Fidler, 2010).

In addition to these key features, has recently been proposed 4 distinctive hallmarks of neoplastic cells.

- **Genome instability and mutation**: The acquisition of the hallmarks enumerated above depends primarily on a succession of alterations in the genomes of neoplastic cells. The extraordinary ability of genome maintenance systems to detect and resolve errors in the DNA ensures that rates of spontaneous mutation are usually very low during each cell generation. In the course of acquiring the roster of mutant genes needed to develop tumorigenesis, cancer cells often increase the rates of mutation (Negrini et al., 2010; Salk et al., 2010). This characteristic is achieved through increased sensitivity to mutagenic agents, through a breakdown in one or several components of the genomic maintenance machinery, or both. In addition, the accumulation of mutations can be accelerated by compromising the surveillance systems that normally monitor genomic integrity and force genetically damaged cells into either senescence or apoptosis (Jackson & Bartek, 2009; Kastan, 2008; Sigal & Rotter, 2000).

- **Tumor-promoting inflammation:** some tumors are densely infiltrated by cells of both the innate and adaptive arms of the immune system (Dvorak, 1986). Tumor-associated inflammatory response have the paradoxical effect of enhancing tumorigenesis and progression, helping incipient neoplasias to acquire hallmark capabilities. In fact inflammation can contribute to tumorigenesis by supplying bioactive molecules to the tumor

microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate invasion, and metastasis, and inductive signals that lead to activation of EMT and other hallmark-facilitating programs (DeNardo et al., 2010; Grivennikov et al., 2010; Qian & Pollard, 2010; Karnoub & Weinberg, 2007).

- **Evading immune destruction:** cells and tissues are constantly monitored by an ever-alert immune system, and that such immune surveillance is responsible for recognizing and eliminating the vast majority of incipient cancer cells and thus nascent tumors. However cancer cells may evade immune destruction by disabling components of the immune system that have been dispatched to eliminate them:
    1. Reduction of the expression of MHCI, so $CD8^+$ cells are not able to recognize these cells and, consequently, to be activated against them.
    2. Increase glycocalyx production to prevent the access of the antibodies to tumor cell's antigens.
    3. abundant secretion of TGFβ and IL 10, which are the only immunosuppressive cytokines and have inhibitory action on macrophages and T lymphocytes.
    4. Maintenance of peripheral tolerance against tumor cells, because on their surface maintain many self-antigens.

- **Metabolic reprogramming:** The chronic and often uncontrolled cell proliferation that represents the essence of neoplastic disease involves not only deregulated control of cell proliferation but also corresponding adjustments of energy metabolism in order to fuel cell growth and division.

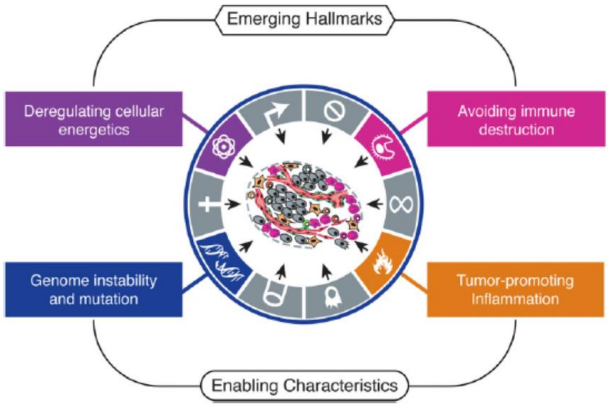


Fig. 7: Representation of the four additional hallmarks of cancer involved in the pathogenesis of some and perhaps all tumors (Hanahan & Weinberg, 2011).

2.1 Metabolic features of tumor cells

All cells need a source of energy to maintain a state of homeostasis. Cellular maintenance involves nonspontaneous energy-consuming processes such as DNA repair, cytoskeletal dynamics protein turnover and generation of concentration gradients. While maintaining homeostasis, proliferating cells have additional energetic requirements for growth and division. Therefore proliferating cells must acquire many nutrients, convert them into biosynthetic building blocks, and coordinate the reactions necessary to transform them into the macromolecules essentials for constructing new cells (Lunt & Vander Heiden, 2011). In pluricellular organisms, cells are exposed to a constant supply of nutrients: but a control system that prevent aberrant cell proliferation is present. Through this system mammalian cells do not normally take up nutrients from their environment unless stimulated to do so by growth factors. Cancer cells overcome this growth factor dependence by acquiring genetic mutations that functionally alter receptor-initiated signaling pathways. There are many evidences that some of these pathways constitutively activate the uptake and metabolism of nutrients that both promote cell survival and fuel cell growth (Hsu & Sabatini, 2008; Deberardinis et al., 2008). The metabolic phenotype of cancer cells must represent a solution that regulates

metabolic pathways to achieve a balance between ATP production and biomass production; anyway this strategy is attributable not only to tumor, as many phenotypes found in cancer cells also exist in normal proliferating cells and fast-growing unicellular organisms. The principle source of energy and new mass is glucose: this is metabolized via glycolysis to pyruvate, which can be oxidatively metabolized to CO_2 in the tricarboxylic acid (TCA) cycle to generate large amounts of ATP through the process of oxidative

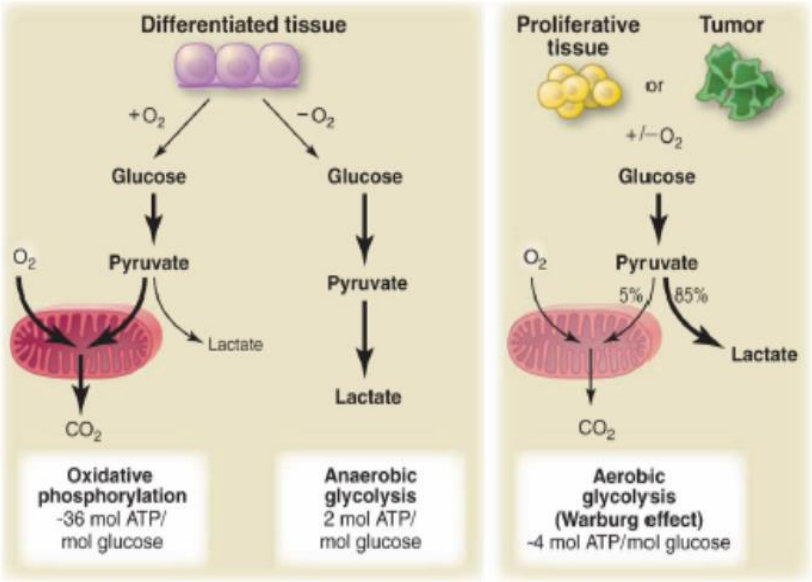


Fig. 8: Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis (Warburg effect) (Vander Heiden et al., 2009)

phosphorylation. Pyruvate also can be reductively metabolized to organic acids or alcohols (lactate, acetate or ethanol), a process called fermentation. Glucose fermentation does not require oxygen, but is far less efficient than the TCA cycle coupled to oxidative phosphorylation in generating ATP. Although decreased efficiency in energy production, many fast-growing unicellular organisms depend mostly on glucose fermentation during proliferation regardless of oxygen availability (Lunt & Vander Heiden, 2011). Similar to many unicellular organisms, multicellular organisms also exhibit enhanced glycolysis during fast growth. In 1924, Otto Warburg observed that cancer cells consume higher amount of glucose than normal tissues, and metabolize it primarily through glycolysis, so producing high levels of lactate even in oxygen-rich conditions (Warburg, 1956; Warburg et al., 1924). This phenomenon of aerobic glycolysis, conversion of glucose to lactate even

in the presence of sufficient oxygen to support glucose catabolism via the TCA cycle with oxidative phosphorylation, is not exclusive to cancer cells. Many Non-transformed cells also exhibit high aerobic glycolysis during rapid proliferation: for example proliferating mouse fibroblasts increase the rates of glucose uptake and lactate production during logarithmic growth (Munyon & Merchant, 1959). In this dividing cells, most of the glucose is converted to lactate; little is oxidized to carbon dioxide. This observation led Warburg to suggest that cancer cells arise from a defect in mitochondrial respiration that causes them to depend on an higher glycolysis. However it is now known that respiration is not damaged in most cancer cells (Fantin et al., 2006; Moreno-Sanchez et al., 2007; Zu & Guppy, 2004). Aerobic glycolysis has been observed in a wide variety of tumors that originate from different cell types, but most normal cells in adult tissues from which cancer cells arise generally do not use aerobic glycolysis. Thus, cancer cells revert to a metabolic phenotype that is characteristic of rapidly dividing cells, which suggests that aerobic glycolysis must provide advantages during proliferation (Lunt & Vander Heiden, 2011). Glycolysis is not efficient to produce ATP, because it generates just two ATP molecules per mole of glucose; whereas complete oxidation of one glucose molecule by oxidative phosphorylation can generate up to 36 ATP molecules (Berg et al., 2007). Although its low efficiency in ATP yield, aerobic glycolysis can generate more ATP than oxidative phosphorylation by producing ATP at a faster rate: an inefficient but faster pathway for ATP production may be preferred to meet the high demands of dividing cells (Pfeiffer et al., 2001).

2.2 Metabolic reprogramming

The metabolic changes induced by cell growth signals are largely conserved between normal and cancer cells; anyway, tumor cells activate signaling pathways in the absence of normal extracellular stimuli, thus promoting a metabolic phenotype that allows inappropriate cell proliferation.

- ***The PI3K pathway:*** this pathway is one of the most commonly altered in human cancers. Once activated, PI3K signaling provides high proliferation and survival signals to tumor cells, but also has profound effects on their metabolism (Plas & Thompson, 2005). One of the most important effector downstream of PI3K is *AKT1*: this is an important driver for the glycolytic phenotype and stimulates ATP generation through many mechanisms, ensuring that cells have the bioenergetics ability required to react to growth signals (Elstrom et al., 2004; Fan et al., 2010). For example, AKT can stimulate glucose uptake by increasing

expression of the glucose transporter GLUT1 (Barthel et al., 1999; Frauwirth et al., 2002; Vander Heiden et al., 2001) and maintaining GLUT1 levels on the cell surface by preventing internalization (Wieman et al., 2007). AKT activation enhances flux through glycolysis in part by maintaining hexokinase (HK) association with mitochondria (Elstrom et al., 2004; Gottlob et al., 2001) and activating phosphofructokinase 2 (PFK-2) through phosphorylation (Deprez et al., 1997). Moreover AKT can stimulate signaling through mTOR: this kinase acts as a key metabolic point, coupling growth signals to nutrient availability. Infact mTOR stimulates protein, lipid biosynthesis and cell growth; INOLTRE indirectly causes other metabolic changes by activating transcription factors such as hypoxia-inducible factor 1 (HIF1) even under normoxic conditions.

- ***HIF1 and MYC:*** rapidly proliferating cells require close proximity to blood vessels for access to oxygen and nutrients. As tumors grow, cells may encounter hypoxic conditions that lead to induction of HIF1. This transcription factor increases the expression of VEGF, to facilitate the growth of new blood vessels (O'Rourke et al., 1996). Once activated HIF1 amplifies the transcription of genes encoding glucose transporters and most glycolytic enzymes; in addition HIF1, activates the pyruvate dehydrogenase kinase (PDKs), which inactivate the mitochondrial pyruvate dehydrogenase complex and thereby reduce the flow of glucose-derived pyruvate into the tri-carboxylic acid (TCA) cycle. This reduction decreases the rate of oxidative phosphorylation and oxygen consumption, reinforcing the glycolytic phenotype and sparing oxygen under hypoxic conditions (Papandreou et al., 2006; Kim et al., 2006; Lu et al., 2008). In addition to its well-known role in controlling cell growth and proliferation, the oncogenic transcription factor MYC also has several effects on cell metabolism. Elevated expression of MYC has been shown to collaborate with HIF1 in the activation of many glucose transporters and glycolytic enzymes, such as lactate dehydrogenase A 8LDHA) and PDK1 (Kim et al., 2007; Dang et al., 2008).

- ***AMP-activated protein kinase:*** this protein is fundamental for the energy status of the cell. Biochemically AMPK opposes the effects of AKT1 and functions as a potent inhibitor of mTOR: functions as a checkpoint, regulating the cellular response to energy availability. During period of energetic stress, AMPK is activated and is responsible for shifting cells to an oxidative metabolic phenotype and inhibiting cell proliferation. For tumor cells is essential to overcome this checkpoint, in fact several oncogenic mutations and signaling pathways can suppress AMPK signaling, allowing them to divide under abnormal nutrient conditions.

- ***p53 and RAS:*** mutation of p53 is another common genetic event in human cancer, and this protein prevents tumor growth by suppressing metabolic pathways conducive to proliferation stressed or damaged cells. p53 promotes mitochondrial respiration by repressing glucose transporters, inhibiting the glycolytic enzyme phosphoglycerate mutase, and decreasing the activity of PFK-1 (Cheung & Voudsen, 2010; Levine & Puzio-Kuter, 2010). RAS, another oncogene widely implicated in human cancer, also promotes glucose metabolism by enhancing glucose uptake (Yun et al., 2009).
- ***Piruvate kinase:*** PK catalyzes the final step in glycolysis converting phosphoenolpyruvate (PEP) in pyruvate, with the production of ATP. Four PK isoform with different kinetic properties exist in mammals (L, R, M1, and M2). The L and R isoforms are expressed in the liver and red blood cells, respectively. The M1 is found in many adult tissues, whereas the M2 isoform take place in rapidly proliferating tissues (Mazurek et al., 2011). Many cancer cells exclusively express the M2 isoform of PK, and its presence is important for tumor growth (Mazurek et al., 2005; Christofk et al., 2008a). PKM2 is less active than PKM1, and inactivation of PKM2 seems to be mediated by phosphotyrosine-catalyzed release of FBP from the enzyme (Christofk et al., 2008b; Hitosugi et al., 2009). Cancer cells may revert to the highly regulated M2 isoform because it can switch between active and inactive forms, depending on different cell cycle phases or physiological contexts. In fact, active PK promotes oxidative phosphorylation, on the contrary, the inactive form would support increased biosynthesis during periods that require higher concentrations of triose phosphates for amino acid, lipid and nucleotide synthesis (Lunt & Vander Heiden, 2011).

However, glycolysis is not the major contributor of ATP in most cells: a compilation of data for 31 cancer cell lines/tissue from studies that determined oxidative ATP production (by measuring O_2 consumption) and glycolytic ATP production (by measuring lactate excretion) shows that the average percentage of ATP contribution from glycolysis is about 17%. Glycolytic ATP contributions are entirely dependent on cell context and cell/tissue type (Zu & Guppy, 2004). Cells exhibit different metabolic phenotypes depending on the growth environment and phase of the cell cycle. Moreover, recent data have highlighted limitations of the Warburg effect and offered compelling evidence for greater complexity within tumor metabolism. Dewhirst et al., has proposed that as the tumor grows, cancer cells establish metabolic symbiosis. a relationship between two population of metabolically distinct cancer cells (Sonveaux et al., 2008). In fact, during tumor growth, inefficient formation of blood vessels produces areas of normoxia and hypoxia within the tumor. Hypoxic cells generate two ATP per mole of glucose via glycolysis, while oxygenated cells

can produce a greater number of ATP from oxidative phosphorylation. Hypoxic cells import glucose via GLUT1 and generate two molecule of pyruvate from one of glucose: in this conditions PDH is inhibited by PDK and LDH-5 converts pyruvate to lactate. Lactate from hypoxic cells is exported through monocarboxylate transporter 4 (MCT4). Oxygenated cells import lactate via MCT-1, and convert the lactate back to pyruvate, via LDH-1 (Bonuccelli et al., 2010; Migneco et al., 2010). Lisanti has described both in breast and colon cancer that this "metabolic cooperation" could also occur between tumor cells and stromal fibroblast (Pavlides et al., 2009). Similarly, macrophages infiltrating tumors could act as lactate suppliers, and T lymphocytes undergo a metabolic switch upon activation, using mainly glycolysis for ATP needs, even in presence of oxygen (Kubota et al., 1992; Roiniotis et al., 2009; Jones & Thompson, 2011).

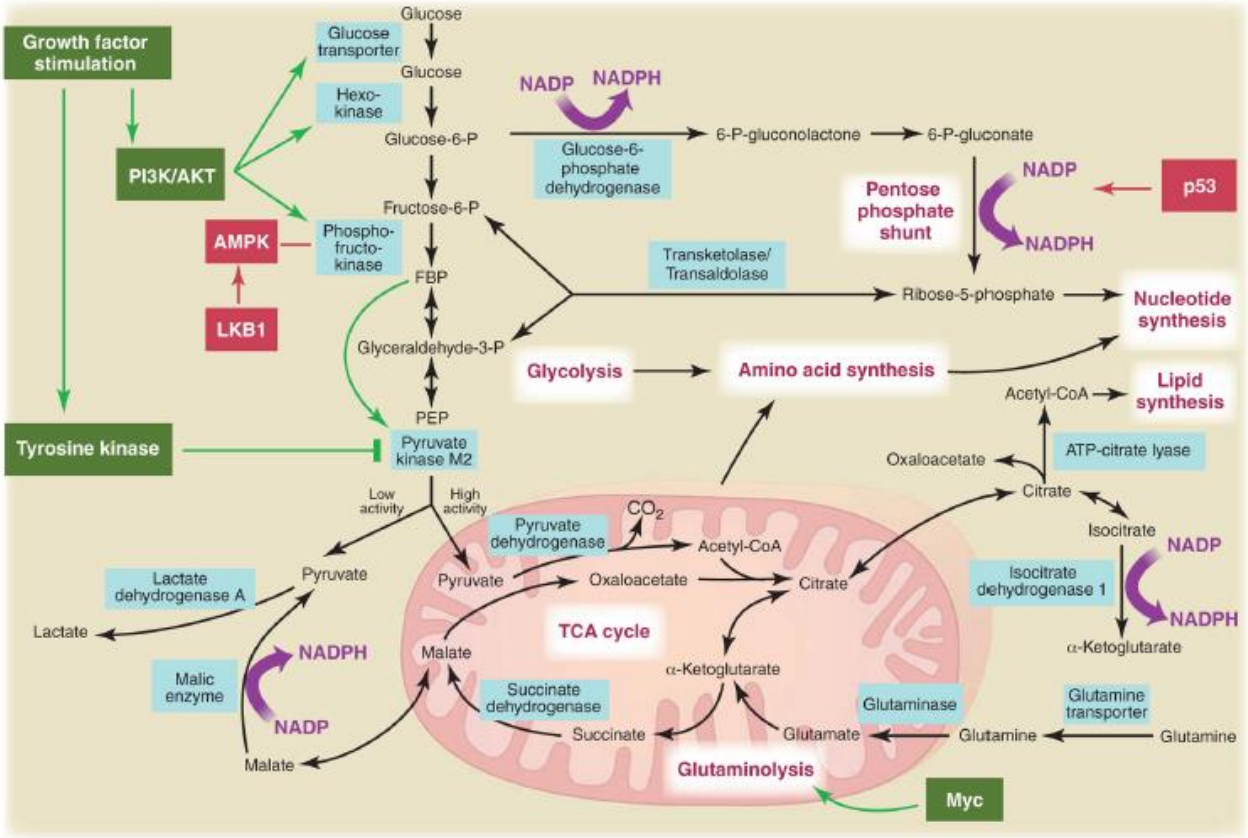


Fig. 9: Schematic representation of our current understanding of how glycolysis, oxidative phosphorylation, the pentose phosphate pathway, and glutamine metabolism are interconnected in proliferating cells (Vander Heiden et al., 2009).

