The crossroad of phosphatase and kinase signaling in cancer

Alessandra Valéria de Sousa Faria

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The Crossroad of Phosphatase and Kinase Signaling in Cancer

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Thesis

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Chapter 1

Introduction and aim of the thesis

Introduction and aim of the thesis

Cellular communication is essential for the thriving of an organism as a whole. Behavioral cues for individual cells come from the cellular microenvironment as well as other cells or soluble mediators. A wide range of surface receptors is responsible for relaying these cue, including G-protein coupled receptors, tyrosine kinase-associated receptors and integrins. Activation of these receptors by their ligands triggers cellular effects through activation of intracellular signal transduction pathways, which in turn relies heavily on post-translational modification of proteins. In particular protein phosphorylation is a pivotal modification mechanism, based on fast and reversible covalent binding of a phosphoryl moiety to specific amino acid residues in proteins (Tamura et al, 2004; Jailkhani et al, 2011). The class of enzymes known as kinases is able to transfer a phosphate group onto a protein, generally derived from and at the expense of an adenosine triphosphate (ATP) molecule, while the class of phosphatases is able to remove this phosphate group through hydrolysis, resulting in the generation of water (**Figure 1**). Phosphorylation of a protein may have several consequences: it might create new recognition sites to allow protein-protein interaction; it can control protein stability and, most importantly, might regulate enzymatic activity of phosphorylated proteins. As such, the overall tyrosine, serine and threonine phosphorylation, carefully balanced by kinases and phosphatase, plays a major role in signaling to lead to survival, proliferation, differentiation and cell death (Jailkhani et al, 2011). In the specific case of cell proliferation and survival signaling, integrins and tyrosine kinase-associated receptors have been highlighted as major players for cell growth signaling (Butti et al, 2018). The tyrosine kinase-associated receptors include many growth factor receptors, which promote activation of mitogenic-activated protein kinase (MAPK) and phosphatidyl-inositol 3'-OH kinase (PI3K)/AKT pathways. The MAPK family members include the effector kinases p38, JNK and Erk. In general, p38 and JNK activate apoptosis and inflammatory pathways upon activation by stress signals from extracellular environment, while Erk signal transduction is associated with proliferation and cellular differentiation (Yang et al, 2007; Lee et al, 2020; Paton et al, 2020). In the same direction, the PI3K/AKT pathway plays an important role in cell survival, proliferation, migration and cell cycle initiation. Rather than a protein phosphatase, PI3K is a lipid phosphatase able to convert the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which allows recruitment and activation of the protein kinases PDK1 and AKT. The main function of AKT is to phosphorylate tuberous sclerosis protein (TSC)-1 and TSC-2,

promoting their disassociation and releasing their negative modulation of mTOR kinase, thereby allowing protein synthesis (Huang et al, 2018) - (see **Figure 2**). Signaling through integrins is associated with activation of focal adhesion kinase (FAK) and Src family kinases, some of the most well-described proteins associated with cell motility and survival (Mitra and Schlaepfer, 2006). Most signaling pathways are not completely independent, with many molecules associated with one pathway able to activate molecules canonically associated with another signaling cascade. For instance, non-canonical MAPK signaling converges on PI3K activation, as tyrosine kinase-associated receptors induce KRAS activation, which promotes both MAPK and PI3K signaling (Janku et al, 2018). Additionally, FAK is known to activate MAPK/PI3K pathways, while Src itself is also a known FAK target able to stimulate MAPK/PI3K signaling.

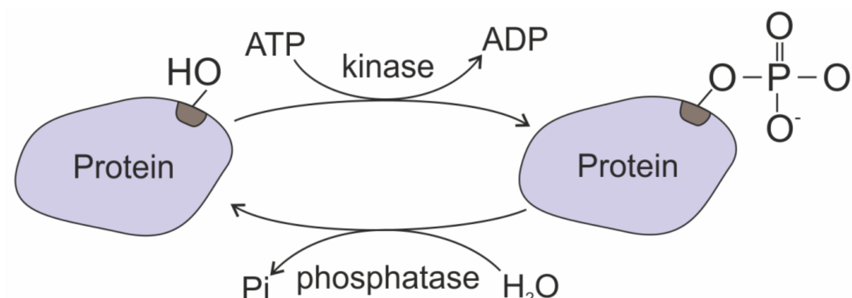


Figure 1. General scheme of protein phosphorylation. The protein kinases have the function to add a phosphate group (PO_4^{2-}) on protein-specific site, while protein phosphatases are able to remove this phosphate group. A fictional: protein is represented with a phosphorylation site (brown color).

Protein kinases are classified as serine/threonine, tyrosine, or dual specificity depending on the amino acid site that will receive the phosphate group. There are around 520 protein kinases in the human genome, emphasizing their importance for cellular functioning. Conversely, abnormal activity of these enzymes is seen in a diverse range of diseases, including diabetes, obesity, inflammation, neurodegenerative diseases and neoplasia (Mustelin et al, 2005; Souza et al, 2009; Jailkhani et al, 2011; Lee et al, 2015), and more than 300 of the tyrosine kinase genes have been implicated in carcinogenesis (Arena et al, 2005; Jacob et al, 2005; Julien et al, 2011; Ferreira-Halder et al, 2019). Indeed, enhanced activation of many kinases and receptors is seen in various cancers (Turner and Grose, 2010; Rajaram et al, 2017; Roskoski et al, 2018).

Using cancer as model, several signal transduction pathways have been singled out for their contribution to survival and proliferation signaling, with the MAPK and PI3K/AKT pathways and their components emerging as arguably two of the most important pathways and

therapeutic targets. The MAPK family is associated with cell proliferation signaling, and this cascade is frequently rendered constitutively activated (i.e. independent of growth factor stimulation) in for instance colorectal cancer, through mutations in *KRAS* (30%) or *BRAF* (17%) genes (Huang et al, 2018; Tate et al, 2019; Martini et al, 2020). PI3K/AKT pathway activation, also associated with cellular survival, is rendered active through for instance mutations in the *PI3KCA* gene (in 15% of colonic cancers) and leads to tumor survival advantage (Tate et al, 2019). The principal negative modulator of PI3K/AKT signaling is the lipid phosphatase PTEN (Phosphatase and Tensing Homologue deleted on chromosome 10), which reverts the actions of PI3K by reverting PIP3 to PIP2, consequently limiting the pathway transduction (Keniry and Parsons, 2008; Álvarez-Garcia et al, 2019). Partial or total loss-of-function of PTEN is frequently observed in several types of cancer, impacting directly on tumorigenesis and cancer progression via PI3K pathway activation (Álvarez-Garcia et al, 2019). A role for Src in tumorigenesis has also been described, and in fact was the first proto-oncogene described in animal cells. The Src kinase family is well-known to be over-activated in several cancers, where it positively regulates survival and proliferation. For instance, 80% of colorectal cancer patients are suggested to have increased Src activity in their tumor cells (Chen et al, 2014). Of note, only up to 17% of colorectal cancers harbor activating Src mutations indicating that Src activity in tumors may also be a consequence of upstream signaling activities. It is of interest to note that the expression of several of the Src kinase family members (including Lck, Fyn and Lyn) is restricted to hematopoietic cells. Thus, it is perhaps not surprising that Src kinase signaling is of particular importance in hematological malignancies. For instance, one of the main characteristics of chronic myeloid leukemia (CML) is the presence of a fusion protein, Bcr-Abl, which arises from translocation of t(9;22)(q34;q11) chromosomes called Philadelphia chromosome (Mahon et al, 2008). This fusion product constitutes a novel, tumor-specific kinase, which activates Src family kinases, in addition to the PI3K and other pathways.

The most advanced treatment development in cancer is based on targeting kinases. Novel kinase inhibitors are used in the clinic to improve cancer outcomes (Kannaiyan and Mahadevan, 2018), and 52 kinase inhibitors have been approved for cancer treatment by the FDA to date (Roskoski, 2019). One striking example is the use of a targeted Bcr-Abl inhibitor, imatinib, which has proven immensely successful for the treatment of CML. Another example is the use of the *BRAF* inhibitor vemurafenib. As *BRAF* mutation has direct implications on MAPK and PI3K/AKT activation, the inhibition of these kinases may be a strategy to reduce tumor growth (Dankner et al, 2018; Huang et al, 2018). Protein (kinases or

receptors) mutations and/or gain-of-function of molecules downstream of the original treatment target have been highlighted as a major contribution to therapeutic resistance. For instance, the crosstalk between the BcrAbl and Src kinase can lead to a more malignant phenotype in CML (Rubbi et al, 2011) impairing the imatinib treatment efficiency (Ferreira et al, 2012; Linev et al, 2018). Thus, dual targeting compounds have been investigated and therapeutic strategies against both of these kinases have appeared promising over the last decade (Quintás-Cardama et al, 2006; Musumeci et al, 2018) Similarly, vemurafenib-treated tumors may acquire resistance to the therapy by the acquisition of additional MAPK mutations, something that appears to be a frequent occurrence in colorectal cancer (Ahronian et al, 2015). Subsequent use of kinase inhibitors targeting the downstream pathways may then be of use. But also non-kinase treatments affecting signaling may be used in the clinical management of tumors, as in the case of Sonic hedgehog (Shh) pathway inhibitor vismodegib (Sekulic et al, 2012), which blocks the Smoothened receptor downstream of Shh signaling. Nevertheless, also vismodegib has occasionally been highlighted as ineffective, potentially due to activation of additional signaling pathways by Hedgehog signaling.

Based on the importance of kinases for cellular function and disease, a wish to investigate this family of enzymes on a wider scale has risen in the last decades. Kinome profiling has emerged as an effective strategy to screen activity of a large amount of kinases simultaneously, and investigate how these are differently modulated in several biological systems. Using an array-like platform (Peppelenbosch et al, 2016), canonical and non-canonical pathways associated with specific signals and/or ligands can be investigated, including potential targets for overcoming cancer. Using the kinome profiling approach, the global upregulation of kinase activity in cancer can be screened and might reveal new potential targets to overcome resistance beyond classical targets.

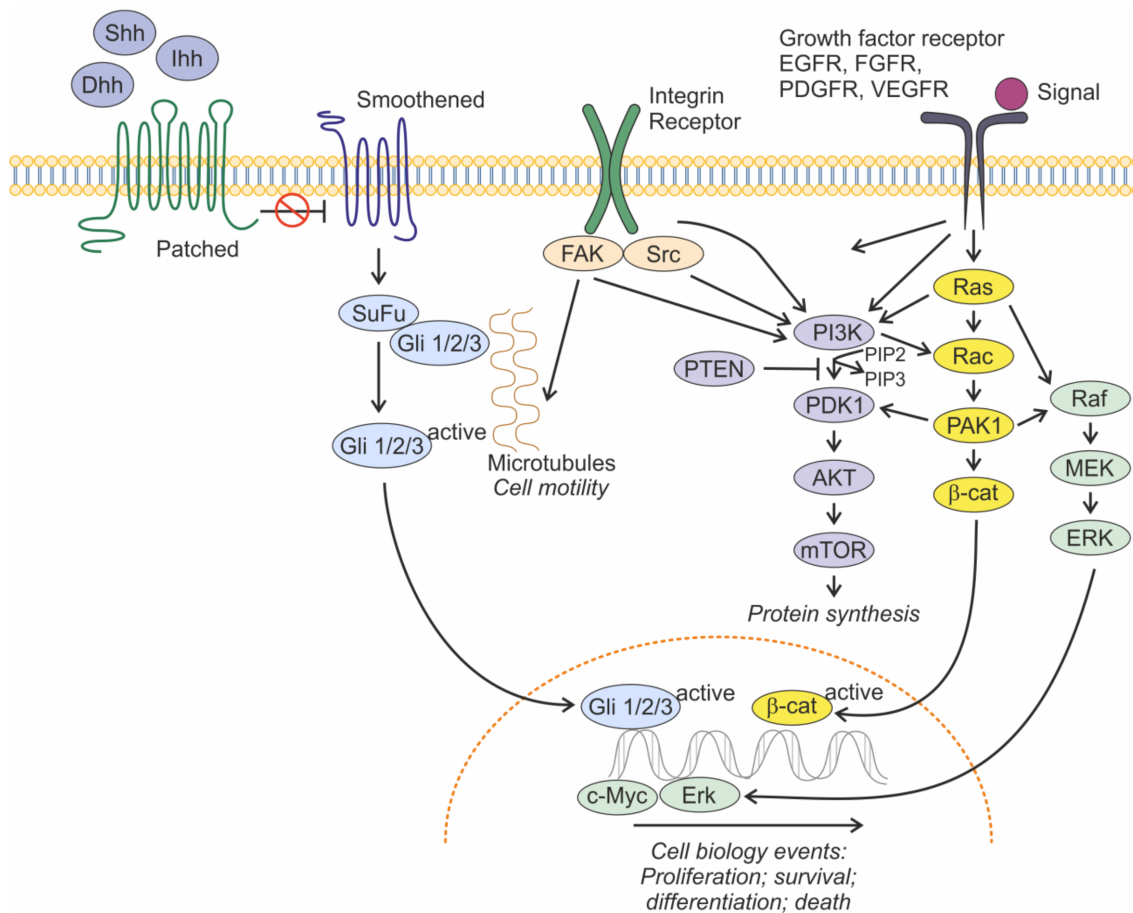
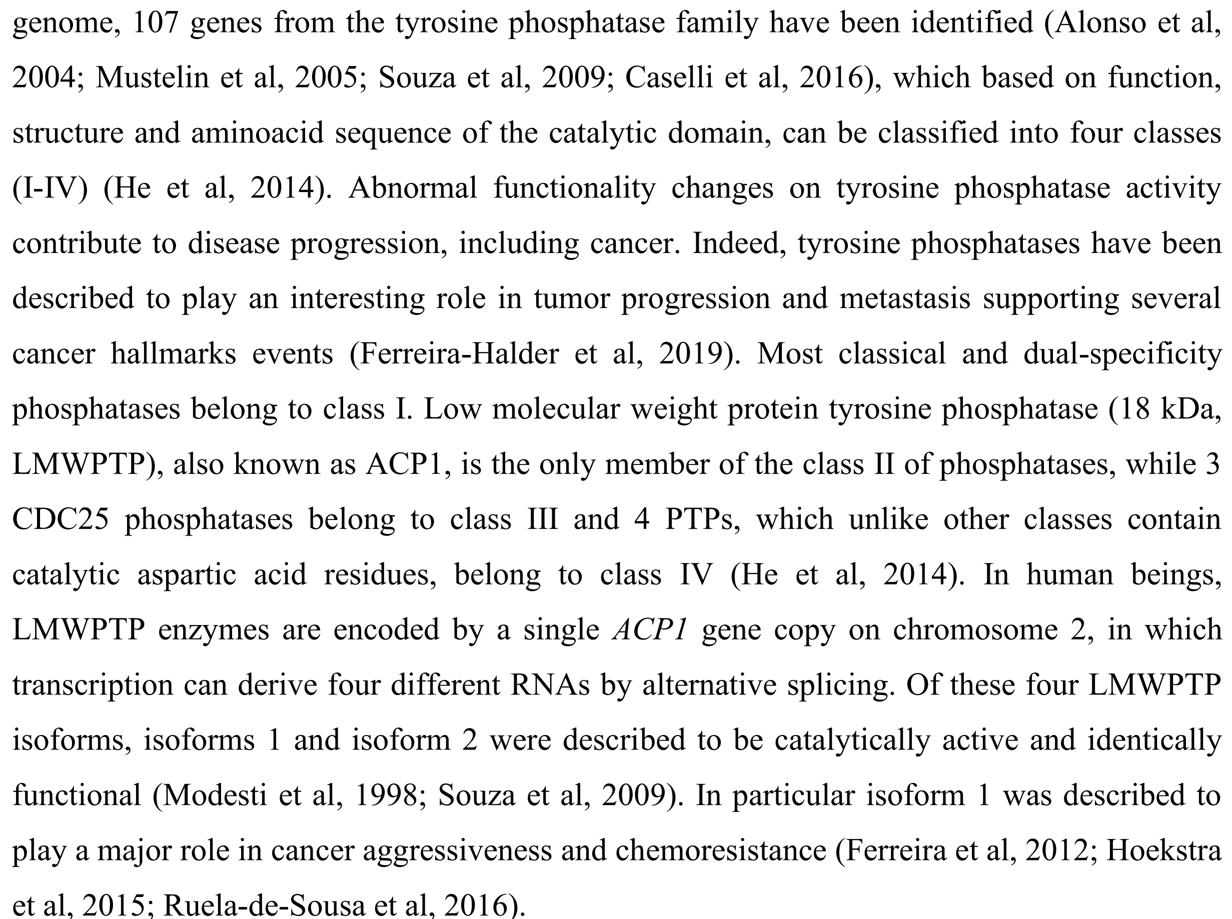


Figure 2. The main signal transduction pathways in normal cells, which are often deregulated in cancer. Hedgehog, Src Family Kinase, FAK are commonly associated with cell motility and survival; PI3K/AKT, Ras-Rac, MAPK pathways play important role in cancer progression by sustaining cell motility (migration profile), proliferation and survival events together with cell death resistance in colorectal cancer.

Despite the widespread knowledge regarding the role of kinases in cancer, the contribution of phosphatases to tumor progression still needs to be largely investigated. While kinases are generally seen as positive regulators of signaling and cancer, phosphatases are mostly regarded as negative regulators and tumor suppressors. For instance, as described above, PTEN loss is commonly seen in cancer, and was associated with energetic metabolism rewiring, anoikis resistance, invasion and metastasis. Based on such examples, phosphatases were largely associated with tumor suppressors (Ortega-Molina and Serrano, 2013; Ferreira-Halder et al, 2019). However, the end result of phosphatase activity may depend on whether the dephosphorylation site is activating or inhibitory. And thus, phosphatase activity may in some context actually activate rather than inhibit downstream signaling. Based on the function, structure, sequence, specificity, sensitivity to activators and inhibitors, the phosphatases are general classified in three families: serine/threonine phosphatases, tyrosine phosphatases and dual-specificity phosphatases (Aoyama et al, 2003). Within the human

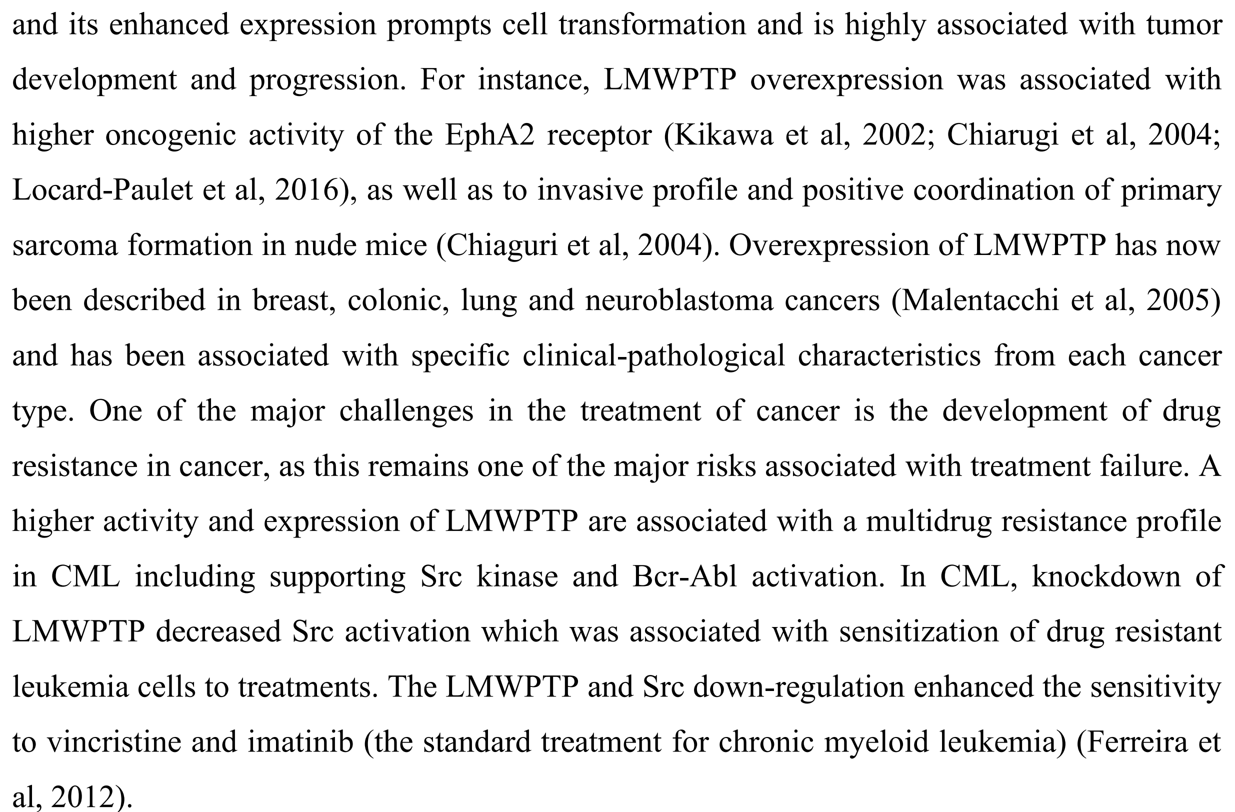


genome, 107 genes from the tyrosine phosphatase family have been identified (Alonso et al, 2004; Mustelin et al, 2005; Souza et al, 2009; Caselli et al, 2016), which based on function, structure and aminoacid sequence of the catalytic domain, can be classified into four classes (I-IV) (He et al, 2014). Abnormal functionality changes on tyrosine phosphatase activity contribute to disease progression, including cancer. Indeed, tyrosine phosphatases have been described to play an interesting role in tumor progression and metastasis supporting several cancer hallmarks events (Ferreira-Halder et al, 2019). Most classical and dual-specificity phosphatases belong to class I. Low molecular weight protein tyrosine phosphatase (18 kDa, LMWPTP), also known as ACP1, is the only member of the class II of phosphatases, while 3 CDC25 phosphatases belong to class III and 4 PTPs, which unlike other classes contain catalytic aspartic acid residues, belong to class IV (He et al, 2014). In human beings, LMWPTP enzymes are encoded by a single *ACP1* gene copy on chromosome 2, in which transcription can derive four different RNAs by alternative splicing. Of these four LMWPTP isoforms, isoforms 1 and isoform 2 were described to be catalytically active and identically functional (Modesti et al, 1998; Souza et al, 2009). In particular isoform 1 was described to play a major role in cancer aggressiveness and chemoresistance (Ferreira et al, 2012; Hoekstra et al, 2015; Ruela-de-Sousa et al, 2016).

Cellular function of LMWPTP is the dephosphorylation/regulation of many tyrosine kinase receptors and other molecules involved in signal transduction (Caselli et al, 2016). Normal function of LMWPTP has been associated to (i) cell motility and spreading coordinated by FAK dephosphorylation on several Tyr sites, in mouse fibroblast model; (ii) immune response modulation by dephosphorylation of Zap-70 Tyr292 (inhibitory site), a member of T-cell receptor signaling; (iii) balance between tight cell-cell contacts by co-localization with β-catenin and inhibition of cell-cell adhesion and clustering by negative modulation of ICAM-1; (iv) cytoskeletal remodeling by interaction with EphrinA2 receptor (EphA2) and modulating of Ras-MAPK signaling; (v) decrease cell proliferation by negative regulation of Janus kinase (JAK)-2, as well as Signal Transducer and Activator of Transcription (STAT) family members, such STAT-2, -3 and -5, platelet derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) – (Chiarugi et al, 1995; Chiarugi et al, 1998; Stein et al, 1998; Bottini et al, 2002 a; Kikawa et al, 2002; Taddei et al, 2002; Park et al, 2002; Rigacci et al, 2002; Giannoni et al, 2003; Rigacci et al, 2003; Lee et al, 2007; He et al, 2014; Hoekstra et al, 2015). Under normal cellular conditions, the function of LMWPTP has been extensively characterized in osteoclast and osteoblast cell lines. The contribution of LMWPTP to bone metabolism was first associated with osteoblast differentiation by

modulating Src phosphorylation status. Indeed, LMWPTP expression decreased in a time-dependent fashion during osteoblastic differentiation. The same pattern of activity was observed for the antioxidant glutathione (GSH) suggesting a crosstalk between redox status and LMWPTP activity (Zambuzzi et al, 2008; de Souza Malaspina et al, 2009). Besides that, LMWPTP activity also coordinates the cellular adhesion process by transient dephosphorylation of FAK Tyr397 and Src Tyr416, both activator sites, in osteoblasts (Fernandes et al, 2014). FAK itself also plays a major role in bone integrity and its activity was described to be modulated by secreted phosphoprotein 1 (SPP1)-induced LMWPTP expression (Kusuyama et al, 2017). Further, under cellular architecture modifying hyperosmotic conditions, LMWPTP in keratinocytes was suggested to effect selective Src Tyr416 dephosphorylation, rather than Tyr527 (inhibitory site). This mechanism might be associated with LMWPTP phosphorylation on Tyr132, which increases its affinity to substrates (for LMWPTP regulation modulation, see Souza et al, 2009). On the other hand, a mutual Src/LMWPTP activation during osteoblast differentiation has also been described (Bucciantini et al, 1999; den Hertog et al, 2008; Zambuzzi et al, 2008; Silva et al, 2015).

Besides its importance in normal processes, LMWPTP has been described to play a role in metabolic diseases, such obesity, diabetes, and cancer. In metabolic diseases, a higher expression of LMWPTP was associated with a protective effect on hypertriglyceridemia (Bottini et al, 2002 b). Indeed, the tyrosine phosphatases LMWPTP and Protein Tyrosine Phosphatase 1B (PTP1B) might coordinate lipid overload, and LMWPTP inhibition provoked lipid-induced apoptosis in liver cells (Bourebaba et al, 2020). On the other hand, high LMWPTP levels appear to be less favorable for diabetes, as LMWPTP overexpression leads to insulin resistance in mouse models of obesity (Stanford et al, 2017). Additionally, LMWPTP knockdown in mice was associated with prevention of cardiomyopathy through decreasing cardiac remodeling, fibrosis and hypertrophy (Wade et al, 2015). In the cancer field, LMWPTP was first described as negative regulator of the PDGFR, consequently inhibiting cell growth (Shimizu et al, 2001; Fiaschi et al, 2001). In normal cells, the increase of LMWPTP expression was associated to lower PDGFR phosphorylation and 90% reduction of mitogenic capacity (Ramponi and Stefani, 1997). Indeed, LMWPTP was able to dephosphorylate PDGFR at Tyr857 which was important for catalytic site regulation (Chiarugi et al, 2002). Taking this information together, it was expected that LMWPTP would play a major role as a tumor suppressor. Instead, LMWPTP has since been described as a positive modulator of Ras-MAPK, FGFR and Eph receptor signaling (Stein et al, 1998; Park et al, 2002). Indeed, LMWPTP activates several cancer-associated signal pathway mediators,



and its enhanced expression prompts cell transformation and is highly associated with tumor development and progression. For instance, LMWPTP overexpression was associated with higher oncogenic activity of the EphA2 receptor (Kikawa et al, 2002; Chiarugi et al, 2004; Locard-Paulet et al, 2016), as well as to invasive profile and positive coordination of primary sarcoma formation in nude mice (Chiaguri et al, 2004). Overexpression of LMWPTP has now been described in breast, colonic, lung and neuroblastoma cancers (Malentacchi et al, 2005) and has been associated with specific clinical-pathological characteristics from each cancer type. One of the major challenges in the treatment of cancer is the development of drug resistance in cancer, as this remains one of the major risks associated with treatment failure. A higher activity and expression of LMWPTP are associated with a multidrug resistance profile in CML including supporting Src kinase and Bcr-Abl activation. In CML, knockdown of LMWPTP decreased Src activation which was associated with sensitization of drug resistant leukemia cells to treatments. The LMWPTP and Src down-regulation enhanced the sensitivity to vincristine and imatinib (the standard treatment for chronic myeloid leukemia) (Ferreira et al, 2012).

The research groups of Prof. Dr. Ferreira-Halder and Prof. Dr. Peppelenbosch, together with Dr. Fuhler, have been collaborating to better understand the contribution of LMWPTP to cancer biology (Ferreira et al, 2006; Bispo de Jesus et al, 2008; Souza et al, 2009; Ferreira et al, 2012; de Abrantes et al, 2013; Hoekstra et al, 2015; Ruela-de Sousa et al, 2016). In this context, these groups have pointed out the relevance of LMWPTP for chemoresistance and metastasis in several tumor models: chronic myeloid leukemia (Ferreira et al, 2012) as described before, prostate cancer (Ruela-de-Sousa et al, 2016) and colorectal cancer (Hoekstra et al, 2015). In solid tumors, they also described that LMWPTP overexpression in patient samples is associated with cancer malignancy and patient survival. LMWPTP emerged as a poor prognostic and development stage biomarker, as a correlation between proportional expression of LMWPTP to higher degree of dysplasia and liver metastasis was observed for colorectal cancer (Hoekstra et al, 2015).

Despite the advances in our knowledge regarding kinase and phosphatase signaling in cancer in recent years, several knowledge gaps remain. While aspects of kinases and phosphatases in several cellular aspects of cancer have been investigated, most notably migration and proliferation, several other characteristics, including interaction of tumor cells with stromal cells and their role in chemoresistance, remain underexplored. Further elucidation of the role of kinase and phosphatase signaling in cancerous processes requires further attention in order to develop secondary lines of treatment.

Outline of the thesis

Treatment and survival of cancer has improved remarkably over the last decades. In particular the advent of targeted therapies, including cell cycle inhibitors, kinase inhibitors and others, have improved outcomes of several types of cancer. However, tumor relapse due to resistance acquisition is often observed and a better understanding of cancer signaling in order to devise novel targeted treatment strategies is thus still required. The aim of this thesis was to further investigate phosphorylation events in different malignancies and determine whether LMWPTP could potentially be a target for treatment.