3. LMW-PTP and cancer

Phosphorylation and dephosphorylation processes are finely regulated, considering their involvement in crucial mechanism inside cells. In fact any deregulation of this balance leads to the development of several pathologies (Ventura et al., 2006). There are various studies associating genetic polymorphisms of *ACP1* gene with different diseases, such as systemic lupus erythematosus (Esteves et al., 2008), obesity-related hypertension (Apelt et al., 2009), hypertension (Da Silva et al., 2006) and cancer (Alho et al., 2013; Spina et al., 2008). In previous data was reported a positive association between the *fast* isoform of *ACP1* gene and human cancers, mainly at the level of the uterine cervix and breast (Alho et al., 2013). Currently it is unclear if the differential expression of the two isoforms, due to the polymorphic *ACP1* gene, can have different roles in carcinogenesis and tumor progression, or if different types of cancer cells express different level of LMW-PTP isoforms. There is a clear need to evaluate the different stages of tumor progression and metastatization, in order to understand the relationship between these polymorphisms and the pathophysiology of the disease; for this reason, in vitro studies were conducted in order to better understand the underlying mechanisms. Chiarugi et al., have shown that, while LMW-PTP negatively regulates the proliferation mediated by growth factors in NIH3T3 mouse embryonic fibroblast cultures, in animal models the protein acts as a positive regulator for tumor onset and growth. These authors also show that, despite the fact that these cells overexpressing LMW-PTP present a greater in vitro adhesion and mobility, their in vivo engraft does not lead to metastases (Chiarugi et al., 2004). Malentacchi et al., evaluated the levels of expression of LMW-PTP mRNA, in different human carcinomas: breast, colon, lung, and a group of neuroblastoma samples as examples of neuroendocrine cancer. The results strongly suggest a common model, by which an increased expression of LMW-PTP, indifferently as regards the isoform, is observed in many tumor samples, especially in breast and colon but not in lung cancers. LMW-PTP protein content was in agreement with the observed increase of mRNA, confirming that the overexpression of LMW-PTP mRNA leads to overproduction of LMW-PTP protein. Based on these results, the authors conclude that the *ACP1* gene can be considered an oncogene (Malentacchi et al., 2005). Marzocchini et al. confirmed these data in rat model treated with dimethyl-hydrazine (Marzocchini et al., 2008). This overexpression of LMW-PTP in different types of cancer is consistently found in cancer cells that have high levels of un-phosphorylated Ephrin A2 receptor (EphA2). This receptor has been largely associated with tumors, and its expression levels on tumor cells correlate with the degeee of tumor malignancy (Kinch & Carles-Kinch, 2003). Kikawa et al., in 2002 demonstrated that LMW-PTP

can regulate the expression of EphA2, the function or both and, in fact, the overexpression of LMW-PTP in metastatic cancer cells causes the dephosphorylation and up-regulation of EphA2 (Kikawa et al., 2002; Walker-Daniels et al., 2003). EphA2 elevated levels are observed in some types of cancers, such as breast, prostate and aggressive melanomas. This overexpression seems to have an important role in the regulation of adherens junctions, being important in cell-cell adhesion processes, and recent studies have reported the destabilization of adherens junctions through overespression of EphA2 in breast tumors. Fang et al., have shown that EphA2 overespression does not affect the phosphorylation status of E-cadherin, p120 or catenins, but seems to result in the up-regulation of RhoA GTPase activity via p190RhoGAP and LMW-PTP (Fang et al., 2008). From all the known molecules apparently regulated by LMW-PTP, EphA2 seems to be the most closely involved in the tumorigenic processes. The action of LMW-PTP on EphA2n highlights the fact that PTPs are not just suppressors of tyrosine phosphorylation-dependent signaling: they act as specific regulators of signaling pathways (Alho et al., 2013). Many other protein have been identify as LMW-PTP substrates***: p190RhoGAP*** (Fang et al., 2008; Chiarugi et al., 2000), ***β-catenin*** (Taddei et al., 2002), ***Focal Adhesion Kinase*** (FAK) (Rigacci et al., 2002; Abdelsaid et al., 2012), ***Janus Kinase*** (JAK) (Lee et al., 2007), and signal transducer and activator of transcription ***STAT*** (Rigacci et al., 2003; Rigacci et al., 2008). LMW-PTP inhibits JAK, thus stimulating STAT1/STAT3 and promoting survival pathways via inhibition of apoptosis: moreover inhibits FAK, decreasing the number of focal adhesion. The fast isoform dephosphprylates and inhibits p190RhoGAP and consequently potentiates Rho action which, through E-cadherin, destabilizes adherens junctions. An important molecule in this processes is Src: the kinase can be activated by ROS, and so associates with the fast isoform of LMW-PTP. This association induces tyrosine phosphorylation and activation of LMW-PTP, which promotes Src activation. Src is able to inhibit the association between E-cadherin and β-catenin, promoting β-catenin signaling action and consequently increasing the expression of mesenchymal proteins, enhancing migration and decreasing cell adhesion (Alho et al., 2013). Hoekstra et al., studied the effect of LMW-PTP in two different type of cancer cells: colorectal cancer (CRC) and prostatic cancer (PCa). In the first one they observed that LMW-PTP expression is not only significantly increased in CRC, but also follows a step-wise increase in different levels of dysplasia. Chemical inhibition of LMW-PTP significantly reduces CRC growth, and its down-regulation leads to a reduced migration ability and sensitization to the chemotherapeutic agent 5-FU. This study shows that LMWPTP is not only overexpressed in colorectal cancer, but it is correlated with the malignant potential of this cancer (Hoekstra et al., 2015). In the second study, on prostatic carcinoma, they show that the expression of LMW-PTP is increased in the primary tumors, which leads to a worse clinical outcome. Moreover the

overexpression of the phosphatase affects cytoskeletal signaling, increases anoikis resistance, and enhances migratory potential. Considering all these data, the authors proposed that LMW-PTP could potentially be used as biomarker and a future treatment target both for for CRC and PCa (Ruela-de-Sousa et al., 2015).

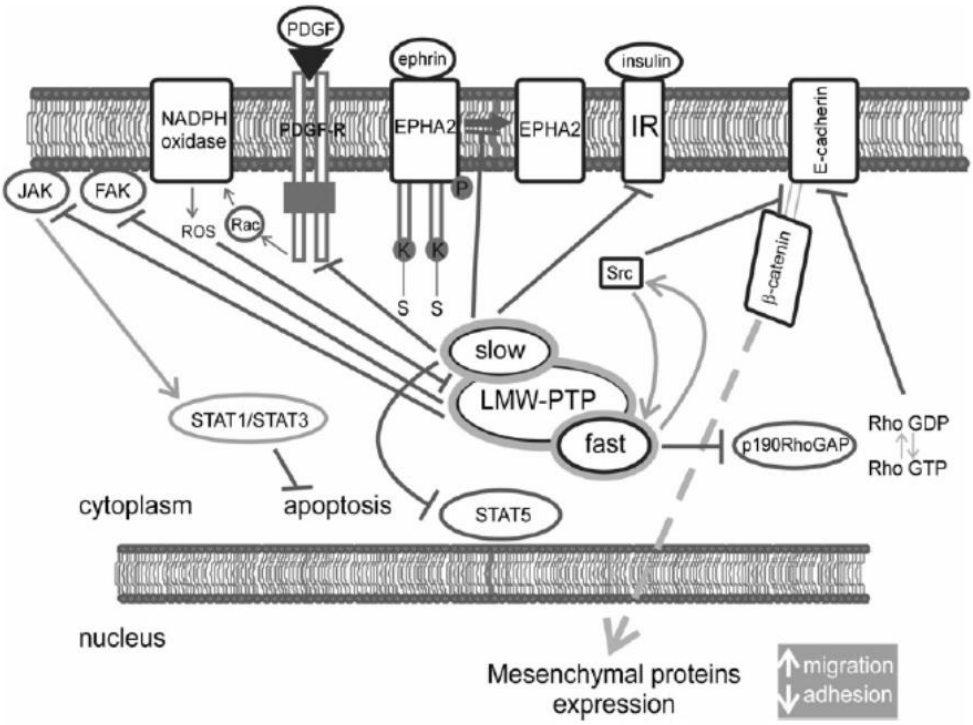


Fig. 10: Interactions between LMW-PTP and cancer-associated molecules. LMW-PTP inhibits important molecules that may be involved in cancer processes 8Alho et al., 2013).

3.1 Drug therapy of cancer: chemotherapy

Currently the pharmacological therapy for the treatment of cancer is *chemotherapy*: the administration of cytotoxic or antineoplastic drugs that inhibit the proliferation of neoplastic cells. This is divided into neoadjuvant and adjuvant chemotherapy:

- ***Neoadjuvant chemotherapy***: it is used before surgical treatment and radiotherapy of the primary tumor in order to decrease the size of the tumor mass and to destroy any micrometastases.
- ***Adjuvant chemotherapy***: it is used after surgery to increase the chances of recovery, to destroy neoplastic foci residues and avoid relapses.

Furthermore there are also a *mono-chemotherapy* based on the use of only one type of drug (until it loses its effectiveness and is replaced with another), and a *poly-chemotherapy* that uses multiple drugs with different characteristics, with the purpose to delay as much as possible the chemoresistance onset. The choice of the best drug to use is closely related to several factors such as location, size, isotype, tumor stage, patient's clinical characteristics (age, gender), and most importantly, the phase of the cell cycle, with a distinction between:

- ***phase specific antineoplastic drugs:*** active in a specific phase of the cell cycle;
- ***phase a-specific antineoplastic drugs:*** drugs active at any stage of the cell cycle;
- ***cycle specific antineoplastic drugs:*** active mainly in the proliferative phase.

Among the most important and used antineoplastic drugs there are: alkylating agents, anti-metabolites, inhibitors of the mitotic spindle, topoisomerase II inhibitors.

Alkylating agents exert their higher cytotoxic action on DNA causing structural and functional alterations. They are active on the proliferating cells in the G1-S phase, blocking the cell cycle and causing the death of cells in G2 phase. We can distinguish mono and polifunctional alkylating agents: the second cause reactions of alkylation at the level of DNA bases, favoring the formation of bridges or crosslinks between the two helixes, which will be unable to separate during the duplication and transcription processes. Some polifunctional agents are: cisplatin, carboplatin and oxalilplatin. The first, instead, bind to DNA destabilizing the guanines, favoring their loss, leading to helixes fragmentation. ***Antimetabolites*** have a chemical structure similar to the normal

metabolites, inhibiting various enzymatic reactions of purines and pyrimidines. Generally they act in S-phase of the cell cycle, hindering the synthesis of DNA and RNA. Antimetabolites can be further divided into folic acid antagonists (Methotrexate), purine analogs (6-mercaptopurine) and pyrimidine analogs (5-Fluorouracile). ***Inhibitors of the mitotic spindle*** have affinity for tubulin and other proteins of the spindle microtubules, thus inhibiting their formation. Some examples are: vinka alcaloids (vinblastine) and taxans (docetaxel). The ***topoisomerase II inhibitors***, such as antraciclin, intercalate between adjacent bases of DNA, inhibiting the enzyme normally involved in helixes opening. Through this mechanism they promote breakage and fragmentation of DNA, with consequently G2 stoppage and release of free radicals oxygen.

3.2 Drug-resistance mechanism

Drug resistance towards antineoplastic agents is the main obstacle to the clinical effectiveness of chemotherapy treatment. This resistance, namely the lack of capacity of a drug to cause toxicity and thus cell death, can be classify in:

- ***Innate chemoresistance:*** refers to tumors that before being treated are already resistant. It is assumed that this process is derived from random cytogenetic changes, creating resistant cells (Skeel, 1999).
- ***Acquired chemoresistance:*** it occurs after exposure to the drug. It may be due to several factors such as the cell cycle kinetics, biochemical or pharmacological causes (Goldie & Coldman, 1979).

Recently "*Mechanisms of Chemoresistance " (MOC)* were divided into five main groups, based on the causes of this process:

- **MOC-1:** deficiency of drug intracellular transport system leading to an uptake reduction of the drug (*MOC-1a*) or increase of its extrusion (*MOC1-b*); alterations of the binding between the drug and protein transporters.
- **MOC**-2: decreased drug intracellular activation because of a deficiency of the enzymes that metabolise it.

- **MOC-3**: increase in drug inactivation mechanisms linked to the intervention of antimetabolites that inhibit the active metabolite and increased detoxification of free radicals by glutathione s-transferase.
- **MOC-4:** increased cancer cell's ability to repair DNA damage caused by the drug (Marin et al., 2010).
- **MOC-5**: alteration of the genes that regulate apoptosis. For example there is an overexpression of the anti-apoptotic Bcl-2 gene *(MOC-5a)*, and p53 mutation that instead promotes apoptotis (*MOC-5b*).

Two recents studies address the role of LMW-PTP in cancer treatment: one is based on the chemoresistance of cancer cells and the potential of LMW-PTP to be involved in this mechanism (Ferreira et al., 2012); the other considered LMW-PTP as an emerging target for the design of novel therapeutic agents (Maccari & Ottanà, 2012). Ferreira et al., showed that LMW-PTP seems to be involved in Multi Drug Resistance (MDR) development through Src and Bcr-Abl. LMW-PTP knock-down in a leukemia resistant cell line completely revert the sensitivity to vincristine and imatinib treatment, followed by a decrease of phosphorylation in the active sites of Src and Bcr-Abl, thus inactivating both kinases. On the contrary, if it is performed an overexpression of LMW-PTP in the non-resistant cell line, this lead to the acquisition of the resistant phenotype and cells appear to be less sensitive to the treatments (Ferreira et al., 2012). Moreover, Maccari and Ottanà highlight the importance of developing new compounds that are isoform specific, given that selective inhibitors of LMW-PTP isoforms are not only considered of medical interest but can also be useful to further investigate the molecules, pathways, and cellular mechanisms in which these proteins are involved, both in physiological and pathological processes (Maccari & Ottanà, 2012).

MATERIAL AND METHODS

Cell culture and transfections.

A375 melanoma cells and DU145 prostate cancer cells were purchased from ATCC (Manassas, USA) and cultured in Dulbecco's Modified Eagles Medium (DMEM, Sigma Aldrich, St. Louis, USA), supplemented with 10% Fetal Bovine Serum ,100 U/ml penicillin, 100 mg/ml streptomycin (Euroclone), and propagated at 37°C in a 5% CO_2 humidified atmosphere. A375 cells ($1x10^5$ cells/mL) were grown for 24h and then transiently transfected with LMW-PTP siRNA (Target sequence CCCATAGTGCACACTTGTATA), using Hiperfect Trafection Reagent (Qiagen) according to the manufacturer's instructions. Briefly the cells were trasfected for 24, 48 or 72h with siRNA at final concentration 20nM. To test the specificity of LMW-PTP transfection, control cells were transfected with a Scramble Sequence (AllStars Negative Control siRNA, final concentration 20nM, Qiagen).

DU145 cells transiently transfected with pRcCMV-C12S-LMW-PTP expressing the dominant-negative (dn) Cys-12 to Ser mutant of LMW-PTP (Chiarugi et al., 1995). Briefly, 10 μgr of plasmidic DNA was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 28h cells were lysed and the efficiency of transfection were assessed by Western Blotting.

Western Blotting

Cells were lysed on iced in 1X Laemli Buffer (0,5 M TrisHCl pH 6,8, 10% SDS, 20% Glicerol, β-Mercaptoetanol, 0,1% bromophenol blue) and samples were boiled for 10 minutes. Cell extracts were resolved by SDS-PAGE and transferred to PVDF membranes (BioRad). Membranes were incubated overnight at 4°C with the appropriate primary antibody: rabbit polyclonal anti-LMW-PTP were produced in our laboratory, Bcl-2, Bim, Caspase3, Annexin A1, PKM2, TIM, GAPDH,

Enolase, GLUT-1, HesokinaseII and Actin were obtained from Santa Cruz Biotechnology. Monoclonal anti-caveolin-1 (pY14) phospho-specific antibody and rabbit polyclonal anti-caveolin-1 were purchased from BD Transduction Laboratories (Lexington, KY, USA). MG132 were purchased from CalbioChem. After washing in TPBS-Tween 20 (0,1%) membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1h. Proteins were detected using Clarity Western ECL (Biorad) by UVP.

Sample preparation and 2-D gel electrophoresis

Cells ($3x10^5$) were transfected with LMW-PTP siRNA. After 28h cells were lysed on ice in RIPA buffer (50mM TrisHCl pH 7,5, 150 mM NaCl, 100 mM NaF, 2 mM EGTA, 1% Triton X-100, 10µL/ml Protease and Phosphatase inhibitor, Sigma). Protein extracts were clarified by centrifugation at 8000 g, 4 °C for 15 min; then they were precipitated overnight with two volume of cold acetone (-20°C) for 2h. Dry pellets were dissolved in 8M Urea, 4% (w/v) 3-cholamidopropyl dimethylammonium-1-propane sulfonate (CHAPS), 50 mM dithioerythritol (DTE). Isoelectric focusing (IEF) was carried out on nonlinear wide-range immobilized pH gradient (pH 3–10; 18-cm-long IPG strip; Bio-Rad) and achieved using an Ettan IPGphor™ system (GE Healthcare). Protein sample was cup-loaded near the anode in the Ettan IPGphor Cup Loading Manifold™ (GE Healthcare) after the rehydration of the IPG strips with 350 µL of rehydration solution (8 M urea, 2% (w/v) CHAPS, 0.5% (w/v) DTE) supplemented with 0.5% (v/v) carrier ampholyte (Bio-Rad) and a trace of bromophenol blue, overnight at room temperature. The strips were focused at 16 °C according to the following electrical conditions: 200 V for 1 h, 300 V for 1 h, from 300 to 3500 V in 30 min, 3500 V for 4 h, 5000 for 2 h, from 5000 to 8000 V in 30 min, and 8000 V until a total of 100,000 V/h was reached, with a limiting current of 50 µA/strip. After focusing, strips were equilibrated in 6 M urea, 2% (w/v) SDS, 2% (w/v) DTE, 30% (v/v) glycerol and 0.05 M Tris–HCl pH 6.8 for 10 min and, subsequently, for 10 min in the same urea/SDS/Tris–HCl buffer solution where DTE was substituted with 2.5% (w/v) iodoacetamide (IA). he equilibrated strips were placed on top of 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) and embedded in 0.5% (w/v) heated low-melting agarose in SDS electrophoresis running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). SDS-PAGE was performed in a PROTEAN II xi cell gel electrophoresis unit (Bio-Rad) at 10 °C and at 40 mA/gel constant current, until the dye front reached the bottom of the gel, according to Hochstrasser et al. (D.F. Hochstrasser, M.G. Harrington,

A.C. Hochstrasser, M.J. Miller, C.R. Merril Methods for increasing the resolution of two-dimensional protein electrophoresis Anal Biochem, 173 (1988), pp. 424–435). Gels were stained with colloidal Coomassie blue silver (G. Candiano, M. Bruschi, L. Musante, L. Santucci, G.M. Ghiggeri, B. Carnemolla, et al. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis Electrophoresis, 25 (2004), pp. 1327–1333).

Immuno and co-immunoprecipitation.

Cells ($3x10^5$) transfected with LMW-PTP siRNA or dn mutant. After 28h cells were lysed for 20 min on ice in 300 µL of RIPA buffer (50mM TrisHCl pH 7,5, 150 mM NaCl, 100 mM NaF, 2 mM EGTA, 1% Triton X-100, 10µL/ml Protease and Phosphatase inhibitor, Sigma). Lysates were centrifugated at 4°C, 14000 rpm for 15 min: supernatants were collected. After protein quantification with Bradford Assay, 400 ugr of proteins were immunoprecipitated overnight, at 4°C with the specific primary antibodies (1:100). Then Protein A/G PLUS-Agarose (Santa Cruz) was added and incubated at 4°C for 1h. Immunocomplexes were collected and analyzed by western blotting.

Glucose uptake

Cells ($1,8 X 10^6$) were seeded in 35 mm dishes and transfected with siRNA. After 28h 1 µL /ml µCi/mL (U-^{14}C)deoxy-D-Glucose (PerkinElmer) was added and dishes were incubated for 30 min at 37°C. Then cells were washed twice with cold PBS and lysed with 0,1M NaOH. The amount of (U-^{14}C)deoxy-D-Glucose incorporated was evaluated by a scintillator analyzer.

Cell viability assay

$2x10^4$ cells were seeded in 24 wells plate: after 24, 48 or 72h 5mM MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added and incubated for 2h at 37°C. Cells were suspended in 200 µL of Dimethyl sulfoxide: wavelength measuring was performed at 595 nm using a spectrophotometer.

Cell cycle analysis

Cells were stained with Sodiumcitrate 0,1% containing Propidiumiodide 50μgr/mL and NP 40 0,1% and measured by citofluorimetric analysis. Cell cycle distributions were analyzed by *ModFit*.

Apoptosis evaluation

Apoptosis was determined using Annexin-V-FLUOS Staining kit from Roche according to manufacturer's instructions. Briefly, $1x10^6$ cells were collected, washed and incubated with 100μL of Annexin-V-FLUOS labelling solution, for 10-15 min at room temperature. Cells were analyzed by flow cytometry BDFACS Canto.

Colony Formation Assay

Briefly cells were seeded in six well plate and treated with Morin or transfected with siRNA at the indicated concentration. After 24h cells were detached and re-plated 1000 cells/well and incubated at 37°C for 1-3 weeks. Subsequently cells were fixed and stained with a solution containing 1% Crystal Violet and 20% Methanol. Colonies were counted and photographed using Nikon Digital SIGHT.

Plating Efficiency and Surviving Factor were extrapolated using the following formulas:

PE= n° of colonies/n° of cells seeded X 100

SF= n° of colonies/n° of cells seeded X PE

Cell detachment assay

Confluent monolayers of cells were washed with PBS and incubated in calcium and magnesium free PBS containing 0,05% Trypsin , for 10 min at 37°C. Then 0,5 mg/ml soybean trypsin inhibitor was added and cells were incubated at 37 for 5 minutes: the dishes were washed twice with PBS. Attached cells were quantify with Cristal Violet Staining. Briefly, cells were incubated for 5 min at

37°C with Cristal Violet: fixed cells were washed with PBS and solubilized with 0,1 M Sodium Citrate, pH 4,2. The assorbance was evaluated at 595 nm.

Wound Healing Assay

Cells were seeded in 6 well plates and grown to confluence: a scratch wound were made with a pipette tip. After washing the cells, the dishes were incubated in starvation medium: photos were taken at 0, 24, 48 and 72h, using Nikon Digital SIGHT and analyzed by NIS Elements Imaging Software.

MDR transporter activity

Briefly, $2x10^4$ cells/ml were seeded in 24 well plates: 1µL/ml of Fluorescein isotiocianate (Sigma) were added. Cells were incubated in the dark at 37°C for 3h. After 3 washing with PBS, cells were lysed with 1 ml of H_2O bidistillate. The fluorescence at 488 nm was evaluated with a Fluorimeter.

Adhesion assay

$3x10^5$ cells/well were seeded in 6 well plates: after siRNA transfection o Morin treatment at the indicated doses, cells were detached and plated again in 35 mm dishes. At 120, 150 and 180 minutes the number of adherent cells was evaluated with Cristal Violet Staining.

Gelatin zimography

Culture medium was collected: 25 µl was added to 4X Laemli sample Buffer, without β-mercaptoetanol and run on a 7,5% SDS gel containing 1mg/ml gelatine. After electrophoresis the gel was washed twice with 2,5% Triton X-100, and then with a Reaction Buffer (50 mM Tris-HCl pH 7,5, 200 mM NaCl, 5mM CaCl2). The gel was incubated overnight at 37°C in Reaction Buffer: then it was stained with Comassie Briliant Blue and destained with a 50% methanol and 10% acetic acid solution.

Oxygen Consumption

Cells were collected by centrifugation and re-suspended in starvation medium (10^6 cells/ml). Oxygen measurement were carry on using a Clark-type O_2 electrode from Hansatech. The instrument is first calibrated using water saturated with oxygen, according to manufacturer's instructions. Then, 1 ml of the medium containing the cells is added inside the chamber: the measurements were made after 15 min. nmol of oxygen consumed in function of time (minutes) were calculated by Oxygraph software. The values were normalized on cells number and protein concentration.

Lactate secretion

Measurement of Lactate production was determined using L-Lactic Acid (L-Lactate) from Megazyme, according to manufacturer's instruction. Briefly medium culture were collected and incubated in a buffer containing NAD+, D-GTP, and L-LDH. Measurements were done at 340 nm.

Boyden Invasion Assay

Invasion assay was performed with $1x10^5$ cells on 8-μm-pore Transwell (Corning) coated with 50ug/cm2 of Matrigel for 16h. Invading cells were stained with Diff Quick (Medion Diagnostic). Chemotaxis was evaluated by counting the cells migrated to the lower surface of the filters (six randomly chosen fields).

RESULTS

Experimental part I

1. LMW-PTP is highly expressed in cancer cells

As already assessed, high level of LMW-PTP expression can be observed in different tumor tissues such as breast cancer, CRC and prostate (Malentacchi et al., 2005; Hoekstra et al., 2015). Moreover, at least in CRC and prostate cancer, expression level positively correlates with tumor aggressively (Malentacchi et al., 2005; Marzocchini et al., 2008; Ruela-de-Sousa et al., 2016). In this study we focused our researches on human melanoma cell line, A375 for two different reasons: the first one is that malignant melanoma is one of the most aggressive types of skin cancer with a poor response to therapies, and an high rate of drug-resistance

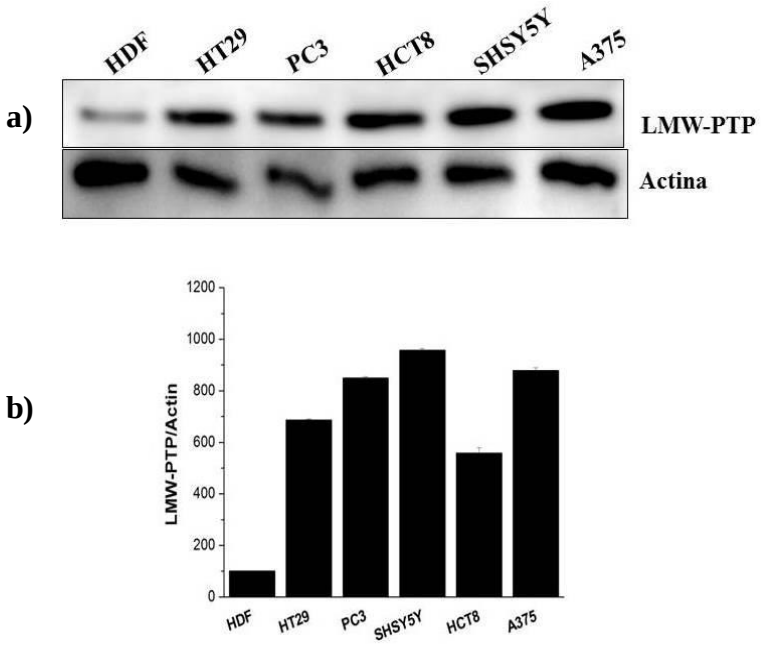


Fig.1 LMW-PTP expression level in different tumor cell lines: a) whole cell extract of many cancer cells were analyzed by western blot, and compared to a normal and few proliferating cell line, HDF. **b)** Densitometric evaluation of LMW-PTP; Actin was used as internal control.

The second one is due to the fact that there are not informations on the correlation between melanoma and LMW-PTP: therefore it will be useful to characterize the role of the phosphatase also in this kind of tumor. First, we evaluated the expression levels of LMW-PTP in A375 Melanoma cells, and several other cell lines, in comparison with Human Dermal Fibroblast (HDF). In Fig.1 we show that while in HDF cells we can hardly detect LMW-PTP, this phosphatase is highly expressed in A375 cells. Expression level is comparable to that of other tumor cell lines of different origins, such as PC3 (from prostate carcinoma), HT29 and HCT8 (from colon carcinoma) and SHSY5Y (neuroblastoma). In order to better study the role of LMW-PTP in tumor onset, we transiently silenced the ACP1 gene, using a specific siRNA: in this way we are able to analyzed possible changing of different phenotypes involved in carcinogenesis. Fig.2 shows that between 24 and 48h after siRNA transfection (20nM) we obtained the maximum level of silencing. On the basis of this result all the following experiment have been performed 24h after transfection.

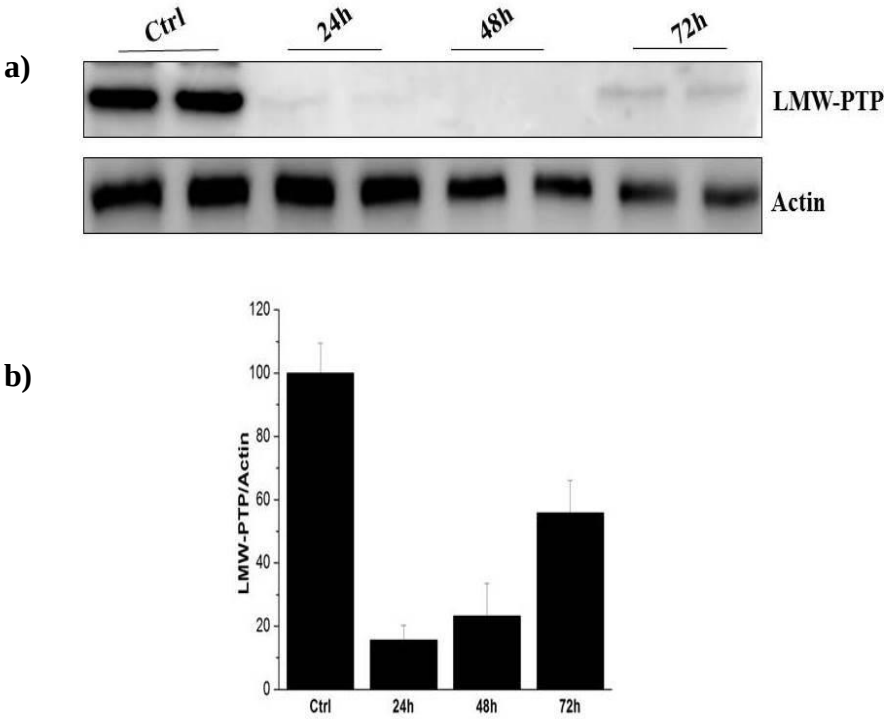


Fig.2 Time course analysis: a) Melanoma cells were transfected with specific siRNA. LMW-PTP level was analyzed by western blot at 24h, 48h and 72h**. b)** Densitometric analysis of LMW-PTP, normalized on Actin.

2. LMW-PTP overexpression induces resistance to apoptosis.

There are several indication that LMW-PTP may affect apoptosis and chemoresistance. (Chiarugi et al., 2004; Ferreira et al., 2012; Hoekstra et al., 2015). We decided to test this parameter is visible in this cell lines too. We evaluated apoptosis rate in two conditions: after siRNA transfection alone, and combining it with a chemoterapic agent treatment, such as 5FU. A375 cells treated with 20μM 5-FU are quite resistant to 5-FU treatment, showing only a little increase in the apoptotic rate. LMW-PTP knocking-down alone has some effects in enhancing apoptosis, but the same treatment on cells in which LMW-PTP has been silenced show a dramatic increase of the apoptotic rate (Fig.3).

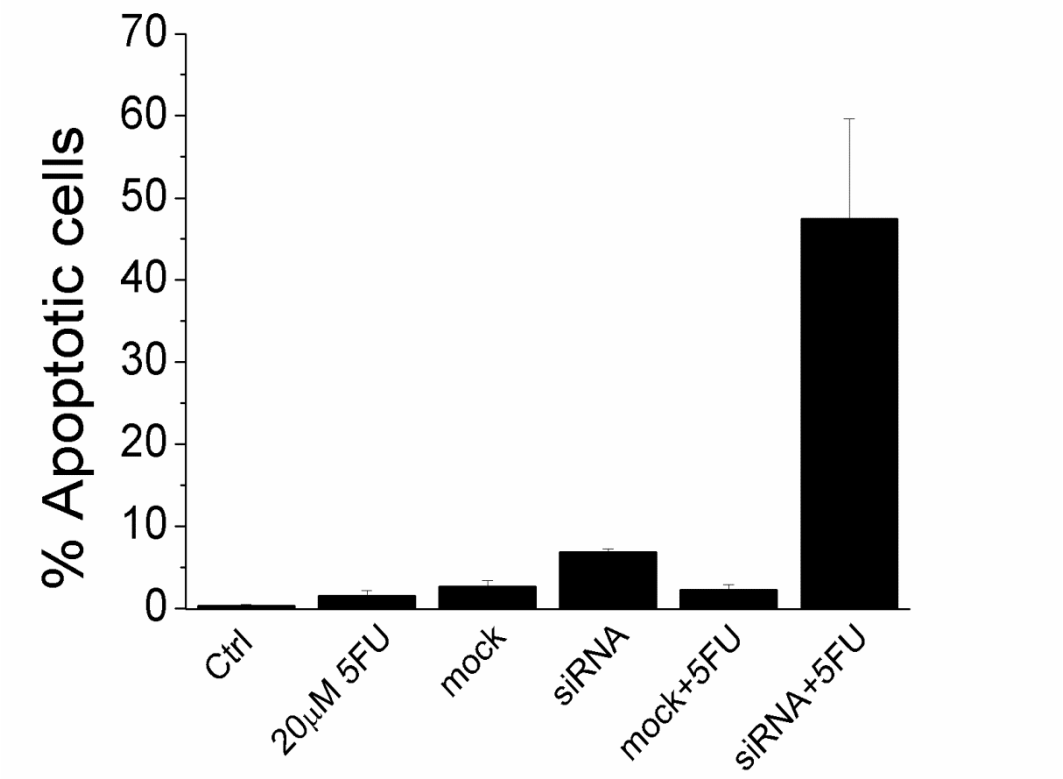


Fig.3 LMW-PTP influences apoptotic response of melanoma cells: FACS analysis with PI/Annexin V staining on A375 cells silenced for LMW-PTP and treated with 20μM 5FU causes an high apoptotic response. Viability of cells treated only with 5FU was not significantly affected by drug treatment.

2.1 LMW-PTP overexpression influences apoptotic markers.

In order to study in depth the effect of LMW-PTP on chemo-resistance and apoptosis, we analyzed the expression level of some apoptosis markers such as, Bcl2 and Caspase3. Fig.4 show how the expression of the active form of Caspase3 is not detectable in untreated cells. On the contrary, samples where the phosphatase is knocked-down showed an increase of this protein levels: obviously the activation of Caspase3 is even greater where there is the treatment with 5FU. Notably, the level of the inactive form of the Caspase (Pro-Caspase3) is comparable in all samples. Then we evaluated the levels of the anti-apoptotic protein: Bcl-2. Western Blot analysis demonstrated that, in control sample, this protein is present at high levels. When LMW-PTP is silenced its expression start to decrease, becoming quite undetectable when cells are even treated with 5FU. The pro-apoptotic Bim showed a similar behavior: in transfected cells, with or without 5FU treatment, its expression levels were increased respect to control samples.

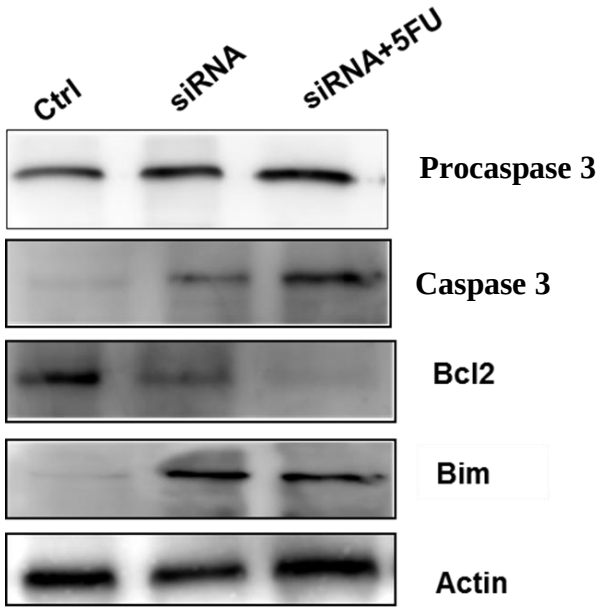


Fig.4 LMW-PTP is involved in pro and anti-apoptotic proteins balance: A375 cells were transfected with siRNA and co-treated with 20μM 5-FU. After 24 h, total protein was extracted for western-blot analysis. The relative expression level of Bcl-2, Bim, Caspase-3 from each independent experiments are shown; Actin was used as internal control. The alteration of pro-apoptotic proteins level are well visible in samples transfected with siRNA, promoting chemosensivity.

2.2 LMW-PTP may act on apoptosis through Caveolin-1 dephosphorylation.

Caselli et al., have shown that Caveolin-1, a membrane protein, is direct substrate of LMW-PTP: in fact it dephosphorylate Caveolin-1 in the residue Tyr14 (Caselli et al., 2007). Moreover Shajahan et al., demonstrated that Caveolin-1 forms a complex with JNK, activating it, and leading to Bcl2 phosphorylation and consequent inactivation. Using a mutant enable to be phosphorylated in Tyr14, they demonstrated that the cells can prevents apoptosis. Then these researchers proposed that the phosphorylation on Tyr 14 is an essential element in response to chemotherapy treatment (Shajahan et al., 2012). Therefore, considering our results, where LMW-PTP down-regulation seems to sensitize Melanoma cells towards apoptosis, almost in part through Bcl-2 de-regulation, we investigated Cav-1/Bcl2 pathway. In order to verify this hypothesis, we analyzed the levels of Cav-1 phosphorylation in LMW-PTP silenced cells. Western blot analyses revealed that down-regulation of LMW-PTP causes a strong increase of Cav-1 phosphorylation on tyrosine 14 residue (Fig. 5). We suggested that this phosphatase, through its interaction with Caveolin-1, may be involved in apoptosis resistance. In fact, the over-expression of LMW-PTP, causes the dephosphorylation on Tyr14 of Caveolin-1 which in turn, can't interact with JNK, and degrade Bcl2 and BCLXL. This pathway may represent at least one way by which LMW-PTP overexpression in tumor cells induces resistance to chemotherapy , with a decreased susceptibility to programmed cell death.

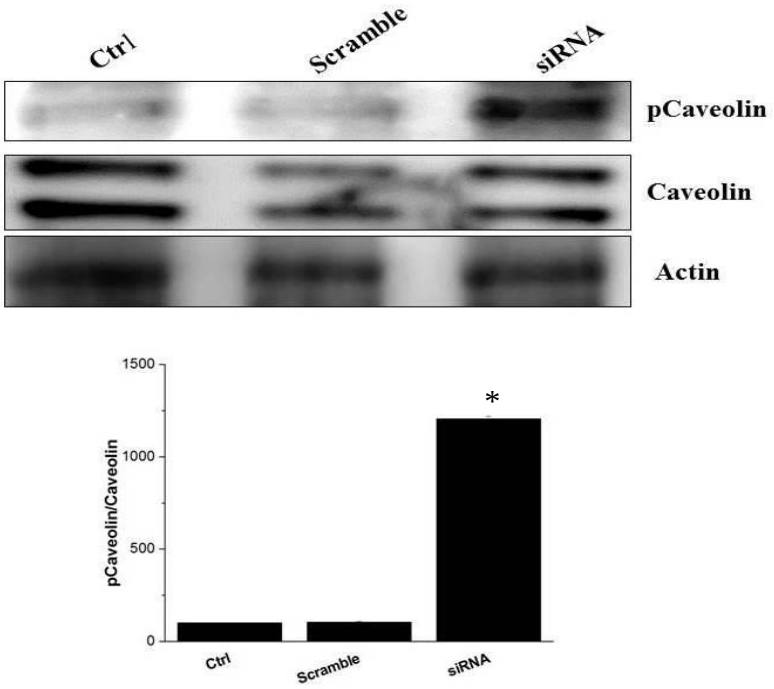


Fig.5 Caveolin-1 is a direct substrate of LMW-PTP: after 24h from siRNA transfection, cells were lysed and level of pCaveolin were analyzed. The phosphorylation on Tyr residue is higher in samples where LMW-PTP was knocked-down, confirming an interaction with Caveolin-1.
*p<0,05

2.3 LMW-PTP overexpression induces chemoresistance

Multiple drug resistance (MDR) describes a phenomenon whereby resistance to one drug is accompanied by resistance to drugs whose structures and mechanisms of action may be completely different: nowadays is a major factor in the failure of many forms of chemotherapy (Bolhuis et al., 1997). This resistance can develop by numerous mechanisms including decreased drug uptake, increased drug efflux, activation of detoxifying systems, activation of DNA repair mechanisms, evasion of drug-induced apoptosis. Resistance to therapy has been correlated to the presence of at least two molecular "pumps" in tumor-cell membranes that actively expel chemotherapy drugs from the interior. This allows tumor cells to avoid the toxic effects of the drug or molecular processes within the nucleus or the cytoplasm. The two pumps commonly found to confer chemo-resistance in cancer are P-glycoprotein and the so-called multidrug resistance–associated protein (MRP). Because of their function and importance, they are the targets of several anticancer efforts (Gillet & Gottesman; 2010). To investigate whether LMW-PTP exert its chemoresistance action through the membrane transporters, we performed a test measuring the activity of these proteins. We used

Fluorescein, a compound that enter inside cells using the same ABC transporters of chemoterapics. In this way it was possible to highlight the activity of the proteins that cause extrusion of anticancer molecules. Results are shown in Fig.6: the percentage of fluorescence inside silenced cells is higher than that of control sample, suggesting that when the phosphatase is knocked-down there is much more retention of the drug inside the cells, due to the fact that ABC transporters activity is decreased.

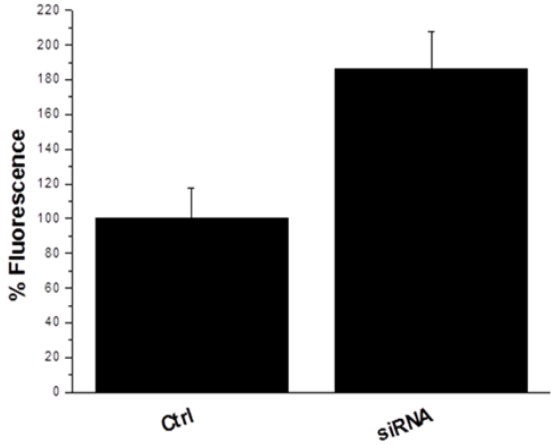


Fig.6 LMW-PTP silencing affect MDR activity: A375 cells were incubated for 3h with Fluorescein, and then lysed. The amount of fluorescein incorporated by the cells was quantify using a Fluorimeter. Silenced cells accumulate more compound than the control, suggesting a possible role of LMW-PTP through these transporters.

2.4 LMW-PTP overexpression influences radioresistance.

Another important aspect of tumor cells is the acquisition of radio-resistance, a characteristic that can heavily influence the success of anti-tumoral treatments. This phenotype is particularly important for melanoma, since for this kind of tumor radiotherapy represent, together with surgery, the major therapeutic tool. For this reason we performed experiments in order to assess a possible sensitization towards radiation, through LMW-PTP silencing. A375 cells were transiently transfected and treated with a 2 Gray irradiation (1Gy = 100 rad), a doses normally used in melanoma therapy. After 48h we evaluated cells viability (Fig.7b): control cells do not show any damages after the treatment. In contrast silenced samples show a reduction of viability, around 50%.