In **CHAPTER 2**, we employed kinome profiling to investigate phosphoprofiles of cells treated with the targeted anti-cancer drug vismodigib. We demonstrate that kinomic activity is modulated by this treatment, but that non-canonical pathways exist which may render cancer cells unsusceptible to these treatments. Thus, finding alternative treatment targets remains imperative. In **CHAPTER 3**, we turn our attention to the phosphatase class of enzymes. The phosphatase LMWPTP is highly overexpressed in malignant hematopoietic cells. Again employing kinome profiling, we demonstrate that this phosphatase modulates phosphoprofiles in hematopoietic cancer cells, which confers metabolic changes associated with increased drug resistance and survival of cells, and in **CHAPTER 4**, we link these metabolic changes to LMWPTP-dependent autophagy modulation. Our data suggest that LMWPTP may enhance this interaction, which confers further survival and growth advantage to tumor cells. After introducing the concept of platelets as tumor-promoting aging-dependent agents in **CHAPTER 5**, we further investigate the role of LMWPTP in tumor-platelet interactions in **CHAPTER 6.** We demonstrate that LMWPTP is overexpressed in gastric and colonic cancers. In addition, our data suggest that LMWPT may enhance the interaction between cancer cells and platelets, which confers further survival and growth advantage to tumor cells. In **CHAPTER 7**, we review signal transduction events in platelets themselves. As for cancer cells, much is known regarding the role of kinase activities in platelets, while phosphatases have been relatively less well studied. In **CHAPTER 8**, we show for the first time that in platelets contain active LMWPTP enzyme, which is modulated by platelet agonists and arguably plays a role in their activation as we demonstrate that the platelet antagonist 3-bromopyruvate inhibits enzymatic activity of LMWPTP.

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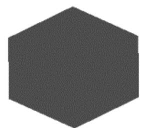

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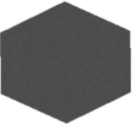

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Chapter 2

Smoothened-dependent and independent pathways in mammalian non-canonical Hedgehog signaling

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Abstract

Hedgehog proteins are pivotal morphogens acting through canonical pathway involving first activation of ligand binding to Patched followed by alleviation of Smoothened receptors inhibition, leading to activation of Gli transcription factors. Noncanonical Hedgehog signaling remains poorly characterized, but is thought to be mainly dependent on Smoothened. However, Smoothened inhibitors have yielded only partial success in combating Hedgehog signal transduction–dependent cancer, suggesting that noncanonical Smoothened-independent pathways also are clinically relevant. Moreover, several Smoothened-dependent effects (e.g. neurite projection) do not require transcriptional activation, further suggesting biological importance of noncanonical Smoothened-dependent pathways. We comprehensive characterized the cellular kinome in Hedgehog-challenged murine wildtype and Smoothened-/- fibroblasts, as well as Smoothened agonist–stimulated cells. A peptide assay–based kinome analysis (in which cell lysates are used to phosphorylate specific kinase substrates), along with endocytosis, Lucifer yellow–based, and immunoblotting assays, identified an elaborate signaling network of both Smoothened-dependent and -independent pathways that mediates actin reorganization through Src-like kinases, activates various proinflammatory signaling cascades, and concomitantly stimulates Wnt and Notch signaling, while suppressing bone morphogenetic protein (BMP) signaling. The contribution of noncanonical Smoothened-independent signaling to overall effects of Hedgehog on cellular physiology appears to be much larger than previously envisioned and may explain the transcriptionally independent effects of Hedgehog signaling on cytoskeleton. The observation that Patched-dependent, Smoothened-independent, noncanonical Hedgehog signaling increases Wnt/Notch signaling provides a possible explanation for the failure of Smoothened antagonists in combating Hedgehog-dependent but Smoothened inhibitor–resistant cancer. Our findings suggest that inhibiting Hedgehog–Patched interaction could result in more effective therapies as compared to conventional Smoothened-directed therapies.

Introduction

Cell fate is determined by morphogens, molecules whose non-uniform distribution governs the pattern of tissue development [1,2]. Notable examples of morphogens include Hedgehog, Wingless-related integration site (Wnt) and Bone morphogenetic protein (BMP) [3-5]. The intracellular signaling resulting from engagement of morphogens with their cognate receptors is involved in many physiological and pathophysiological processes, including embryogenesis, tissue regeneration, and carcinogenesis. Fully understanding morphogen signaling is therefor of the utmost importance [6]. Unfortunately, morphogen signaling is often extremely complex, a special case to point being signal transduction initiated by Hedgehogs [7].

Hedgehog proteins are a highly conserved family of intercellular signalling molecules. Originally identified as a Drosophila segment polarity gene required for embryonic patterning, several vertebrate homologues have been discovered—Indian (Ihh), Desert (Dhh) and Sonic Hedgehog (Shh), the latter being most extensively characterised [8]. Hedgehog signals are fundamental regulators of embryonic development, as illustrated by embryological malformations seen when accurate timing of Hedgehog signals during gestation is corrupted [9]. Hedgehog remains active in the post-embryonic period, maintaining histostasis in a variety of tissues, including the gastrointestinal tract and the immune system [10]. Continuous hedgehog signalling is an essential permissive factor for many cancers and causative in basal cell carcinoma of the skin [11]. In humans, one-allelic loss of the inhibitory hedgehog receptor Patched is sufficient to produce the so-called Gorlin syndrome [12], which is associated with rhabdomyosarcoma and the development of multiple basal cell carcinomas.

Despite the importance of Hedgehog signalling for human physiology and pathophysiology, the molecular details underlying this signalling pathway remain only partly characterized. The primary receptor for Hedgehogs is Patched, an unconventional receptor, as it does not convey the Hedgehog signal to the intracellular components of the pathway itself. Rather, binding of Hedgehog to Patched alleviates the inhibitory effect of Patched on another membrane receptor, Smoothened. The Patched inhibition alleviation is probably caused by internalization of Patched following Hedgehog binding, but the signaling mechanisms involved remain obscure [13]. Subsequently, Smoothened mediates the activation of the latent transcription factor glioma-associated oncogene (Gli) via a process which involves the kinase Fused (Fu), the Suppressor of Fused protein (Su(Fu)) [14, 15] and inhibition of Gli proteolysis. Gli proteins are considered the final transcriptional effectors of Hedgehog signaling, both in normal vertebrate development as well as oncological disease [16].

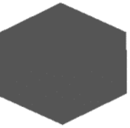
Together this signalling cascade may be termed the canonical hedgehog pathway. It is obvious that enhanced knowledge of the signaling elements involved in this pathway should prove exceeding useful in defining novel rational therapy directed at disease emanating from aberrant activation of canonical Hedgehog signaling.

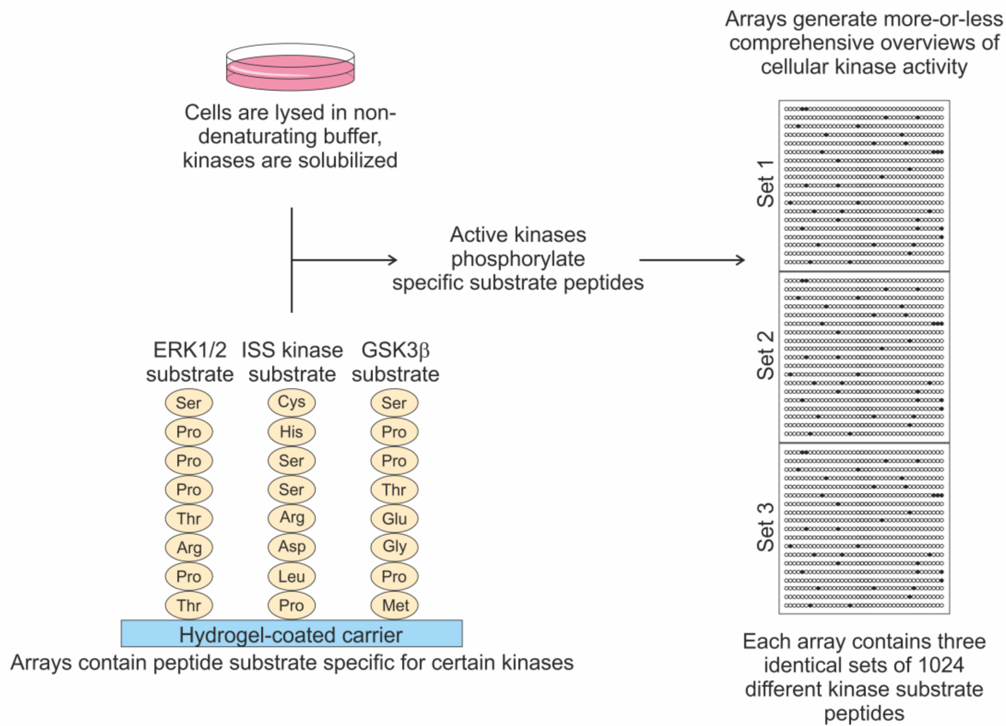
In addition to canonical Hedgehog signalling, a role for transcription-independent signalling via Hedgehog has also been suggested [17-19]. Tantalizingly, the presence of canonical and non-canonical Hedgehog signaling opens the theoretical possibility to uncouple the anti-cancer effect of Hedgehog signaling on cancer in general [20] and the trophic effect of Hedgehog signaling on specifically cancer stem cells. In the absence, however, of knowledge on the molecular pathways that mediate these non-canonical effects of Patched-dependent but Smoothened-independent Hedgehog signaling, this possibility remains hypothetical only. In an effort to address this issue, here we endeavor to characterize the signaling pathways involved.

Results

Hedgehog stimulation provokes rapid and marked reorganization of the cellular kinome

We set out to characterize the kinase activities associated with Hedgehog challenge in general, as well as those specifically associated with Patched activation or Smoothened activation in isolation. To this end we exploited the power of peptide array-based kinome profiling, which allows the generation of comprehensive descriptions of cellular kinase activities [21-23]. The general approach to this study, both technically and biologically is provided through Figure 1. We characterized the kinase signatures associated with Hedgehog stimulation of mouse embryonic fibroblasts (MEFs), which we have recently shown to constitute a powerful model for delineating signal transduction events [24]. We established that under our experimental conditions, these cells do not endogenously release Hedgehog (not shown). Cells were incubated for 10 min with either 2 μg/mL Shh or a vehicle control, and the cell lysates were employed for in vitro phosphorylation of peptide arrays using 33P-γ-ATP. Arrays consisted of 1024 different undecapeptides, of which 48 are various technical controls, whereas the remaining 976 peptides provide kinase substrate consensus sequences spanning the entire mammalian kinome and which we have shown earlier to provide comprehensive insight in cellular signal transduction [25]. On each separate carrier, the array was spotted three times, to allow assessment of possible variability in substrate phosphorylation. As a control for the specificity of the reaction 33P-α-ATP was used; no incorporation of radioactivity was seen (data not shown). We then calculated the mean phosphorylation level for all substrates before and after the treatment (total number of data points is 9 for each group). The technical quality of the profiles was good, and we only allowed experiments in which the Pearson product moment correlation coefficient was more as 0.95 for the technical replicas. Results were collapsed on elective signal transduction categories (see experimental procedures and [25]).





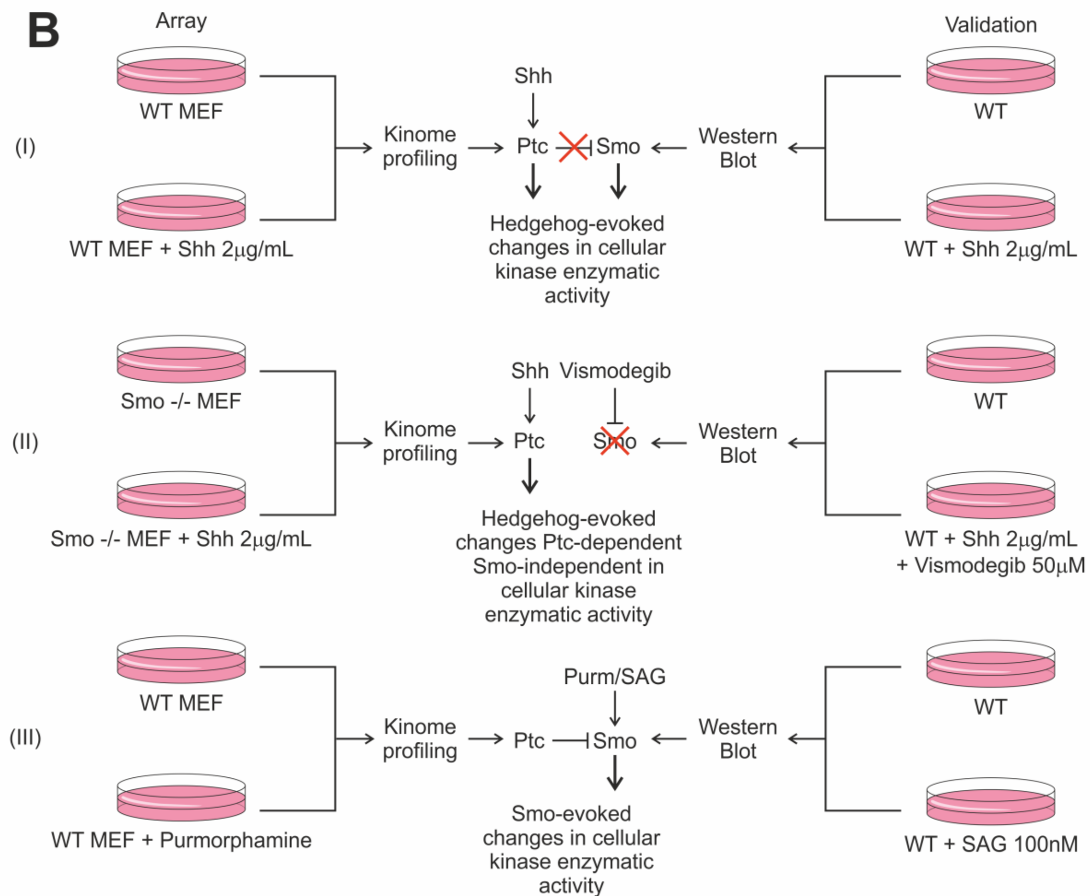


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Figure 1. Outline of the study. A. Technical approach – kinome profiling. In this study we aim to comprehensively characterize cellular kinase enzymatic activities. To this end appropriately stimulated cell cultures are washed with ice-cold PBS and lysed in a non-denaturing complete lysis buffer so as to solubilize cellular kinases. Lysates are the transferred to arrays consisting of a substrate peptide library, spotted in triplicate to assess technical reproducibility, which are spotted on a hydrogel-coated glass carrier. Upon addition of radioactive ATP and an activation mix, kinases –if enzymatically active- will phosphorylate substrate peptides. Incorporation of radioactive ATP into a substrate peptide is taken as measure of enzymatic kinase activity towards a particular substrate. The broad variation in specific substrates used (see also supplementary data) allows obtaining a more-or-less complete description of cellular signaling, the so-called kinome. **B.** Biological approach. In this study we first generate a description of the effects of Shh challenge on cellular signaling in general by comparing kinome profiling results of cultures challenged and not challenged by the morphogen. To identify signal transduction events that are downstream of Ptc but do not involve Smo, the Hedgehog provoked effects on the cellular kinome are studied in fibroblasts genetically deficient for Smo. Finally, to identify events that are solely dependent on the activation of Smo, we study the effects of the Smo agonist purmorphamine (purm). Several kinome profiling results are subsequently validated using a second approach, in which MEFs were stimulated with Shh and subjected to Western blot analysis. To simulate Ptc-dependent effects, cells are treated with the Smoothened inhibitor (Vismodegib) prior to Shh stimulation. To simulate Smo-dependent effects, cells are treated with the Smoothened agonist SAG.

The results are shown in Figure 2A and detailed in Supplementary table 1. They show that Hedgehog challenge provokes fast and substantial remodeling of cellular signaling. Particularly notable is the upregulation of mTOR signaling. mTOR is a key component of Hedgehog signaling and is a putative target for treating Hedgehog-driven cancers [26]. Other interesting points include an upregulation of G-protein-coupled receptor kinase enzymatic activity, which is able to control Smoothened activity [27, 28]. This is also in line with the fact that Smoothened itself is such a receptor and the observation that PKC enzymatic activity is upregulated, conform the canonical mode of action of G-protein coupled receptors. Strong regulation of PKA, a proposed regulator of Hedgehog signaling [29], is also seen. We observed activation of a variety of pro-inflammatory signaling modules (including Lyn, Fyn and peptides that are consensus substrates for Bruton's tyrosine kinase), but as embryonic fibroblasts are not immunological cells, the importance of this observation is uncertain. In our untransformed epithelial model system, Hedgehog stimulation reduced Wnt signaling. These data are in line with studies shown that Hedgehog acts as an inhibitor of Wnt signaling in colon cells [30] although an activating role for Hedgehog on Wnt signaling has been proposed in cancer stem cells [31]. Lastly, the upregulation of substrate peptides for p21-activated kinase (Pak) activity and related molecules indicates that Hedgehog stimulation stimulates

actin reorganization and morphological changes. Together, these data show that the effect of Hedgehog on the cellular kinome is rapid and profound.

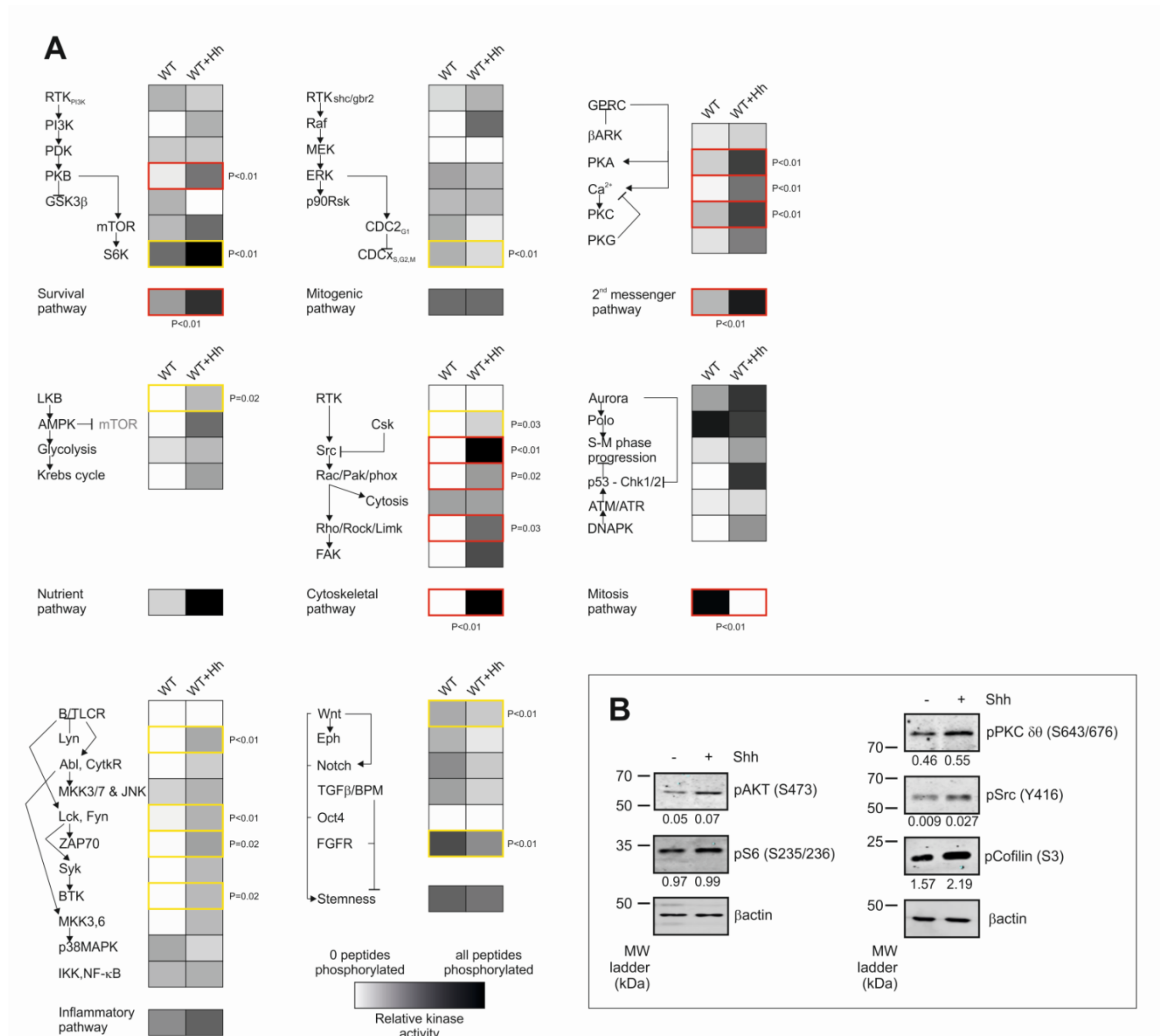


Figure 2. Effects of Hedgehog stimulation on cellular signaling as determined by kinome profiling. (A) Murine fibroblasts were stimulated with 2 µg/mL Shh. Subsequently cells were lysed and the resulting lysates were used to phosphorylate arrays of different kinase substrates employing ^{33}P-γ-ATP and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements. The picture depicts the number of peptides significantly phosphorylated (which means the number of peptides that received a Markov "on" call - see experimental procedures) for each element. A darker color reflects more kinase activity towards substrate elements and the results reveal the effects of Hedgehog stimulation on cellular signal transduction, thus a black color means all peptides were significantly phosphorylated, whereas a white color means that no peptides allotted to this signal transduction in this experimental condition were phosphorylated. Results were first statistically tested by a dichotomal analysis based on the number of Markov "on" calls observed in vehicle-and Shh-stimulated cultures. If statistically significant differences were noted the signal transduction category is highlighted with a red border and the level of significance observed is indicated in red. For signal transduction elements in which this very robust analysis fails to detect a statistically significant difference, a parametric

test was performed. If this proved significant, the category is highlighted with an orange color and corresponding level of significance is depicted as well. The results provide a wealth of data on the effects of Hedgehog stimulation on cellular signaling. (B) MEFs were grown in 6 wells plates. To simulate Smo and Ptc-dependent signaling, cells were treated with Shh (2 μg/mL) for 10 minutes and compared to unstimulated cells. Cells were lysed and proteins resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with antibodies against the indicated phosphorylated proteins. Blots were reprobed with antibodies against ß-Actin to confirm equal loading.

Despite the great sensitivity and efficiency of array kinome profiling, we validated several of the key pathways by western blot (Figure 2B). Consistent with canonical Shh signaling, phosphorylation of PKC was observed (intentsity of α-phosho-PKCδ/θ increased by a factor 1.22), showing the validity of these models. Secondly, we show an increased activity of the mTOR-PKB/Akt-S6 pathway upon Shh stimulation (intentsity of α-phosho-Akt staining increased by a factor 1.75, $p<0.05$). Furthermore, in agreement with the Shh-induced cytoskeletal remodeling seen in kinome experiments, we observed an increase in Cofilin (intentsity of α-phosho-cofilin staining increased by a factor 1.86, $p<0.05$) and Src family phosphorylation (intentsity of α-phosho-Src staining increased by a factor 1.19). Although these changes in phosphorylation are more modest as those observed in the kinome array, they do support the peptide array data. As Western blot measures the sum of kinase and phosphatase activity, whereas the kinome array measures only kinase activity the Western blot data indicate the presence of compensatory mechanisms counteracting increased phosphorylation of substrate proteins. Hence, these data validate the robustness and validity of the kinome data.

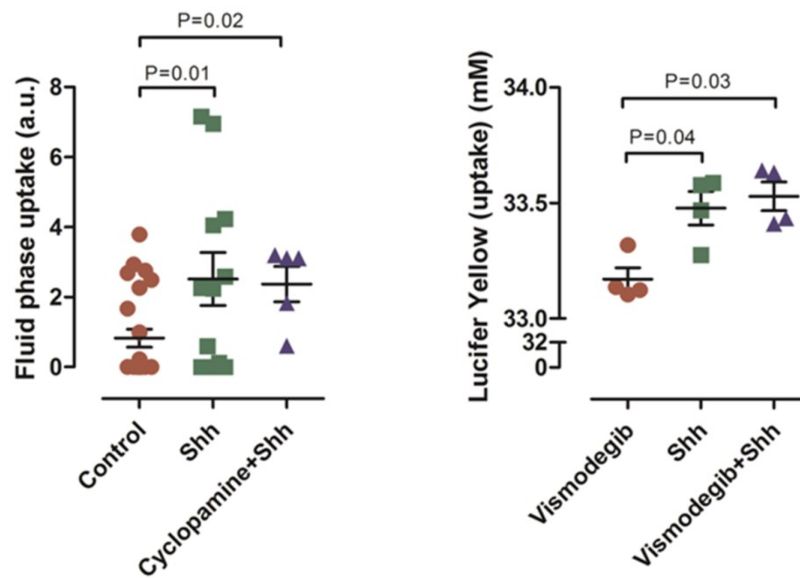
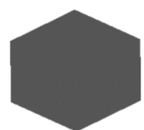


Figure 3. Effects of Hedgehog on endocytosis and the influence of Smoothened inhibition thereon. (A) Fibroblast cultures were grown in twenty-four-wells plates and incubated in a 1 mL containing 200 nCi of [^{3}H]-sucrose in the presence or absence of either 1 µg/mL Shh and 10 µM cyclopamine or appropriate vehicle control. At the end of the experiment cells were extensively washed with ice-cold PBS and lysed in NP-40 for subsequent scintillation counting. As sucrose can only enter cells through fluid phase uptake, this provides a reliable measure of cellular endocytosis. We observe that Hedgehog stimulates fluid phase uptake and this effect does not require Smoothened as it is not sensitive to the Smoothened inhibitor cyclopamine. (B) Similarly, fluorescence spectrophotometry indicated that fibroblasts grown in 96 wells plates and treated with Shh (2 µg/mL) for 6 hours still show uptake of Luciferin Yellow (35 µM) even in the presence of the smoothened inhibitor Vismodigib (50 µM), indicative of a Ptc-dependent, Smo-independent cellular process.

Patched-dependent Smoothened-independent effects on cellular kinase activity

The existence of Patched-dependent Smoothened-independent signal transduction is supported by various observations [32] and appears highly relevant in that it is essential for cancer stem cell survival in colorectal cancer [31]. To test whether such signaling is present in our model system, we incubated embryonic fibroblasts with 3H-sucrose (which is membrane impermeable and is only taken up via endocytosis in most cell types) and challenged the cells with either a vehicle control or 2 µg/mL Shh, in the presence or absence of the Smoothened inhibitor cyclopamine (Figure 3A). We observed strong accumulation of radioactivity in Hedgehog-challenged cells, as well as in cells challenged with Hedgehog in the presence of cyclopamine, indicating that Smoothened-independent cellular function is present in Hedgehog-stimulated fibroblasts. As a control tomatidine (an alkaloid similar to cyclopamine that has no action on Smo) was used but no effect was observed (not shown). To confirm our observation using a more specific, clinically relevant Shh signaling inhibitor, we used

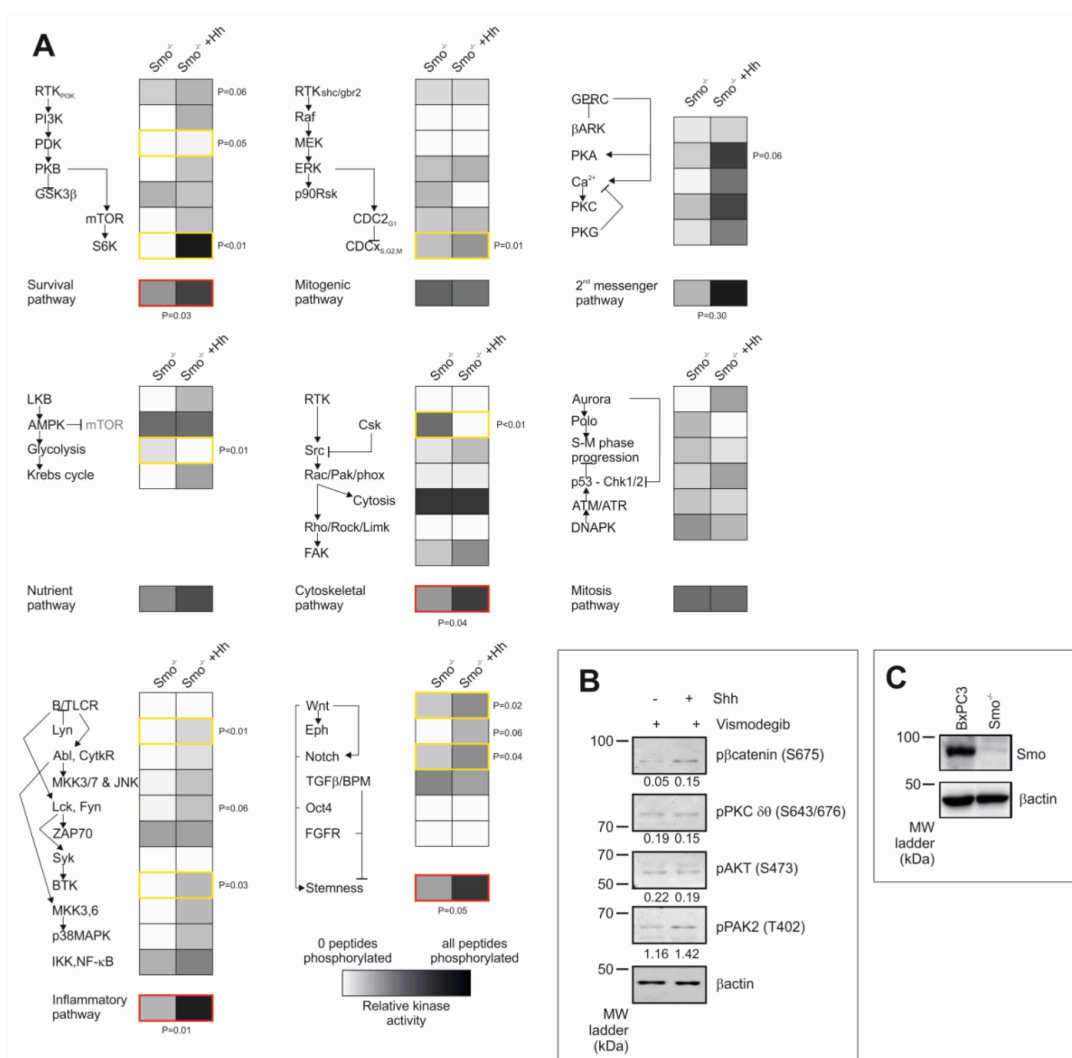
Vismodegib. Vismodegib is described to be a specific Smoothened inhibitor and was FDA approved in 2012 for the use of advanced basal-cell carcinoma [33]. Vismodigib-treated cells were stimulated with Shh (2µg/mL), and incubated with Lucifer Yellow, a classic fluorescent molecule that can be used to quantify pinocytosis [44]. Lucifer Yellow uptake in the presence of Shh was not decreased by inhibition of Smoothened (Figure 3B). We thus concluded that endocytosis following Hedgehog stimulation does not require Smoothened activity and that hence our model system was suitable for investigating at least certain aspects of Smoothened-independent signal transduction.