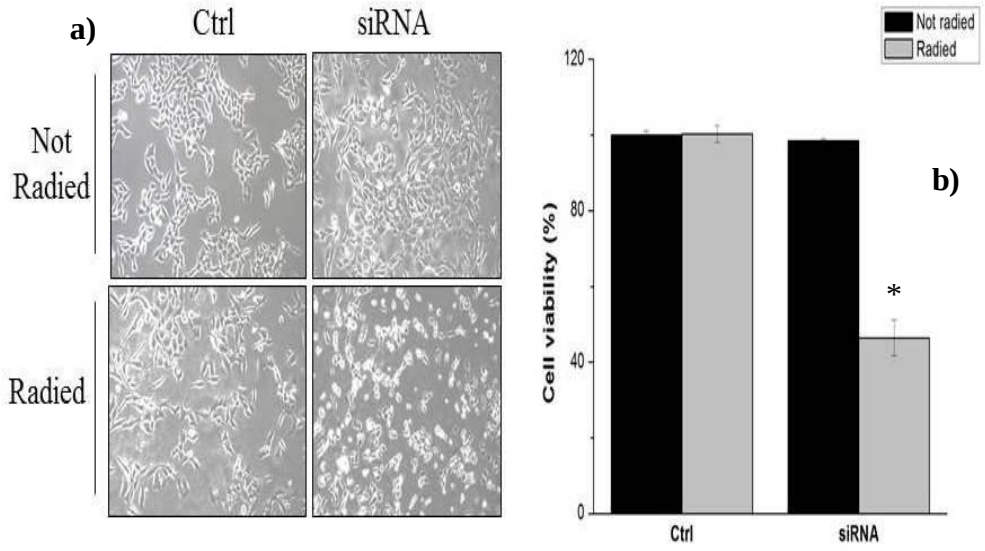


Fig.7 LMW-PTP sensitize melanoma cells towards irradiation: cells were radiated with 2Gy. After 48h cells were photographed **(a)** and viability analyzed with MTT test **(b).** *p<0,05

2.5 LMW-PTP overexpression influences self-renewal capability.

Self-renewal capability is one the most important properties of cancer stem cells. For this reason we investigated the ability of A375 melanoma cells to form colony after LMW-PTP silencing, performing a Colony Formation Assay (CFA). Through this test is possible to analyze the effectiveness of a treatment (chemo or radiotherapy) to eliminate the cells with the more aggressive characteristics. Fig.8 shows how cells silenced show a phenotype very similar to the control: cells are able to re-form colonies after 10 days. Cells treated with 5FU form colonies, but with a particular morphology: the colonies are bigger than the other, probably because the treatment select the more aggressive cells. In contrast, LMW-PTP knocking-down in co-treatment with 5-FU, clearly prevents the formation of new colonies, suggesting that in these conditions cells are losing some characteristics of self- renewal, fundamental for metastasis. Considering that this kind of

experiment is useful to test also radiotherapic efficacy, we repeat it incubating cells with 2 Gy (Fig.9). Similar to the results obtained with 5FU treatment, radiation exposition lead to the selection of colonies with a different morphology: interestingly, the association with LMW-PTP silencing decreases the number of colonies from 150 (control) to 20. This results show that the sensitization effect of LMW-PTP silencing is more effective when combined with drug treatment, respect to the irradiation.

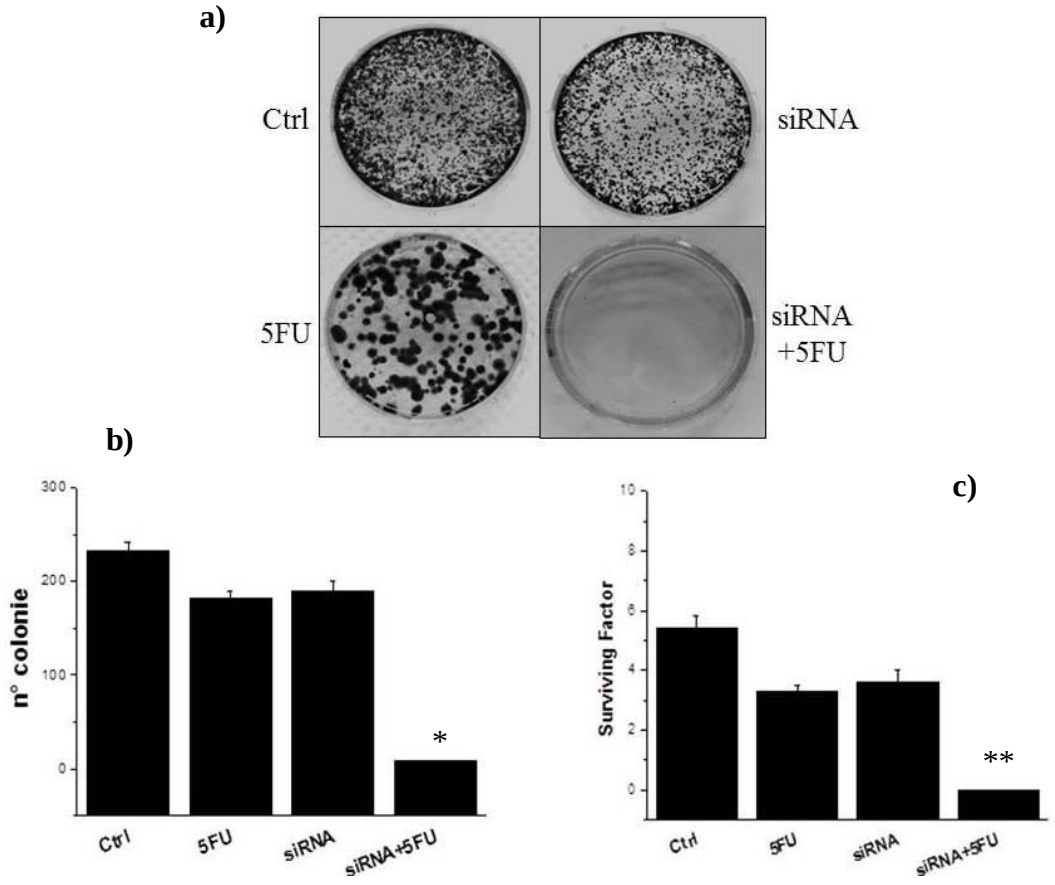


Fig.8.1 LMW-PTP is involved in self-renewal traits acquisition: a) after transfection and/or 5FU treatment the ability to form new colonies was analyzed. Images were taken and *n° of colonies* and *Surviving Factor* were determined **b)** and **c)** (see Methods). * and **p<0,05

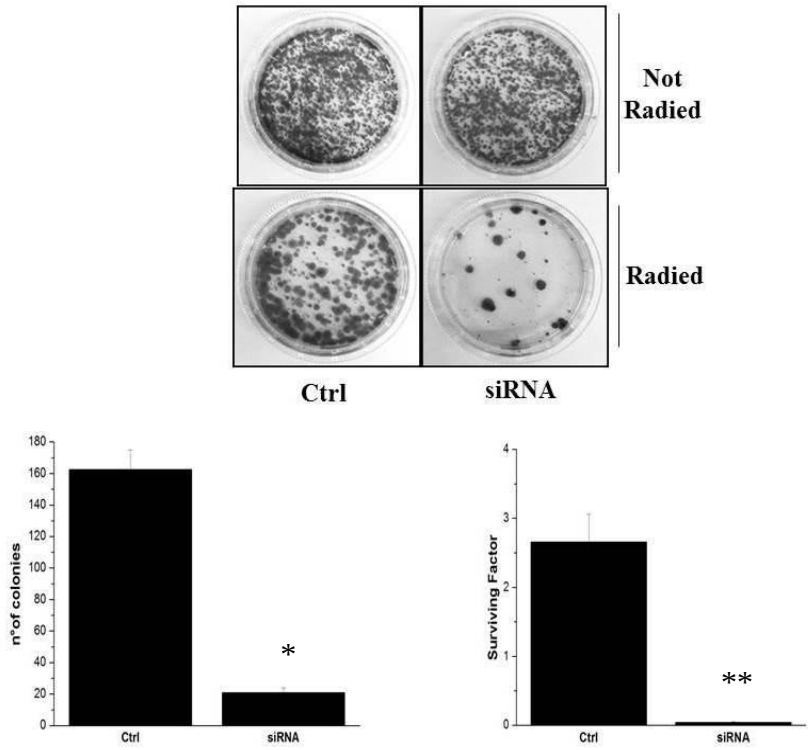


Fig. 8.2 LMW-PTP is involved in self-renewal traits acquisition: CFA was performed after 2Gy exposition, showing similar results. * and **p<0,05

3. LMW-PTP overexpression influences cell migration and invasiveness.

Considering the high capability of Melanoma cells to migrate and invade, becoming very aggressive, we investigated the possible role of LMW-PTP in this phenotype. Confluent dishes of A375 cells were scratched using a sterile pipet tip: the number of cells migrated into the wound were quantify after 12 and 20h. Cells where LMW-PTP was silenced showed a lower ability to move into the scratch respect to the control (Fig.9). To further investigated the effect of LMW-PTP silencing in cell migration we performed a Detachment Assay. Confluent monolayers of A375 cells (transfected or not) were incubated for 10 min at 37 °C with 0.05% trypsin. 0.5 mg/ml soybean trypsin inhibitor was added, and the cells were incubated at 37 °C for 5 min. Attached cells were evaluated by crystal violet staining. Fig.10a shows how PTP knock-down causes an increase of cells adhesion, mostly between 24 and 48h where the transfection effect is maximum. To confirm this results we performed also an Adhesion test: control and silenced cells were detached and plated

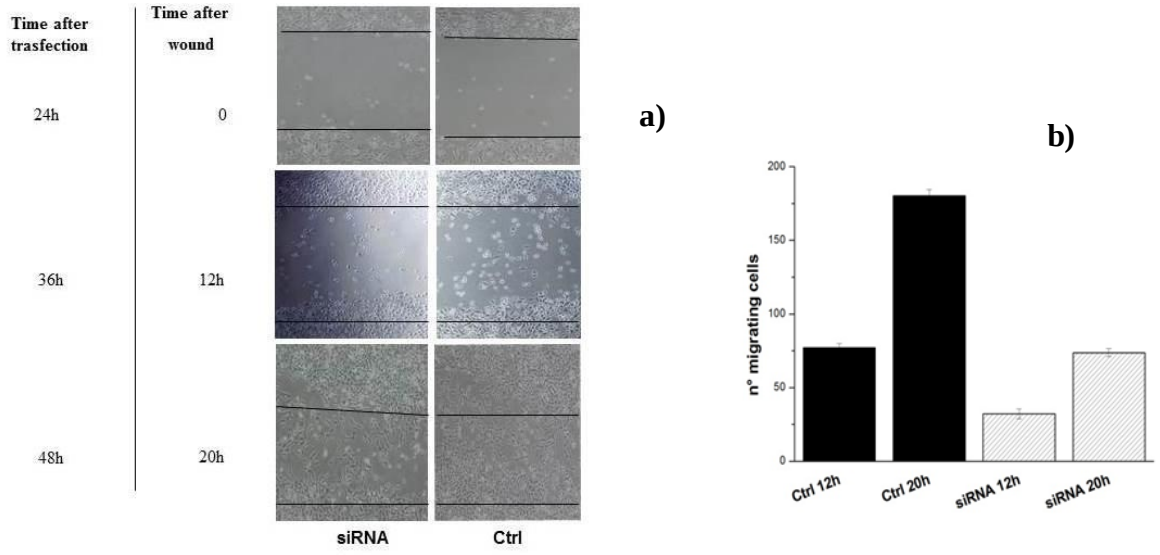


Fig.9 LMW-PTP affect cell migration: a) a small area of confluent cell monolayer have been disrupted by scratching a line through the layer, using a sterile tip. The open gap is then inspected microscopically over time as the cells move in and fill the damaged area. Images were acquired at t0, 12 and 20h after scratch. Silenced cells show a lower migratory ability respect to the control. **b)** Number of migrating cells have been calculated from morphometric analysis of static images shown in a). Data are representative of three independent experiments.

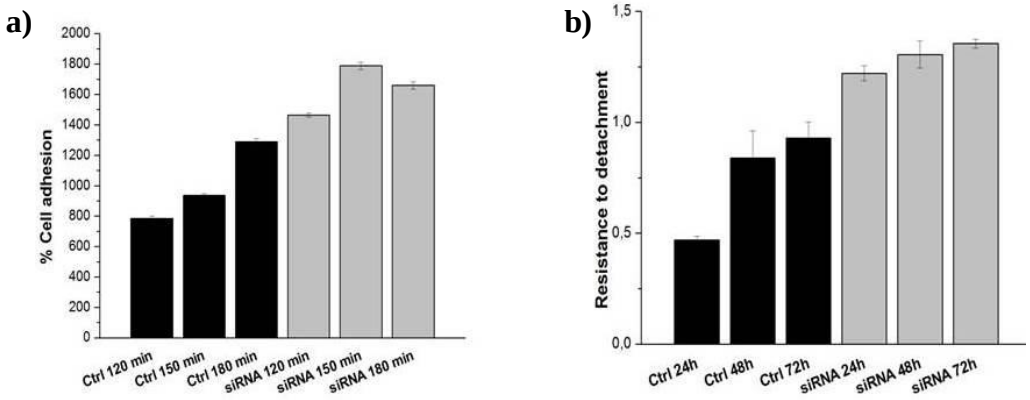


Fig.10 LMW-PTP and cell adhesion: a) To analyses the effect of siRNA transfection on the strenght cell adhesion a Detachment test was performed using trypsin on confluent monolayers of A375 cells. Value in ordinate represent the ratio between the cells attached after and before the treatment. Data show a clear difference between control and silenced samples. **b)** To confirm our results, we performed also an Adhesion Test. In agreement with our previous experiment, silenced cells are more able to establish connections.

again at the same time. After 120 min, 180 min and 150 min we evaluated the number of the cells in the dishes (Fig.10b).The results demonstrate how the phosphatase is involved in cell migration and focal adhesion formation, because silenced cells are able to form new adhesions already 120 minutes after from the detachment.

Then we evaluated the role of LMW-PTP in invasion: MMP secretion is one of the principal biomarker investigated for EMT. We tested cell ability to secret MMP after phosphatase knock-down by gelatin zimographic analysis. Fig xxx demonstrated how the level of MMP decreases when LMW-PTP is silenced. This result was confirmed by Invasion Assay using Boyden Chamber (Fig.11). Control cells are able to invade digesting matrigel. On the contrary when LMW-PTP is silenced, the invasiveness of cells is reduce about 50%.

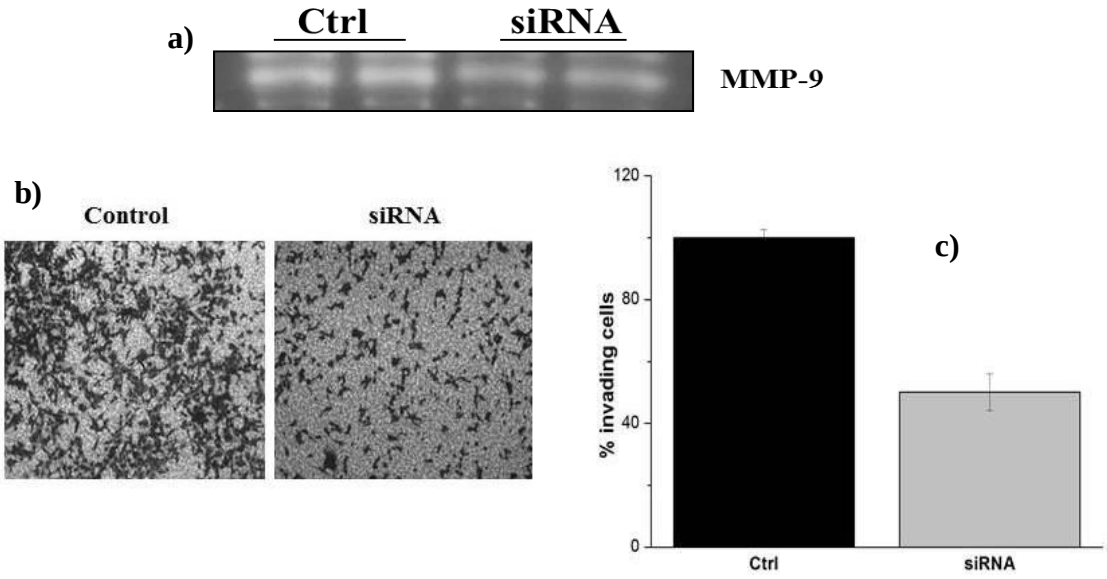


Fig.11 LMW-PTP and invasion: a) MMP secretion was evaluated by gelatin zimography. LMW-PTP knocking-down at 24h, lead to a decrease of MMP production. **b)** and **c)** Invasiveness was evaluated also with a Boyden Chamber Assay. Accordingly with zimography result, after PTP silencing, melanoma cells appear less able to digest matrigel, and then to invade.

4. Morin mimics the effects of LMW-PTP silencing upon 5FU treatment.

Previous works have demonstrated that Morin, a natural compound belonging to the Flavonoids family is an inhibitor of LMW-PTP (Paoli et al., 2013). Considering the crucial role of this phosphatase in tumorigenesis, and the phenotypes induced by its silencing, we decided to verify if the flavonoid is able to reproduce similar results. First we tested the effect of Morin co-treatment with 5FU. A375 cells were pretreated for 4h with 50μM Morin in starvation medium and then treated with 20μM 5-FU, for further 24h. Apoptotic cells were stained with Annexin V and Iodure Propide (PI). Fig.12 clearly shows that Morin treatment, in combination with chemotherapeutic administration is capable to induce apoptosis: co-treatment with 5-FU induces a strong increase in cell death percentage (up to 40%) with respect to the treatment with 5-FU alone (1,4 %). Morin treatment apparently replicate the effect observed upon LMW-PTP silencing. Interestingly, incubation with Morin alone didn't affect cells viability (0.4 % of apoptotic cells), indicating that the natural compound is not toxic *per se*.

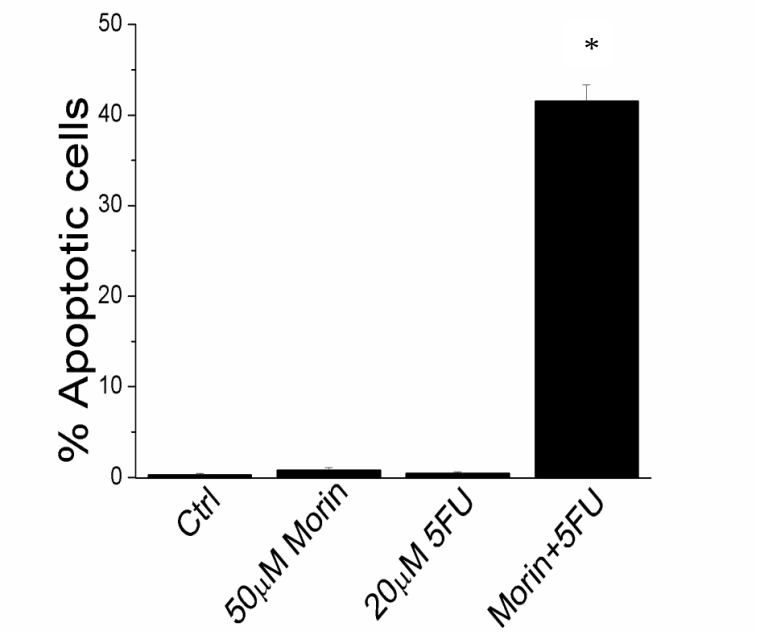


Fig.12 Morin sensitizes Melanoma cells to 5FU treatment: A375 cells were pretreated with 50 μM Morin for 4h and then exposed to chemotherapeutic agents for 24 h. Apoptosis was measured by PI/Annexin V staining: Morin treatment increases cyto-toxic effect of 5FU, respect to control. *p<0,05

It is important to assess whether co-treatment with Morin and a chemotherapeutic agent affect cell viability in non-tumoral cells. Differentiated mouse myoblast cells (C2C12) were chosen for this

assay. Cells were pre-treated for 4h with a Morin concentration spanning from 0.01, 0.1, 10, 50 and 100μM and then treated for 24 hours with 20μM 5-FU (Fig.13). Analysis for cell viability with MTT assay revealed that C2C12 cell sensitivity to this chemotherapeutic agent is very low and is not enhanced when cells are pre-treated with Morin, even at maximal concentration. These results suggest that the action of Morin on cancer cells may be selective.

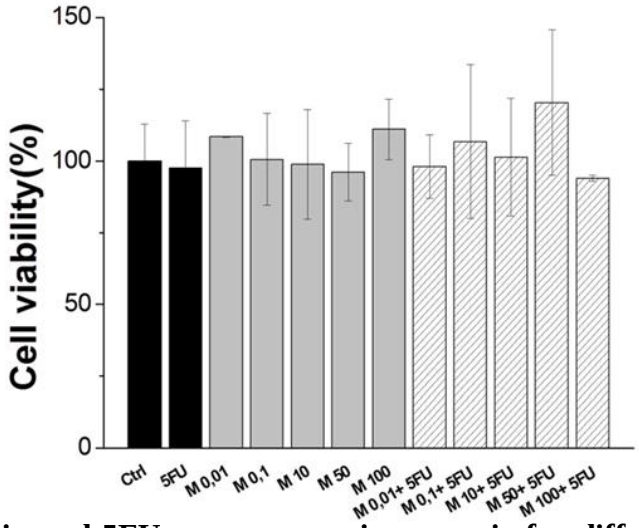


Fig.13 Morin and 5FU co-treatment is not toxic for differentiated cells: C2C12 myoblast cells were treated with different concentration of Morin, and then with 20μM 5FU. In differentiated cells there is no potentiating effect of Morin co-treatment.

5. Morin affect LMW-PTP stability.

To better assess the possible mechanisms responsible for these effects, we have investigated whether Morin plays a role in LMW-PTP stability in the cell. Melanoma cells were incubated for 4h in starvation medium with 50μM Morin, like in the previous experiment. Western blot analysis was carried out with the use of specific LMW-PTP antibodies. In these conditions we can observe a strong down-regulation of LMW-PTP content inside the cell (Fig.14). Time course analysis shows that this effect is very quick: LMW-PTP decrease is clearly detectable already after 1h of treatment

with Morin, with a minimum reached 4 hours after treatment. Seven hours after treatment, a recovery of the protein can be observed. A dose/response analysis was carried out using different Morin concentrations. A375 cells were incubated for 4h at the indicated Morin concentration (Fig.15a). Protein decrease can be clearly observed already using Morin at a concentration of 1μM. Such a sudden decrease suggests that the mechanism may be mediated by protein degradation more than through transcriptional or translational regulation. One of the main pathway in protein turnover involves digestion at proteasome.

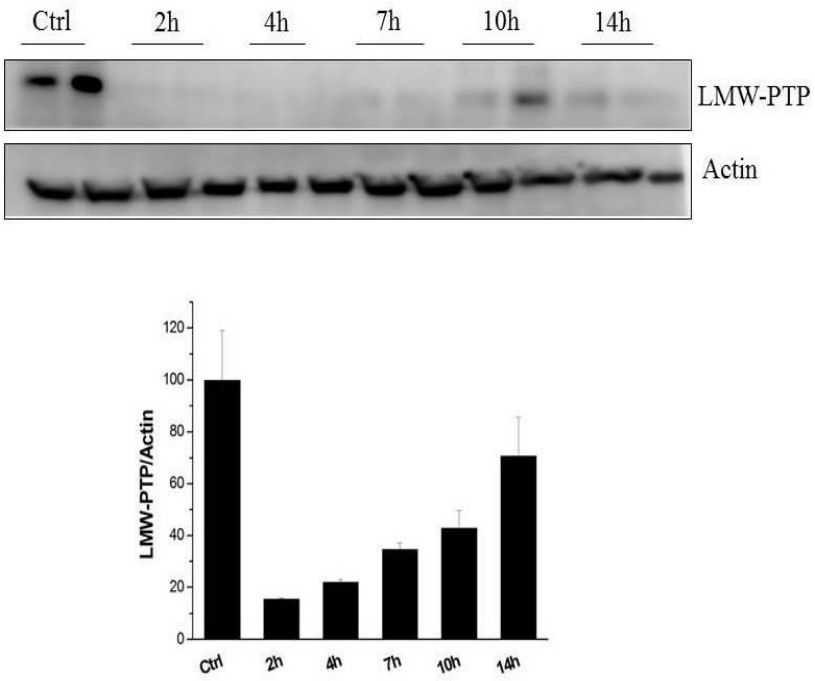


Fig.14 Morin treatment induce LMW-PTP down-regulation: A375 cells were incubated with 50μm Morin for the indicated time. Western Blot were carried on using specific antibodies; Actin was used as internal control.

In order to assess this point we used a specific proteasome inhibitor, namely MG132. Cells were pre-treated for 1h with 10μM MG132, and then with 50μM Morin for 4 additional hours. Fig.15b clearly shows that in presence of MG132 the level of LMW-PTP remain stable also upon Morin treatment, strongly suggesting that proteasome in actually involved in this mechanism.

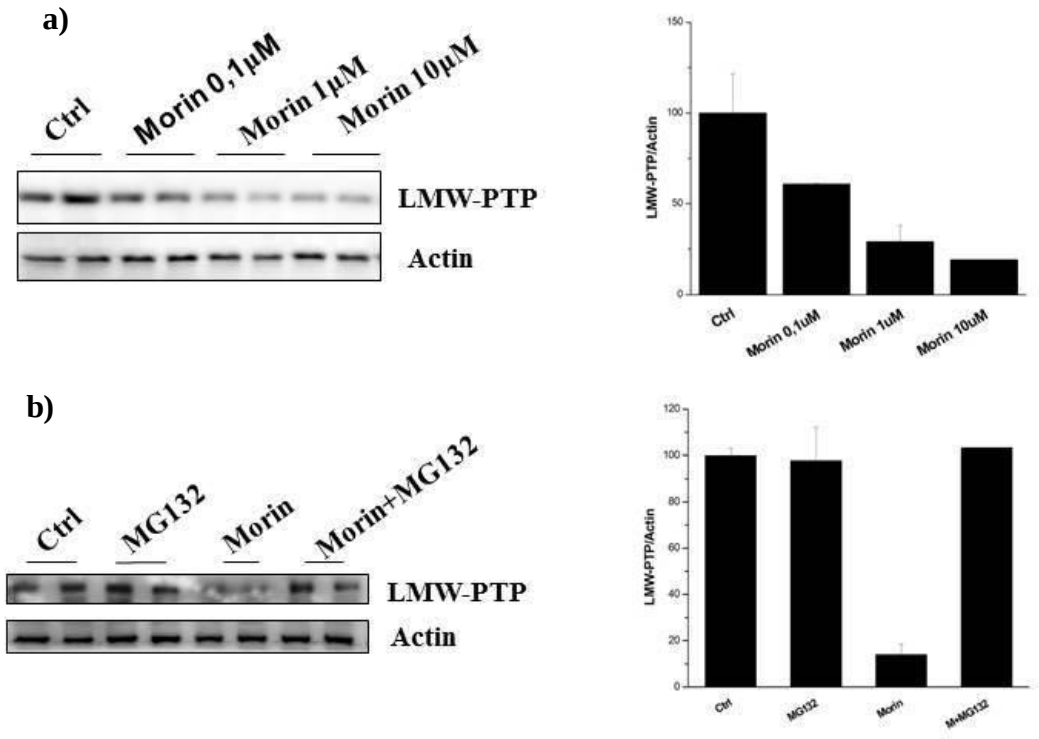


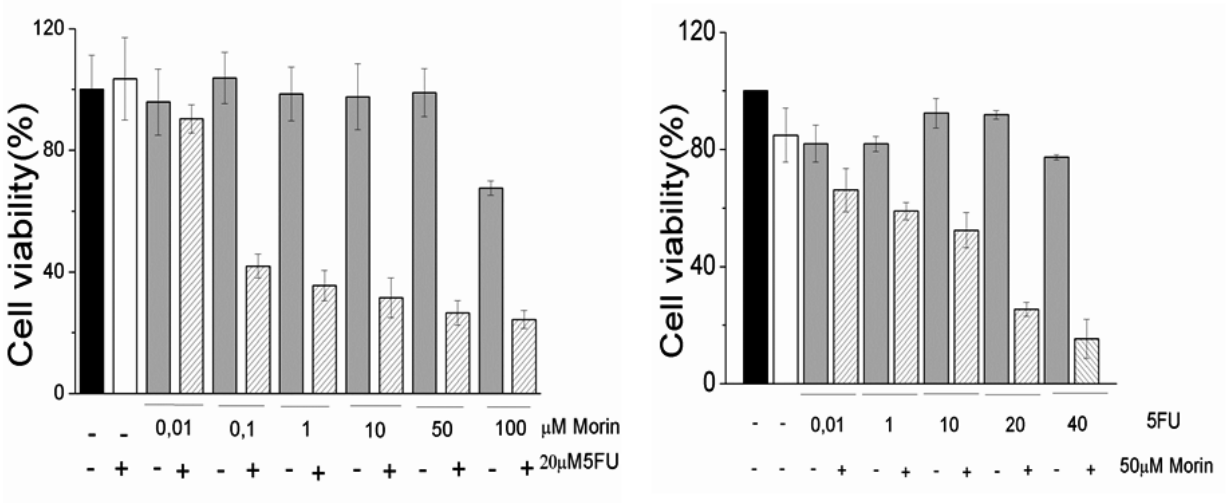
Fig.15 Morin acts at low concentration, through proteasome mechanism: a) LMW-PTP down-regulation is detectable also at very low doses, such as 1µM. **b)** Melanoma cells were pre-incubated with MG132, and then with 50µM Morin. When the inhibitor is present, there is no PTP degradation.

6. Morin in effective also at low concentration

Considering that Morin is capable to affect LMW-PTP protein level inside cell, even at 1nM concentration (Fig.15a), we wanted to assess whether such low concentration is still effective in enhancing the effects of a chemotherapeutical agents. Melanoma cells were incubated for 4h in starvation medium with Morin at the indicated concentration ranging from 0.001 to 100 µM. This pre-treatment was followed by 20µM 5-FU administration. After 24h cell viability was evaluated with MTT assay. Results (Fig.16a) indicate that Morin can enhance melanoma cells sensitivity to 5-FU treatment, in a dose dependent manner, being highly effective already at 1uM concentration. Samples treated only with Morin show a very limited decrease of cell viability only at the highest concentration (100µM): this effect is probably due to a cell cycle arrest, In fact a cell cycle analysis showed that after Morin treatment there is G2 arrest (fig. 16c). We also performed a dose/response experiment, in order to determine the minimal effective concentration of 5-FU in combination with Morin pretreatment. A375 cells were pre-treated with 50µM Morin for 4 hours. Different

concentration of 5-FU were then added (0.1, 1, 10, 20 and 40µM). Results (Fig.16b) indicate that while Melanoma cells are not affected by 5-FU treatment, even at the maximal concentration, co-treatment with Morin strongly enhances chemotherapic toxic effects, with a 5-FU concentration spanning from 0.1 to 40 µM. At higher concentration, after 24h, we can observe a cell survival around 20%.

a) **b)**



c)

Control	G1	G2	S
24h	50,3	21,6	28,1
48h	50,9	20,7	20,7
72h	47,3	28,6	24,1

Morin 50µM	G1	G2	S
24h	38,14	28,96	32,89
48h	61,82	11,9	26,2
72h	69,6	11,3	19,1

Fig.16 Morin pre-treatment sensitize Melanoma cells to 5FU: a) A375 cells were pre-incubated with different Morin concentration, then with 20µM 5FU, leading to cell death. The flavonoid alone is not toxic. **b)** Cells were incubated with 50µM Morin and treated with different concentration of 5FU. A reduction of cell viability is visible starting from 1µM. On the contrary 5FU does not damage melanoma cells. Cell viability was measured with MTT test. **c)** Cell cycle analysis of A375 melanoma cells: Morin treatment causes a G1 phase arrest.

7. Morin affect stemness of tumor cells.

Then we tested if Morin treatment and co-treatment with 5FU can reduce stemness of cancer cells, performing a Colony Formation Assay (Fig.17). Morin treatment alone didn't affect the ability of melanoma cells to form new colonies: the numbers of the colonies are similar to the control. Instead Morin co-treatment with 5FU, prevents colony formation, hindering that the more resistant and aggressive cells produce a new tumor clone.

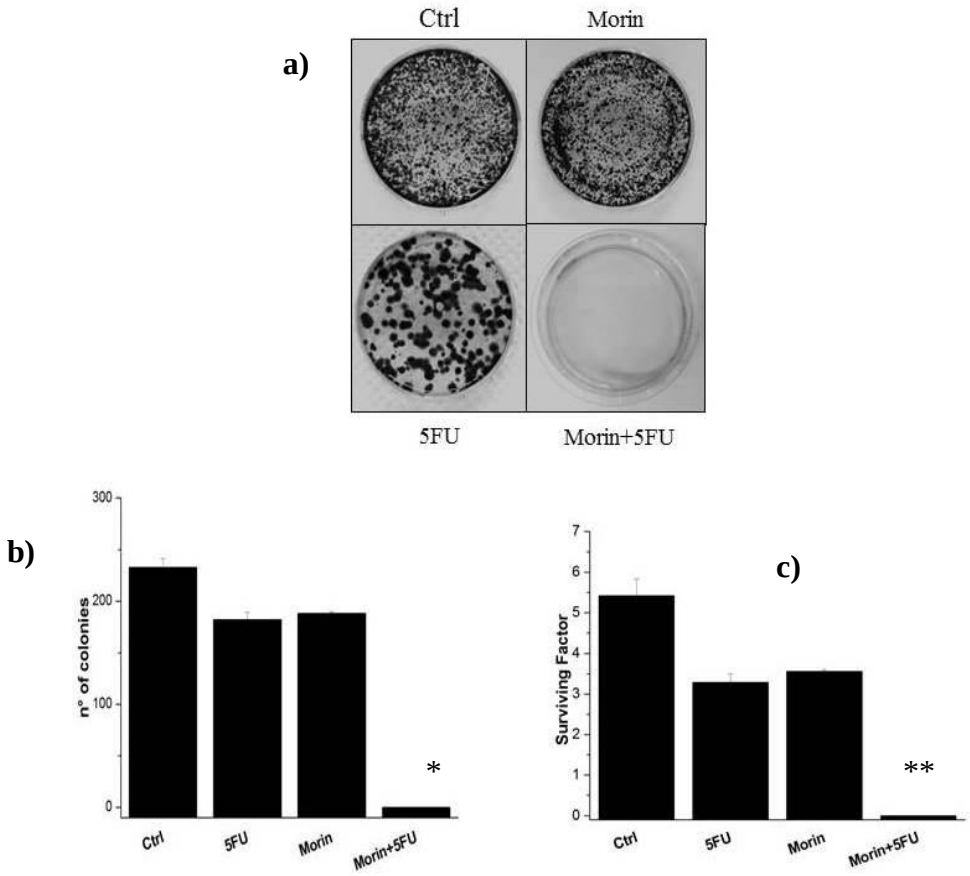


Fig.17 Morin can affect stemness of cancer cells: a) after Morin treatment and/or 5FU treatment the ability to form new colonies was analyzed. Images were taken and n° of colonies and Surviving Factor were determined, **b** and **c** (see Methods). Flavonoid and 5FU combination can affect stemness of cancer cells. * and **$p<0,05$

7.1 Morin sensitize Melanoma cells towards irradiation.

We repeated this test also irradiating cells (Fig.18). Similar to the results obtained with LMW-PTP silencing, the sensitizing action of phosphatase down-regulation is a little less, when the cells are exposed to radiation, respect to drug administration. Anyway Fig. 19a show that the ability of Morin at 2,5µM concentration, to sensitize A375 cells towards apoptosis after 2Gy exposition is very strong. Interestingly, as in the previous dose/response experiments, the effectiveness of Morin start from very low concentration, such as 1µM. Moreover we can obtain these results using different doses of irradiation (1, 2 Gy), commonly used in therapy, suggesting a possible application (Fig.19b).

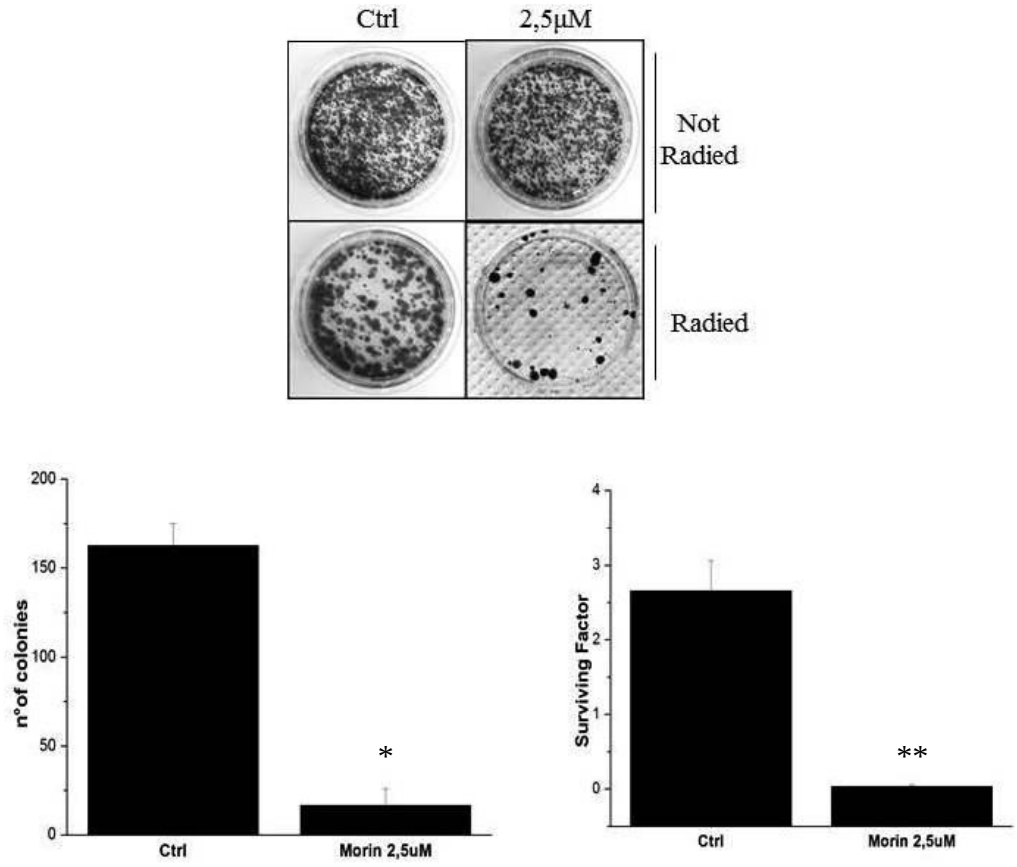


Fig.18 Morin can enhance irradiation damages, even at low concentrations: Colony Formation Assay was performed after 2Gy radiation. n° of colonies and Surviving Factor were calculated as described in Methods. Flavonoid pre-treatment is able to eliminate the more aggressive cells. * and **p<0,05

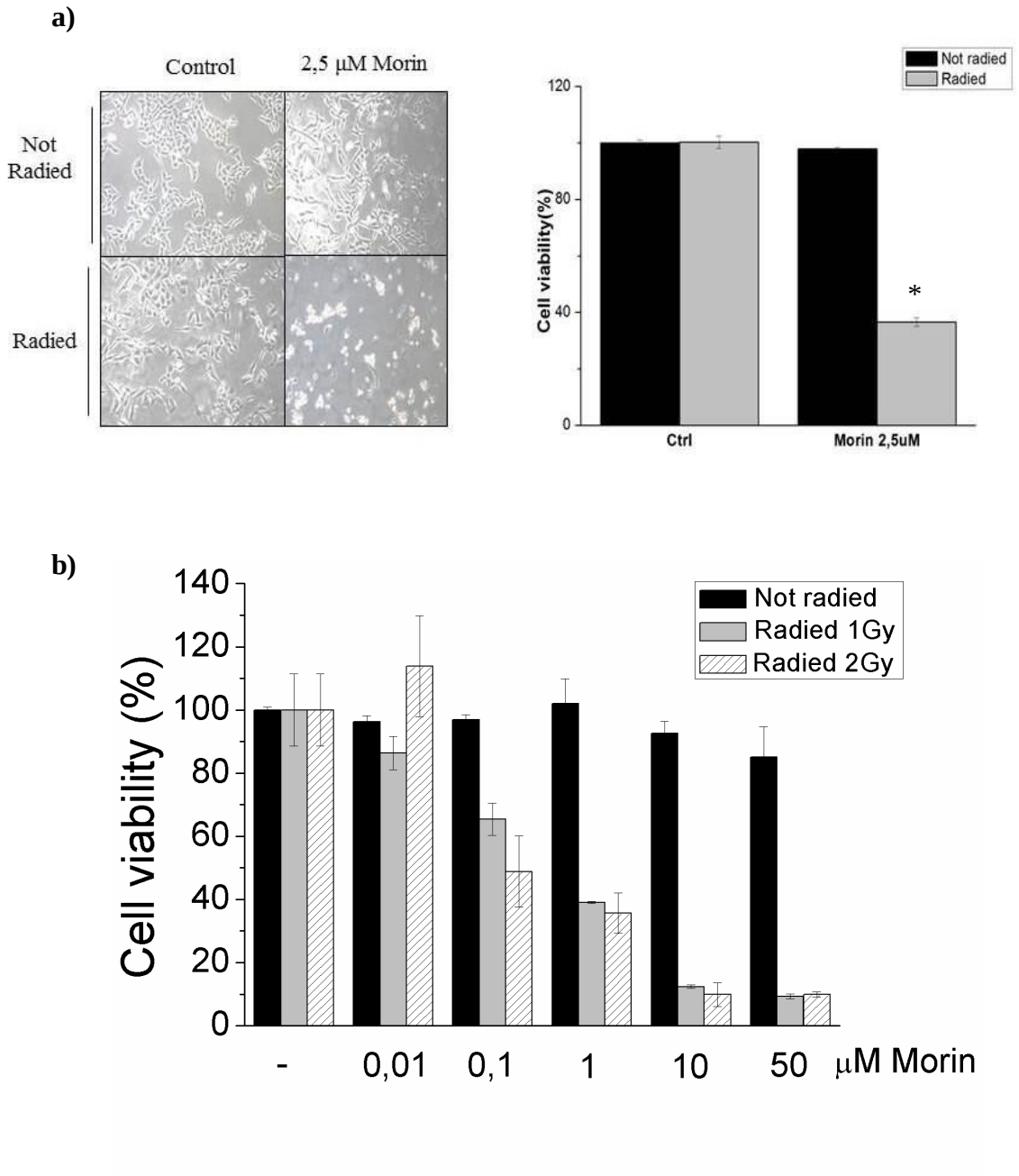


Fig. 19 Morin radio-sensitization is effective even at low concentration **a)** A375 cells were radiated with 2Gy: after 48h cell images were taken and viability was evaluated with Cristal Violet staining. Images show how Morin is able to sensitize cells to radiation. **b)** A375 cells were incubated with different Morin concentration, and then treated with 1Gy or 2Gy. Using therapeutically doses of irradiation and flavonoid administration, cells viability is drastically reduced. *p<0,05

8. Morin mimics the effects of LMW-PTP on cell migration.

Finally we verify the effect of Morin treatment, towards migratory phenotype. Confluent dishes of A375 cells were treated with 50μM Morin in starvation medium; then a scratch was made using a sterile pipet tip: the number of cells migrated into the wound were quantify after 24, 48 and 72h. As shown in Fig.20 cells treated with Morin are less able to refill the scratch: in fact the number of migrated cells after 24, 48 or 72 is very similar.