To further characterize these aspects we performed kinome profiling of Smoothened-/- fibroblasts (originally obtained from Drs. James Chen and Philip Beachy and previously described by Varajosalo et al [35]), challenged with either a vehicle control or 2 µg/mL Shh for 10 min. The results are summarized in Figure 4A and Supplementary table 1 and reveal that the influence of Smoothened-independent Hedgehog-induced signaling on cellular kinase activity is substantial. Lacking however, is G protein-coupled receptor-associated signal transduction, which is obviously in line with the absence of Smoothened-dependent events. In particular, activation of cytoskeletal remodeling is seen following addition of Hedgehog, which correlates with a reduced activity of the negative Src activity regulator, Csk. This may relate to the observed Smoothened-independent effects of Hedgehog on endocytosis described above, especially as kinase enzymatic activity directed against FAK-responsive peptides is observed to be co-activated in our profiles, which fits canonical signaling on endocytosis [36]. Another prominent effect upon Hedgehog in Smoothened-/- fibroblasts is increased mTOR activation, whereas inflammatory signal transduction was also activated. Hedgehog in wild type fibroblasts provokes similar effects (see above) and thus these effects of Hedgehog signaling appear at least partially to stem from Smoothened-independent signaling. Similarly, activation of Wnt and Notch signaling is also seen and thus this aspect of Hedgehog signaling seems also independent of Smoothened. Interestingly, in the absence of Smoothened, Hedgehog activates rather than inhibits PKA, and it is tempting to speculate that this effect may relate to activating phosphorylation of Smoothened by PKA that has been described in Hedgehog signaling [37]. In conjunction, these results reveal that an unexpectedly large proportion of Hedgehog signal transduction towards the cellular kinome is mediated though non-canonical Patched-dependent Smoothened-independent signaling.
To simulate these Patched-dependent, smoothened independent effects, we also treated cells with Vismodigib in the presence and absence of Shh (Figure 1, 4B), and show that Wnt signaling (as measured by β-Catenin activity) was also indeed activated independently of

smoothened in this system, as were PAK and S6 phosphorylation. Although the changes in phosphorylation observed on Western blot are more modest as those observed in the kinome array, they do support the peptide array data. As Western blot measures the sum of kinase and phosphatase activity, whereas the kinome array measures only kinase activity the Western blot data indicate the presence of compensatory mechanisms counteracting increased phosphorylation of substrate proteins. In addition we verified the nature of the Smoothened-/- fibroblasts by Western blot (Figure 4C).

These results, demonstrating the presence of a Smoothened-independent activation, suggest that treatment with Smoothened inhibitors may lack the potential to attenuate full Shh signaling and may provide some explanation as to why, while efficacious in some tumor types, the use of Vismodigib in other Shh-activated tumors (e.g. prostate cancer) shows less promise [38].



(Legend on next page)

Figure 4. Effects of Hedgehog stimulation on cellular signaling in Smo-deficient fibroblasts. Murine $Smo^{-/-}$ fibroblasts were stimulated with 2 µg/mL Shh. Subsequently cells were lysed and the resulting lysates were used to phosphorylate arrays of different kinase substrates employing ^{33}P-γ-ATP and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements and a darker color reflects more kinase activity towards substrate elements and the results reveal the effects of Hedgehog stimulation on cellular signal transduction. Results were first statistically tested by a dichotomal analysis based on the number of Markov "on" calls observed in vehicle-and Shh-stimulated cultures (highlighted with a red border). For signal transduction elements in which this very robust analysis fails to detect a statistically significant difference, a parametric test was performed (highlighted in orange). The results reveal an intricate web of Patched-dependent Smoothened-independent non-canonical signal transduction events. (B) Smo-independent signaling was investigated by treating cells in the presence of both Shh (2 µg/mL) and the Smoothened inhibitor Vismodigib (50 µM, 30 minutes pre-incubation). Cells were lysed and proteins resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with antibodies against the indicated phosphorylated proteins. Blots were reprobed with antibodies against ß-Actin to confirm equal loading. C. Validation of the nature of the $Smo^{-/-}$ culture. BxPC3 cells were used as $Smo^{+/+}$ control. Cells were lysed and proteins resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with an antibody against Smo. Blots were reprobed with antibodies against ß-Actin to confirm equal loading.

Cellular kinase response to selective Smoothened activation

Next, we decided to investigate the effects of selective Smoothened activation in MEFs. To this end we challenged cells with purmorphamine, a purine derivative that acts as a direct agonist of Smoothened [39]. The results are provided through Figure 5A and Supplementary table 1. We observe that purmorphamine results in inhibition of PKA. As Hedgehog stimulation in both WT and Smoothened-/- cells was increased, PKA activity appears dominated by Patched-dependent, Smoothened independent signaling. Intriguingly, purmorphamine results in a downregulation of ROCK, which is important for a variety of cellular processes, but in particular for cytoskeletal reorganization [40]. It was earlier established that Smoothened is a powerful mediator of chemotactic responses, but only so when not located at the primary cilium [30]. At the primary cilium, Smoothened loses its capacity to stimulate chemotaxis. The apparent downregulation of ROCK activity following purmorphamine stimulation is thus best explained by a purmorphamine-dependent recruitment of Smoothened to the primary cilium. The strong canonical responses to purmorphamine stimulation observed by others would agree with this notion, as would the marked downregulation of PKA activity in our profiles. We also employed the Smoothened agonist SAG to confirm some of these effects by Western blot analysis (Figure 5B). While generally lower than Shh (Figure 5B), SAG induced Src, Pak, PKB/S6 and Wnt signaling in MEFs. Although these changes in phosphorylation observed on Western blot are more modest

as those observed in the kinome array, they do support the peptide array data. As Western blot measures the sum of kinase and phosphatase activity, whereas the kinome array measures only kinase activity the Western blot data indicate the presence of compensatory mechanisms counteracting increased phosphorylation of substrate proteins.

In some aspects, the rapid Smoothened-independent effects and rapid Smoothened-dependent effects on cellular kinase activities studied in our experimental set up, are similar, as both provoke mTOR activation and, in our model system, activation of Wnt signaling. In this sense, non-canonical signaling downstream of Patched and Smoothened may converge to produce the final phenotype. It is important to stress that our set up does not allow for studying the effects of canonical Hedgehog signaling, which requires transcriptional responses. Generally speaking canonical signaling and non-canonical signaling by morphogens counteract each other and the effects observed in this study partially substantiate that notion for Hedgehog signaling as well. Not seen downstream of specific Smoothened stimulation were strong pro-inflammatory responses, which therefore seem mainly Patched-dependent. Generally speaking, Patched-specific signaling events (i.e. the effects of Hedgehog stimulation on Smoothened-/- fibroblasts) were more pronounced as those provoked by purmorphamine stimulation as also evident from the number of peptides that became significantly phosphorylated (see experimental procedures), i.e. 180 peptides in Hedgehog-stimulated Smoothened-/- fibroblasts and 134 in purmorphamine-stimulated wild type fibroblasts. It thus appears that the major branch of non-canonical Hedgehog signaling is downstream of Patched but not of Smoothened (See Figure 6 and Table 1 for overview).

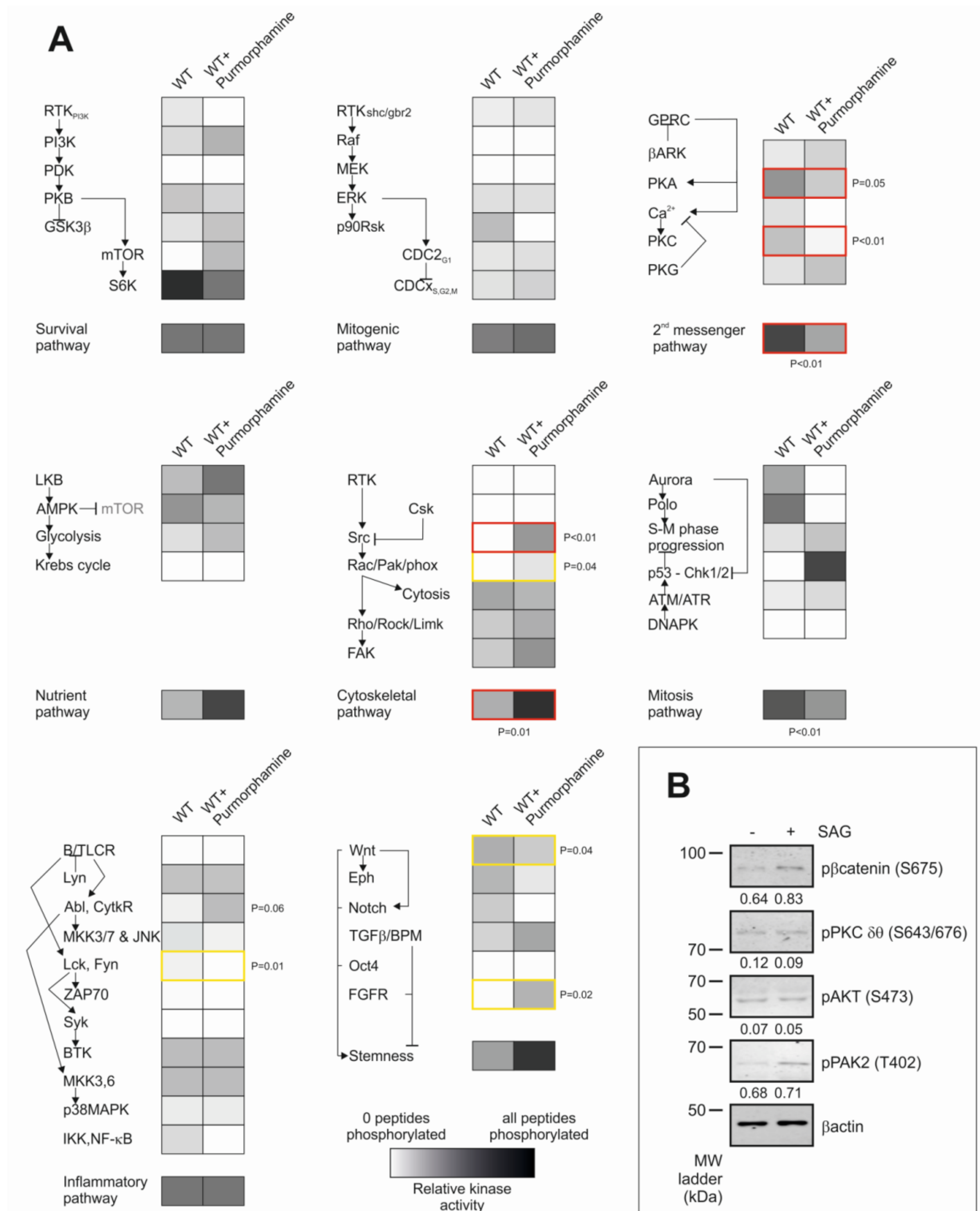


Figure 5. Effects of selective Smoothened activation by purmorphamine stimulation on cellular signaling in fibroblasts. (A) Murine fibroblasts were stimulated with purmorphamine. Subsequently cells were lysed and the resulting lysates were used to phosphorylate arrays of different kinase substrates employing ^{33}P-γ-ATP and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements and a darker color reflects more kinase activity towards substrate elements and the results reveal the effects of Hedgehog stimulation on cellular signal transduction. Results were first statistically tested by a dichotomal analysis based on the number of Markov "on" calls observed in vehicle-and Shh-stimulated cultures (highlighted with a red border). For signal transduction elements in which this very robust analysis fails to

detect a statistically significant difference, a parametric test was performed (highlighted in orange). The results reveal a web of Smoothened-dependent signal transduction events clearly distinct from Patched-dependent signaling. (B) To investigate Ptc-independent signaling, cells were subjected to treatment with the Smo agonist SAG (100 nM) for 10 minutes. Cells were lysed and proteins resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with antibodies against the indicated phosphorylated proteins. Blots were reprobed with antibodies against ß-Actin to confirm equal loading.

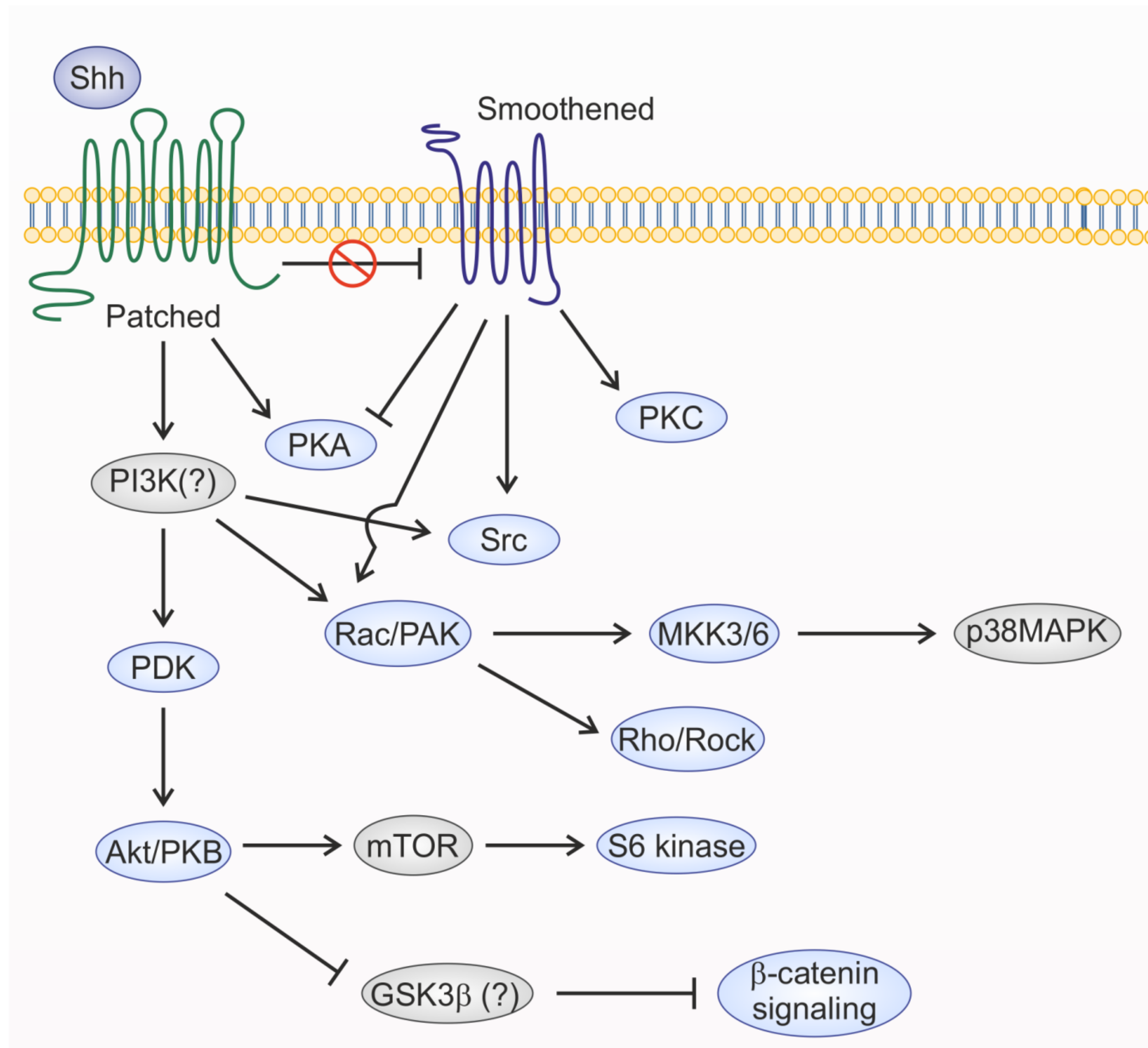


Figure 6. Selected kinome profiling-detected Shh-provoked signal transduction events and the role of Patched and Smoothened therein. Blue elements are confirmed, whereas gray elements showed a trend but did not reach Bonferoni-corrected statistical significance. The results reveal that the role of Patched-dependent Smoothened-independent signal transduction is more prominent in transcription-independent cellular effects of Hedgehog as previously thought.

Discussion

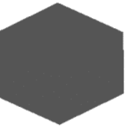
Hedgehog signal transduction is highly unusual, containing many features unique to this signaling system (e.g. [41, 42]). Apart from canonical Hedgehog signaling, Hedgehog effects in physiology and pathophysiology also depend on so-called non-canonical signaling. For most morphogens, non-canonical signaling has been identified and effects observed are in general contrasting the effects derived from canonical signaling. An example is BMP signaling, which generally acts as a tumor suppressor in the colon [5]. In the presence of canonical BMP-signaling abrogating SMAD4 mutations, a non-canonical BMP-induced signaling pathway becomes evident that stimulates epithelial-to-mesenchymal transition and metastasis via activation of Rho and ROCK and furthers the colon cancer process [9]. Likewise, non-canonical Wnt signal transduction mediates important aspects of the action of this morphogen in the body through activation of small GTPases like Rac, Rho and Cdc42 to regulate the activity of ROCK, MAPK and JNK as well as Ca^{2+} signaling, also an effect important for colon cancer metastasis [43]. For Hedgehog also various modes of non-canonical signaling have been described, both downstream of Patched and independent of Smoothened as well as downstream of Smoothened. The most prominent example of the former concerns colorectal cancer stem cells [31]. Whereas canonical Gli-dependent Hedgehog signaling negatively regulates Wnt signaling in the normal intestine and intestinal tumors [30], Hedgehog signaling in colon cancer stem cells activates a non-canonical Patched-dependent but Smoothened-independent signaling that is required for survival of these cancer stem cells.

Apart from Patched-dependent Smoothened-independent non-canonical Hedgehog signaling, Smoothened-dependent Gli-independent non-canonical Hedgehog signaling has also been described and likewise the molecular mechanisms involved are only partly understood. The interaction of Hedgehog with Patched stimulates the translocation of Smoothened to the primary cilium, which is required for the transcriptional Hedgehog response [26]. This translocation involves activation of phospholipase A2 following Smoothened activation and results in the enzymatic release of arachidonic acid from plasma membrane phospholipids. Arachidonic acid metabolites are powerful actin cytoskeleton remodeling agents [44] and while located outside the primary cilium, Smoothened also mediates transcription-independent actin reorganization and chemotactic responses through the production of these metabolites [17-19]. The physiological importance of this non-canonical response to Hedgehog signaling is illustrated by its pivotal role in Hedgehog effects in directing neurite projection [18]. It has been shown that non-canonical Hedgehog effects on axonal guidance

involve activation of Src-like kinases [19], and our data now yield a plethora of information regarding the signaling pathways contributing the non-canonical signaling induced by Hedgehog. The changes in kinase activity measured may derive from either altered expression of kinases or altered activity of the individual kinase enzymes involved. As the stimulation period of the experiments is very short (10 minutes) we feel the latter explanation the most probably but until experiments in the presence of translation inhibitors have been performed, other possibilities should be kept in mind. Similarly, it should be noted that there is a disconnect between effect size on Western blot and kinome array, suggesting that part of the kinase effects observed are counteracted by compensatory phosphatase activity, thus the importance of our observations for phenotypic cellular activities such as proliferation, viability, migration will remain to be investigated in other studies. Nevertheless, the final effect of Hedgehog in physiology and pathophysiology is resultant from the integration of both canonical and non-canonical Hedgehog signaling [32]. The potential of pharmacological inhibitors of Hedgehog signaling in the treatment of disease has received substantial attention and various trials employing pharmacological inhibitors of Hedgehog signaling have been conducted. Especially Vismodegib and Sonidegib have met with success in diseases driven by canonical Hedgehog signaling, in particular dermatological cancer [33]. Despite the evidence, however, that Hedgehog signaling is important for many gastrointestinal cancers [46], trials in this type of disease have not yet proven successful. In view of our data presented above that Patched and not Smoothened is a major mediator of non-canonical Hedgehog signaling and the momentum-gaining notion that especially non-canonical Hedgehog signaling may be important for maintaining gastrointestinal cancer [31], this may not be surprising. Vismodegib and Sonidegib target Hedgehog signaling at the level of Smoothened and leave Patched-dependent non-canonical Hedgehog signaling unaffected. Especially in view of the Patched-dependent Smoothened-independent Wnt signaling, one can easily imagine that especially the non-canonical branch of Hedgehog signaling is important in supporting growth in the gastrointestinal compartment. An implication of our results is thus that future Hedgehog-based therapy with respect to gastrointestinal cancer should be directed at counteracting the interaction of Patched with Hedgehog rather than the current strategy of targeting Smoothened. Obviously, proof of this notion awaits experimentation in cancer cells that are insensitive to Smoothened inhibitors but require extracellular Hedgehog.

Conclusions

Here we characterise the non-canonical aspect of Hedgehog signaling. We observe that such non-canonical signaling mainly involves Patched-dependent Smoothened-independent signaling, with especially activation of cytoskeletal remodeling and the activation of Wnt signaling being prominent elements. Thus, for efficient targeting of Hedgehog-dependent signaling it may prove essential to target such signaling at the level of Patched and not Smoothened.



Experimental procedures

Materials

Cyclopamine was from Biomol (Hamburg, Germany). Purmorphamine was from EMD Biochemicals (Darmstadt, Germany) and was dissolved in ethanol (final concentration 0.2 %). Recombinant Sonic Hedgehog was from R&D Systems (Minnesota, USA). Sonic Hedgehog inhibitor Vismodegib (GDC-0449) was from Selleck Chemicals (Texas, USA) and reconstituted in DMSO (final concentration 0.025%). Shh agonist SAG (SML1314-1MG; #14454) was from Sigma-Aldrich (Missouri, USA) and Recombinant Murine Sonic Hedgehog (Shh) (315-22, 0513521) was from PeproTech, Inc.

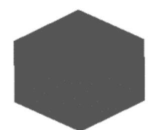
Cell culture

Smoothened$^{-/-}$ fibroblasts (provided by Dr. Taipale) and wild-type mouse embryonic fibroblast (provided by Dr. Scott) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, California, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen, California, USA), and propagated at 37°C in a 5% CO_2 humidified atmosphere. For experiments a confluence of 50% cells was allowed to grow in six-wells plates. Stimulations were done, if appropriate, with 2 μg/mL Shh for 10 minutes. Each experiment consisted of three biological replicas of experiments containing three technical replicas.

Kinome Profiling

For peptide array analysis, we employed the Pepchip kinomics array. The protocol and associated analysis has been described in detail elsewhere [25] and is based on the original protocol of van Baal et al. [47]. In short, cells were washed in ice-cold PBS and lysed in a non-denaturing complete lysis buffer (cells were lysed in 50μL lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM MgCl2, 1mM glycerophosphate, 1mM Na3VO4, 1mM NaF,1μg/mL Leupeptin, 1μg/mL Aprotinin, 1mM PMSF). Subsequently the cell lysates were cleared by centrifugation and peptide array incubation mix was produced by adding 10 μL of activation mix (50% glycerol, 50 μM ATP, 0.05% v/v Brij-35, 0.25 mg/ml bovine serum albumin) and 2 μL [γ-33P] ATP (approx. 1000 kBq (Amersham AH9968). Next, the peptide array mix was added onto the chip, and the chip was kept at 37°C in a humidified stove for 90 minutes. Subsequently the peptide array was washed twice with Tris-buffered saline with Tween 20, twice in 2M NaCl, and twice in demineralised H_2O and then air-dried. The chips were exposed to a phosphor screen for 72 h, and the density of the spots was measured and

analyzed with array software (ScanAnalyze). Using grid tools, spot density and individual background were corrected and spot intensities and background intensities were analyzed. Data from at least 9 independent data points were exported to an excel sheet for further analysis. Control spots on the array were analyzed for validation of spot intensities between the different samples. Inconsistent data (i.e., SD between the different data points >1.96 of the mean value) were excluded from further analysis. For each peptide the average and standard deviation of phosphorylation was determined and plotted in an amplitude-based hierarchical fashion. For data analysis, first every peptide was given an "on" call or "off" call (Markov state analysis). To this end, first an average signal was calculated for each peptide using the three biological replicates (each consisting of two technical replicates) yielding an aggregate dataset for each the hematopoietic subsets. Subsequently, for each of the aggregate datasets, either "on" calls or "off" calls were given to each peptide substrate (Markov state analysis). In order to do this, we assumed that the subset of signals representing the 1-e-1 fraction of peptides having the lowest phosphorylation of all peptides contained pure noise and did represent meaningful phosphorylation. The distribution of this noise was fitted as a single exponent, using the amplitude-sorted row number of these substrates as the X domain of the distribution and this single exponent was assumed to describe noise for the entire dataset. Now for all data points within the subset, when the actual amplitude observed minus 1,96 the standard deviation was in excess of the value expected from distribution describing the noise, a substrate was given an "on" cal ($p < 0.05$) in this Markov analysis. Subsequently results were collapsed on elective signal transduction categories and subjected to dichotomal significance analysis, contrasting Shh-stimulated cultures to parallel vehicle cultures or Purmorphamine-stimulated cultures to parallel unstimulated cultures. If a significant result ($p < 0.05$) was detected, we considered the result as robust evidence of differential activation of signal transduction between Hedgehog-stimulated and unstimulated cultures and in the depiction of results the corresponding signal transduction categories have been highlighted with a red border. For those signal transduction categories in which using this dichotomal testing based on number of Markov state "on" peptides did not result in statistical significance, the relative levels of phosphorylation were also tested using a paired T test, directly parametrically comparing phosphorylation of the corresponding spots. As we considered thus-discovered statistically significant differences between the relevant experimental conditions less robust, in the depiction of the results they have been highlighted with an orange border. Note that due to differences in the number of peptides allotted to the signal transduction categories apparently large differences in phosphorylation not always



yield statistically significant results, while smaller differences can produce such results if the number of substrates in such categories is large.

Endocytosis assay

Cells were grown on 24-well plates to 70% confluence and were stimulated with either 1 μg/mL Shh or vehicle control (0.1% BSA/PBS) and or cyclopamine (Biomol, Plymouth Meeting, Pennsylvania, United States) for 1 hour. After extensive washing with ice-cold PBS, cells were lysed in 1% Nonidet P-40 and the lysate was transferred to 4 mL of scintillation fluid and activity was determined on a Packard Tri-Carb scintillation counter (PerkinElmer, Wellesley, Massachusetts, United States). Values were corrected for solvent control treated cells on ice.

Lucifer Yellow assay

Mouse embryonic fibroblast were plated at a density of $3.5x10^3$cells/well. After 24 hours, Vismodegib was added (50μM DMSO 0.25%) for 15 minutes, followed by Shh treatment at 2μg/mL for 15 minutes. Stock solution of Lucifer Yellow CH dilithium salt (Sigma Aldrich, Germany) was prepared in PBS, and working solution in culture medium. The assay was performed using 35mM of Lucifer Yellow, incubated for 6 hours, at 37°C, 5% CO_2. After that, the supernatant was removed and the Lucifer Yellow fluorescence was measured by spectrophotometer CytoFluor MultiWell Plate 4000 (PerSeptive Biosystems, USA) with excitation 430nm and emission at 530nm. The concentration was calculated using a Lucifer Yellow curve.

MEF treatment

MEFs were seeded at $1x10^3$ cells/well and the next day, cells were incubated with Vismodegib (50μM DMSO 0.25%) for 1 hour. After, Shh at 4μg/mL and SAG at 100nM were added for 7 minutes, and western blot samples were prepared, as described below.

Western blot

After treatment, the samples were prepared by adding 2X Laemmli buffer (100 mM Tris–HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) and samples were boiled for 95°C, 10 minutes. Cell extracts were resolved by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Merck chemicals BV, Amsterdam, the Netherlands). Membranes were blocked in 50% Odyssey Blocking Buffer (LI-COR

Biosciences, Lincoln, NE) in TBS and incubated overnight at 4°C with primary antibody. Primary antibodies: From Cell Signalling: phospho-Akt (Ser473) (#4060S); phospho-PKA C (Thr197) (#4781); phospho-Src Family (Tyr416) (#2101); phospho-PKCδ/θ (Thr638/641) (#9376); phospho-S6K Ribosomal (Ser235/236) (#4858); phospho-β-Catenin (Ser675) (#9567); phospho-PAK2 (Ser20) (#2607). From Santa Cruz: β-Actin (C4) (sc-47778). From SignalWay: phospho-cofilin (Ser3) (#11139) and phospho-ROCK2 (Ser1379) (#13005). Goat polyclonal anti-Smo C-17 was obtained from Santa Cruz. After washing in TBS-T, membranes were incubated with IRDye® antibodies (LI-COR Biosciences, Lincoln, NE) for 1 hour. Detection was performed using Odyssey reader and analyzed using manufacturers software.

Statistical analysis

Statistical analysis details for each experiment are described at the legend. Furthermore, statistical methods were: a) unpaired and paired t-student, confidence interval at 95%, two-tailed and b) one-way ANOVA repeated measures test, significance level alpha 0.05 (95% confidence interval), followed by post-test Turkey, (*, **) indicates significance $P<0.05$.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Authors' contributions: The head group leaders M.P.P., M.F.B., M.J.B and C.A.S contributed for the article conceptualization; The principal group researchers K.P. and G.M.F were responsible to design the methodology; A.V.S.F., A.I.A, W.C. and L.B. performed the research investigation; A.I.A. was responsible for the original writing, and A.V.S.F., M.P.P., G.M.F., and M.F.B were responsible for reviewing and editing; The work was supervised by M.P.P and C.A.S. All authors read and approved the final manuscript.

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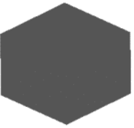

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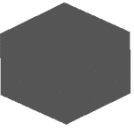

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The abbreviations used are: BMP, Bone morphogenetic protein; Dhh, Desert Hedgehog; Fu, Fused kinase; Gli, Glioma-associated oncogene; Hh, Hedgehog; Ihh, Indian Hedgehog; Shh, Sonic Hedgehog; SuFu, Suppressor of Fused protein; Wnt, Wingless-related integration site; ROCK, Rho-associated coiled-coil-containing protein kinase; MAPK, Mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MEF, Mouse embryonic fibroblast; mTOR, Mammalian target of rapamycin kinase; PKA, Protein kinase A; PKC, Protein kinase C; Pak, p21-activated kinase; FDA, Food and Drug Administration; PKB, Protein kinase B; FAK, Focal adhesion kinase; SAG, Smoothened Agonist.

TABLE 1. Summary of pathways analyzed using kinome. Cross comparison as a short description of kinome profiling showing the major pathways and statistical comparing the conditions: canonical and non-canonical pathways (Patched-dependent –Ptc-, and Smoothened-dependent – Smo-).

Pathway	Shh canonical	Statistics	Ptc-dependent	Statistics	Smo-dependent	Statistics
Survival	+	<0.01	+	0.03		
Mitogenic						
2nd messenger	+	<0.01			+	0.01
Nutrient						
Cytoskeletal	+	<0.01	+	0.04		
Mitosis	-	0.02				
Inflamatory			+	0.01		
Stemness			+	0.05		

Chapter 3

Oncophosphosignaling favors a glycolytic phenotype in human drug resistant leukemia

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Abstract

In chemoresistant leukemia cells (Lucena-1), the low molecular weight protein tyrosine phosphatase (LMWPTP) is about 20-fold more active than in their susceptible counterpart (K562). We found this phosphatase ensures the activated statuses of Src and Bcr-Abl. Since phosphorylation and dephosphorylation of proteins represent a key post-translational regulation of several enzymes, we also explored the kinome. We hereby show that LMWPTP superactivation, together with kinome reprogramming, cooperate towards glucose addiction. Resistant leukemia cells present lower levels of oxidative metabolism, in part due to downexpression of the following mitochondrial proteins: pyruvate dehydrogenase subunit alpha 1, succinate dehydrogenase and voltage-dependent anion channel. Those cells displayed higher expression levels of glucose transporter 1 and higher production of lactate. In addition, Lucena-1 siRNA LMWPTP cells showed lower expression levels of glucose transporter 1 and lower activity of lactate dehydrogenase. On the other hand, K562 cells overexpressing LMWPTP presented higher expression/activity of both proteins. In this study, we show that LMWPTP is a pivotal mediator of metabolic reprogramming that confers survival advantages to leukemia cells against death stimuli.

Keywords: Chemoresistant leukemia cells; Kinome; LMWPTP; ACP1, Glycolytic metabolism

Introduction

Among major problems in dealing with leukemic disease is the build-up of resistance against therapy [Martelli et. al., 2003; O'Hare et. al., 2006; Diehl et. al., 2007; Wieczorek et. al., 2016]. This may include resistance associated with decreased drug accumulation in the cell, altered intracellular drug distribution, increased detoxification, diminished drug-target interaction, increased DNA repair, altered cell-cycle regulation and uncoupling of pathways linking cellular damage with programmed cell death [Branger et. al., 2002; Kondo et. al., 2005]. In this context, increased expression of P-gp, encoded by the MDR1 gene, is a well-characterized mechanism for chemoresistance in cancer cells. However, the biochemical mechanism behind drug resistance has yet to be fully understood. Therefore, a better understanding of the mechanisms mediating resistance and novel targets for improved therapeutic options are required. Cellular functions are under tight control of the balance between protein phosphorylation and dephosphorylation. Kinases and phosphatases that control these processes are, therefore, essential regulators of processes such as cell proliferation, adhesion, migration and death. The widespread nature of protein phosphorylation/dephosphorylation underscores its key role in cell-signaling metabolism. In fact, cells are able to give a specific response towards diverse changes in their microenvironment through integrated networks of intracellular signaling pathways. These acts via cascades of sequential phosphorylation or dephosphorylation reactions, which are governed by protein kinases and phosphatases, respectively. Previous studies have revealed that oncosignaling under stress conditions can lead to metabolic reprogramming. Glucose metabolism is reprogrammed in many malignancies as shown by accelerated glycolysis and by the active truncated tricarboxylic acid (TCA) cycle [Liberti and Locasale, 2016]. In addition, glycolysis is also favored in some tumor cells in response to metabolic stress. In the present study, we focus our attention on LMWPTP and its association to a metabolic switch responsible for drug resistance in leukemia. Over the last decade, it has become clear that many protein tyrosine phosphatases are involved in different diseases, including cancer. Specifically, the LMWPTP family, also known as acid phosphatase locus 1 (ACP1), is shown to be upregulated in various human cancers [Malentacchi et. al., 2005; Marzocchini et. al., 2008; Hoekstra et. al., 2015; Ruela-de-Sousa et. al., 2016] and to play a role in leukemia resistance [Ferreira et. al., 2012]. We now show that LMWPTP, together with kinases such as Pyruvate dehydrogenase kinase 1 (PDK1), Src and mTOR, are involved in glucose metabolism reprogramming in resistant leukemia cells providing survival advantages to these cells towards death stimuli.

Materials & methods

Cell line and Antibodies

K562 cells were purchased from the American Type Culture Collection and Lucena-1 cells (counterpart of K562 cells with multiresistant phenotype) were provided by Rumjanek et. al. (2001). Anti-sheep, anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology. HIF-1α (28b) (sc-13515) and LDHA (H160) (sc33781) antibodies were obtained from Santa Cruz Biotechnology, Inc.. p-mTOR (Ser2448) (gtx50258) was purchased from GeneTex Inc.. LMWPTP (ACP1) (ab26232), mTOR (ab63480) and Glut-1 (ab15309) antibodies were obtained from Abcam. PKM2 (#3198), p-PKM2 (#3827), p-Erk1/2 (Thr202/Tyr204) (#9101S), p-P38 (Thr180/Tyr182) (#92165), p-Pax (Tyr118) (#2541) p-JNK (Thr183/Tyr185) (#46685), p-Src (Tyr416) (#2101S), Pyruvate dehydrogenase subunit α1 (C54G1) (#3205), SDHA (D6J9M) (#11998) and VDAC (D73D12) (#4661), p-LDHA (Tyr10) (#8176S), Insulin Receptor β (4B8) (#3025), p-Insulin Receptor β (Tyr1345) (14A4) (#3026), Pan Actin (D18C11) (#8456), β-Tubulin (9F3) (#2128) antibodies were purchased from Cell Signaling Technology.

Cell Culture

K562 and Lucena-1 cells were routinely grown in suspension in Roswell Park Memorial Institute 1640 medium supplemented with 2mM glutamine, 100U/mL penicillin, 100µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS), at 37°C in a 5% CO_2 humidified atmosphere.

Kinome

Kinome arrays were performed essentially as described before [Diks et. al., 2004; Lowenberg et. al., 2006; van Baal et. al., 2006]. Briefly, cells ($2.5x10^5$) were washed in PBS and lysed in a non-denaturing complete lysis buffer. The peptide arrays (Pepscan), containing up to 1024 different kinase substrates in triplicate, were incubated with cell lysates for 2 hours in a humidified incubator at 37ºC. Subsequently, the arrays were washed in 2M NaCl, 1% Triton-X-100, PBS, 0.1% Tween and water. Thereafter slides were exposed to a phospho-imaging screen for 24-72 hours and scanned on a phospho-imager (Fuji). The level of incorporated radioactivity, which reflects the extent of phosphorylation, was quantified with specific array software (EisenLab ScanAlyze, version 2.50). Datasets from chips were then analyzed statistically using PepMatrix, as described by Milani et. al. (2010). Basically, spot replications

were scrutinized for consistency using two indexes: one being the standard deviation:average (SD/A) ratio and the other being the ratio between the average and the median (A/M) of all three replications for each chip. Parameters applied to the indexes were SD/A<50% and 80%<A/M<120%. The fold change in phosphorylation between control and experiment cells was assessed using Student's t-test, with $p<0.05$ indicating significance.

Western blotting analysis

Cells (3×10^7) were lysed in 200µL of lysis buffer (50mM Tris–HCl pH 7.4, 1% Tween 20, 0.25% sodium deoxycholate, 150mM NaCl, 1mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1mM Na_3VO_4, 1mM NaF and protease inhibitors [1µg/ml aprotinin, 10µg/ml leupeptin, and 1mM 4-(2-aminoethyl) benzenesulfonyl-fluorid-hydrochloride] for 2 hours on ice. Protein extracts were cleared by centrifugation and protein concentrations were determined using the Bradford reagent (Sigma-Aldrich). Twice the volume of sodium dodecyl sulfate (SDS) gel loading buffer (100mM Tris–HCl pH 6.8), 200mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to the samples, which were subsequently boiled for 5 minutes. Cell extracts were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% fat-free dried milk or bovine serum albumin (BSA) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4°C with the appropriate primary antibody at 1:1000 dilution. After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse and anti-goat horseradish peroxidase-conjugated secondary antibodies, at 1:5000 dilutions, in BSA 1% in TBS-Tween 20 (0.05%) for 2 hours. Proteins were detected using enhanced chemiluminescence in Alliance 6.7 (UVITEC).

Isolation and preparation of mitochondria for western blotting analysis

For mitochondria isolation, 2×10^7 cells (K562 and Lucena-1) were washed with saline solution (0.9% NaCl) in triplicate using a centrifuge tube at 850×g for 2 minutes each wash at 4°C. Afterwards, the procedure consisted in following the protocol of the mitochondria isolation kit (Thermo Scientific). Briefly, after the last centrifugation, the supernatant was discarded and the cell pellet was re-suspended in 800µL of Mitochondria Isolation Reagent A, mixed using a vortex mixer at medium speed for 5 seconds and incubated on ice for no longer than 2 minutes. Then, 10µL of Mitochondria Isolation Reagent B was added and mixed again for 5 seconds at maximum speed. The tube was kept on ice for 5 minutes repeating the mixing

for each minute at maximum speed. On the next step, the addition of 800µL of Mitochondria Isolation Reagent C was done followed by the inversion of the tube several times to mix (without vortexing). The mixture was then centrifuged at 700×g for 10 minutes at 4°C. The supernatant was transferred to a new tube and centrifuged at 3,000×g for 15 minutes and 4°C. At this point, the supernatant is the cytosolic fraction and the pellet contains isolated mitochondria. The pellet was then re-suspended in 500µL of Mitochondria Isolation Reagent C and centrifuged at 12,000×g for 5 minutes at 4°C. For western blotting assays the mitochondria was lysed with 50 mM Tris pH 8, 2mM $MgCl_2$, 1mM EGTA pH 8, 1mM DTT, 10% glycerol, 0.2% NP-40. Reagents A, C and lysis buffer were supplemented with Halt Protease Inhibitor Cocktail, EDTA-Free (Thermo Scientific). Protein concentrations of mitochondria and cytosol fractions were determined using the Lowry method (DC Protein Assay, Bio-Rad).

Oxygen consumption

The oxygen consumption assay of non-permeabilized cells was performed in Krebs-Ringer buffer (Sigma-Aldrich) pH 7.4 at 37ºC in the presence of glucose (25mmol/L) and HEPES (10mmol/L). After, we added viable cells ($3x10^6$ cells/mL) and signal stabilization, the chamber was closed and O_2 flow (Jo) recorded. The ATP synthase inhibitor oligomycin A (1µg/mL) and the mitochondrial uncoupler CCCP (Carbonyl cyanide m-chlorophenylhydrazone – 1µM) were used as internal control [Pesta and Gnaiger, 2011; Teodoro et. al., 2014].

Cell Viability

Cells were plated at $1x10^5$ cells/mL and treated with H_2O_2 for 24 hours. After treatment, cell viability was assessed by trypan blue dye exclusion.

MTT assay

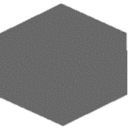
$1x10^5$ cells/well was seeded into a 12-wells plate for 48 hours and the medium was removed from the cells after each incubation period. 1mL of MTT (Sigma Aldrich) solution (0.5mg/mL in FBS free culture medium) was added to each well. After incubating for 3 hours at 37°C, the MTT solution was removed and the formed formazan crystals were solubilized in 100µL of ethanol. The plate was shaken for 10 minutes and the absorbance was measured at $\lambda = 570$ nm with a microplate reader (Synergy HT, BioTek).

Lactate assay

The medium was collected from cells after each incubation period and used for extracellular lactate measurement by Lactate kit (BioClin) following manufacturer's instructions.

Transfection of K562 Cells with LMW-PTP Plasmid

K562 cells ($1x10^5$ cells/mL) were grown for 24 hours and the transfections were done using the Effectene transfection kit (QIAGEN) according to the manufacturer's instructions. Briefly, the cells were transfected with 2.4μg of pcDNA3.1/V5-His-TOPO vector with or without an insert containing the sequence of human LMWPTP after a cytomegalovirus promoter. Overexpression was always verified by western blotting.



Transfection of K562 and Lucena-1 Cells with LMW-PTP siRNA

Lucena-1 cells ($1x10^5$ cells/mL) were grown for 24 hours and subsequently transiently transfected with LMWPTP siRNA (QIAGEN). Transfections were done using the Hiperfect transfection kit (QIAGEN) according to the manufacturer's instructions. Briefly, the cells were transfected with LMWPTP siRNA (final concentration: 5nM) for 72 hours. The efficiency of transfection was assessed based on the expression of LMWPTP by western blotting analysis.

Data Analysis

All experiments were performed in triplicate and results were shown in the graphs as means ± standard error of the mean (S.E.M.). Soluble lysates were matched for protein content and analyzed by western blotting and all bands were compared with their respective internal control. Kinome profile data were assessed using Student's t-test, with $p<0.05$ considered significant. MTT and lactate statistical analyses were analyzed using one-way analysis of variance (ANOVA) followed by Tukey posttest, with $p<0.05$ considered significant. All data were analyzed using GraphPad Prism Software, Version 5.0.

Results

Kinome profiling of K562 cells and its multidrug-resistant counterpart Lucena-1

Erythroleukemic cell line K562 and its derivative line Lucena-1 have been described to be a useful model for investigating the acquisition of multidrug resistance, the former being sensitive to a variety of chemical agents whereas the latter displays significant resistance to such treatment [Queiroz et. al., 2010; Moreira et. al., 2014]. Previously, we have shown that LMWPTP is highly expressed in Lucena-1 cells, and that this tyrosine phosphatase is essential to keep Src and Bcr-Abl signaling [Ferreira et. al., 2012]. In order to explore to what extent signaling pathways were being rewired, we sought to understand the kinome activity of K562 and Lucena-1 cells using a kinase array of 1024 consensus peptides [Diks et. al., 2004; Lowenberg et. al., 2006; van Baal et. al., 2006]. Profiling quality was considered good as technical replicates showed an average Pearson product-moment correlation coefficient of 0.93 (Figure 1A). Single data analysis of arrays with K562 cell lysates resulted in the statistically significant phosphorylation of 78 peptides, whereas lysates of Lucena-1 cells resulted in phosphorylation of 107 peptides. Subsequent statistical analysis of signal intensity showed that phosphorylation of 30 peptides was significantly altered between Lucena-1 and K562 cells ($p<0.05$) (Figure 1B). In general, all kinases with higher activities in Lucena-1 cells are involved in signalling pathways responsible for cellular proliferation and survival. CK2 and PDK1, in particular, were two of the most activated kinases, and both of them can modulate glucose metabolism. Accordingly, an insulin receptor that displays intrinsic kinase activity was also more active in Lucena-1 cells. Even though kinome profiling is a robust and reproducible technology, western blotting analysis was performed to validate peptide array data. As shown in Figure 2, Lucena-1 cells exhibit increased activation of p21Rac targets in cytoskeletal remodelling (Src and Paxillin) as well as downstream targets of this GTPase including, ERK1/2, p38 and JNK. In addition, a strong activation of mTOR kinase was observed. Thus, these results agree with data from kinome profiling and hence confirm that the transition from a chemotherapy-sensitive to a chemotherapy-resistant phenotype in erythroleukemia is accompanied by a distinct set of changes in the activation status of specific signal transduction pathways.

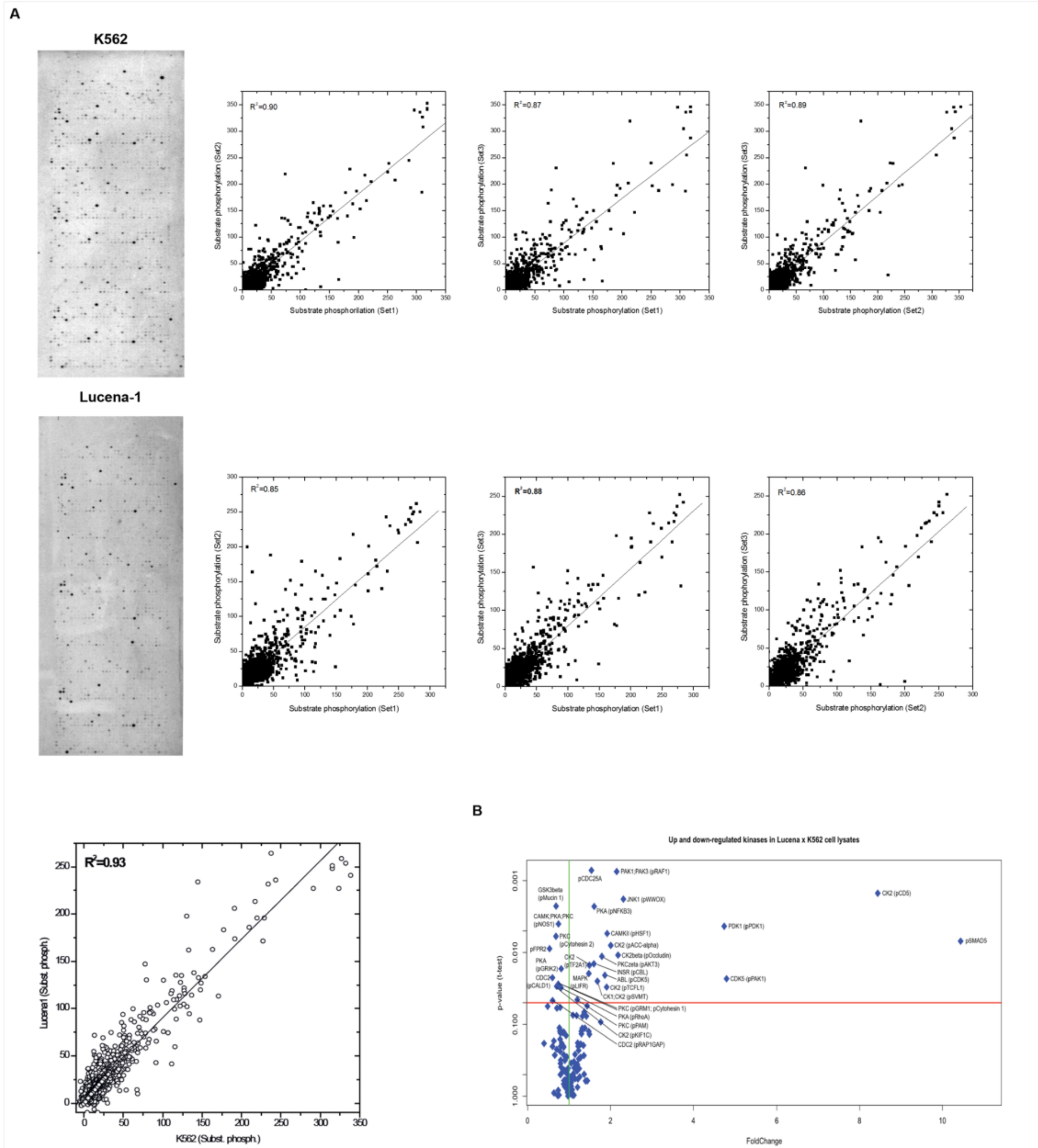


Figure 1. Comparison of kinome profiling between K562 and Lucena-1 cells. (A) Scatter plots showing the correlation between pepchip array replicates for K562 and Lucena-1 cells. (B) Comparison between statistically significant phosphorylated spots in K562 and in Lucena-1 cells. This graph shows the correlation between fold change and p-values for statistically significant phosphorylated spots in both cell lines. Fold changes in Lucena-1 cells were assessed using Student's t-test, with $p<0.05$ indicating significance. Vertical axis is in log-scale.

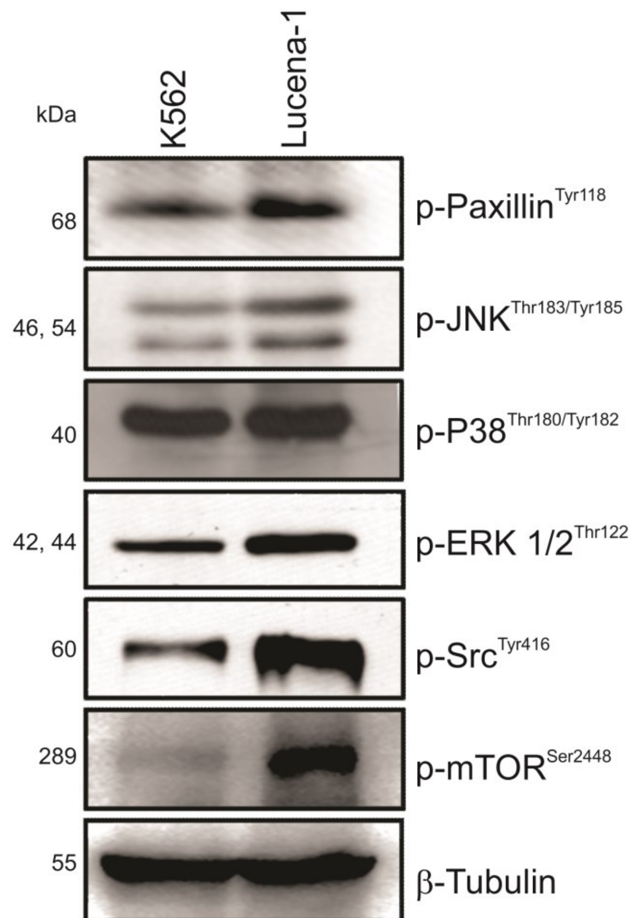


Figure 2. Validation of kinome profiling. Lysates of K562 and Lucena-1 were submitted to western blotting and probed with anti-phosphosite specific antibodies.

A glycolytic switch is related to the maintenance of the drug resistant phenotype

Accordingly, when we looked at proteins involved in glucose transport and metabolism, we detected a) higher expression levels of Glucose transporter 1 (Glut-1) and HIF-1α; b) higher activity of Insulin Receptor β and Lactate Dehydrogenase subunit A (LDHA) and c) a strong inhibition of PKM2, all of which indicate a higher glycolytic rate in Lucena-1 cells (Figure 3A). Furthermore, the amount of lactate produced by those cells was also higher (Figure 3B). Since it is known that key metabolites, such as NADPH (pentose phosphate pathway), are produced by tumor cells via accelerated glycolysis and a truncated TCA cycle [Liberti and Locasale, 2016], we investigated whether Lucena-1 and K562 cells would have different responses to hydrogen peroxide. Indeed, when both cells were treated with this compound for 24 hours, we observed that Lucena-1 cells were less sensitive (Figure 3C).

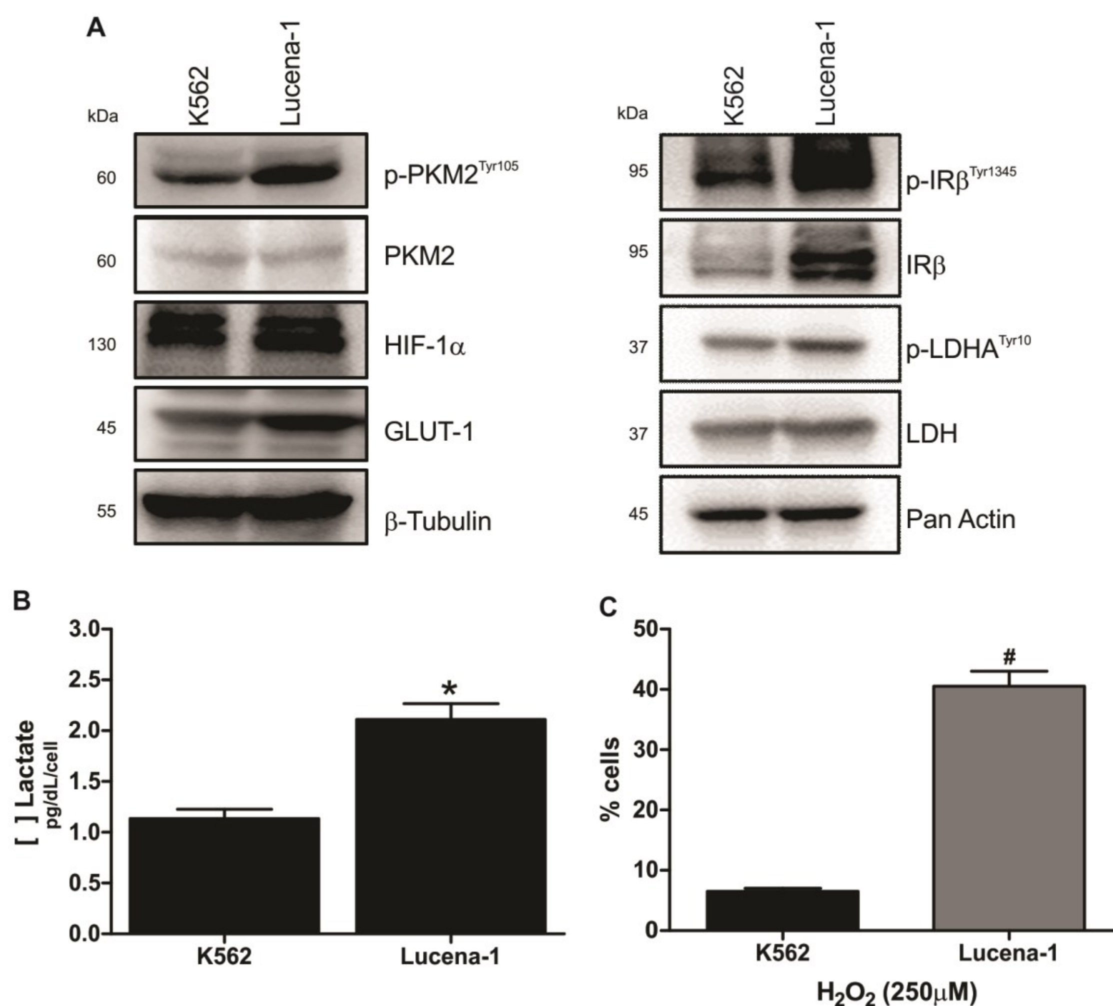


Figure 3. Glycolytic metabolism is predominant in resistant leukemia cells. (A) Lysates of K562 and Lucena-1 were submitted to western blotting and probed with specific antibodies against proteins involved in glucose uptake and regulation of glucose metabolism. (B) Lactate production was checked by measuring this metabolite in culture medium. (C) Leukemia cells (1 x 10^5 cells/mL) were treated with 250 µM hydrogen peroxide for 24 hours and viable cells quantified by trypan blue. Data are represented as mean ± S.E.M. and one-way analysis of variance (ANOVA) followed by Tukey posttest. (#), (*) $p<0.01$ Lucena-1 vs K562 cells.

After we established that Lucena-1, a cell line that organically acquired resistance to chemotherapy, displays high levels of Glut-1 expression and lactate production in comparison to K562 cells, we sought to examine the mitochondrial function of those cells. Accordingly, we found some mitochondrial proteins (PDHα1, SDH and VDAC) were strongly downregulated in Lucena-1 cells (Figure 4A). In addition, when we compared the ability of Lucena-1 and K562 in reducing MTT, Lucena-1 cells also were less efficient (Figure 4B). Next, we monitored oxygen consumption. As shown in Figure 4C, basal oxygen consumption by Lucena-1 was significantly lower when compared to K562. When both cell lines were incubated with oligomycin, an FO-ATP synthase inhibitor, Lucena-1 cells consumed 50% less

oxygen (Figure 4C). This result suggests that Lucena-1 cells have a lower mitochondrial function compared to K562. Also, when Lucena-1 and K562 cells were submitted to CCCP, a potent uncoupler of mitochondrial oxidative phosphorylation, Lucena-1 presents a lower oxygen consumption rate compared to K562 (Figure 4C). All of these results suggest that the oxidative metabolism is not the main energy supplier in resistant cells, mainly due to downregulation of key mitochondrial proteins.

LMWPTP contributes to the Warburg effect in resistant human erythrocytic leukemia cells

Previously we reported that inhibition of LMWPTP in Lucena-1 cells culminates in reducing both Src and Bcr-Abl activities and increasing chemotherapeutic sensitivity [Ferreira et al, 2012]. We now show that Lucena-1 cells also display a highly active glycolytic metabolism. Therefore, we wondered if LMWPTP could contribute to the Warburg effect. To address this question, we silenced LMWPTP and examined the expression and/or activity of proteins involved in glucose metabolism.

Lucena-1 siRNA LMWPTP cells showed lower expression levels of Glut-1 and lower activity of LDHA, once this enzyme was less phosphorylated at Tyrosine (Tyr10), an activator residue. On the other hand, K562 LMWPTP cells presented higher expression of Glut-1 and higher activity of LDHA (Figure 5).