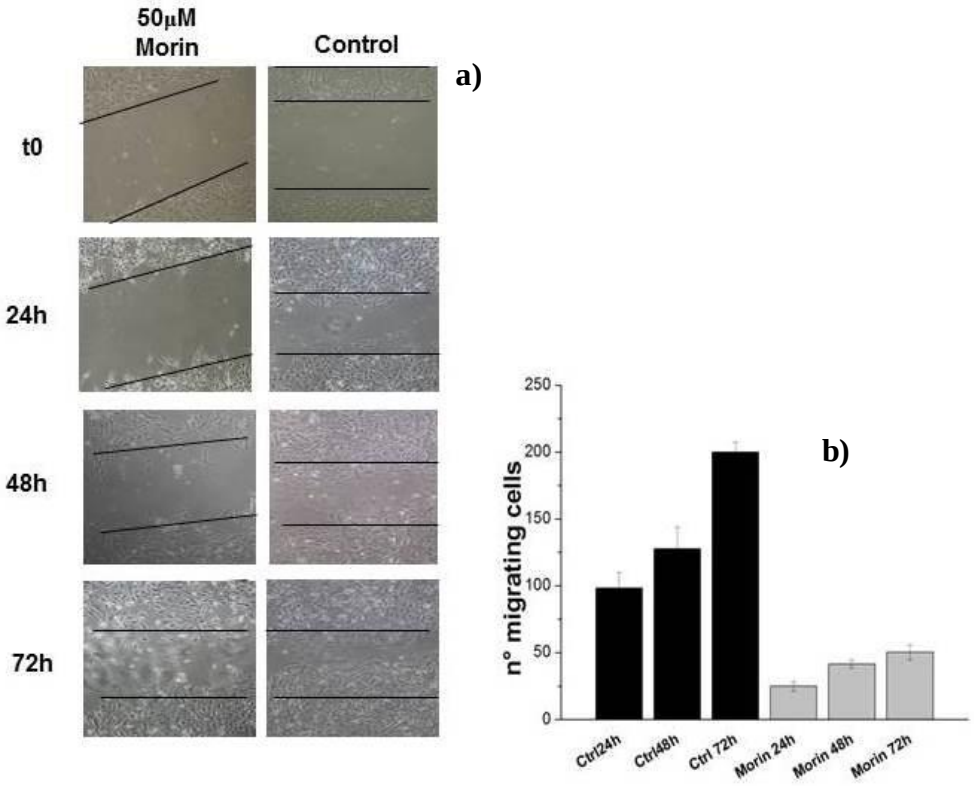


Fig.20 Morin affects cell migration of Melanoma cells: a) A375 cells were treated with 50μM Morin for the indicated times. Then a wound was made using a sterile pipette tip. As shown in **b)** Morin decrease melanoma cells ability to migrate and refill the scratch.

Interestingly Morin treatment induce a changing in cell morphology: A375 cells changes from mesenchymal form to epithelial form in a dose-dependent manner (Fig.21a). Considering this effect, we tested the adhesion capacity of melanoma cells, in this conditions.

We performed the Detachment assay at different times from those used in silencing condition, shown in Fig.10, because the action of Morin is faster than siRNA transfection. A375 cells were treated with 50μM Morin, incubated for 2h, 4h and 6h, then incubated for 10 min at 37 °C with 0.05% trypsin. 0.5 mg/ml soybean trypsin inhibitor was added, and the cells were incubated at 37 °C for 5 min. Attached cells were evaluated by crystal violet staining (Fig.21c).

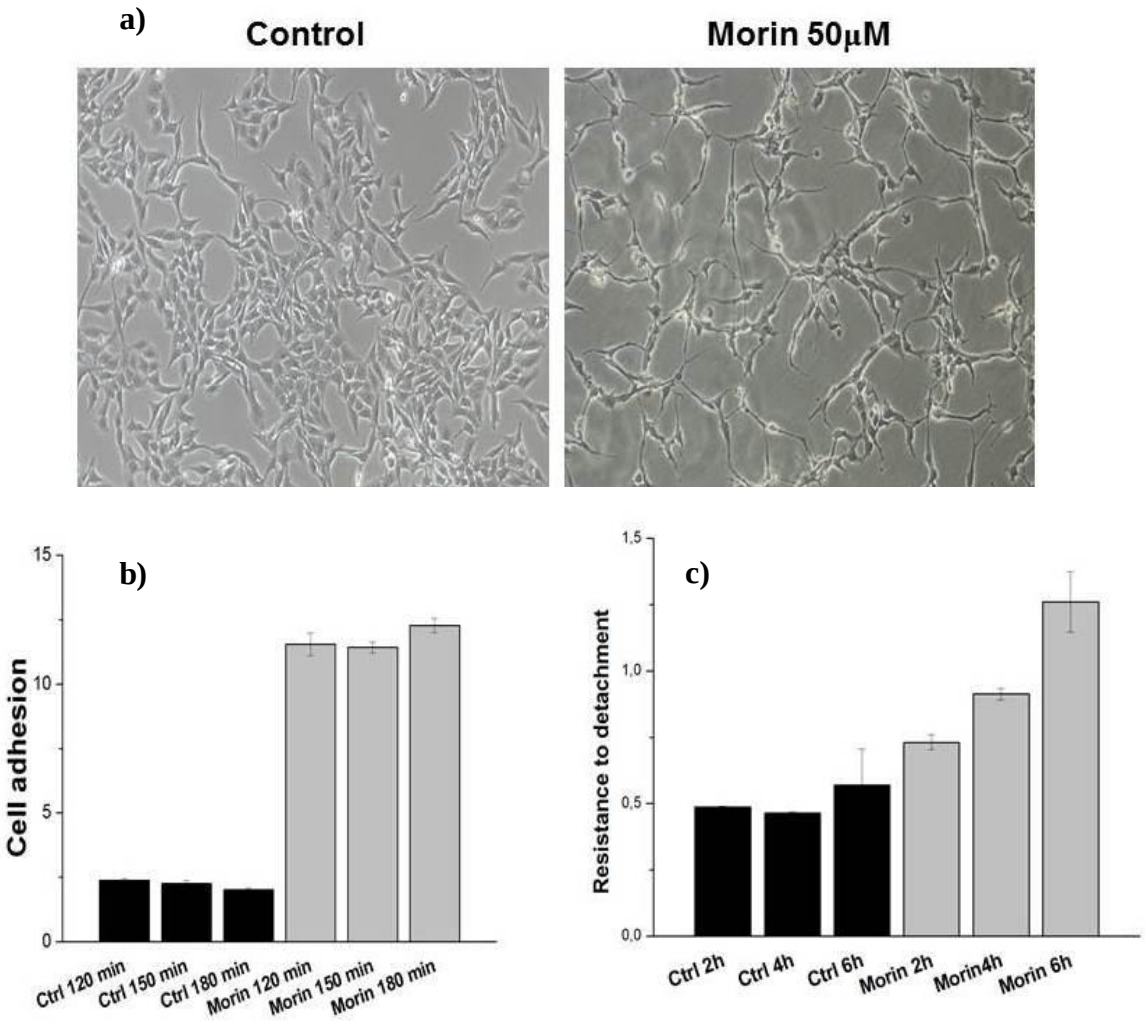


Fig.21 Morin is involved in cytoskeleton remodeling: a) after 50μM Morin treatment A375 cells showed a morphology changing, similar to dendrites. This remodeling leads to an higher ability to form focal adhesion, and remain adhese to a substrate **b)** and **c)**.

Morin induces cells to remain more attached to the dishes, suggesting that this effect can pass through LMW-PTP inhibition and degradation. Then we performed also the Adhesion test, as described for transfection conditions. As shown in Fig.21b, accordingly to previous results, cells incubated with the flavonoid are able to reform cell adhesions in a more rapidly, respect to the control. In order to evaluate the influence of Morin on Invasiveness of Melanoma cells, we carry on

a gelatin zimography. A375 cells were treated with 50µM in starvation medium, for 24h. Results shown in Fig.22a shown how the polyphenol is able to act on MMP secretion, leading to a reduction of MMP levels. We analyzed also the invading ability of A375 cells, performing a Boyden Chamber Assay. Fig.22b shows that after Morin treatment, cells had a decreased Invasiveness ability: in fact they were less able to digest matrigel.

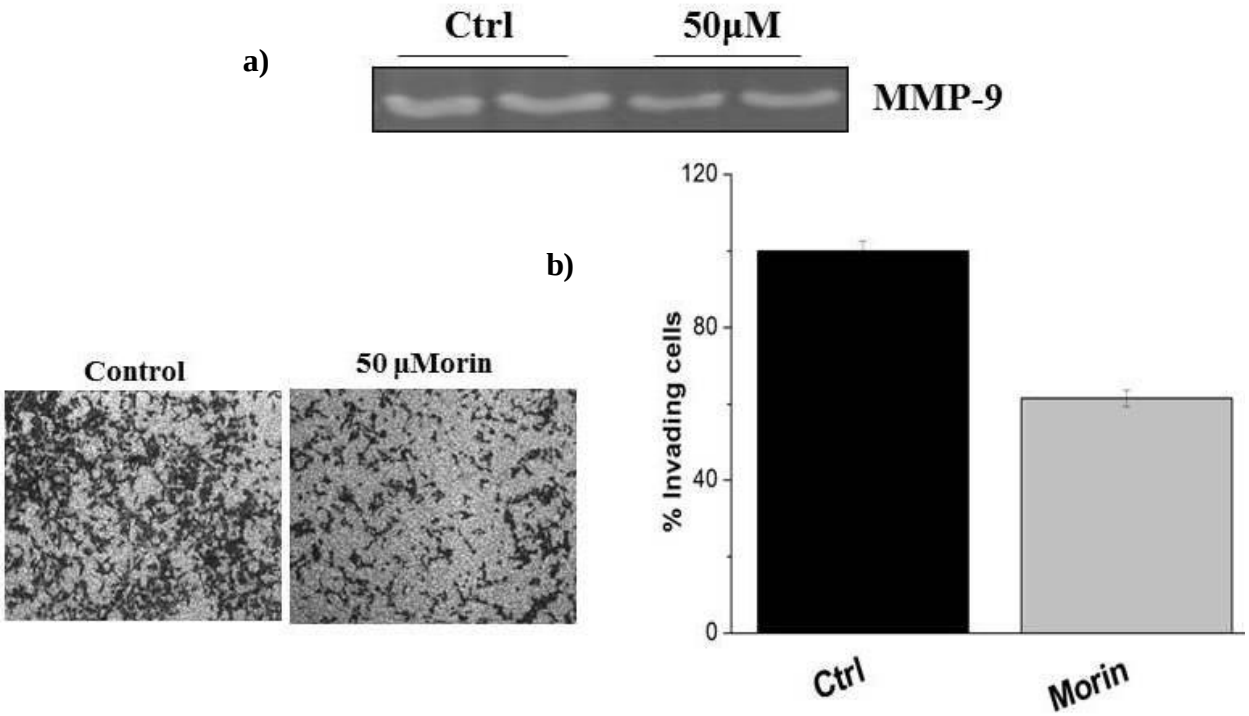


Fig.22 Morin affects Invasiveness of Melanoma cells: a) Zimographic analysis show a reduction of MMP-9 expression, after Morin treatment. This result was confirmed by Boyden Chamber Invasion Assay, **b)**. Down-regulation of LMW-PTP caused by Morin incubation, decrease invasiveness of A375 cells.

RESULTS

Experimental Part II

1. Proteomic analysis of A375 melanoma cells.

Many works have demonstrated how LMW-PTP exerts a crucial role in tumorigenesis, and that its overexpression is correlated with a worse prognosis and reduced survival. In fact this phosphatase is becoming a new possible target to contrast tumor onset. For this reason it is very important to study in deep both the proteins with which it interacts and the pathway in which LMW-PTP is implicated. Therefore we decided to perform a phospho-proteomic analysis on melanoma cells, silenced for the LMW-PTP: the aim was to identify new potential substrates of the phosphatase, clarifying the molecular mechanisms in which it may be involved. The rational was that a putative LMW-PTP protein substrate should result iper-phosphorylated when LMW-PTP is silenced. To be able to perform this analysis we needed to identify the best time, after the cell transfection, where it was possible to achieved the maximum of LMW-PTP silencing. We carry out a time course analysis: by western blot we evaluated the protein levels at 18, 28 and 36h, after siRNA transfection. Fig. 1 shows how the best knock-down is obtained after 28h: for this reason the two-dimensional analysis was conducted using this timing. The two-dimensional electrophoresis is the result of two different electrophoretic processes, which separate the sample according to different and not related, chemical-physical properties of the biomolecules: the first involves the separation of proteins according to their isoelectric point in a pH gradient (first dimension or isoelectric focusing, IEF), while the second uses a separation by SDS-PAGE according to their molecular mass (second dimension).

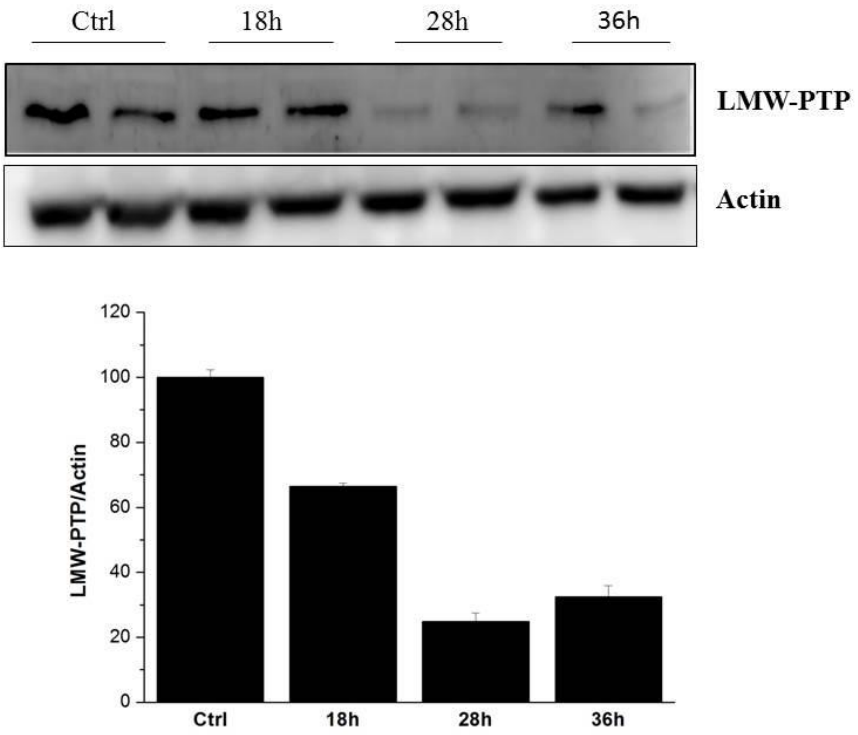


Fig.1 Transfection time course: A375 cells were silenced with siRNA or scramble. Western blot were conducted at 18h, 28h and 36h after transfection. Actin were used as internal control.

This analysis was conducted on A375 melanoma cells: a control sample (transfected with scramble) and a silenced sample. In order to understand which proteins appear to be regulated by Tyr-phosphorylation, these samples were transferred onto PVDF membrane and incubated with anti-phosphotyrosine antibody (4G10, Merck-Millipore), highlighting the tyrosine phosphorylated proteins. Western blot images (Fig.2a and 2b) were analyzed with ImageMaster 2D Platinum 6.0 program (GE-Healthcare) to quantify the intensity of the phosphorylation signal, expressed as a percentage of the volume spot (volume of the single spot respect to the total volume of all the spots present in the western image).

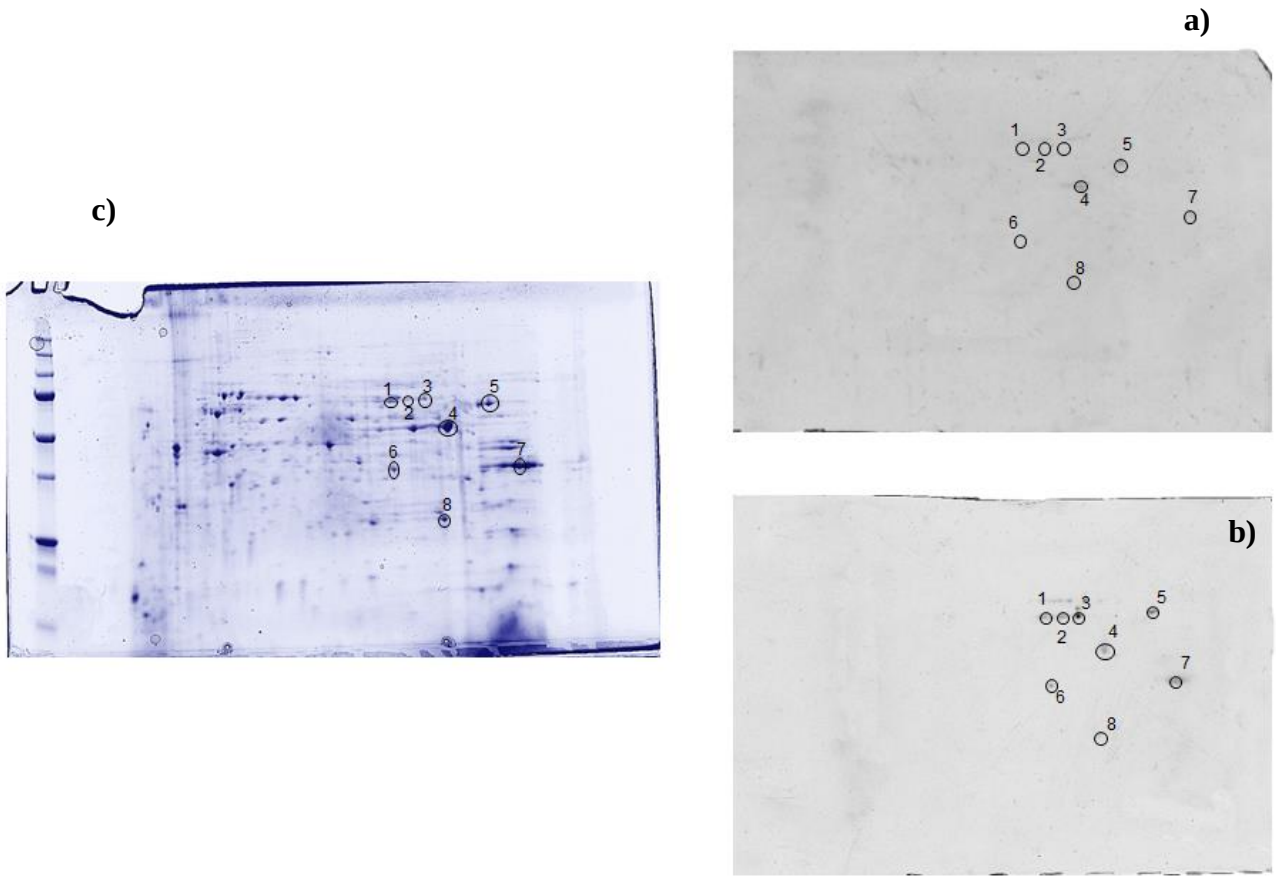


Fig.2 Two-dimensional electrophoresis analysis: a) Control and **b)** siRNA, the phosphorylation levels of each proteins is represented by spots; every spot is circled and numbered. After two-dimensional separation of the samples were transferred to PVDF membranes and incubated with anti-phosphotyrosine antibodies**. c)** Two-dimensional gel stained with colloidal Comassie: this gel has been used to normalize the intensity of the spots.