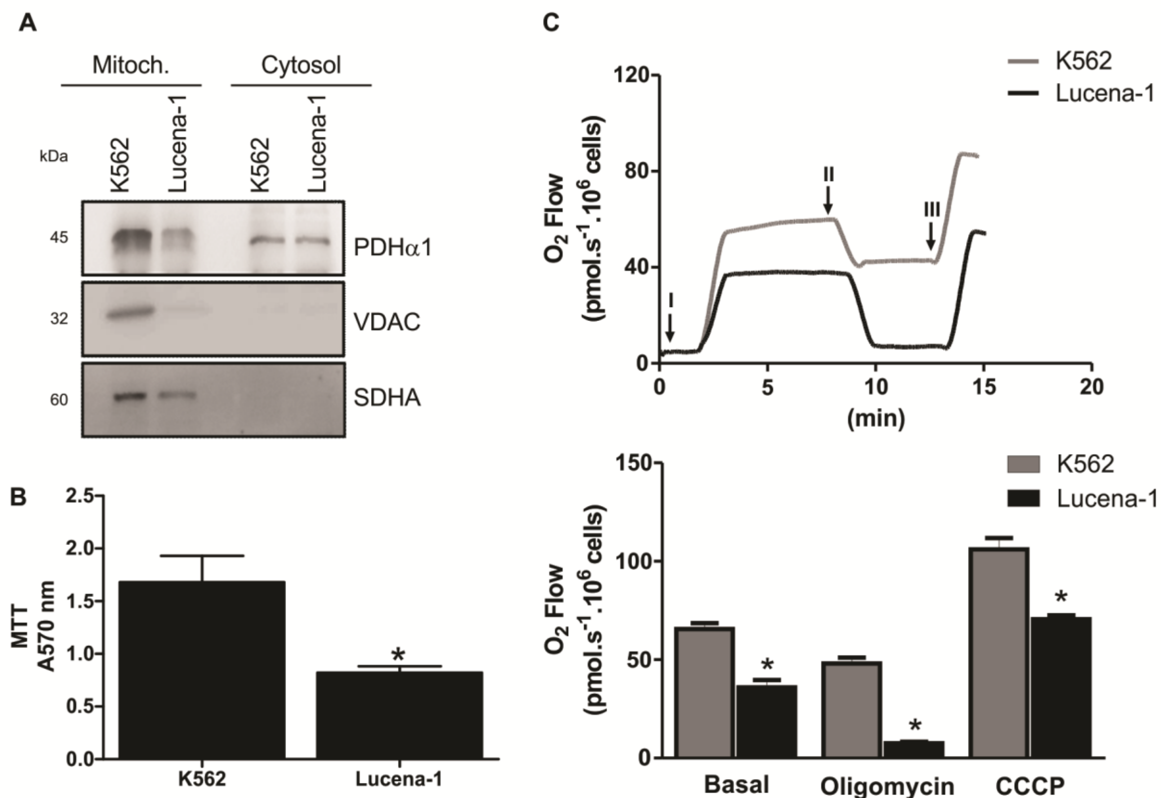


Figure 4. Mitochondrial function in leukemia cells. (A) Mitochondrial lysates of K562 and Lucena-1 were submitted to western blotting and probed with specific antibodies against mitochodrial proteins. (B) Cells were incubated with MTT solution for 3 hours at 37°C. The MTT solution was removed and formazan crystals were solubilized in ethanol. Data are represented as mean ± S.E.M. and one-way analysis of variance (ANOVA) followed by Tukey posttest. (#) $p<0.01$ Lucena-1 vs K562 cells. (C) Oxygen consumption assay of non-permeabilized cells ($3x10^6$ cells/mL) was performed in Krebs-Ringer buffer, pH 7.4 at 37ºC in the presence of glucose (25mM) and HEPES (10mM). After signal stabilization, the chamber containing the cell suspension was closed and O_2 flow (Jo) recorded. ATP synthase inhibitor oligomycin A (1µg/mL) and the mitochondrial uncoupler CCCP (1µM) were used as internal controls. Data are represented as mean ± S.E.M. and (*) $p>0.05$ Lucena-1 vs K562 cells.

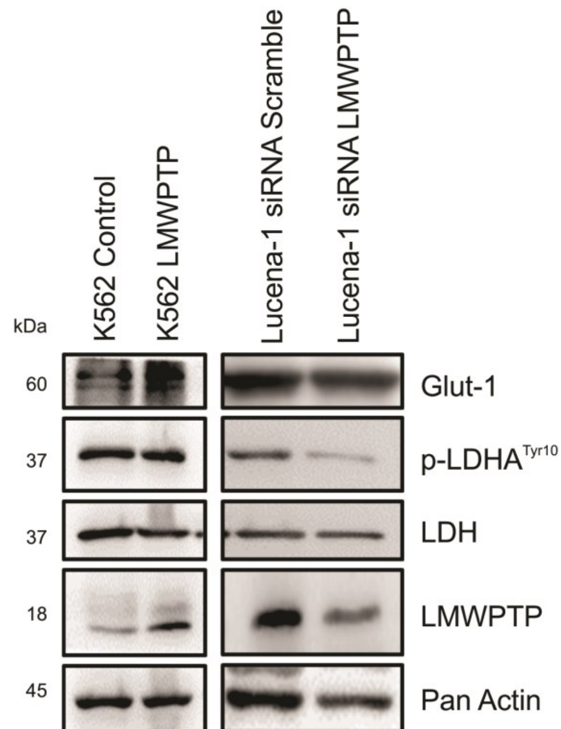


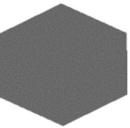
Figure 5. Low molecular weight protein tyrosine phosphatase (LMWPTP) contributes to the Warburg effect in resistant human erythrocytic leukemia cells. LWMPTP was overexpressed in K562 cells and knocked down in Lucena-1 cells. LMWPTP expression was checked by western blotting. Lysates of K562 control and K562 LMWPTP, as well as Lucena-1 siRNA scramble and Lucena-1 siRNA LMWPTP were submitted to western blotting and probed with specific antibodies against Glut-1 and LDHA proteins.

Discussion

Changes in cellular biochemistry associated with acquisition of the multidrug resistant phenotype remain only partially understood. Therefore, efforts in building a metabolic atlas to better understand cells with the resistant phenotype will open new avenues leading to improved therapy. Among the major problems in dealing with leukemic disease is the build-up of resistance against therapy [Martelli et. al., 2003; O'Hare et. al., 2006; Diehl et. al., 2007]. The biochemical aspects behind drug resistance are still far from being fully revealed. Since metabolic pathways depend on a tightly controlled balance between phosphorylation and dephosphorylation of proteins, kinases and phosphatases have a key function in the cancer resistance process [Abrantes et. al., 2014]. An earlier report from our group has shown a significant association of LMWPTP activity to chemoresistance of leukemia cells [Ferreira et. al., 2012], which was partly due to the maintenance of Src and Bcr-Abl activation. Therefore, in order to correlate phosphorylation-driven signaling with changes in cellular metabolism associated to the resistant phenotype, we evaluated the kinome of K562 cells and Lucena-1 cells. Kinome profiling provided us with a global overview of kinase activity that accompanies the acquisition of multidrug resistance. A fairly coherent picture emerged in which the resistant phenotype exhibits more activity of glycolytic signalling as demonstrated by: a) increased activity of the insulin receptor and its downstream mediators Akt/mTOR; b) diminished GSK-3 activity; c) activation of p21 Rac, leading to both cytoskeletal responses as well as activation of stress-activated kinases JNK, PAK, CK2 and PDK1. Accordingly, kinome profiling and pulldown experiments also revealed that PAK1 is more active in resistant leukemia cells. It is known that PAK1 also regulates glucose homeostasis by stimulating Glut-4 translocation and glucose uptake [Chiang et. al., 2014] and modulating Phosphoglycerate mutase (PGM), an enzyme in the lower end of the glycolytic pathway that catalyzes the interconversion of 3- and 2-phosphoglycerate. Gururaj and colleagues (2004) demonstrated that when PAK1 phosphorylates threonine 466 of PGM, it significantly increased its enzymatic activity. Beyond that, kinome profiling showed that PDK1 is more active in Lucena-1 cells. PDK1 is a key negative regulator enzyme of glucose metabolism through phosphorylation of Pyruvate Dehydrogenase (PDH) [Fan et. al., 2014]. PDH belongs to a multienzyme complex that catalyzes the oxidative decarboxylation of pyruvate, generating acetyl-CoA and, consequently, favouring the oxidative pathway in mitochondria [Saunier et. al., 2016]. Therefore, since PDK1 is more active in Lucena-1 cells, use of pyruvate by mitochondria is inhibited. In addition, PDH is inhibited by Src [Jin et. al., 2016], a kinase that is much more active in Lucena-1 cells [Ferreira et. al., 2012]. This explains the

higher production of lactate observed in those cells. Furthermore, Lucena-1 presents a lower enzymatic activity of Pyruvate kinase isoform 2 (PKM2) when compared to K562. Shinohara and colleagues (2015) have reported that a higher activity of Src kinase promotes PKM2 inhibition. Furthermore, PKM2 catalyzes the conversion of phosphoenolpyruvate to pyruvate. Higher lactate production in Lucena-1 cells is likely guaranteed by Pyruvate kinase isoform 1 (PKM1) [Taniguchi et. al., 2016]. Furthermore, the activity of PKM2 determines the level of cellular energy, redox homeostasis and proliferating ability [Filipp, 2013; Iqbal et. al., 2013].
In order to explain the predominance of a glycolytic metabolism in Lucena-1 cells, we focused our attention on the investigation of mitochondrial function in both K562 and Lucena-1. Glucose metabolism is reprogrammed in many malignancies, as shown by accelerated glycolysis rates to provide energy/biosynthetic precursors and by the active truncated TCA cycle to produce intermediates for tumor cells. It is already known that alterations in glucose metabolism are closely associated with therapeutic resistance and clinical outcome [Liberti and Locasale, 2016]. Lucena-1 cells displayed lower oxygen consumption, which is in accordance with higher production of lactate. Based on western blotting analysis, we also showed that key mitochondrial proteins (SDHA, PDHα1 and VDAC) were strongly downregulated. SDHA, Succinate dehydrogenase complex, subunit A, belongs to the SDH protein complex of the inner mitochondrial membrane, which catalyzes the conversion of succinate to fumarate (TCA reaction) and also takes part on electron transport in the mitochondrial complex II. Dysfunctions of SDH have been associated with the development of cancer, as the excess of succinate inhibited prolyl hydroxylases dependent on α-ketoglutarate, leading to HIF-1α stabilization. Consequently, HIF-1α is able to regulate the expression of genes involved in glucose metabolism including Glut-1 [Kirches, 2009; Wallace, 2012; Morin et. al., 2014]. HIF-1α and Glut-1 increase glycolysis and decrease mitochondrial function in tumors, and this axis is critical for cells to overcome metabolic stress. Another important player in the regulation of mitochondrial function is VDAC, which constitutes an ionic transport channel protein family that acts as a selective pore for anions and cations, like calcium, and promotes the communication between mitochondria and the rest of the cell. Therefore, downregulation of this protein in Lucena-1 cells suggests mitochondria are not the main energy supplier. Besides, the low concentration of VDAC also contributes for lower TCA efficiency, since this protein is important for the uptake of TCA subtrates [Maldonado and Lemasters, 2014].
Our findings so far allow us to propose the following hypothesis: Lucena-1 siRNA LMWPTP cells take up less glucose due to the lower expression of Glut-1. All these findings suggest

that inhibition of LMWPTP switches cell metabolism from glycolytic to oxidative. Furthermore, K562 LMWPTP presented increased Glut-1 expression and also LDHA activity. In the last four years, we have reported the importance of the low molecular weight protein tyrosine phosphatase for aggressiveness of different tumors (cancer cell lines and patient biopsies). Specifically, in relation to chronic myeloid leukemia, high activity of this phosphatase correlates to a resistant phenotype, since inhibition of LMWPTP in Lucena-1 cells culminates in reducing both Src and Bcr-Abl activities and increasing chemotherapeutic sensitivity [Ferreira et. al., 2012]. In this study, we show that LMWPTP is a pivotal mediator of metabolic reprogramming that confers survival advantages to leukemia cells against death stimuli, such as hydrogen peroxide. Indeed, we have observed that Lucena-1 cells (resistant phenotype) are much more resistant to the toxic effect of peroxide than K562, its non-resistant counterpart.



Acknowledgements

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Conflicts of interest

None.

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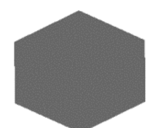

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Chapter 4

LMWPTP modulates the antioxidant response and autophagy process in human chronic myeloid leukemia cells

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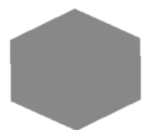
Molecular and Cellular Biochemistry, 2020

Abstract
In the last decade, several reports highlight the importance of the low molecular weight protein tyrosine phosphatase (LMWPTP) in cancer aggressiveness and resistance. Specifically, in chronic myeloid leukemia, we have reported that high expression of the LMWPTP maintains Src and Bcr-Abl kinases in an activated status and the glucose metabolism is directed to lactate production, and in turn favoring the pentoses pathway (one of the key process for antioxidant and protective responses). In this present study, we investigated the possible correlation between the LMWPTP and autophagy. In resistant chronic myeloid leukemia cells, the antioxidant response is supported by the glycolytic metabolism and antioxidant enzymes such as SOD and catalase, both favored by the LMWPTP. Therefore, when the cells were challenged by hydrogen peroxide treatment, the LMWPTP level goes down as well as SOD, and in turn, autophagy process was stimulated. The findings presented here, reveal a novel aspect by which LMWPTP cooperates for the resistance of CML towards stressor stimuli.

Keywords: LMWPTP, ACP1, autophagy, leukemia, antioxidant response.

Introduction

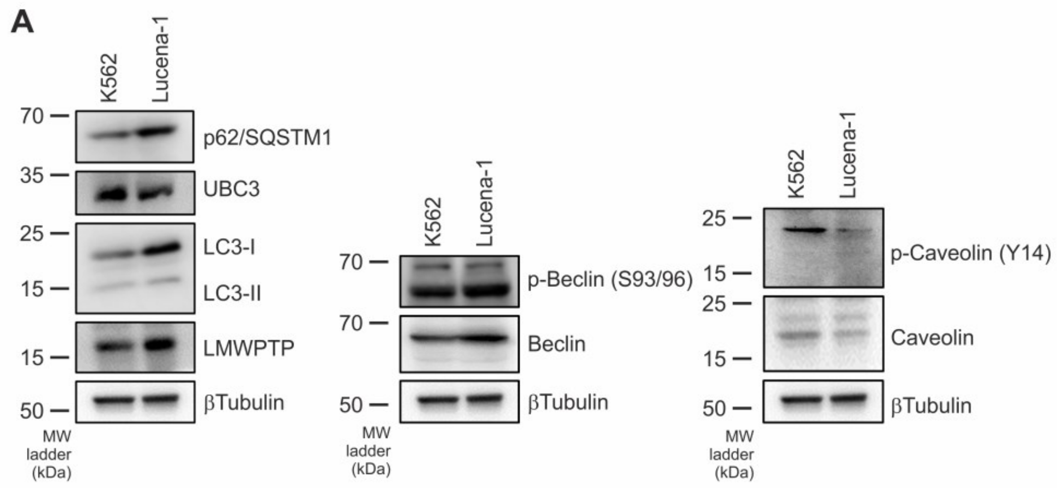
Autophagy is a cellular recycling process crucial for homeostasis and stress adaptation. Therefore, it can confer protection and promote survival in response to metabolic/environmental stress, such as nutrient deprivation, hypoxia, and chemotherapies [1]. There is preclinical evidence that targeting autophagy can enhance the efficacy of cancer therapies [2, 3]. However, it has been described that autophagy activation seems to be reduced in human acute myeloid leukemia blasts and loss of key autophagy genes leads to leukemia initiation and progression in mouse models [4, 5, 6, 7]. These observations prompted us to investigate whether the LMWPTP could influence the autophagy process in chronic myeloid leukemia (CML) cells (K562 and Lucena-1). In the last years, our research group conducted several large-scale analyses to identify biochemical differences between two leukemic cells lines K562 and a multidrug resistant phenotype Lucena-1 [8, 9, 10]. Based on that, the Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) was identified overexpressed in chemoresistant leukemia cells. LMWPTPs are a group of 18kDa-tyrosine phosphatase class II, encoded by ACP1 gene and have been reported to play a major role in leukemia as well as solid cancers [8, 9, 11, 12]. We reported that the high expression and activity of the LMWPTP in resistant CML cells (Lucena-1 cell line) kept Src and Bcr-Abl active [9]. More recently, we showed that the LMWPTP switches the glucose oxidative metabolism off, and in turn, favoring a potent antioxidant defense [10]. However, the mechanisms by which this tyrosine phosphatase contributes for the basal protection response towards environmental stresses is not completely understood. In the present study, we report that in Lucena-1 cells, the basal autophagy process is down-modulated which is in part due to the control of autophagy mediators and up regulation of the antioxidant response by the LMWPTP.

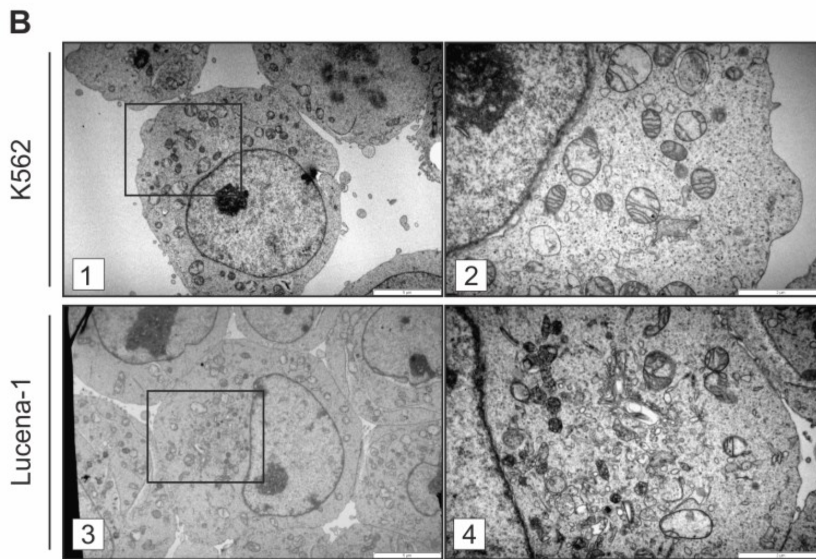


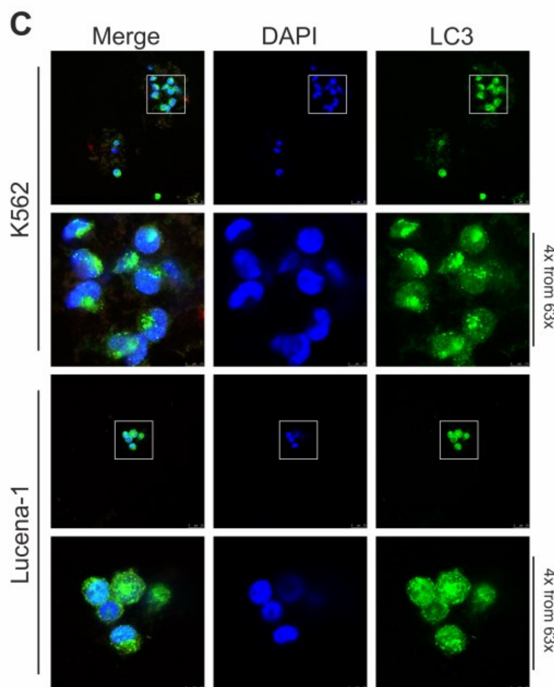
Results

Crosstalk between the LMWPTP and autophagy in chronic myeloid leukemia cells

In order to explore the basal autophagy process in CML cells, we analyzed the expression/phosphorylation status of protein involved in nucleation phase: beclin and the phosphorylated form phospho-beclin (Ser 93/96), in elongation phase: LC3 I and II, Caveolin-1 and phosphorylated form Caveolin-1 (Tyr14), and in autophagy process execution (UBC3, an ubiquitin protein). As it can be seen in Figure 1, cell with higher amount of LMWPTP also display higher amount of p62/SQSTM1 and LC3I. In addition, the phospho-caveolin/caveolin ratio was much lower in Lucena-1 compare to K562 cells (Figure 1A). These findings indicate that autophagy process is not favored in Lucena-1 cells. Accordingly, we do observe a huge number of vacuoles in Lucena-1 cells, which might be autophagosomes, that means the autophagy process starts but is not completed (Figure 1B). Also, it was observed higher LC3 accumulation on Lucena-1 cells compared to K562 (Figure 1C), as a confirmation of our findings on western blot. We next examined whether LMWPTP silencing in Lucena-1 cells would affect intracellular level of P62/SQSTM1 and caveolin-1. Interestingly, p62/SQSTM1 is lower expressed and Caveolin is more phosphorylated in knockdown cells (Figure 2). Caveolin phosphorylation has been reported to be important, among other processes, for endosome formation and autophagy activation [13- 15].







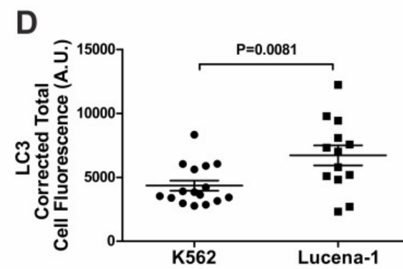


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Figure 1. Autophagy in CML cells. (A) Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) expression at chronic myeloid leukemia cells and autophagy-related proteins. Lysates of cells were submitted to Western blotting and probed with specific antibodies against LMWPTP and proteins involved in autophagy process. β-tubulin was used as the internal housekeeping, and the densitometry is represented by histograms on Supplemental Figure 1A. The number under the western blot picture is the indication of densitometry. The densitometry indicated below the blot is related to the ratio between phosphorylated and total protein forms. (B) Electron microscopy showing K562 (1 and 2 – zoom from the highlighted area) and Lucena-1 (3 and 4 – zoom from the highlighted area). (C) Representation of immunofluorescence microscopy of K562 and Lucena-1 cells targeted LC3 (green) expression. Nuclei are stained in blue (DAPI). Representative images are shown at 63X, and 4X from 63X magnification. (D) Immunofluorescence quantification of LC3 on K562 and Lucena-1. Statistical analysis: $P<0.05$ K562 vs. Lucena-1.

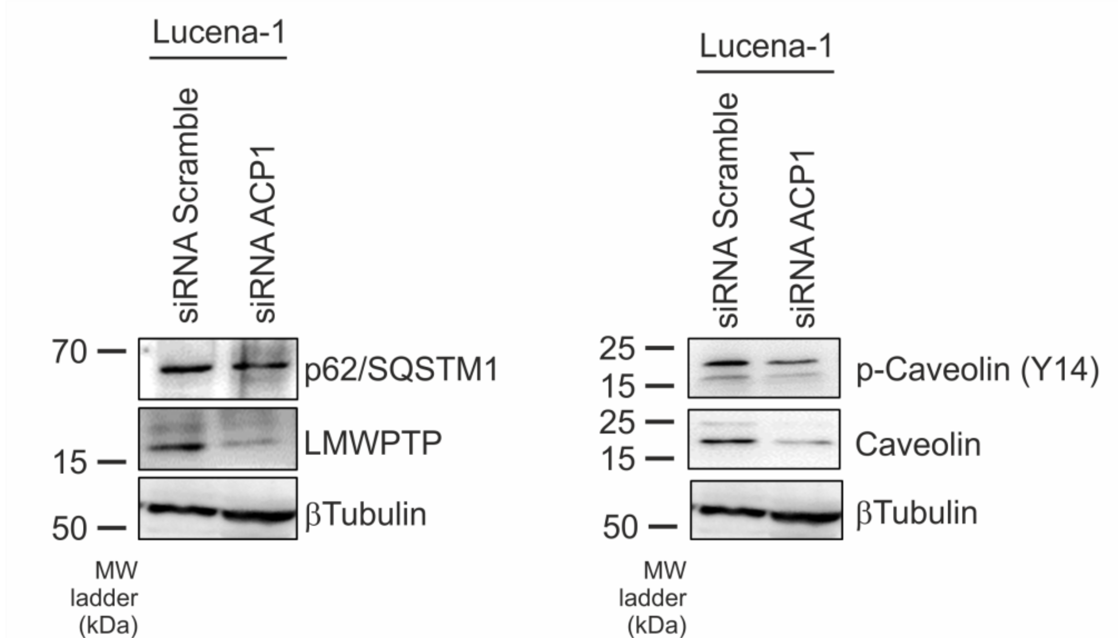


Figure 2. LMWPTP expression coordinates autophagy. Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) expression at Lucena-1 control (Scramble) and siRNA LMWPTP and autophagy-related proteins. Lysates of cells were submitted to Western blotting and probed with specific antibodies against LMWPTP and proteins involved in autophagy process. β-tubulin was used as the internal housekeeping, and the densitometry is represented by histograms on Supplemental Figure 1B.

Short time challenged Lucena-1 cells with hydrogen peroxide decreases LMWPTP and p62/SQSTM1 amounts

Previously, it has been shown that Lucena-1 cells display high resistance towards hydrogen peroxide treatment compared to K562 [10, 16]. Therefore, we decided to check if the challenging of Lucena-1 cells with subtoxic concentration of hydrogen peroxide would affect the LMWPTP and p62/SQSTM1. In other words, we wonder if the environmental stress would favor an extra protective response, for instance by reactivating autophagy. For that,

K562 and Lucena-1 cells were treated with 5mM hydrogen peroxide up to 60 minutes. Interestingly, autophagy- and antioxidant-associated proteins levels decreased during hydrogen peroxide treatment of K562 cells (Figure 3A), while in Lucena-1 cells, LMWPTP amount decreased, as well as the total p62/SQSTM1 (Figure 3B). On the other hand, SOD2 was upregulated at 30 minutes and dramatically downregulated after 60 min of hydrogen peroxide treatment, which correlates with catalase downregulation (Figure 3C). These findings reinforce that the LMWPTP contributes for autophagy process inhibition in Lucena-1 cells, which is reverted when these cells are treated with oxidant agent.

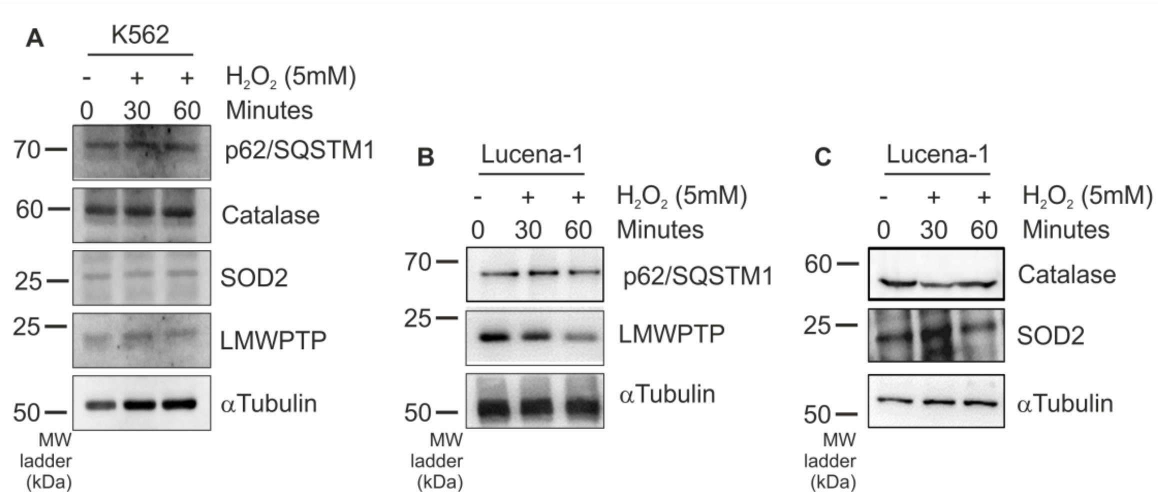


Figure 3. The antioxidant profile of K562 and Lucena-1. CML cells ($1x10^5$/mL) were treated with 5mM of H_2O_2 for 30 and 60 minutes. Lysates of cells K562 (A) and Lucena-1 (B,C) were submitted to Western blotting. α-tubulin was used as the internal housekeeping, and the densitometry is represented by histograms on Supplemental Figure 1C,D.

Discussion

K562 and Lucena-1 cells are useful models of chronic myeloid leukemia which have been characterized molecularly [16, 17]. Comparing the both models, our group described that the high amount of the LMWPTP in Lucena-1 cells (resistant cells) is important for keeping Src and Bcr-Abl activated [9]. Specifically, in relation to antioxidant response, Src has been highlighted to promote Warburg effect, which among other effects, stimulates the pentose pathway, a key cascade for producing NADPH, essential for antioxidant reaction. Recently, Jin and co-workers (2016) reported that the activation of Src decreased pyruvate dehydrogenase (PDH) activity, also the generation of reactive oxygen species (ROS) [18]. In opposite, Src inhibitors activated PDH and increased cellular ROS levels. PDH has a critical role in defining the fate of pyruvate produced by glycolysis pathway, once this enzyme catalyzed the conversion of pyruvate in Acetyl-CoA. In other words, PDH is the gatekeeper enzyme that strategically links glycolysis to mitochondrial oxidation. Later on, our group showed that LMWPTP is also connected to the glycolytic profile of Lucena-1 cells [10], which is important for the resistance of these cells towards hydrogen peroxide treatment. These findings prompted us to compare the autophagy pathway in Lucena-1 (resistant) and K562 (sensitive) cells, once this process contributes to remove the irreversibly oxidized biomolecules from cells, and to find out whether there is any connection between the LMWPTP and autophagy process. Indeed, the autophagy process is inhibited in Lucena-1 cells. Hydrogen peroxide treatment of Lucena-1 caused a wave response in LMWPTP, SOD2 and p62/SQSTM1. These proteins were upregulated at 30 min, followed by downregulation at 60 minutes. It means that, at first Lucena-1 recruits antioxidant players in order to overcome the oxidant stimulus, which it is not sustained for longer hydrogen peroxide exposure. Consequently, the autophagy process is required for controlling the ROS excess and/or removing damage organelles and macromolecules. On the same direction, K562 cell line, which has lower LMWPTP expression, has lower antioxidant capacity, as it was observed on catalase and SOD2 amount along hydrogen peroxide treatment time frame. These findings suggested that Lucena-1 cells do not keep autophagy process activated under basal condition. The explanation for that might be, in part, due to an efficient antioxidant response supported by: glycolytic metabolism that guarantees NADPH production [19]. However, when these cells were challenged with an oxidant agent, the LMWPTP, SOD and p62/SQSTM1 amount dropped. Consequently, this redox imbalance favors autophagy occurrence (Figure 4). It has been reported that autophagy triggered by reactive oxygen species (ROS) is prevented in the presence of ROS scavengers [20, 21].

Overall, we showed, for the first time, that the LMWPTP is also linked to cell redox stasis and autophagy regulation, kinds of cellular strategies to withstand an unfavorable environment. In addition, the findings presented here brought out new aspect of LMWPTP relevance for leukemia resistance, once keeping Src kinase active enables resistant leukemia cells to counteract the harmful effect of hydrogen peroxide.

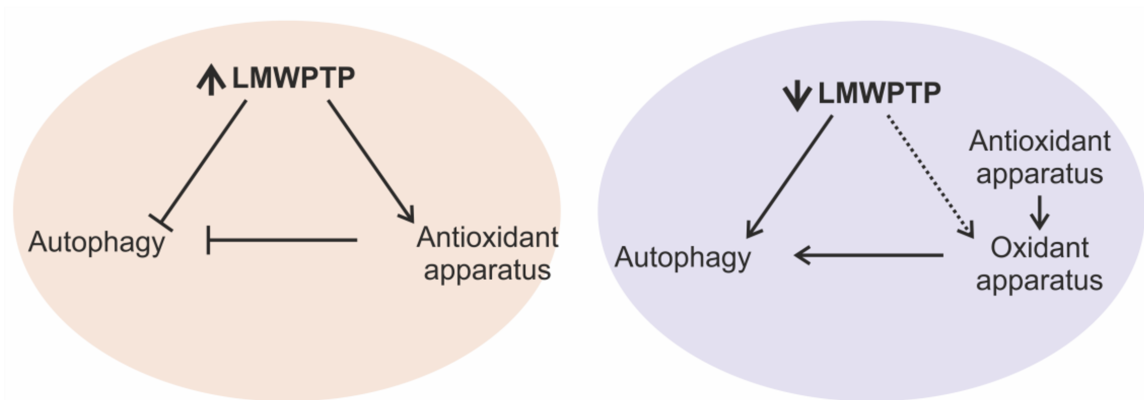
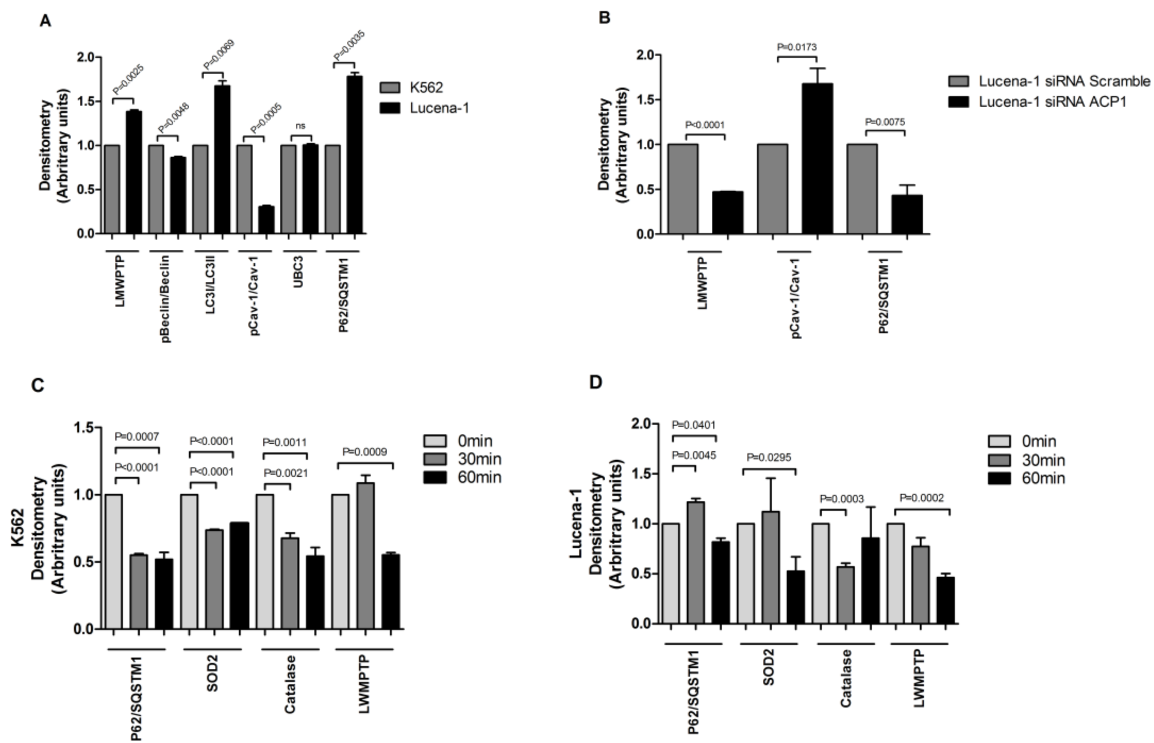


Figure 4. LMWPTP supports antioxidant defense in Lucena-1 cells. LMWPTP for an effective antioxidant arsenal and consequently autophagy process is not favored. However, when Lucena-1 cells are treated with H_2O_2, the LMWPTP amount goes down, and in turn autophagy can occurs.



Supplemental figure 1. Histograms for Western blots represents western blot from Figure 1 (A), Figure 2 (B) and Figure 3 (C,D). It was considered the housekeeping control for total proteins, and the ratio between phosphorylated/total forms for phosphorylated proteins. Statistical analysis: Figure 1 (A), P<0.05 K562 vs. Lucena-1; Figure 2 (B), P<0.05 Lucena-1 shScramble vs. Lucena-1 shACP1 and for figure 3 (C,D), P<0.05 Control vs. H_2O_2 treatments.

Experimental procedures

Cells line, antibodies, and reagents

Leukemic cells K562 cells were purchased from the American Type Culture Collection and Lucena-1 cells (counterpart of K562 cells with multiresistant phenotype) were provided by prof. V.M. Rumjanek [14] – for details, see Table 1. Polyclonal antibodies against α-Tubulin (#2144), Beclin (#3738S), phospho-Beclin (Ser 93/96) (#12476S), LC3 (#2775), Catalase (#14097), UBC3 (#4997), Caveolin-1 (#3238) and phospho-Caveolin (Tyr 14) (#3251) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). SQSTM1 (D3) p62 (sc28359) antibody was obtained from Santa Cruz Biotechnology, Inc. (St. Louis, MO, USA). LC3 B (MAP1) (18725-1) and SOD2 (1080) antibodies were obtained from Bioss (Boston, MA, USA). LMWPTP (ACP1) (ab26232), β-Tubulin (ab15568) antibodies were obtained from Abcam (Cambridge, UK). Anti-sheep, anti-rabbit, and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Hydrogen peroxide 30% (H_2O_2) was purchased from Merck Millipore Corporation (Darmstadt, Germany).

Cell culture

K562 and Lucena-1 cells were routinely cultured as described in [9]. Briefly, cells were grown in suspension in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 2mM glutamine, 100U/mL penicillin, 100μg/ml streptomycin and 10% fetal bovine serum (FBS). For Lucena-1 cells, vincristine (final concentration: 60nM) was routinely added culture medium. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. In the present study, the basal condition was considered as cell cultured in medium contained FBS, glutamine and antibiotics. The stress condition was under 5mM H_2O_2 treatment.

Table 1. Cell lines characteristics

Cell line	K562	Lucena-1
Tissue	Bone marrow (human)	Bone marrow (human)
Disease	Chronic myeloid leukemia	Chronic myeloid leukemia
MDR profile	Negative	Positive [16]
Vincristine resistance	Negative	Positive [16]
Bcr-Abl mutation	Positive	Positive [9, 16]
Obtained from	ATCC	Derived from K562 Described at Rumjanek et al, 2001 [16]
Cell culture	Suspension	Suspension

Transmission electron microscopy

The transmission electron microscopy was performed as described before [22, 23]. Briefly, the K562 and Lucena-1 cells were fixed with 2.0% phosphate-buffered glutaraldehyde. The cells were then postfixed in 1% phosphate-buffered OsO_4, and embedded in Spurr's resin. Thin sections (0.12μm) were cut, double stained with $UO_2(CH_3COO)_2$ (uranyl acetate) and $Pb_3C_{12}H_{10}O_{14}$ (lead citrate), and visualized with a Philips TECNA10 transmission electron microscope (TEM). Fifty cells from randomly chosen TEM fields were analyzed for each cell line.

Immunofluorescence Microscopy

CML cells were cultured ($1x10^5$ cells/well – 1000 μL), on 24-well plate for 3 hours. After, cells were fixed with 4% PFA for 10 minutes, washed with PBS, permeabilized with Triton x-100 (0.1%) for 10 minutes and blocked with 3% BSA for 1 hour. Cells were washed 3X with PBS and incubated overnight at 4°C in a humidified chamber with the following antibody LC3 (#2775) Cell Signaling Technology (Beverly, MA, USA). After, cells were stained with Alexa-Fluor® - Invitrogen (Thermo Fisher Scientific, MA, USA) 488 Rabbit secondary antibodies at 1:500 dilution for 1 hour. Cells were subjected to a standard staining with DAPI-Invitrogen (Thermo Fisher Scientific, MA, USA) at 1:1000 dilution. Cell suspensions were mounted onto glass slides covered with poly-lysine (Sigma-Aldrich, Missouri, USA) with a coverlips. Images were acquired on a LEICA TCS SP5 II confocal microscope (Leica, Wetzlar, Germany) at Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP) - using a 63X objectives. Images format 1024x1024 and 4x optical zoom. Images were analyzed using Image J software (NIH, USA). The analysis of expression was performed using: Corrected Total Cell Fluorescence = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

Transfection of Lucena-1 cells with LMWPTP siRNA

The transfection of Lucena-1 cells was performed as described before [9]. Briefly, Lucena-1 cells ($1x10^5$ cells/mL) were grown for 24 hours at 37°C in a 5% CO_2 humidified atmosphere and subsequently transiently transfected with LMWPTP siRNA (QIAGEN #SI02776851). Transfections were done using the Hiperfect transfection kit (QIAGEN) according to the manufacturer's instructions. Briefly, the cells were transfected with LMWPTP siRNA (5nM) for 48 hours and then lysed with a specific buffer for Western blotting procedure. The

efficiency of transfection was assessed based on the expression of LMWPTP by Western blotting analysis.

Cell Viability and Treatment

CML cells were plated at $1x10^5$ cells/mL density into a 6-well plate and treated with H_2O_2 (5mM) for 30 and 60 minutes. After treatment, cell viability was assessed by trypan blue dye exclusion. The cells were harvested and western blot samples were prepared.

Western blotting

Western blotting was performed as described before [10]. Briefly, cells were lysed for 2 hours on ice in cell lysis buffer (50mM Tris–HCl pH 7.4, 1% Tween 20, 0.25% sodium deoxycholate, 150mM NaCl, 1mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1mM Na_3VO_4, 1mM NaF and protease inhibitors [1µg/ml aprotinin, 10µg/ml leupeptin, and 1mM 4-(2-aminoethyl) benzenesulfonyl-fluorid-hydrochloride]. Protein extracts were cleared by centrifugation and protein concentration was determined using the Bradford reagent (Sigma-Aldrich, Missouri, USA). Twice the volume of sodium dodecyl sulfate (SDS) gel loading buffer (100mM Tris–HCl pH 6.8), 200mM DTT, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to the samples, which were subsequently boiled for 5 minutes. Cell extracts were resolved by SDS-polyacrylamide gel (12%) electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in bovine serum albumin (3%) in Tris-buffered saline (TBS)-Tween 20 (0.05%) and incubated overnight at 4°C with the appropriate primary antibody at 1:1000 dilutions, in bovine serum albumin (1%) in TBS-Tween 20 (0.05%). After washing in TBS-Tween 20 (0.05%), membranes were incubated with anti-rabbit, anti-mouse, and anti-sheep horseradish peroxidase-conjugated secondary antibodies, at 1:10000 dilutions, in bovine serum albumin (1%) in TBS-Tween 20 (0.05%) for 2 hours. Proteins were detected using enhanced chemiluminescence in Alliance 6.7 (UVITEC, Cambridge, UK).

Data Analysis

All experiments were performed in triplicate. Soluble lysates were matched for protein content and analyzed by Western blotting and all bands were compared with their respective internal control. Student's t-test was performed (paired, 95% confidence intervals, two tailed) using GraphPad software (version 5.0, GraphPad Inc, San Diego, CA, USA).

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Authors' contributions

The head group leader CVFH contributed for the article conceptualization; AVSF, SPC, PSFO, KCSQ, MPP and CVFH were responsible to design the methodology; AVSF, SPC, PSFO, KCSQ performed the research investigation; AVSF was responsible for the original writing, and SPC, PSFO, KCSQ, and CVFH were responsible for reviewing and editing; the work was supervised by MPP and CVFH. All authors read and approved the final manuscript.

Footnotes

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Chapter 5

Platelets in aging and cancer – “double edged sword”

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Abstract

Platelets control hemostasis and play a key role in inflammation and immunity. However, platelet function may change during aging, and a role for these versatile cells in many age-related pathological processes is emerging. In addition to a well-known role in cardiovascular disease, platelet activity is now thought to contribute to cancer cell metastasis and tumor-associated venous thromboembolism (VTE) development. Worldwide, the great majority of all patients with cardiovascular disease and some with cancer receive anti-platelet therapy to reduce the risk of thrombosis. However, not only do thrombotic diseases remain a leading cause of morbidity and mortality, cancer, especially metastasis, is still the second cause of death world-wide. Understanding how platelets change during aging and how they may contribute to aging-related diseases such as cancer may contribute to steps taken along the road towards a 'healthy aging' strategy. Here, we review the changes that occur in platelets during aging, and investigate how these versatile blood components contribute to cancer progression.

Keywords

Platelet function, platelet reactivity, aging, cancer.

1. Introduction

Physiological changes occur in all organ systems during aging, and are a reflection of changes that occur on a molecular level in individual cells. Diverse animal and yeast models have shown that aging is associated with tissue-specific changes in transcriptomes as well as intra- and extracellular metabolite changes [1]. Cellular senescence, a block in cellular proliferation as a result of (amongst others) telomere shortening and loss of DNA damage repair, plays an important role in the process of aging [2]. In addition to telomere attrition, genomic instability, and cellular senescence, other hallmarks of cellular aging include stem cell exhaustion, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, and altered intercellular communication [3]. Not all cells become senescent, and removal of senescent cells may reduce aging on an organismal level [4]. However, cellular communication is mediated in part via the release of vesicles known as exosomes, which can carry cellular components from one cell to another across large distances. Senescent cells also release such exosomes and these have been speculated to play a significant role in age-related phenotypes including age-related diseases [5]. Connecting all known cellular alterations to biological aging remains challenging, and finding ways to promote “healthy aging” remains a holy grail [3].

Thus far, aging is often studied in the context of stem cell capacity and longevity, but cellular changes in individual cell types have also been investigated for neurons, skin fibroblasts and keratinocytes, bone and bone marrow (bone-proximal osteoblastic niche), and many other tissues and cell types [6–8]. One more cellular component to be added to this mix are platelets, as a role for these blood constituents in aging and age-related diseases is now emerging [9]. Like many systems in cellularmetabolismand catabolism, the biology/function of platelets appears to be altered in the elderly. In addition, altered platelet function and clinical conditions such as cancer create a complex chain of cause and effect, which can culminate in systemic responses responsible for the main causes of death in the world, namely, (1) inappropriate blood clot formation known as thrombosis and (2) cancer metastasis, responsible for more than 90% of cancer-related deaths [9,10]. Thrombotic risk in the elderly is associated with genetic factors, but also with lifestyle, obesity, and diseases such as cancer [11, 12], creating a complex feedback loop. Other examples of the interrelationship between platelet function and pathological conditions can be seen in the acquisition of bleeding disorders such as hemophilia or Von Willebrand syndrome [13], or the involvement of platelets to neurological disorders such as Alzheimer disease (for review, see [14]). In this latter condition, the microenvironment sensitizes platelets to activation and renders them less

sensitive to inhibition, most likely due to increased sensitivity to some platelet activation agonists, such as thrombin and collagen, leading to an increase in β-amyloid production by platelets [15, 16]. Large-scale omics studies have demonstrated age-specific proteomic changes in platelets from childhood to adulthood [17], and miRNA patterns associated with age in individuals ranging from 18 to 46 years old [18]. It is conceivable that such cellular changes may predispose an individual to aging-related diseases. In this review, we summarize the impact of aging on platelet function, and investigate how such altered platelet functionality can contribute to aging-related diseases, with particular emphasis on cancer.

2. Aging-associated changes in platelet phenotype and function(s)

Since the lifespan of platelets is around 7 to 10 days in the bloodstream, changes in platelet functions may be correlated with megakaryocyte matura t ion, adhes ion, and thrombopoiesis, as changes in megakaryocyte maturation during aging lead to altered proplatelet formation and release of platelets with an altered content [19]. Some of these events appear to be driven by β-adrenergic signals coming from a senescent microenvironment [19–21]. As such, megakaryocyte aging, aging of platelets in the circulation, and cues from an aged microenvironment to megakaryocytes and nascent platelets during organismal aging can all contribute to changes in platelet biology in elderly individuals. Under normal conditions, there is a gradual loss of RNA content over the course of a platelet lifespan, while in aged organisms, distribution of megakaryocyte content to platelets is altered. However, there are also clear differences between “aged platelets” and “platelets in aged individuals.” Hepatic clearance of senescent platelets from the circulation of adult organisms is dependent on the loss of sialic acid residues of glycoproteins in the cell membrane. Activation of the pro-apoptotic BAX–BAK pathway in aged platelets results in caspase-dependent surface exposure of phosphatidylserine, which serves as a recognition signal for phagocytic cells. In terms of functionality, senescent platelets have impaired adhesion and aggregation responses. On the other hand, platelets in senescent organism might be primed to increase their responsiveness to agonists (hyper-reactive platelets) [22, 23].

Several recent studies have investigated the effect of aging on platelet morphology and function. During the course of life, platelet size increases [24], which directly affects platelet content, including granules and pro-coagulation factors. Other morphological changes seen in platelets from older individuals include an irregular, less smooth plasma membrane with more frequent ruptures, and an increase of slender pseudopodia [25]. The number of circulating platelets is thought to decrease with advanced age. While a study of over 5000 participants

suggested that platelet count in individuals of > 65 years is not affected by subsequent age differences [26], two large studies investigating over 25,000 and 40,000 individuals, respectively, showed that platelet numbers drop from early childhood, are relatively stable in adulthood, and drop again over the age of 60 years old, irrespective of gender and ethnicity [27, 28]. Careful consideration of the age groups studied is essential, and for the purpose of this review, we therefore aimed to compare young adults (18–39 years), middle-aged (40–59 years), old-aged (60–79 years), and very-old-aged (> 80 years) groups, where possible (Figs. 1 and 2). While the cause of reduced platelet numbers during aging remains to be clarified, some studies have suggested changes in hematopoietic stem cells as a pivotal cause of lower platelet counts in advanced age [29–31].

Despite a lower platelet count in older individuals, bleeding times are reduced during aging, which is thought to contribute to an increased risk of blood clot formation [32, 33]. Bleeding time (i.e., time before efficient blood clotting occurs) is dependent on platelet count and vessel contractibility, as well as platelet function, and platelets in the elderly are indeed hyper-reactivated, especially in subjects with associated comorbidities (for review, see [31, 34]). For instance, spontaneous platelet aggregation is higher in very old subjects as compared with old adults [35, 36], and a higher sensitivity to ADP stimulation [10, 37, 38] and thrombin receptor–activating protein (TRAP6) [39] is seen. Several other platelet agonists, including ristocetin, thrombin, and collagen, have received attention but whether responsiveness of platelets towards these agonists is increased or decreased during aging remains disputed (Fig. 1).

Whether overactivation of platelets is a failed compensation mechanisms to make up for the loss of platelet count remains speculative. The mechanisms contributing to higher platelet activity in elderly individuals are still under investigation. It has been suggested that age-related inflammatory and metabolic changes contribute to an increased platelet function in the elderly [40]. Mouse models have shown an increase of hydrogen peroxide concentration in blood, which directly increases platelet activity during aging [41]. In humans, oxidative stress markers in platelets increase from young to middle-aged individuals [35, 42, 43]. Hydrogen peroxide accumulation in platelets could be the result of NADPH oxidase and superoxide dismutase activity, which are associated with an increased integrin αIIbβ3 activity in platelets [44, 45]. Indeed, the expression of surface markers such as integrin αIIb and αIIbβ3 is increased during the course of aging [46, 47]. Thus, overall increased oxidative stress is generally seen during the aging process, contr ibuting to the concept that platelet alterations in aging are associated with an increasing inflammatory state. The oxidative burst

triggers activation of the signaling molecule mTOR, a key regulator of lifespan and aging [48]. mTOR activation in turn results in an increased platelet production by megakaryocytes [49]. Moreover, mTOR hyper-activation during aging is associated with increased platelet aggregability and aging-related venous thrombosis risk in mice [50]. Thus, mTOR plays a dual role in platelet hyper-aggregability by increasing the activity of platelets, while oxidative stress further increases platelet reactivity, resulting in an enhanced risk of thrombi formation in the elderly (Fig. 2).

Association between activated platelets and monocytes, as would occur during blood clotting, enhances the formation of aggregates. While there is no impact of age on platelet-monocyte aggregation per se in healthy adults [51], higher levels of platelet-monocytes aggregates were seen in patients with acute coronary syndrome [52], and platelet hyper-activation may thus be further exacerbated in disease states. Others have shown that the age-related increases of platelet- derived β-2-microglobulin levels in the serum cause monocyte differentiation towards a less regenerative phenotype, providing a further link between platelet changes during aging and the aging process [53].

A clear association between platelet hyper-reactivity and the occurrence of thromboembolic events exists and may contribute to cardiovascular comorbidities in the elderly [54]. In addition to the direct effect of aging on platelet aggregation described above, this phenomenon has also been attributed to the fact that the production of anti-coagulation factors does not follow the increasing pro-coagulation factor production during aging [11]. Gleerup and Winther showed that, in addition to an enhancement of platelet aggregability, aging provokes a decrease of fibrinolytic activity, further reinforcing the association between lower fibrinolytic activity forming stable thrombus formation and accumulation, an imbalance between thrombotic versus fibrinolytic events [55]. The same research group described that adrenaline and sub-concentration ADP-induced canonical platelet activation is enhanced in old and very old individuals, as is the synergistic effect of serotonin on adrenaline-/ADP-induced platelet activation. Adrenaline levels were also augmented in the old and very old groups [56, 57]. This might be a compensatory mechanism for the fact that β-adrenoreceptors from older individuals show higher ligand affinity. This receptor reduces platelet aggregation through the production of cAMP, and a reduced signaling capacity through this receptor may thus contribute to an enhanced platelet aggregation in the elderly; however, the levels of cAMP in plasma did not change significantly during aging [56, 57]. Endothelial dysfunction during aging may further increase platelet responsiveness [58]. For instance, it has been speculated that platelet activation and aggregation caused by dysfunctional lung epithelium in

virally infected individuals may cause depletion of thrombocytes, and contribute to the thrombocytopenia observed in COVID-19 patients infected with SARS-CoV-2 [59, 60].

In addition to blood clotting, it is increasingly recognized that platelets play an important role in wound healing. While wound healing is not absolutely impaired, delayed closure rates and weaker wound repair are commonly seen in subjects of advanced age [61]. During wound healing, many different cell types, including fibroblasts and immune cells such as macrophages and lymphocytes, cooperate to restore tissue architecture. Activated platelets trapped in the blood clot release mediators to attract these cells and express P-selectin which acts as cell adhesion molecule for passing lymphocytes [62]. Furthermore, the secretion of several growth factors, such VEGF, PDGF, EGF, and TGFβ, may modulate T cells to induce keratinocyte regenerative capacity and enhance proliferation of regenerative cells such as fibroblasts [63, 64]. However, while reduced serum levels of these platelet-derived factors could theoretically contribute to decreased wound healing rates, age-related variations in cytokine levels appear most pronounced in early adulthood, disputing their relevance for wound healing delay in the very old individuals [25, 65].

Data collection on platelet function during aging is complicated by several issues. For one thing, platelet aging may be gender-specific, as studies have indicated that aging-related loss of interaction with the adhesion molecule von Willebrand factor (vWF) is more pronounced in women as compared to men [28, 66]. Thus, hormonal changes may contribute to platelet alterations in older subjects [67]. Levels of steroids such as testosterone and dihydrotestosterone in older individuals are negatively associated with platelet activation markers, and these steroids can directly inhibit collagen-induced aggregation in vitro [68]. Secondly, recent data suggest that changes that occur during aging are complicated and were not always found to be continuous during aging. Spontaneous aggregation was increased in elderly individuals compared with younger subjects, while ristocetin or collagen-induced aggregation was decreased (pointing towards platelet exhaustion) [35]. However, these trends did not follow linear relationships with changes most pronounced in the very old (80+ years) [35]. Other platelet activation markers (soluble P-selectin, integrin αIIb, caspase 3, oxidative stress)were shown to increase from young to old individuals, but decrease again in the very old [43]. However, it should be noted that others found no differences in basal membrane-bound P-selectin between individuals < 45 years and > 65 years old [69, 70], while the percentage of platelets expressing P-selectin upon stimulation with TRAP-6 was actually higher in younger individuals [39]. Differences in age groups, methods, and stimuli used vary per study and may account for conflicting results. It should further be noted that the effects

observed are sometimes small, and small group sizes may hamper interpretation of results. While many studies point towards disturbances in platelet functionality during aging, the direct consequences on coagulation in healthy aging may not always be clear [68, 71], and may be more pronounced under pathological conditions.

Platelet bioactive lipids in aging

A detailed study on platelet lipid production and aging was reported in 1986 [72]. This study investigated platelet cholesterol and phospholipids content, and observed a slight increase of cholesterol/phospholipids molar ratio upon aging within a range of 20 to 69 years old [72, 73]. It is important to highlight that platelets are not able to produce their own cholesterol, which must be obtained during their genesis (from megakaryocytes) or derived from plasma. The cholesterol/phospholipid molar ratio is important to maintain platelet membrane fluidity, and, consequently, the platelet capacity to change its shape during activation. In addition, activation of platelets via agonist-receptor activation in many cases requires localization of receptors and downstream signaling molecules in cholesterol-rich lipid rafts [74]. The lipid composition is also affected by aging [75], with increased fatty acids 16:0 phosphatidylcholine and sphingomyelin, and a decrease of linoleic acids 18:2, 20:4, and 20:3 in older subjects [72]. It is important to note that lipid oxidation occurs on platelet LDL, and this phenomenon may have severe consequences for cardiovascular diseases. One study showed that older males at risk for coronary heart disease due to dietary habits (55–73 years old) showed higher platelet aggregation in response to epinephrine as compared with younger individuals (28–54 years old) and males at lower risk for heart disease, indicating that age-related platelet changes associated with phospholipid content may be a risk factor for cardiovascular diseases [76].

Besides the platelet membrane lipid composition, the most important bioactive lipids relevant to platelet function are the signaling lipids derived from the eicosanoid pathway. Briefly, upon stimulation of cells, membrane-anchored arachidonic acids (AA) are released from the membrane phospholipids by phospholipases (phospholipase A2), after which they are enzymatically converted to prostanoids by COX1/2 enzymes. This process results in production of platelet stimulatory thromboxane (TxA2, mainly produced via COX1 [77]) or platelet antagonistic prostaglandins (PG), PGI2, prostacyclin), PGD2, and PGE2 (mainly via COX2) [78, 79]. Alternatively, AA can be converted to leukotrienes through lipoxygenases activity. Eicosanoids are impor tant mediators of inflammation, and, indeed, eicosanoid biosynthesis is higher on advanced age [57, 80, 81], which in turn may contribute to enhanced

inflammatory state during aging [78, 80, 82]. Platelet interaction with peripheral blood mononuclear cells directly modulates inflammatory responses, potentially through their production of PGE2 [62, 63]. In this case, PGE2 decreases the effectiveness of myeloid cell differentiation and affects their responses [83].

However, both increased TxA2 as well as PGE2 and prostacyclin excretion were seen in older humans or rats, which begs the question of how this balance would affect platelet activity [57, 84, 85]. While TxA2 is produced by platelets, the major source of prostacyclins is endothelial cells. While some studies showed no differences in prostacyclin secretion by arterial endothelial cells for donors of different ages [83], others demonstrated reduced prostacyclin expression in aorta endothelia from older individuals, suggesting that perhaps the TxA2 effectwins out during aging. It is of interest to know that dietary restriction, known to prolong healthy aging, is associated with an enhanced prostacyclin/TxA2 ratio in rats [86, 87]. Indeed, increased TxA2 excretion appears to be associated not only with age-related diseases such as atherothrombosis but also with metabolic disease [88, 89]. Obesity and decompensated glucose metabolism increase not only platelet activation but also inflammation (for review, see [90]). In this case, the persistent TxA2-dependent platelet activation increases systemic inflammation [89, 91]. Inflammation-induced endothelial events may play a major role in aging comorbidities. For instance, glycemia-mediated TxA2-receptor activation was associated to disturbed blood-brain barrier integrity in diabetes [92]. Furthermore, TxA2 is a P2X1 ion channel agonist and both platelets and P2X1 are required to maintain vascular integrity in a mouse colitis model [93, 94].

Taken together, a clear change in platelet morphology and function is seen during aging, which may have severe consequences for aging-related physiology. The most relevant changes in platelet biology were highlighted in Figs. 1 and 2.