The percentage value of each spot was then normalized with respect to the percentage of the same spot present in two-dimensional gels stained with colloidal Coomassie (Fig 2c). Then the percentage values of normalized volume of each spot present in the silenced sample, were compared with the values obtained in the control one. In Fig. 2a and 2b the spots highlighted with a circle and number are those that have obtained a p-value <0.05, after the statistical analysis.

Spot N[a]	Protein	AC[b]	Gene	Mascot Results		
				Score[c]	Matched Pept.[d]	Seq. coverage (%)[e]
1	Prelamin-A/C	P02545	LMNA	66	14/50	22
2	Prelamin-A/C	P02545	LMNA	130	18/34	27
3	Prelamin-A/C	P02545	LMNA	155	22/47	31
4	Alpha-enolase	P06733	ENO1	58	8/43	24
5	Pyruvate kinase	P14618	PKM	204	30/83	61
6	Annexin A1	P04083	ANXA1	137	13/38	42
7	Glyceraldehyde-3-phosphate dehydrogenase	P04406	GAPDH	78	10/45	29
8	Triosephosphate isomerse	P60174	TPIS	110	14/40	38

Fig.3 Proteins identified by mass spectrometry MALDI-TOF:
a number of the spot (see figure 2a and 2b)
b protein identification expressed as Accession Number in Swiss-Prot/UniprotKB (http://www.uniprot.org/)
c MASCOT MS score (Matrix Science, London, UK; http://www.matrixscience.com). MS score higher than 56 corresponds to a p-value <0.05.
d number of peptide that allowed protein identification.
e Sequence coverage: (number of amino acidic residues identified/total amino acidic residues)X 100

In order to identify the spots with a statistically different Tyr-phosphorylation , western blot images were place on two-dimensional gel stained with colloidal Coomassie, using ImageMaster 2D Platinum 6.0. Fig.2c depicts the stained gel with Coomassie dye: it is visible the total protein of the samples, and the corresponding spot are indicated with circle. The spots were then taken from the gels and the proteins, contained in it, have been treated in order to identified them by mass spectrometry Maldi TOF. Results are shown in Table 3.

To validate LMW-PTP knock-down, in the samples subjected to two-dimensional electrophoresis, we performed western blot analysis. Results are shown in Fig 4.

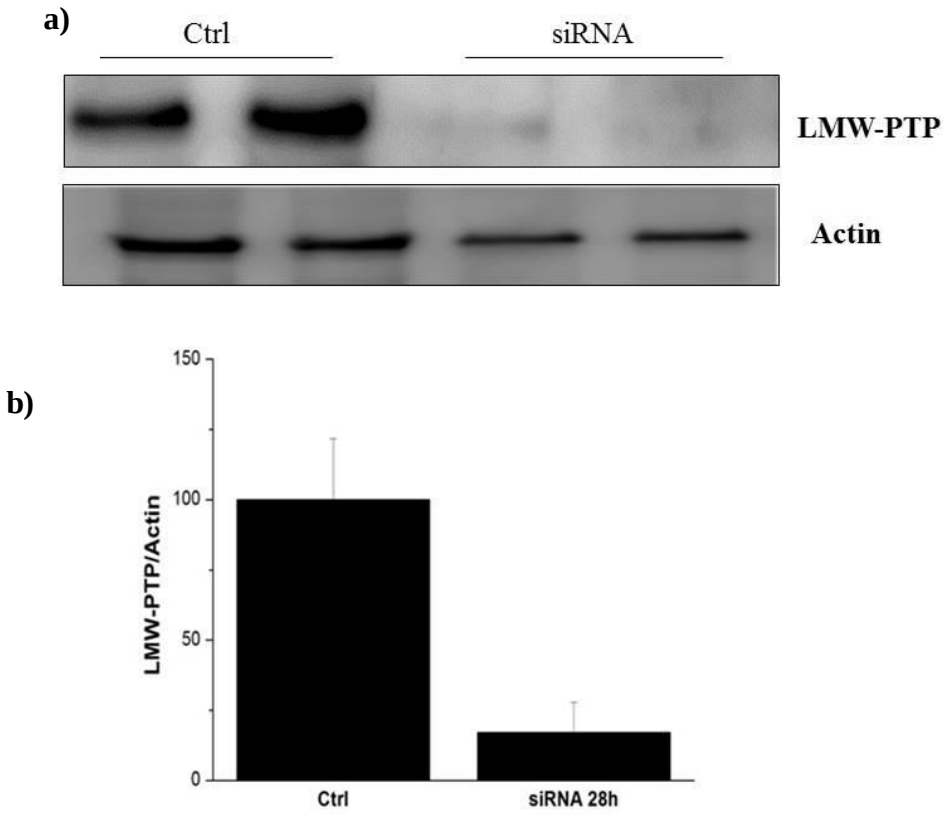


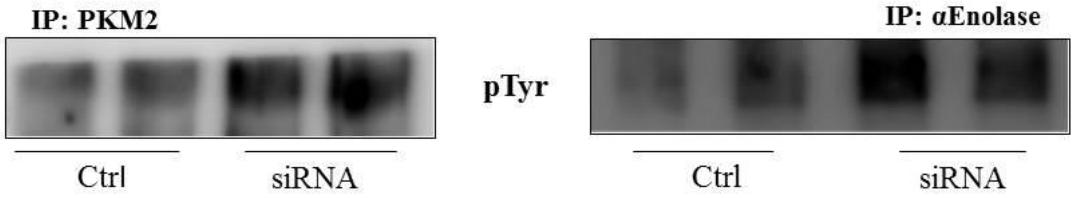
Fig. 4 Validation of LMW-PTP silencing: a) the same samples analyzed with two-dimensional electrophoresis , were test by immunoblotting with specific anti-LMW-PTP antibody. Actin was used as internal control. **b)** Densitometric evaluation of LMW-PTP.

2. Substrate validation: Immunoprecipitations.

To validate if the proteins identified by Mass Spettrometry are real substrates of LMW-PTP, we performed Immunoprecipitation assays. A375 cells were transfected with LMW-PTP siRNA, after 28h the selected proteins, mentioned above have been precipitated and, using 4G10 antibody, we have analyzed the different level of Tyrosine phosphorylation in these proteins. We focused our analysis on these proteins: ***PKM2, GAPDH, Enolase, Triosephosphate isomerase (TPIS)*** and ***Annexin A1***; for two different reasons.

The first one is that there are four enzymes belonging to glycolysis pathway, and the second one is that Annexin A1 is linked to apoptosis signaling, where LMW-PTP seems to be involved. Fig 5 and 6 show how all of these enzymes have an higher Tyr-phosphorylation level, respect to the control samples, when the phosphatase is down-regulated. These results confirm that PKM2, Enolase, TIM and Annexin A1 may be new identified substrates of LMW-PTP.

a)



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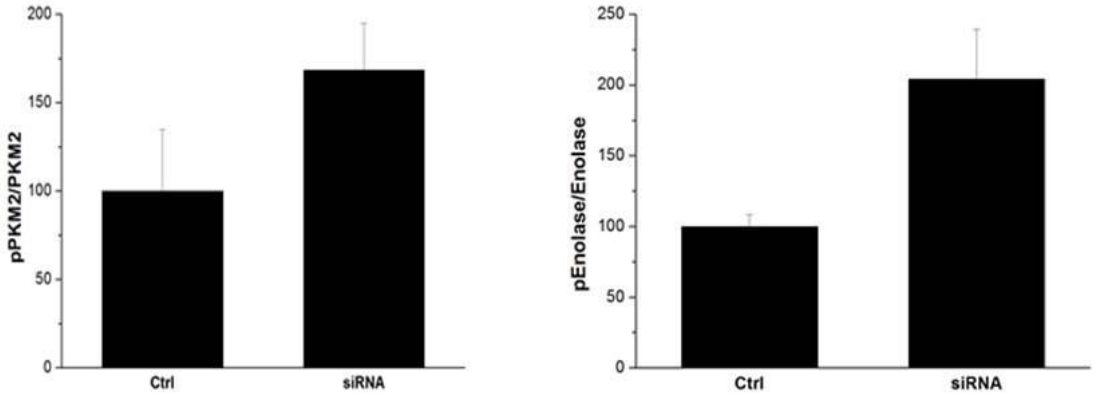
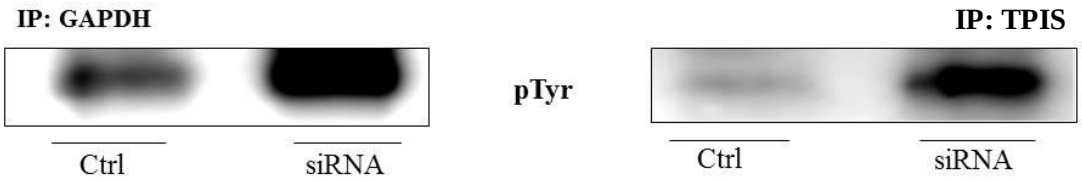


Fig.5 Evaluation of pTyr level in PKM2 and αEnolase: a) Western blot on PKM2 and αEnolase immunoprecipitates. The detection of pTyr residues were obtained with anti-pTyr antibodies, 4G10: anti-PKM2 and anti-αEnolase were used for normalization.
b) Densitometric analysis of pTyr-PKM2/PKM2 and pTyrαEnolase/αEnolase

a)



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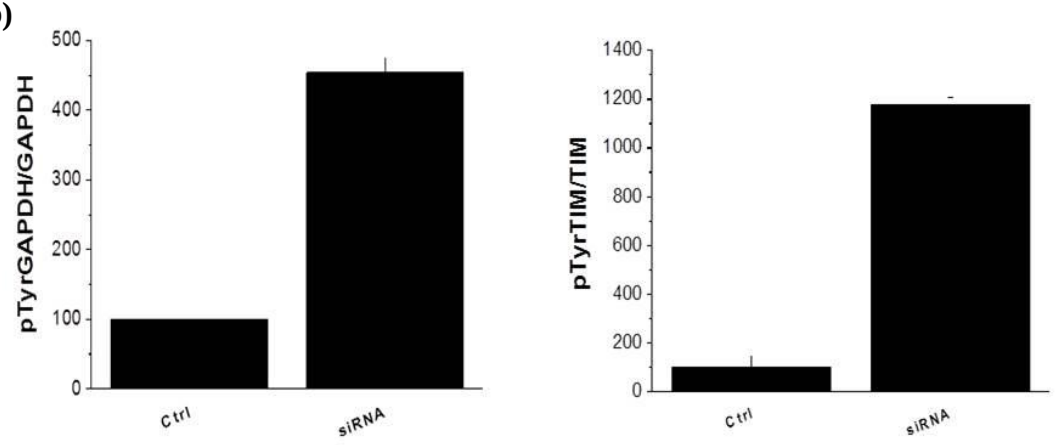
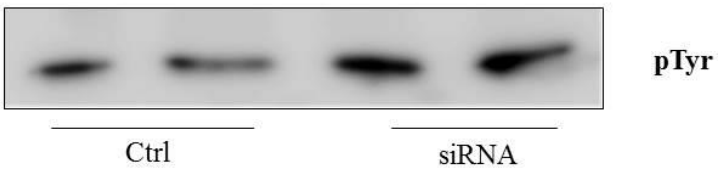
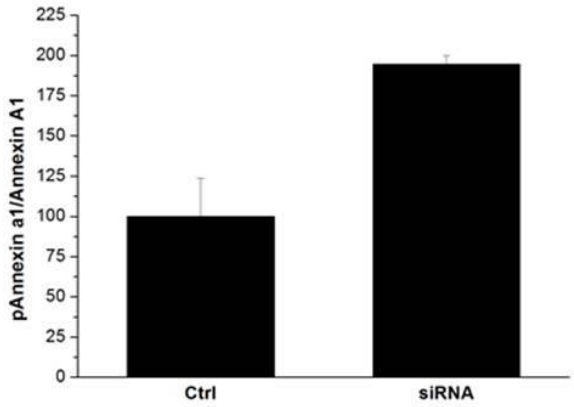


Fig.6 Evaluation of pTyr level in GAPDH, TIM and AnnexinA1: a) and **c)** the phosphorilation in Tyr was revealed by western blot analysis on GAPDH , TIM or AnnexinA1 IP, using an anti-pTyr antibody.Anti-GAPDH, anti-TIM and anti-AnnexinA1 were used for normalization**. c)** and **d)** Densitometric analysis of pTyr-GAPDH /GAPDH, pTyrTPIS/TPIS and of pAnnexinA1/AnnexinA1

c)



d)



2.1 Enzyme-subtrate interaction: Co-immunoprecipitations

However, in order to investigate whether these proteins is direct or indirect substrates of the phosphatase we must perform co-immunoprecipitation experiments. In this way it is possible to precipitate a protein of interest (PKM2, Enolase, GAPDH, TPIS or AnnexinA1) and by western blot verify if inside the immuno-complex there is LMW-PTP. To be able to carry out these analysis it was necessary to use the *PTP dominant negative (Dn)*, which is a mutant carrying a modification in the Cys present in the active site. This mutant is able to interact with its substrates, but it can not catalyze any reaction of dephosphorylation. Using this form of the phosphatase we can detect any enzyme-substrate complexes formed, if the two proteins interact. Considering that we have to over-expressed the mutant inside the cells, we need a sperimental model where PTP level is not high. For this reason the co-immunoprecipitation assay were carry out on a different cell lines: the human

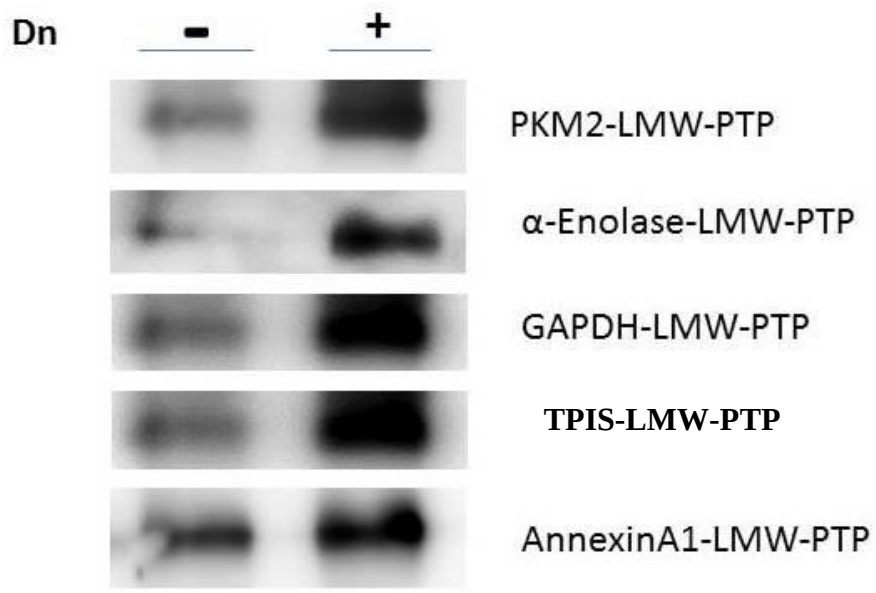


Fig.7 Co-immuno precipitation analysis: after Dn overexpression, LMW-PTP association with the identify proteins was revealed by western blot on PKM2, αEnolase, GAPDH, TPIS or Annexin A1 IP, respectively, using anti-LMW-PTP antibody. DU145 not overexpressing Dn was used as control.

prostate carcinoma, DU145. After 24h from trasfection, cells were lysed and the proteins immunoprecipitated: these cell lysates were anlyzed by western blotting, using specific primary antibody against LMW-PTP. Fig. 7 show that all of the new substrates directly interact with LMW-PTP.

3. Metabolic analysis

Our previous results show that LMW-PTP may be involved in metabolism of cancer cells, considering that 4 of the identified proteins belong to glycolisis. To further investigate the metabolic role of LMW-PTP, we performed experiments to test if these phosphatase exert some phenotypic effects on this pathway. First we evaluated the glucose up-take in silenced cells, respect to the control samples. 28 hours after siRNA or scrambled RNA transfection, A375 cells were incubated for 30 minutes with (U-^{14}C)deoxy-D-Glucose, and then lysed with 0,1M NaOH. The amount of Glucose incorporated from the cells was measured using a scintillator analyzer. Fig.8 show that when the phosphatase is knocked-down, and so the Tyr-phosphorylation of its substrates is higher, the percentage of glucose uptake is approximately 100% higher than the control.

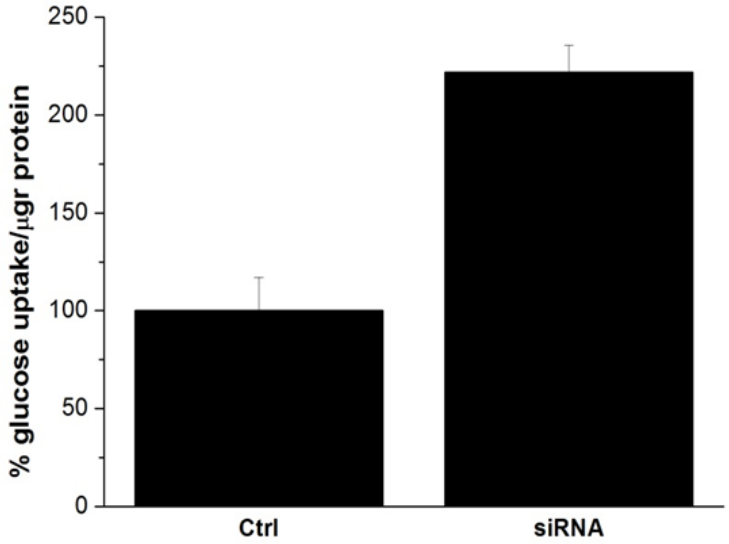


Fig.8 Glucose uptake evaluation: 28h after transfection, A375 cells were incubated with (U-^{14}C)deoxy-D-Glucose. The values obtained were normalized on proteins μgr.

Cancer cells are able to modify their metabolism, depending on several conditions such as oxygen level, presence of nutrients, and cell cycle phase. In 1924 Warburg assesed that some cancer cells consume large quantity of glucose, and metabolize it predominantly through glycolisis, thus producing high level of lactate, even in oxygen-rich conditions (*Warburg Effect*). For this reason we have evaluated first the quantity of lactate produced by our cells, and then their respiration rate. Using a specific kit (Megazyme), we are able to measured the amount of Lactate present in the medium of cell cultures.

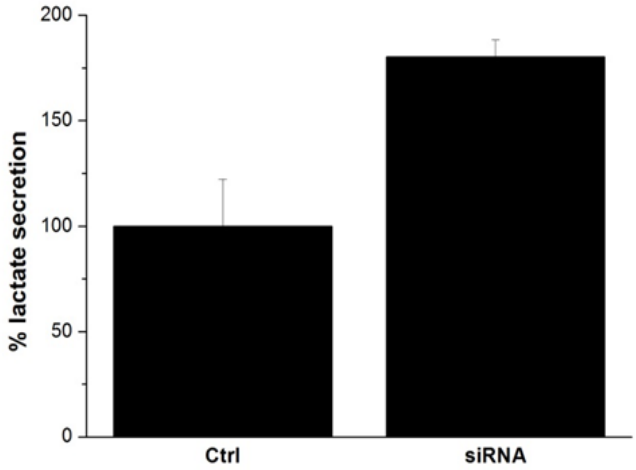


Fig.9 Lactate production analysis: percentage of lactate secretion in melanoma cells silenced for LMW-PTP and in control samples (scramble). The values were normalized on cells number.

Fig.9 confirm our previosu results, because silenced cells import more glucose than the control, and so produce higher level of lactate, incrementing glycolisis rate.

An higher rate of glycolysis, inevitably, results in a lower oxygen consumption, so we measured also this parameter. Using a Clark-type O2 electrode from Hansatech, we can register the renspiration rate of cells. From Fig.10 is highlighted that the rate of oxygen consumption, after LMW-PTP knocking down, is lower than the control samples, confirming our ipothesis.