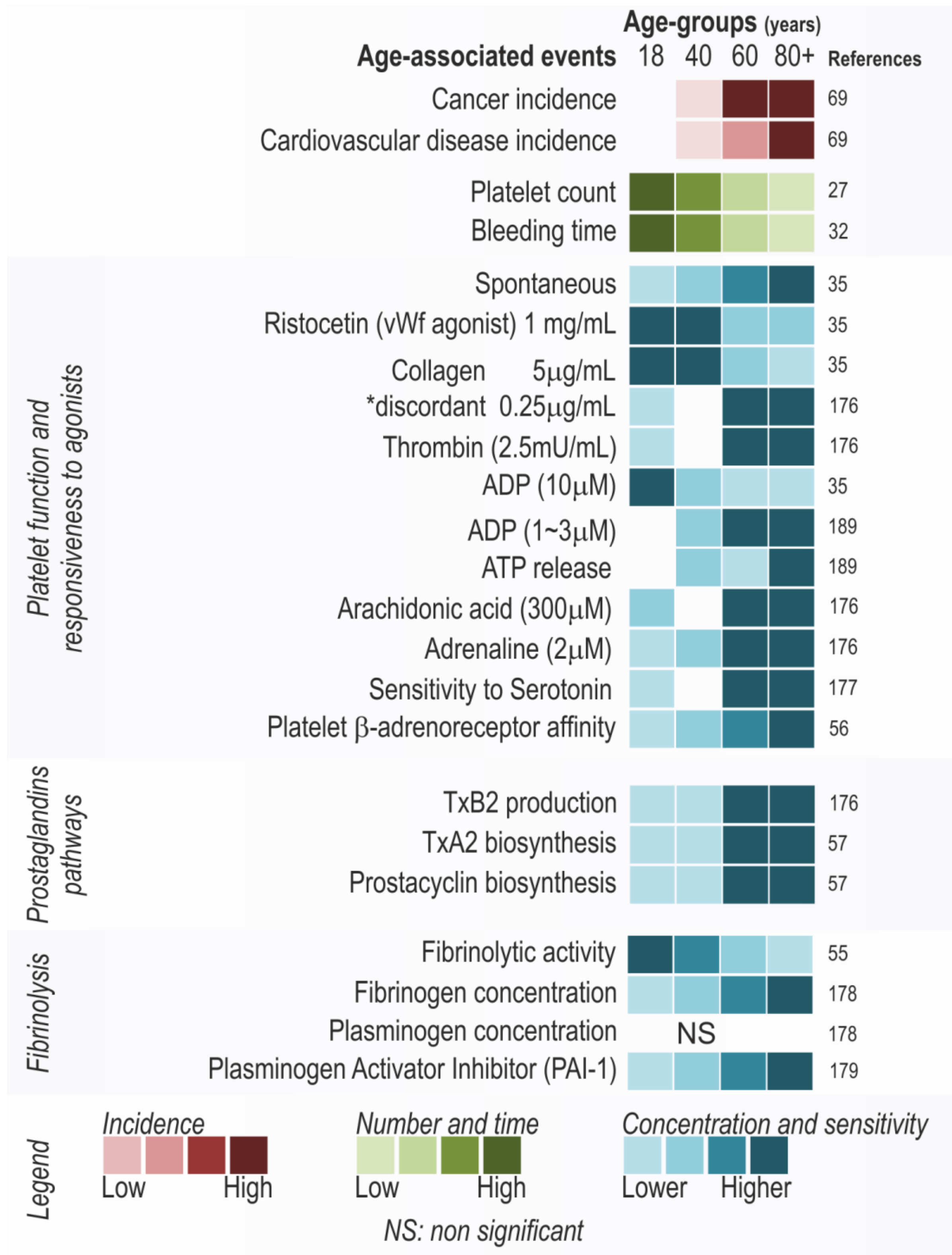

Figure 1. Age-associated changes in platelets function. Platelet function of aggregation, tissue repair and remodeling changes discriminated on age-groups. The concept of age-groups is based on young adults (18-39 years), middle-age (40–59 years), old-age (60–79 years), very-old-age group (>80 years).

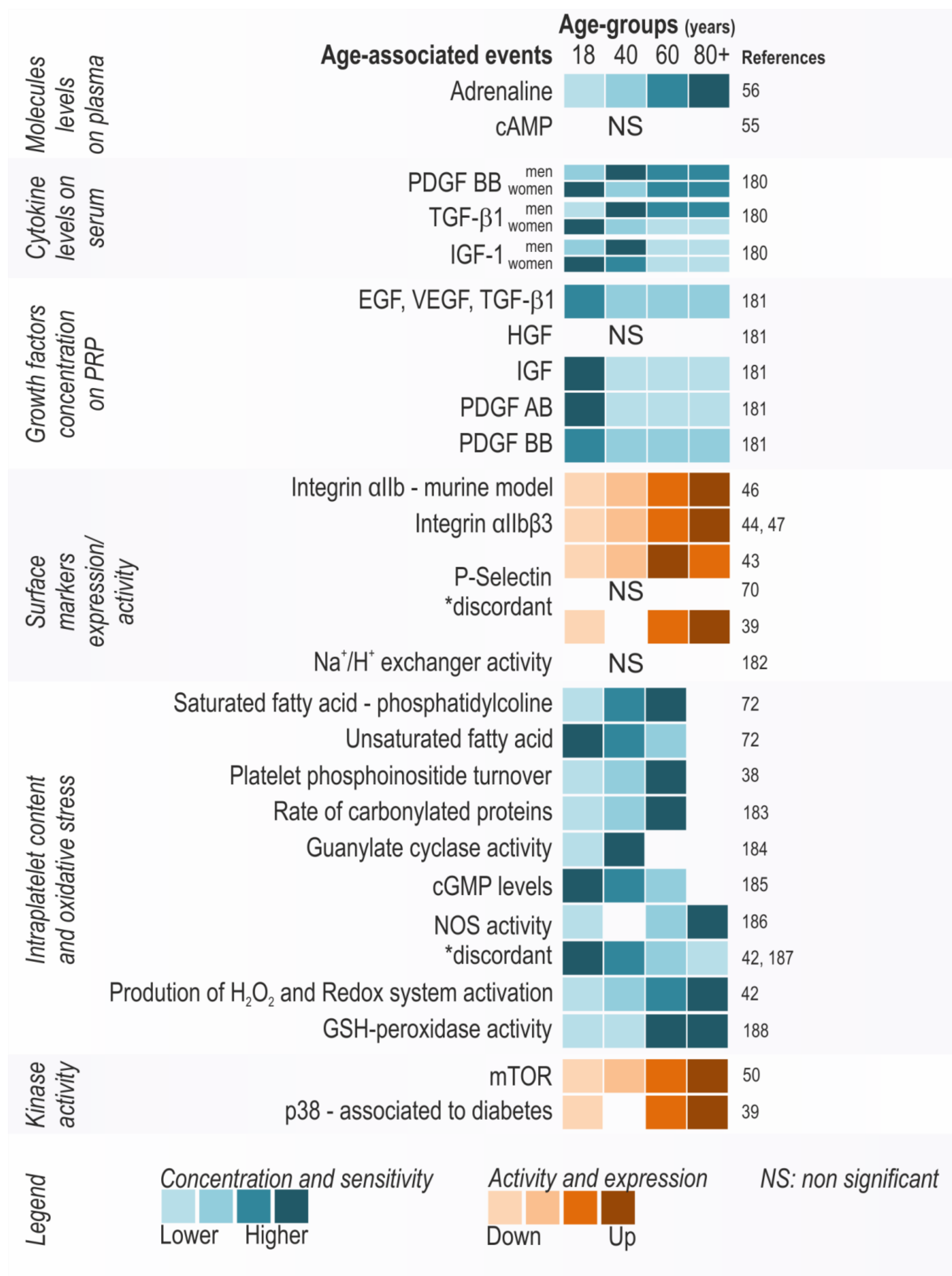

Figure 2. Age-associated changed in platelet markers. Platelets present several changes during the aging process on their content (cytosolic and membrane) and release thereof. The concept of age-groups is based on young adults (18-39 years), middle-age (40–59 years), old-age (60–79 years), very-old-age group (>80 years).

3. Platelets in cancer – "double edged sword"?

As described above, platelet hyper-reactivity during aging is associated with an increased risk of formation of embolisms. Nevertheless, despite cancer being an age-related disease, thrombocytopenia is a common event in these patients. The risk of bleeding in thrombocytopenic cancer patients is difficult to predict [95], and platelet counts must be carefully monitored. In particular, cancers of the bone marrow (platelet production from megakaryocytes) or spleen (platelet clearance), where hematopoiesis is affected, are prone to lead to loss of platelet counts. For instance, thrombocytopenia in patients with bone dyscrasias is directly related to bleeding events [96]. However, the most common cause of bleeding due to platelet loss in cancer patients arises as a result of
myeloablative chemotherapy [97] and cytopenia may therefore be a bystander effect rather than a pathogenic event. In fact, the role of platelets in cancer appears to be ambiguous, as enhanced blood clotting represents a major risk factor in cancer patients.

Patients with cancer (but also those with cardiovascular diseases including diabetes, hyper-cholesterolemia, and hypertension) can develop an increased platelet activity, which may be either age-related or disease-specific. The hyper-aggregability observed in these diseases appears to be related to higher platelet reactivity towards agonists or in- creased circulation of these agonists (such as thrombin and factor Xa), and is a primary cause of thrombotic events, in particular venous thromboembolismevents (VTE) and arterial thrombosis (AT) [98, 99]. These events partially overlap, with shared risk factors, and similar incidence in cancer patients [100, 101].

The first report of a platelet-related disorder in cancer came from Armand Trousseau, who described a higher risk of thrombotic events in cancer patients [102], which has subsequently been termed Trousseau syndrome. As the second cause of death, VTE poses a significant comorbidity in cancer patients, and a common cause of hospitalizations, thereby significantly contributing to cancer-associated health care costs [103]. Several cancers are associated with increased VTE risk, including renal carcinoma [104]; hepatocellular carcinoma [105]; lung cancer [106]; and esophageal and stomach cancer [98]. Moreover, VTE in esophageal or gastric cancer patients has been associated with decreased survival: patient survival without VTE is 18 months compared with 13.9 months with VTE [107]. While the risk of VTE appears to be especially high in patients suffering from stomach and pancreatic cancer, up to 20% of all cancer patients may develop thromboembolisms, including pulmonary and venous events. For AT, the overall incidence of events in patients with cancer is increased 2-fold [101].

Enhanced platelet activation as determined by mean platelet volume (MPV) is seen in cancer patients, andmay correlate with tumor stage [108, 109]. BothMPV and increased soluble P-selectin levels correlate with VTE development in cancer patients [110–112]. Age does not predict VTE risk for all cancer types, suggesting that at least for some cancer types, tumor cells themselves increase platelet reactivity and VTE risk [113]. Indeed, higher platelet P-selectin expression was found in mouse models of breast cancer, which in turn was associated to lung metastasis [114]. In addition, MPV, which is enhanced in malignant tumors, drops upon treatment [115], enforcing the direct link between tumor burden and platelet activation. Thus, cancer cell–mediated platelet hyper-reactivity contributes to increased VTE risk. While to date, there is no method available and validated to monitor the clinical implication of platelet hyper-aggregability in cancer patients; this may be a promising avenue of investigation [116].

Multiple mechanisms may underlie the tendency of platelets from cancer patients to aggregate. Tumor cells can stimulate platelet aggregation through direct interaction via adhesion molecules or via the delivery of extracellular vesicles and/or secreted factors. This phenomenon, described as tumor cell–induced platelet activation (TCIPA), was already identified decades ago [116]. It has now been shown that single tumor cells are capable of attracting and activating platelets to form fibrin clots [117]. Furthermore, platelets from cancer patients differ from platelets from healthy controls in their mRNA profiles, with mRNA transcripts undergoing alternative splicing under influence of tumor-derived stimuli [118,119]. Platelets are also capable of taking up tumor content, as determined by the fact that tumor-specific mutations can be identified in platelets upon co-culture with tumor cells. This process appears to be regulated by extracellular vesicles released by the tumor cells, which are subsequently taken up by co-cultured platelets [120]. This alteration of platelets by tumor cells, i.e., tumor education, was shown to contribute to an increased adhesive propensity of platelets [121–123]. Furthermore, cancer cells shed extracellular vesicles containing the adhesion molecule tissue factor (TF), which may contribute to VTE at sites of vessel damage [120, 124].

4. Platelets drive tumor growth, angiogenesis, and metastasis in cancer

Specifically in solid tumors, the interaction of tumor cells and platelets leads to a condition called paraneoplastic thrombocytosis, in which malignant tumors not only hijack or mimic platelet functions but can also increase their production. A cyclic picture emerges, which contributes to the most feared outcome of a malignant neoplasm: metastasis [125].

Metastasis is the principal cause of death in cancer patients and investigation of the molecular mechanisms that coordinate this process is therefore crucial. The process of metastasis requires several steps: invasion of cells in the surrounding matrix, intravasation to the blood circulation, survival at the circulation, extravasation at the secondary site (tissue or organ), micrometastasis formation and colonization [126]. The primary tumor can shed many cells during the growth phase; however, only a few cells are able to colonize a secondary site [121]. Much depends on the survival of these tumor cells in the blood circulation, survival of detachment, and the hemodynamic flux force, as well as escaping the immune system. One of the principal strategies of cancer cells to survive in the circulation is interaction with platelets, and nearly all processes of cancer metastasis appear to be facilitated by interaction of tumor cells with platelets.

Platelets can stimulate expression of metalloproteinases in tumor cells, which in turn contributes to tumor cell invasion by facilitating extracellular matrix degradation [127, 128]. Tumor cell metastasis often requires the acquisition of a different phenotype, termed epithelial-to-mesenchymal transition (EMT). This process is characterized by upregulation of several molecular markers (e.g., expression of SNAIL, vimentin cadherin, and MMPs), and platelet-released TGFβ can significantly enhance the upregulation of these markers in cancer cells [129, 130]. In addition, direct contact between cancer cells and platelets contributes to TGFβ/Smad and NFκB pathway activation, culminating in EMT stimulation. Adherence of cells to the extracellular matrix provides survival signals, which are disrupted upon detachment of cells, thereby leading to anoikis: detachment-induced apoptosis. While cancer cells have several mechanisms to overcome anoikis, it has been demonstrated that interaction of cancer cells with platelets further induces tumor cell resistance against anoikis [115]. Thus, platelet-induced alteration of cancer cell intracellular programs contributes to tumor invasiveness and metastasis [121, 130, 131].

Extravasation of tumor cells from tissue to bloodstream is facilitated by platelet-derived ADP stimulation of P2Y2 receptors on endothelial cells [132]. Once the cancer cell enters the blood circulation, the dissemination efficiency also depends on the interaction with platelets, with many studies showing that platelets facilitate the metastatic process via hematogenous dissemination [129, 133]. Survival of tumor cells in the blood stream is not only enhanced by platelets through mechanic protection from shear force but also by protecting the cancer cells from circulating immune cells, which may target neoantigens, expressed by tumor cells. Interestingly, it has been demonstrated that cancer cells may mimic platelets by expressing megakaryocytic genes and expressing platelet surface markers,

including adhesion molecules such as integrins and selectins [125, 134]. Additionally, coating of tumor cells with platelets allows transferring their major histocompatibility complex (MHC) class I to tumor cells, thereby giving these cells a false “pseudonormal” exterior, and allowing escape from immunosurveillence by natural killer cells [135]. TGFβ released by platelets also downregulates the NK receptor NKG2D on tumor cells, further shielding them from immunosurveillence [136, 137]. Lastly, extravasation of the tumor cells from the blood stream is facilitated by platelets, and appears to require binding of platelets to Integrin αvβ3 expressed on tumor cells [138].

As a solid tumor grows and its oxygen and nutrient demands increase, angiogenesis, the formation of new blood vessels, is essential for its survival. Tumor-induced angiogenesis often results in an abnormal vasculature with suboptimal perfusion. Nevertheless, tumor cells may benefit from this, as this may reduce delivery of therapies and tumor-targeted immune cells [136]. Furthermore, tumor cells may adapt to such ineffective vascularization, and the ensuing hypoxia may favor tumorigenesis by selecting for aggressive and metastatic clones [139]. Supplementation of platelets or their released products stimulates angiogenesis induced by breast tumor cells *in vitro* [122, 140]. In glioblastoma patients, release of VEGF by platelets was shown to contribute to vessel formation [141], although other studies indicated that platelet-induced angiogenesis was independent of VEGF but most likely relied on release of several other factors, including IL6, thrombopoietin, and angiopoietin [142, 143]. Furthermore, animal models indicate that tumor-educated platelets are more efficient at inducing angiogenesis than healthy platelets, suggesting a more efficient delivery of pro-angiogenic factors by tumor-educated platelets [144]. This appears to be supported by findings in humans, showing that levels of VEGF are increased in platelets fromprostate, breast, and colorectal cancer patients [145, 146]. It is of interest to note that vasculogenic mimicry, where tumor cells themselves rather than endothelial cells form vessels, is inhibited by platelets. While counterintuitive, this process is thought to promote metastasis [147]. Thus, platelets tightly coordinate the vascularization process in the context of cancer, and may thereby potentiate malignancies.

Thus far, platelet participation in cancer progression has been associated with vascularization, delivery of growth factors, and hematogenous dissemination [129]. In addition, platelets may directly stimulate cancer cell proliferation through upregulation of oncogenic genes, as was demonstrated for colorectal cancer cells [117]. Thus, platelets play a role in all aspects of cancer progression, something we may do well to take into account when addressing these diseases.

Taking the above into account, it is perhaps surprising to realize that fibrinolysis, the process of dissolving a blood clot, can also play a tumor-promoting role [148]. The main enzyme promoting fibrinolysis is plasmin, while the platelet-derived plasminogen activator inhibitor (PAI) is the main suppressor of this system. Elevated PAI-1 levels are associated with VTE [149], and may explain VTE in pancreatic and glioma cancer patients [150, 151]. As such, inhibition of fibrinolysis is detrimental to cancer patients. On the other hand, plasminogen itself contributes to metastasis by degradation of the extracellular matrix surrounding tumor cells. In addition, the fibrinolytic system contributes to inflammation, angiogenesis, the release of tumor growth factors, and other tumor-promoting functions [148]. Thus, coagulation and fibrinolysis play double roles in cancer, highlighting platelet performance as double-edged sword [152].

In order to target these interactions in healthy aging as well as age-related diseases, detailed knowledge regarding the molecular mechanisms involved may prove essential (Fig. 3). Many of the molecular interactions between cancer cells and platelets depend on their molecular cell surface composition. Platelets can interact with cancer cells via tissue factor (TF), selectins, integrins, and glycoproteins receptors, all of which may activate signaling pathways leading to platelet activation. Thus, platelet membrane components havemultiple functions: they contribute directly to hemostasis during thrombus formation, but can also contribute to multifactorial cancer dissemination. TF expressed by cancer cells stimulates platelet activation and initiation of the coagulation cascade. The fibrin produced by platelets subsequently interacts with integrins from cancer cells as well as platelets themselves, inducing formation of cancer cell–fibrin–platelet clusters, which may enter the circulation [153, 154]. Overexpression of TF on breast cancer cells has been reported, and appears to be linked to the release of TGFβ from activated platelets [155]. Furthermore, in ovarian cancer, platelet-induced increase in TF acts as a metastasis initiator [156].

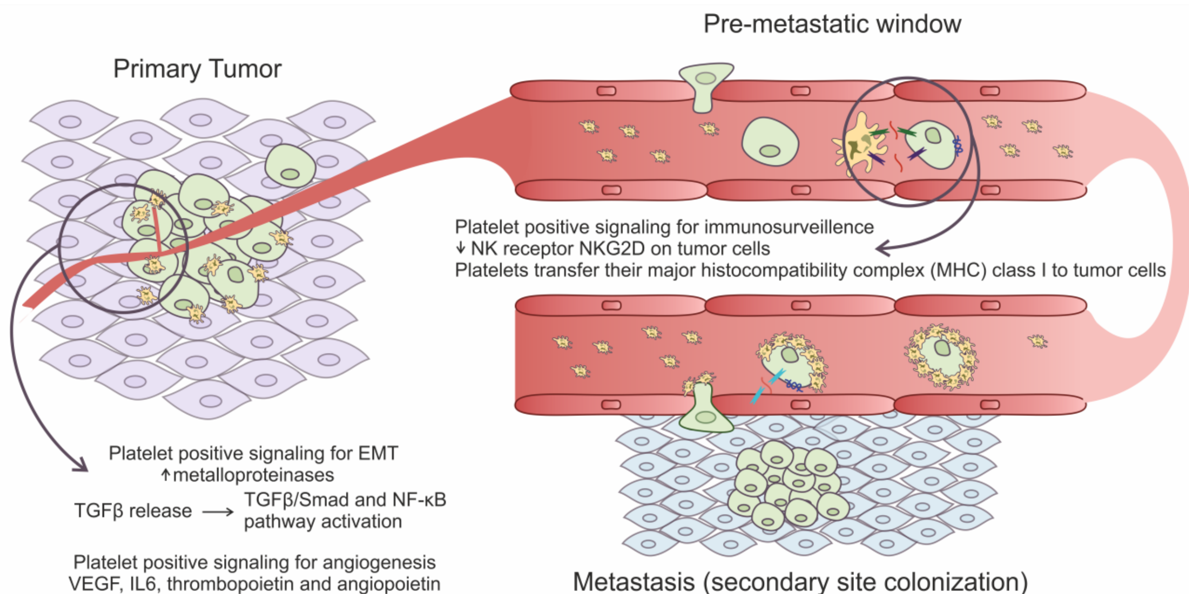

Figure 3. The crosstalk between cancer cells and platelets support metastasis, angiogenesis and tumor growth. Platelets release factors such as TGFβ and VEGF that stimulate epithelial-to-mesenchymal transition (EMT) and angiogenesis. Additionally, platelets contribute to escape from immunosurveillance by covering cancer cells and shielding them from the immune system.

The contribution of integrins to cancer cell–platelet interactions is broad and bidirectional. Platelets express integrins αIIbβ3, αvβ3, α2β1, α5β1, and α6β1, which bind preferentially fibrinogen, vitronectin, collagen, fibronectin, and laminins, respectively, all of which have been described to have adhesive proprieties [136]. Mammadova-Bach and colleagues described that integrin α6β1 from platelets directly binds ADAM9 from tumor cells, a member of the disintegrin and metalloproteinase family. As a consequence of this interaction, platelets are activated and support hematogenous dissemination of cancer cells [157]. Conversely, as already mentioned above, interaction of αvβ3 on platelets was associated with extravasation in aggressive breast cancer [138]. A last class of molecules facilitating the interaction between cancer cells and platelets are selectins, membrane-localized glycoproteins that bind carbohydrates from glycoproteins, glycolipids, and glycosaminoglycan/proteoglycans. Of the selectin family, P-selectin is expressed on platelets and endothelial cells and has already been mentioned above. Platelet dysfunction as a result of P-selectin deficiency limits colon carcinoma and metastasis progression [158, 159]. E-selectin, which is produced by endothelial cells, binds to sialyl-Lewis-x/an, otherwise known as CA19-9, a common tumor marker. The ensuing interaction promotes hematogenous dissemination of colorectal cancer cells [160].

Platelet bioactive lipids are also associated to cancer metastasis (for review, see [161]), and prostanoid synthesis inhibition as a strategy for cancer treatment has been suggested since

1972 [162]. Leukemic cell–induced platelet aggregation is associated with increased TxA2 and decreased leukotriene B4 (LTB-4) production by platelets [163]. TxA2 in turn promotesmetastasis of various tumor models by increasing TCIPA, endothelial cell activation, and recruitment of innate immune cells, all contributing to creating a pre-metastatic niche [164]. Targeting COX1/TxA2 appears efficient to reduce tumor cell metastasis [165, 166]. Conversely, prostacyclin, one of the most potent platelet inhibitors, prevents metastasis in a melanoma model [162, 164]. Endothelial function, essential to tumor cellintravasation/extravasation, is also modulated by prostacyclins. Interestingly, endothelial dysfunction, as characterized (amongst others) by decreased prostacyclin and increased P-selectin levels, was associated with more severe lung cancer stage, but also to patient age [167]. PGD2 can also decrease tumor MMP-2 expression, inhibit EMT inhibition, and reduce tumor cell proliferation [168, 169]. While these latter functions appear to be independent of platelets, some of the prostacyclin-mediated anti-tumor effects may come from inactivation of platelet hyper-reactivity in response to cancer cells, as was shown for melanoma, lung cancer, and breast cancer [165]. However, the anti-tumorigenic effects of prostacyclin and PGD2 may be specific to these prostanoids, as PGE2 did not reduce TCIPA, and COX2 and PGE2 have been associated with enhanced rather than reduced cancer metastasis [170, 171]. Thus, while COX2 inhibitors have been advocated as anti-cancer treatments in the context of inflammation (i.e., prostaglandins are important mediators of inflammation, which in turn may have carcinogenic effects), caution should be taken [172, 173]. Complicatingmatters further is the fact that platelets and their products may actually protect endothelial cells, in particular under inflamed conditions (e.g., platelet dysfunction has been suggested to contribute to endothelial dysfunction in COVID-19 patients) [174]. By strengthening the endothelial barrier, platelets may prevent intra/extravasation of tumor cells, thereby limiting tumor metastasis (reviewed in [175]).

All in all, many different molecular associations underlie platelet–cancer cell interactions and a better insight into these pathways may provide targets for treatment of both cancer and its associated VTE risk in elderly patients. With platelets playing multiple roles in cancer progression, care needs to be taken when using platelet inhibitors [175].

5. Conclusions

It is becoming increasingly clear that aging is associated with changes in platelet ontogenesis/biogenesis and function, and that this may have consequences for physiological aging. With the (relatively late) recognition of the importance of platelets, it has also become evident that age-related diseases such as cancer and cardiovascular disease are associated with platelet alterations (Fig. 4). However, to what extent this is driven by age-related changes or whether these alterations are disease-specific is perhaps unclear and age-matching in platelet investigation is imperative. Nevertheless, evidence showing that tumor cells directly modulate platelet content and functions suggests that while aging may predispose towards platelet dysfunction, specific disease states may further exacerbate platelet dysfunction to a pathological extent. Finding ways to break this pathological interaction while maintaining the balance of hemostasis may prove an important step towards healthy aging.

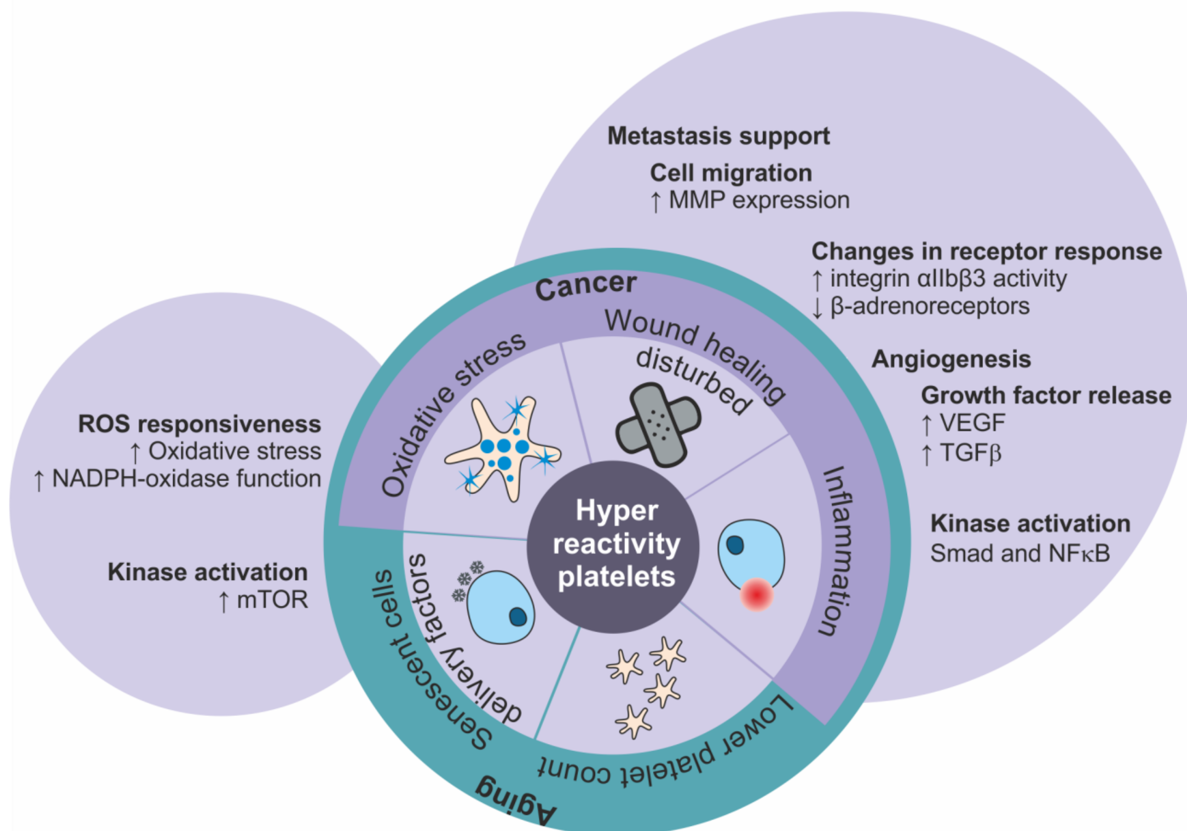

Figure 4. Aging related changes in platelet function and their association with aging related diseases (e.g. cancer). As a crosslink between aging and cancer, oxidative stress, wound healing disturbed, inflammation, lower platelet count and senescent cells delivery factors is highlighted. Platelets support metastasis by augmentation of integrin activity, increasing expression of metalloproteinases and the release of growth factors, which also augment angiogenesis. Furthermore kinase activation, including mTOR pathways, increase platelet activation. Production of reactive oxygen species enhances platelet production.