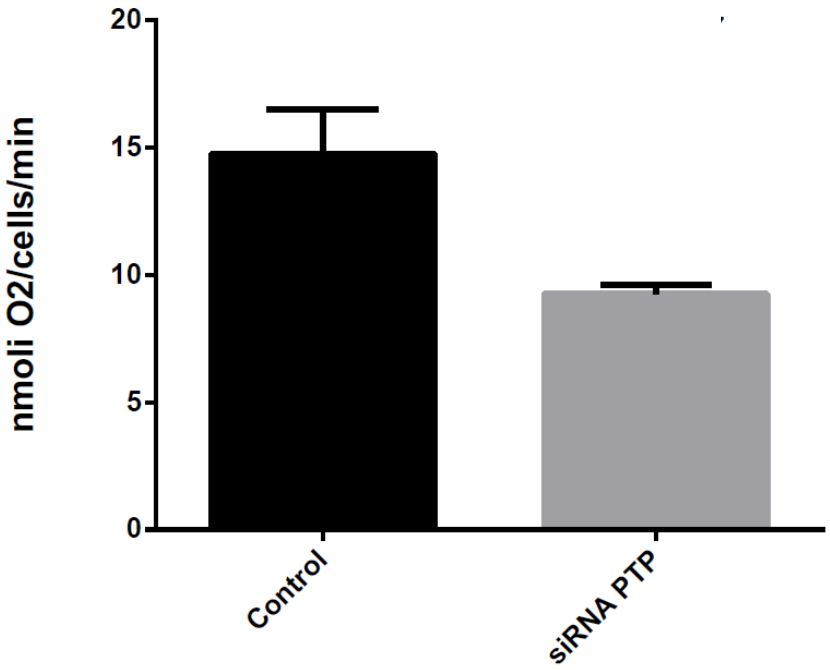


Fig.10 Oxygen consumption evaluation: the amount of O_2 consumed by cells were registered using a Clark-type O2 electrode from Hansatech, and expressed in nmoli. The values were normalized on the number of cells.

Pyruvate kinase controls the final and rate-limiting reaction of glycolys: in contrast to PKM1, which is present in a constitutively tetrameric active form, PKM2 undergoes conformational conversion between a tetrameric/full active and a dimeric/less active state (Anastasiou et al., 2011; Gao et al., 2012). The dimeric form of PKM2 is able to translocates into the nucleus and acts as a transcriptional co-activator of β-catenin and hypoxia-inducible factor 1α (HIF-1α) (Luo et al., 2011; Yang et al., 2011). Considering this duale role of PKM2 we investigate if the increase of glucose upatke may be due to an higher production of glucose transporter or some glycolitic enzyme. We analyze expression level of two proteins: *GLUT1* and *Hesokinase*. Fig.11 show the results: when the phosphatase is silenced we found an increased production of the glucose transporter GLUT1, and of the enzyme Hesokinase II, which is the first rate-limiting enzyme of glycolysis. On the contrary, control samples, where the PTP is able to act, consume less glucose, and so present a lower expression level of these two key proteins.

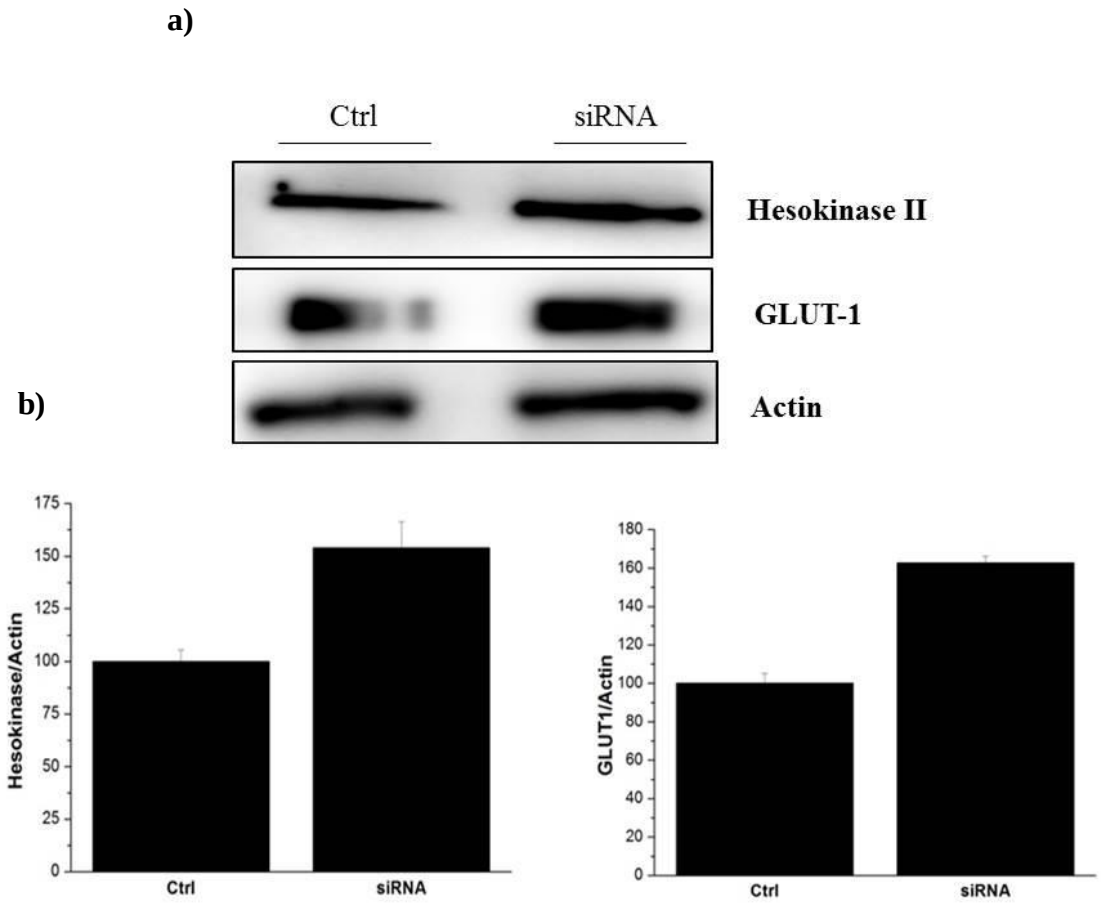


Fig.11 Hesokinase and GLUT-1 Immunoblot: a) A375 cells were transfected (siRNA or scramble), lysed and analyzed by western blot with anti-HesokinaseII and anti-GLUT-1 antibodies. Actin was used as internal control**. b)** Densitometric analysis of HesokinaseII and GLUT-1.

Also PKM2 may be subject to changes in its expression levels, and for this reason we evaluated this parameter too. Accordingly with previuos data, PKM2 appears about 30% higher in siRNA samples, suggesting that the PTP knocking-down causes a post transcriptional modification, but also a modulation in the protein level (Fig.12). Taken together all of these results confirm a role of LMW-PTP in regulating cancer cell metabolism.

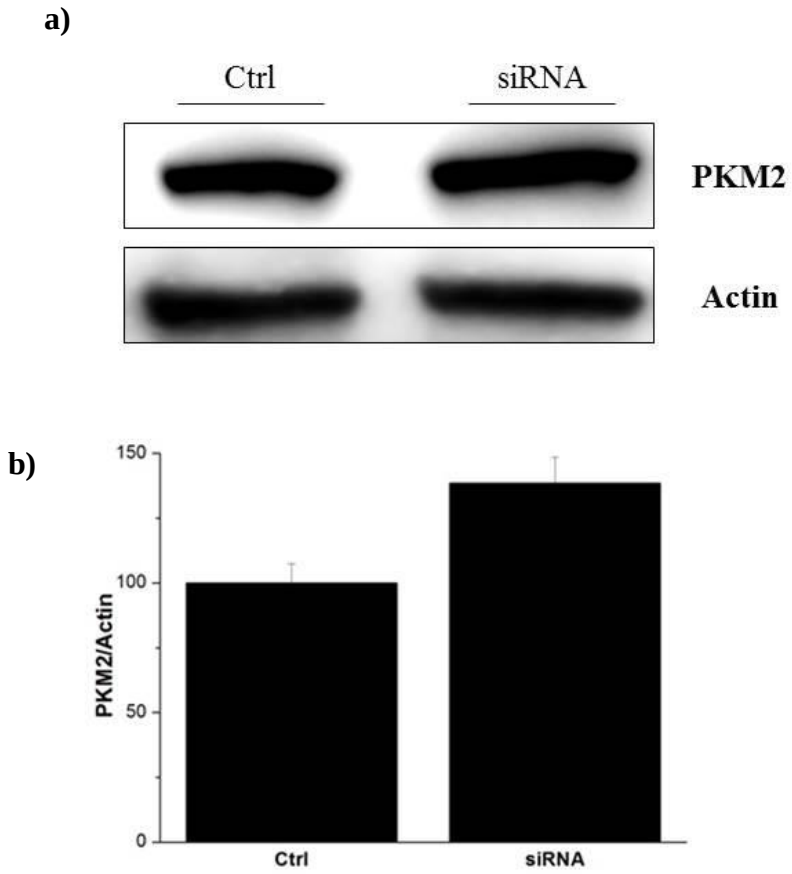


Fig.12 PKM2 expression level evaluation: a) after 28h from silencing, melanoma cells were analyzed by PKM2 immunoblot. Values were normalized on Actin. **b)** Densitometric analysis of PKM2.

DISCUSSION

The mechanism of proteins phosphorylation plays a key role in many physiological processes and it is often deregulated in pathological conditions. A popular paradigm suggests that a balance between tyrosine kinase and phosphatase activities determines the cellular levels of protein tyrosine phosphorylation and thereby governs many cellular processes, such as proliferation, migration, adhesion and survival. It is generally accepted by scientific community that tyrosine phosphorylation is regulated by equal and balanced action of the PTK and PTP but, nevertheless, most of the researches are focused on PTKs. However, in the last few years, new findings have led to the emerging view that PTPs play a dominant and specific role in controlling levels of tyrosine phosphorylation in the cells. It has been found that PTP disorders are linked to many pathological conditions, such as inflammation, diabetes, autoimmunity and cancer. In particular, there are many findings that LMW-PTP plays a key role in oncogenesis and chemoresistance.

Our studies were conducted on a Melanoma cell line, A375 to study in depth the role of LMW-PTP in tumor onset of this tissue, in order to elucidate the molecular mechanism and pathways in which this enzyme is involved and so identify new possible therapeutic targets. Using transient silencing technique, we analyzed the phenotypic changes due to LMW-PTP knocking-down. First we tested the apoptotic response of Melanoma cells: when the phosphatase was silenced cells showed a little increase of early apoptosis (around 10%) compared to control samples. If the transfection is followed with a chemotherapic treatment (20 μM 5FU), apoptotic cells increased up to 50%. This results were confirmed by apoptotic markers: western blot analysis of the active form of Caspase3 showed that LMW-PTP overexpression (control samples) lead to a down-regulation of this protein, crucial for apoptosis initiation. Thus when we silenced the LMW-PTP, caspase3 level became a little higher: obviously activation of Caspase3 is greater when concomitantly 5FU treatment is performed. Which is a possible mechanism linking LMW-PTP to apoptosis? Previous studies demonstrated that LMW-PTP targets the phosphorylated Y14 of Caveolin-1, dephosphorylating it. Phosphorylated Cav-1, acts as scaffold protein recruiting apoptosis effectors such as Bcl2, Bcl-xL, and c-Jun N-terminal Kinase (JNK), which, in turn, phosphorylates anti-apoptotic proteins, leading them to degradation. Our analysis confirmed that LMW-PTP silencing lead to an higher Tyr

phosphorylation of Cav-1. Moreover Bcl-2 results to be down-regulated in silenced samples, in comparison with untreated cells. Accordingly with these data the pro-apoptotic protein Bim and the active form of Caspase3 increased when LMW-PTP is knocked-down, leading cells to an higher sensitivity to apoptosis.

Cancer cells develop different strategies to become resistant to chemotherapics. Several studies have demonstrated how tumor cells can obtain it increasing the activity or the expression level of membrane transporters that actively expel chemotherapy drugs from inside. Our experiments suggest that LMW-PTP may confer resistance against anti-tumoral drugs, increasing the activity of some of these membrane transporters, thus limiting the toxic effect of chemotherapy. Nowadays Melanoma is treated mainly with surgery and radiation: chemotherapy is used when metastasis have been developed. For this reason we evaluated also the effect of LMW-PTP silencing using radiation. When A375 cells are exposed to a 2Gy irradiation, a dose normally used in therapy, after 48h cell viability is not affected at all. On the contrary silenced cells show a dramatic increase of mortality: this result confirm that LMW-PTP has a key-role not only in chemo- but also in radio-resistance. Resistant cancer cells, usually, show common phenotypes: one of this features is self-renewal capability. We investigated if PTP down-regulation can affect this aspect. Colony formation assay demonstrates that melanoma cells are able to reform new colonies, even when cells are exposed to a damage, such as 5FU treatment or 2Gy irradiation. Interestingly these cells survive, but change their morphology, possibly due to the selection of most aggressive clones. When LMW-PTP is knocked-down, cells normally are able to grow again, but when this condition is coupled with 5FU treatment we observe no colony formation. This result suggests that in these conditions cells are losing some characteristics of self- renewal, fundamental for metastasis. Combining phosphatase silencing with irradiation exposition, we obtain similar result, but the effect is less pronounced, probably due to the fact that the acquisition of radio-resistance is a more complex phenomenon. Anyway the loss of the phosphatase makes cells more sensitive to therapy: this result open a view on a new possible target to reduce and counteract radio-resistance.

Previous works have demonstrated that LMW-PTP exerts its action through some molecules implicated in cell migration and adhesion. Considering that Melanoma is characterized by an high invasiveness, we investigated this type of marker. Adhesion and Detachment assay showed that LMW-PTP silencing lead cells to be more adherent to a substrate. Accordingly Wound Healing Assay demonstrated that melanoma cells are able to migrate and refill the wound very quickly: phosphatase knock-down affects cell migration ability. These results were confirmed also by zimography and Boyden Chamber Invasion Assay. Silenced cells showed a decreased ability to invade, respect to untreated cells: in fact LMW-PTP down-regulation lead to a MMP-9 reduction.

Although it has been demonstrated many links between LMW-PTP and chemio/radio-resistance, gene silencing cannot be used in therapy. For this reason we looked for LMW-PTP inhibitors, that are able to reproduce a similar phenotype, as shown by Hoekstra et al., using PLP. Previous works demonstrated that there is a Flavonol compound, called Morin, able to inhibits both isoforms of this enzyme. Apoptosis evaluation of Morin treatment demonstrated that on one hand the flavonol is not toxic for A375 cells, and on the other that its combination with 5FU treatment causes an high apoptosis induction, comparable to what obtained with LMW-PTP silencing. Interestingly when we replicate this test in differentiated cells, such as C2C12 myoblast, there is not toxic effect of Morin and 5FU treatment. We speculate that the enhancement of 5FU action exerts by the flavonol has no effect in cells with a low duplication rate, suggesting that this co-treatment could be specific for cancer cells. It is well known that polyphenols are multi-target molecules that can influence the expression level of several cellular proteins. Analyzing the action of Morin on LMW-PTP, we found that this compound is able to down-regulate the expression level of the phosphatase in a dose and time dependent manner. LMW-PTP decrease start after 2h of incubation with 50μM Morin, but it works even at lower concentration, such as 1μM. Down-regulation can be due to different mechanism: transcriptional regulation or protein degradation. Considering that this effect is evident already 2h after treatment, we suggest that it may be due to degradation. Incubation with Morin together with a proteasome inhibitor (MG132), confirmed our hypothesis: in fact, in this condition, there is no LMW-PTP down-regulation. Chemotherapics treatment cause damage to other tissue and organs, often because of the high concentration used in therapy. Since Morin is able to enhance 5FU action, we tested if, reducing 5FU concentration, we can obtained similar results. Interestingly, even if 40 μM 5FU doesn't affect A375 cells viability, with Morin co-treatment cell death increase around 80%. Moreover Morin treatment is able to affect Self-renewal traits of tumor cells. The results of Colony Formation Assay confirmed those obtained with siRNA transfection: LMW-PTP down-regulation associated with 5FU prevents new colonies formation, hindering metastasis formation. The effectiveness of Morin to sensitize towards anti-tumoral treatment is confirmed with irradiation experiments. A375 cells pre-treated with the flavonol, decreased their viability about 70-80%. Interestingly, we can obtained these results even using low concentration of Morin, such as 0,1μM, and above all, with 1 and 2Gy, doses normally used in therapy. Moreover irradiation exposition didn't influence self-renewal capability of Melanoma cells: on the contrary, Morin treatment before irradiation is able to affect new colonies formation.

The effect of this natural compound is not limited only to apoptosis sensitization, but also in cytoskeleton remodeling. Morin incubation lead cells to change their morphology: they acquired a spindle morphology and showed several branch like dendrites protrusions. Accordingly to these

data the treatment decrease the migratory ability and increase the rate of adhesion to the dishes. In fact LMW-PTP regulates activities of p190Rho-GAP, which is involved in the regulation of cytoskeleton rearrangement as well as in the organization of focal adhesion. Then LMW-PTP down-regulation due to Morin, can explain these results. Morin treatment affect not only the migratory phenotype, but also the invasiveness of melanoma cells, as confirmed by zimography and Boyden Chamber Invasion Assay. Considering all of these results: that Morin is not toxic at all for normal and cancer cells, that is able to produce effects at concentration very close to those that can arrive to tissue and organs, it is possible to suggest a possible future use of Morin in clinic.

To better understand the role of LMW-PTP in tumor onset, it is important not only to identify possible inhibitor, but also find new substrates, in order to develop new strategies against cancerogenesis and metastasis. To analyze this aspect we studied the phospho-proteomic profile of silenced Melanoma cells. Through these analysis we identified different proteins showing an higher levels of Tyr phosphorylation, when LMW-PTP is down-regulated. One substrate is Annexin-A1, a protein involved in apoptosis, confirming our previous results about LMW-PTP and apoptosis resistance. More interestingly, four identified proteins belong to glycolysis pathway, PKM2, α-Enolase, GAPDH and TPIS, suggesting a new role for LMW-PTP in cancer metabolism. Our proteomic results were confirmed by Immunoprecipitation and co-immunoprecipitation analysis, that demonstrated on one hand that these enzymes are real substrate of LMW-PTP, and on the other that are direct substrates. Since "Warburg effect" comports alteration in cancer cell energetic metabolism, leading cells to consume large quantity of glucose, metabolizing it predominantly through glycolysis, and producing high level of lactate, we investigated different metabolic parameters. Our results demonstrate how LMW-PTP silencing lead cells to up-take higher quantity of glucose, and consequently to consume less O2, with respect to untreated cells. This increased glycolysis rate leads silenced cells to secrete more lactate, as described in the experiments. Pyruvate kinase controls the final and rate-limiting reaction of glycolys: in contrast to PKM1, which is present in a constitutively tetrameric active form, PKM2 undergoes conformational conversion between a tetrameric/full active and a dimeric/less active state (Anastasiou et al., 2012; Gao et al., 2012). A number of post-translational modifications have been reported to impair PKM2 enzymatic activity by promoting the tetramer to dimer conversion, including Tyr105 phosphorylation (Hitosugi et al., 2009). Notably, the conversion to a less active state confers to PKM2 "non-metabolic" abilities. Indeed, PKM2 translocate into the nucleus and acts as a transcriptional co-activator of β-catenin and hypoxia-inducible factor 1α (HIF-1α), cooperating to control cell proliferation and glucose catabolism, respectively (Luo et al., 2011; Yang et al., 2009). In fact, the balance between PKM1 and PKM2, and between the dimeric and tetrameric forms of PKM2, allows

cancer cells to adapt to different conditions of oxygen and glucose. Analysis of expression level of the Glucose transporter GLUT-1 and Hesokinase II, the first rate-limiting enzyme of glycolysis, confirmed our previous data. When LMW-PTP is down-regulated both proteins had an increased expression level, explaining, at least in part, the glycolytic metabolism showed by silenced cells. Moreover cancer cells are able to switch their metabolism changing PKM2 phosphorylation state and protein level. In fact when LMW-PTP is silenced PKM2 increased its Tyr phosphorylation, but even its expression level, confirming that the phosphatase is involved in a double modulation. Our results demonstrated that LMW-PTP leads melanoma cells to a less glycolytic metabolism. One of Hallmarks of cancer is *Metabolic Reprogramming*: cancer cells are able to survive in extreme conditions, such as low O2 and nutrients availability, modulating their metabolism. The tumor mass is highly heterogeneous, where "well-oxygenated" cancer cells consume lactate produced by hypoxic, glycolytic cells. Our data, showing the influence of LMW-PTP on melanoma metabolism, suggest that the phosphatase plays a crucial role in tumorigenesis and aggressive phenotype acquisition. However, these studies were conducted only on a Melanoma cell line, thus is not possible to generalize the results for each kind of tumor, because of tumor heterogeneity. Further studies will be conducted to better characterize the role of this enzyme, and its regulation in tumor metabolism.

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