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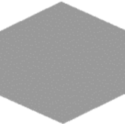

Chapter 6

Platelet-dependent signaling and Low Molecular Weight Protein Tyrosine Phosphatase expression promote aggressive phenotypic changes in gastrointestinal cancer cells

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Abstract

Over the last decades, several members of the tyrosine phosphatase family have emerged as cancer promoters. Among them, the low molecular weight protein tyrosine phosphatase (LMWPTP) has been described to be associated with colorectal cancer liver metastasis and poor prostate cancer prognosis. Of importance in the process of cancer progression and metastasis is the interaction between tumor cells and platelets, as the latter are thought to promote several tumor hallmarks. Here, we examine to what extent LMWPTP expression in tumor cells affects their interaction with platelets. We demonstrate that the gene encoding LMWPTP is overexpressed in upper gastrointestinal (GI) cancer cell as well as colorectal cancer, and subsequently employ cell line models to show that this expression may be further upregulated by platelets. We demonstrate that tumor-platelet interaction promotes GI tumor cell proliferation and show that LMWPTP expression within the tumor cell contributes to a more efficient interaction with platelets by using knock down models. These data are the first to demonstrate that phosphatases play a positive role in the tumor-promoting activities of platelets. Further elucidation the molecular mechanisms underlying these interactions may provide novel treatment strategies for gastrointestinal cancer.

Keywords

Gastric cancer, colorectal cancer, LMWPTP, ACP1, tyrosine phosphatases, gastrointestinal cancer, platelets, tumor microenvironment.

1. Introduction

Over the last few years, an important role for platelets in cancer progression has emerged. Platelets can interact with cancer cells, either directly, or via tumor-cell released factors [1]. This can have several tumor-promoting effects. Firstly, the dynamic crosstalk between platelets and cancer cells promotes cancer cell survival in the blood circulation. Coating of the cancer cell surface with platelets not only protects cancer cells from hemodynamic flux, but may also allow cancer cells to escape immunosurveillance through shielding of tumor antigens and platelet-induced shedding of immune cell ligands from the tumor cell surface [2]. Secondly, owing to their growth factor content, platelets can directly stimulate cancer growth [3,4]. For instance, co-culture of ovarian cancer cells with platelets increased tumor cell proliferation via binding of transforming growth factor beta (TGFβ) released by platelets to its receptor present on tumor cells [5]. Thirdly, a role for platelets in metastasis and cancer angiogenesis has thus far been demonstrated for breast, ovarian and prostate cancers, through platelet-tumor cell interaction and/or factors released by platelets [4,6,7]. Thus, it is clear that tumor cells may derive beneficial effects from interaction with platelets. This interaction appears to be bidirectional, as tumor cells may also cause tumor cell-induced platelet aggregation (TCIPA) [8].

The exact molecular mechanisms governing platelet-tumor cell interactions are still unclear. The receptors contributing to this interaction are relatively well described [9], as is the role of the kinases which promote oncogenic signaling in tumor cells [10]. However, while over the past decade it has become clear that tumor cell over-expression of protein tyrosine phosphatases (PTPs) contributes to tumorigenesis [11]; their roles in platelet-tumor cell interactions are scarcely investigated. We and others have previously shown that the low molecular weight protein tyrosine phosphatase (LMWPTP) is upregulated in various human cancers [12-14], and in turn, contributes to tumor cell invasiveness and chemotherapy resistance [15,16]. In addition, we showed that LMWPTP expression follows a step-wise increase through different levels of dysplasia in colorectal cancer (CRC) [14]. However, it is unclear whether this phosphatase also affects tumor progression by promoting interactions with platelets. Therefore, in the present study, we aimed to increase our understanding of the relevance of a high expression of LMWPTP in colorectal as well as upper gastrointestinal (GI) cancer. We show that LMWPTP expression in tumor cells affects their ability to interact with platelets and proliferate in the presence of platelets, while platelets themselves affect LMWPTP expression in cancer cells, creating a feedback loop. These data show for the first time that cellular levels of LMWPTP may affect tumor-platelet interactions, suggesting that

targeting such phosphatases may not only reduce primary cancer growth, but may also affect tumor survival.

2. Material and methods

2.1.Antibodies and reagents

Antibodies were purchased from Santa Cruz (Dallas, TX), Cell Signaling Technology (Danvers, MA) or SignalWay (College Park, MD). For details, see **Table 1.** Other reagents were purchased from Sigma Aldrich (Saint Louis, MO), Santa Cruz (Dallas, TX), Merck (Kenilworth, NJ), Millipore (Burlington, MA).

Table 1. Antibodies

Acp1 α/β (LMWPTP)	Santa Cruz Biotechnologies	sc-100343
β-actin	Santa Cruz Biotechnologies	sc-47778
Phospho-Akt (Ser473)	Cell Signaling	4060
Phospho-Src family (Tyr416)	Cell Signaling	2101
Phospho-FAK (Tyr925)	SignalWay Antibodies	11123-2
Phospho-p38 (Thr180/Tyr182)	Cell Signaling	4511
Phospho-Cofilin (Ser3)	SignalWay Antibodies	21164
Phospho-Paxillin (Tyr118)	Cell Signaling	2541
Phospho-S6K (Ser235/236)	Cell Signaling	21225
Anti-rabbit IRDye® 800CW	Odyssey	926–32211
Anti-mouse IRDye® 680RD	Odyssey	926-68070

2.2. Cell culture

CRC cell (HCT116) was routinely grown in McCoy 5A culture medium (Lonza, Maryland, USA). GES-1, HCT116 shScramble, HCT116 shLMWPTP, Caco-2 shScramble, Caco-2 shLMWPTP, HT29 wild type and HT29 LMWPTP KD were routinely grown in Dulbecco's Modified Eagles Medium (DMEM, Lonza, Basel, Switzerland), 23132/87, KatoIII wild type and KatoIII LMWPTP KD cells were routinely grown in Roswell Park Memorial Institute medium (RPMI1640, Lonza, Basel, Switzerland). All cell lines were supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, Missouri, USA), and 1% 100 U/mL penicillin, 100 µg/mL streptomycin (Life technologies, Bleiswijk, Netherlands) at 37°C in a 5% CO_2 humidified atmosphere. The gastric cancer cell line 23132/87 was a kind gift from Prof. Dr.

Winand Dinjens of the department of pathology of the Erasmus University Medical Center. The immortalized gastric epithelial cell line GES-1 was a kind gift from Prof. Dr. Yun Yu of the department of Gastroenterology and Hepatology of the Chinese University of Hong Kong. CRC cells were obtained from BCRJ (Rio de Janeiro, RJ, Brasil). HCT116 and Caco-2 (shScramble and shLMWPTP) cells were generated and described by Hoekstra [14]. HT29 wild type, HT29 LMWPTP KD, KatoIII wild type and KatoIII LMWPTP KD were generated through Crispr/Cas9 gene editing using forward oligo CACCGACACACAAACAGCACGGACT and reverse oligo AAACAGTCCGTGCTGTTTGTGTGTc which were annealed and ligated into pX330 vector which was subsequently electroporated into competent NEB5x bacteria. After sequencing individual colonies for verification of correct insertion of the oligo, plasmids were isolated by midiprep (Qiagen, Germany). Cell lines were plated in 6 well plates, grown to 50% confluency and transfected with pX330-ACP1 and GFP-empty vector using Fugene transfection reagent (Promega, USA) according to manufacturer's protocol. After 48h, single GFP-positive cells were sorted into 96 well plates containing 50% conditioned medium and individual cell colonies were tested for the presence of LMWPTP by Western blot analysis to confirm successful knock out of LMWPTP. Clones without successful knockout were taken as control lines, having undergone the exact same procedure as the knock out lines. All lines were routinely checked for mycoplasma.

2.3. Immunohistochemistry

Immunohistochemistry was performed as described before [14]. Briefly, 5µm formalin fixed paraffin embedded (FFPE) tissue sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen-retrieval was performed by boiling the slides in citrate buffer pH 6.0 for 15 minutes. Endogenous peroxidases were blocked by immersing the slides for 10 minutes in 3% H_2O_2 in phosphate buffered saline (PBS). Next, slides were blocked by incubation in PBS containing 10% goat serum in for 1 hour at RT. Primary antibody Acp1 α/β was added 1:100 in blocking buffer (BSA 5% in PBS) - (for primary antibody, see specification in **Table 1**) and incubated overnight at 4°C. Envision goat anti-mouse-horseradish peroxidase (Dako, Heverlee, Belgium) was used as secondary antibody. The slide scoring was based on Allred score (Allred et al, 1998, Hoekstra et al, 2015), taking the sum of intensity of staining (scored 0 to 5) and proportion of positively stained cells (scored from 0 to 3) [14,17].

2.4. Oncomine™ and GEOdata analysis

The Oncomine™ and GEOdata analysis was performed as described before [14]. Expression profiles from publicly available Oncomine™ were used to assess *ACP1* mRNA expression in colorectal, gastric and esophageal cancer, while GEO databases were searched for additional databases not already represented in Oncomine™. Information on *ACP1* expression in gastric cancer was available in 3 additional arrays. GEO Dataset Record GSE13861 based on the Illumina HumanWG-6 v3.0 expression beadchip (Illumina, Inc., California, USA) was used to compare 71 gastric cancer and 19 normal tissue samples [18]. Oncomine™ platform was used in dataset record (transcript 201630_s_at) based on the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, California, USA) to compare 3 gastric cancer and 10 normal tissue samples [19]. Oncomine™ platform was used in dataset record (transcript U25849_at) based on the GeneChip HuGeneFL array (Affymetrix, California, USA) to compare 22 gastric cancer and 8 normal tissue samples [20]. Oncomine™ searching was based on: gene: *ACP1*; analysis type: gastric cancer vs normal analysis; concept type: over-expression (oncomine concepts). GEOdata analysis searching was based on raw RNA data available with RNA discrimination, and search on *ACP1* gene on each file. For colorectal cancer GEO Dataset Record GSE24514 (transcript 201630_s_at) based on the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, California, USA) was used to compare 34 colorectal cancer and 15 normal tissue samples [21].

2.5. Platelet preparation

To obtain Platelet Rich Plasma (PRP), peripheral blood was obtained after signing informed consent (Ethical committee Project NL66029.078.18 approved by Erasmus MC medical and ethical committee, confirming that all methods were carried out in accordance with relevant guidelines and regulations and all experimental protocols were approved by this committee). Platelet isolation was performed as described before [22]. Briefly, whole blood was collected from healthy, drug-free volunteers into 3.2% sodium citrate tubes (BD, New Jersey, USA). Whole blood was centrifuged at 1500 rpm, 10 minutes, 22°C, and PRP was collected. The remaining blood was centrifuged at 2500 rpm, 10 minutes, 22°C, and Platelet-Poor Plasma (PPP) was collected. This platelet preparation was used in co-culture (2D and 3D), colony formation assay, MTT assay, adhesion assay, platelet-cancer cells interaction assays, and confocal microscopy.

2.6.Platelet-Cancer Cells Interaction Assays

Platelet isolation was performed as described before [22]. Briefly, Caco-2 cell lines (shScramble and shLMWPTP) were detached with trypsin-EDTA and washed several times with NaCl 0.9% to remove the excess of trypsin-EDTA. 500 uL of PRP was incubated with tumor cells (1.5×10^4 cells/test in NaCl 0.9%)—(protocol described before [23] with some modifications) at 37°C for 5 minutes. Subsequently, the agonist collagen (2 µg/mL) was added to the samples. An aggregation curve was recorded for 10 minutes after the addition of agonist. Light transmission changes (an indicator of aggregation) were monitored with an aggregometer (Chrono-Log Corp., Pennsylvania, USA) following the method described before [24]. Quality controls of platelets were assessed by aggregation response at the beginning and end of experiments.

2.7. Co-culture

Gastric cells (GES-1 and 23132/87) and CRC cells (Caco-2 shScramble, Caco-2 shLMWPTP, HT29 wild type, HT29 LMWPTP KD) were plated at $4x10^4$ cells/cm^2 at 24-well plate for 24 hours. After that, cells were washed with PBS, and the following conditions were applied: control (without platelets), 5% PRP (platelet stimulation) or 10% FBS (growth factor positive control). After 24 hours, microscopy analyses were made for CRC cells, as described in [22]. Briefly, microscopic images obtained by EVOS XL Core Cell Imaging System (ThermoFischer Scientific, Massachusetts, USA), using 10x magnification, focusing on cells and platelets differently. For gastric cells, platelets were removed by NaCl (0.9%) washing, and only tumor cells were collected for western blot sample preparation.

2.8. Western blot assay

Western blot was performed as described before [25]. In short, cells were plated at a density of $4x10^4$ cells/cm^2 for GES-1,23132/87, KatoIII wild type, KatoIII LMWPTP KD HT29 wild type and HT29 LMWPTP KD. After 24 hours, cells were washed with NaCl 0.9% and lysed in 2× concentrated Laemmli buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) and samples were boiled for 10 minutes. Cell extracts were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membranes (Merck chemicals BV, Darmstadt, Germany). Membranes were blocked in 50% odyssey blocking buffer (LI-COR Biosciences, Nebraska, USA) in TBS and incubated overnight at 4°C with a primary antibody, dilution 1:1000. After washing in TBS-T (TBS with 0.5% Tween 20), membranes

were incubated with IRDye antibodies (LI-COR Biosciences, Lincoln, USA) for 1 hour. Detection was performed using Odyssey reader and analyzed using the manufacturer's software. For antibodies used, see **Table 1**.

2.9. Colony formation assay

Cells (GC and CRC) were plated at 2.0×10^3 cells/well in 6-well plates. After 24h, 250µL of culture medium or PRP was added to the appropriate wells. After 10 days, the incubated medium was removed, and the cells were stained using crystal violet (0.5% water:methanol) for 40 minutes. After, the crystal violet was discarded and the wells were washed 3 times with tap water. Microscopic images were acquired using a Zoom Stereomicroscope (scale bar: 2000µm) - (2x, Nikon, Japan) and the colonies were counted using ImageJ software (NIH, USA).

2.10. MTT assay

Cells were plated at 4.0×10^4 cells/cm^2 (GES-1, KatoIII wild type and KatoIII LMWPTP KD) and 5.0×10^4 cells/cm^2 (23132/87) at 96-well plate for 24 hours. After that, culture medium (control) or PRP (5%) were added at each corresponding well for 24 hours. After, the supernatant with platelets was removed and MTT (0.5 mg/mL) was added, and incubated for 3 hours. Next, cells were resuspended in 100 µL of Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Montana, USA) and optical density (OD) was measured using a spectrophotometer at 595 nm (BioRad, California, USA).

2.11. Adhesion assay

Cells in serum-free medium were allowed to adhere to plates for 30 and 120 minutes in the presence and absence of platelets. The attached cells were stained with DAPI, and the attached cells were counted from microscopic images obtained by EVOS XL Core Cell Imaging System (Thermo Fischer Scientific, Massachusetts, USA).

2.12. Confocal Microscopy

HCT116 were cultured under density 4.5×10^4 cells/well – 500 µL on glass coverslips for 24 hours. Subsequently, cells were incubated with platelets (5%) in medium without FBS, and medium with FBS (control), and cultured for another 24 hours. HT29 cells were grown in 3D cultured based on Souza [26]. Cells were seeded in 6-wells microplates and grown in a 2D model for 24 hours after which they were statically incubated for 24 hours with 60µl of

NanoShuttle (Nano3D Biosciences, Texas, USA) at a proportion of 2µL/$1x10^4$ cells. After 24 hours of magnetization, cells were washed twice with PBS and enzymatically detached with 350 µL of trypsin. Detached cells were suspended with 750 µL of McCoy 5A medium and seeded at $1x10^4$ cells/well – 100 µL on a 96-wells cell repellent microplate. Platelets were mixed with cancer cells, seeded on 96-microplate and placed atop a magnetic drive of 96 neodymium magnets (Nano3D Biosciences, Greiner Bio-One, Brazil) to induce spheroid formation. After 24 hours, the magnetic drive was removed. Images were taken by Luma Scope microscope (Etaluma Inc, California, USA) in a 10X magnification after 120 hours of culturing. Next, the protocol followed as described here [27] with some modifications. Cells were fixed with 4% PFA for 10 minutes, washed with PBS, permeabilized with Triton X-100 (0.1%) and blocked with 3% BSA for 1 hour. Cells were washed with PBS and incubated overnight at 4°C in a humidified chamber with the following antibodies (For primary antibodies, see Table 1). Coverslips were stained with Alexa-Fluor® - Invitrogen (Thermo Fisher Scientific, Massachusetts, USA) 488 Mouse secondary antibody at 1:500 dilution for 1 hour. Coverslips were subjected to a standard staining with DAPI-Invitrogen (Thermo Fisher Scientific, Massachusetts, USA) at 1:1000 dilution and it were mounted onto glass slides. Images were acquired on a LEICA TCS SP5 II confocal microscope (Leica, Wetzlar, Germany) at Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP) - using 100X objectives (scale bar: 25µm). Images format 1024x1024. Images were analyzed using ImageJ software (NIH, USA).

2.13. Statistical analysis

The data is represented by means ± SEM. Statistical analysis was performed using t-student (paired, 95% confidence intervals, two tailed) for Figure 1(A-C,E), 2(E), 3(B,C,E,F,G,I,J), 4(A,C,D,F,H,I,K,M), S2(D) and One-way ANOVA with post-test corrected for multiple testing for Figure 3(K) and 5(C,D,G,H,K) and * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$ using GraphPad (version 5.0, GraphPad Inc, California, USA). All experiments were performed a minimum of three independent times.

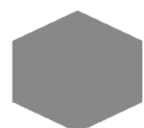
3. Results

3.1. High LMWPTP protein expression in gastric cancer cells affects oncogenic hallmarks

Having previously established a role for LMWPTP in CRC, we first investigated whether these findings also extend to upper GI cancers. Oncomine™ was used to investigate *ACP1* mRNA expression/copy number gain in gastric cancer tissues. GEO database was searched for additional expression databases not already represented in Oncomine™. A comparison of the 15 datasets present in Oncomine™ for gastric cancer showed a borderline significant increase in *ACP1* expression in gastric tumors as compared to normal gastric mucosa (p=0.055, **Supplementary Figure S1A**), which was strengthened by a significant upregulation of *ACP1* mRNA expression in gastric tumors observed in three available additional GEO datasets [18-21] (**Figure 1A-C**). As a proof of concept to demonstrate that enhanced *ACP1* levels also translate to enhanced protein expression, we analyzed LMWPTP by immunohistochemistry and showed a significant overexpression of LMWPTP in a small sample set of gastric cancer tissues as compared to normal gastric epithelium (**Figure 1D, E**). Investigation of three esophageal squamous cell carcinoma (ESCC) studies present in Oncomine™ indicated an enhanced expression in tumor tissue as compared to controls (P=0.001), while a non-significant trend towards *ACP1* upregulation was seen in in esophageal adenocarcinoma (EAC) (P=0.174). No additional GEO datasets were discovered (**Supplementary Figure S2A, B**). As a proof of concept, we also show significantly enhanced expression of LMWPTP by immunohistochemistry in esophageal adenocarcinoma (n=8) as compared to normal squamous epithelium (n=7) (**Supplementary Figure S2C, D)**. These data indicate that upregulation of *ACP1* expression extends to upper GI cancers as well as CRC.

Having established an overexpression of LMWPTP in upper GI cancers, we employed gastric cell lines as a model system to investigate the molecular contribution of this tyrosine phosphatase to carcinogenesis (**Figure 2A**). First we compared LMWPTP expression in the gastric cancer cell line 23132/87 and the non-transformed cell line GES-1, and showed that, corresponding to the immunohistochemistry data, LMWPTP expression is enhanced in 23132/87 cells compared to non-transformed cells (**Figure 2B**). Next, we investigated the phosphorylation pattern of several kinases related to cell proliferation and cytoskeletal remodeling, such Src, FAK and Cofilin, and observed an enhanced activation of these kinases in gastric cancer cells (**Figure 2C-E**). To confirm whether a higher expression of LMWPTP directly contributes to activation of proliferative signaling pathways, we performed genetic

knockout of LMWPTP in the gastric cancer cell line KatoIII. A reduced phosphorylation of cytoskeletal proteins **(Figure 1D)**, as well as decreased proliferation **(Figure 1E)** was observed upon knockout of LMWPTP in these gastric cancer cells. Thus, these data imply that LMWPTP overexpression leads to stimulation of oncogenic signaling in gastric cancer cells.



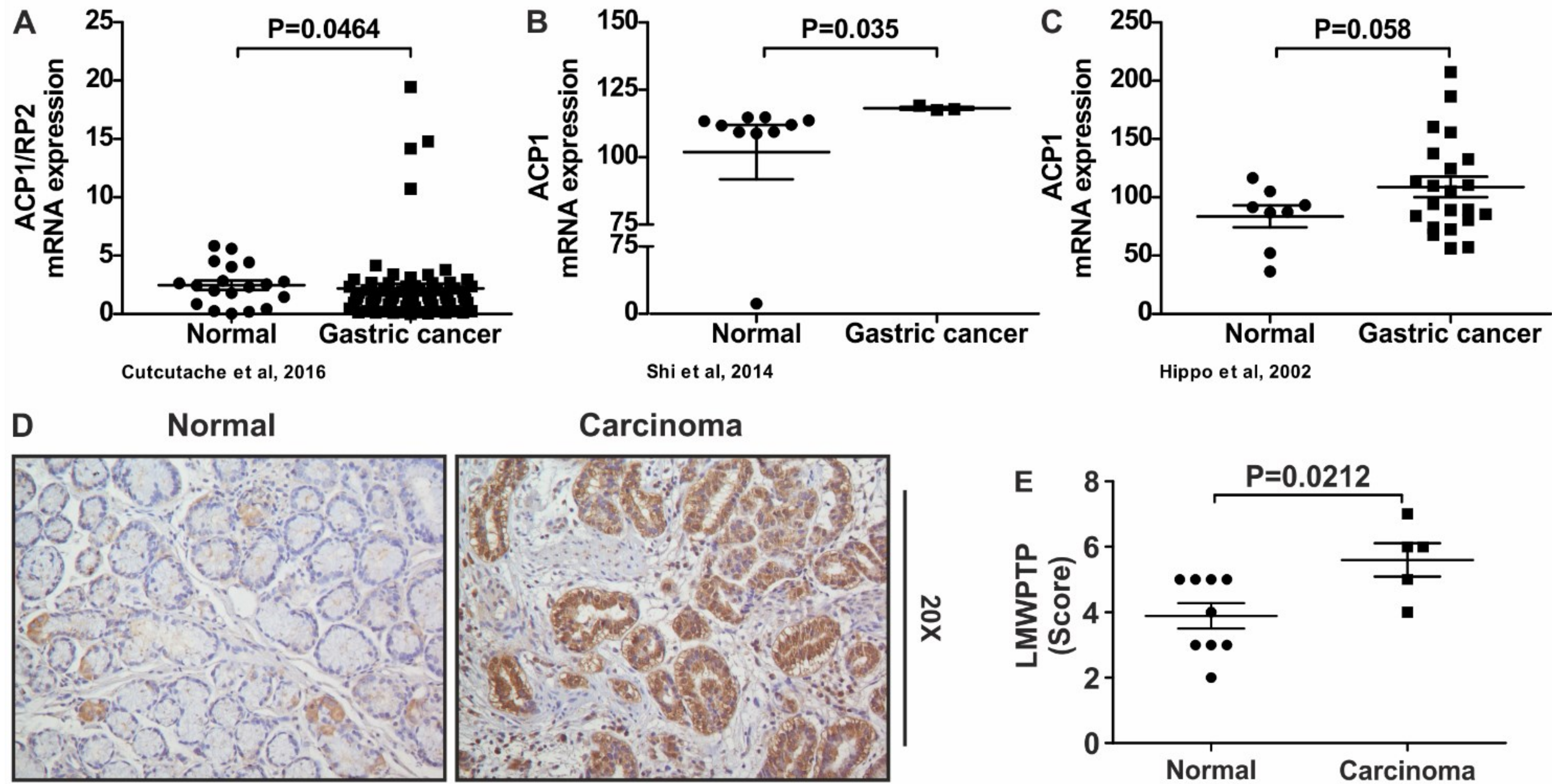


Figure 1. ***ACP1*****/LMWPTP expression is increased in gastric cancer**. **(A-C)** Oncomine and GEOdata analysis from three different studies A [18], B[19], C[20] studies. (**D, E**) Normal gastric (n=9) was compared to gastric carcinoma (n=5) and representative samples (20X) as well as quantifications are shown. The intensity and proportion of the staining were scored and control and carcinoma groups were compared. Dots indicate individual patients or experiments, and mean +/- SEM is shown.

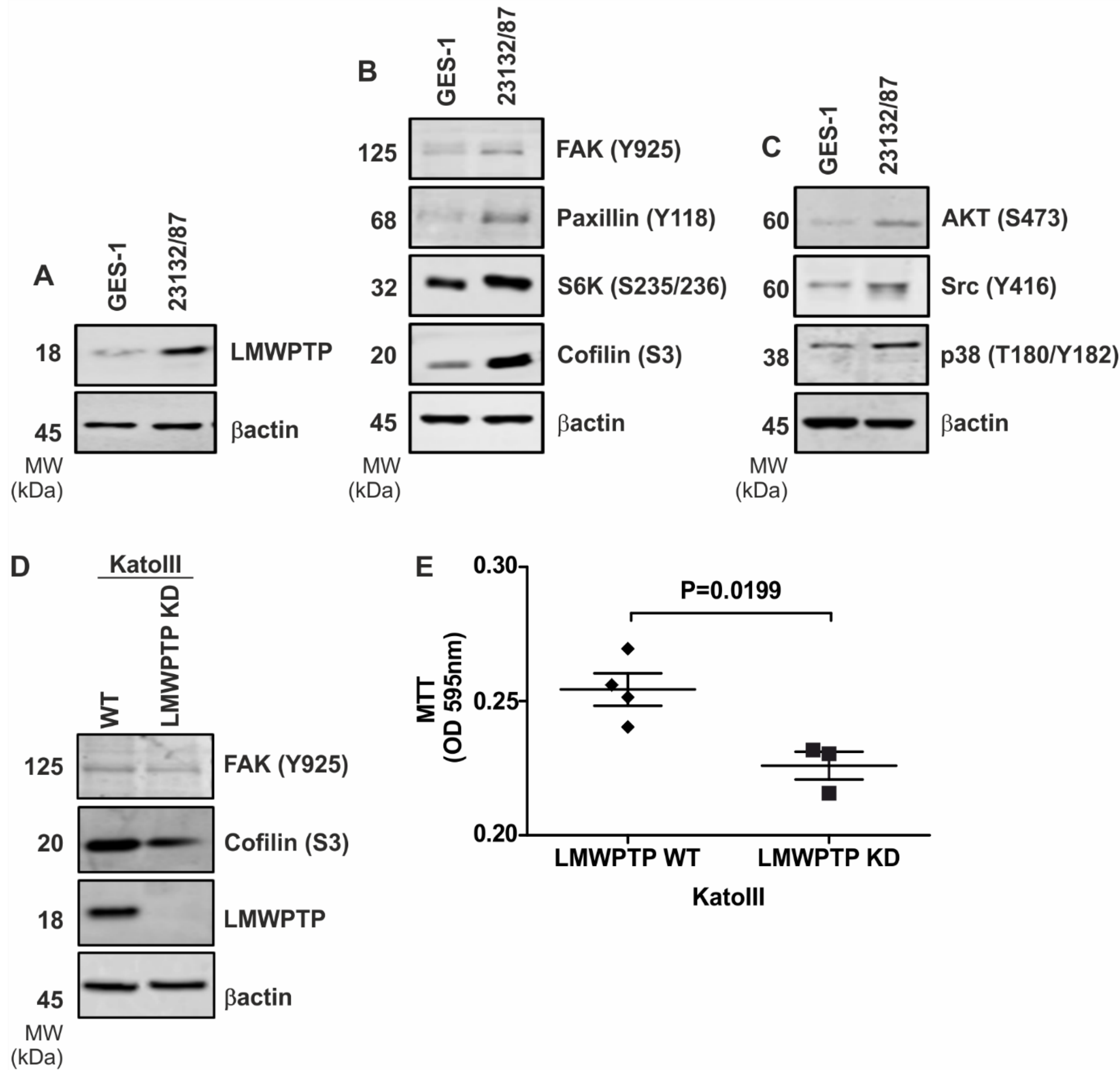


Figure 2. LMWPTP contributes to proliferative and cytoskeletal signaling in gastric cancer cells. (A) LMWPTP expression in the non-transformed GES-1 cell line and gastric cancer cell line 23132/87 as determined by Western blot analysis. β-actin was used as loading control. **(B-C)** Phosphorylation of signaling molecules related to cytoskeletal remodeling (B) and survival (C) were investigated in normal and cancer gastric cell lines. β-actin served as loading control. (**D**) Phosphorylation status of kinases comparing KatoIII wild type (WT) and LMWPTP knockout (KD) cells. (**E**) Proliferation rate using MTT assay comparing KatoIII WT and LMWPTP KD. Dots indicate individual experiments, and mean ± SEM is shown.

3.2. LMWPTP protein expression in gastric cancer cells affects tumor-platelet interactions

Next, we investigated whether LMWPTP-mediated signaling affects gastric cell interactions with platelets. To better understand the molecular mechanisms related to stimulation of cell survival and LMWPTP expression, we first analyzed the protein expression and/or activation in gastric cells co-cultured with platelets. As expected, survival-associated kinases were not activated in GES-1 cells, while in GC cells cultured with platelets, Src and p38 were activated (**Figure 3A**). Interestingly, co-culturing gastric cancer cells with platelets further increased their LMWPTP protein expression, something which was not observed in non-transformed cells (**Figure 3A-C**). When detached gastric cells were incubated with platelets prior to plating, attachment of 23132/87 cells, but not GES-1 cells was reduced (**Figure 3D, E**), suggesting that direct interaction between gastric cells and platelets is more pronounced in tumor cells. Next, we assessed gastric cell proliferation in the presence or absence of platelets by MTT assays. While normal gastric cells (GES-1) were not affected by co-culture with platelets, proliferation of GC cells (23132/87) was significantly increased in the presence of platelets (**Figure 3F,G**). To validate these findings, we further investigated proliferation by colony formation assay. While the size of cancer cell colonies was significantly increased upon co-culture, non-transformed cells showed a decrease rather than increase in size of colonies (**Figure 3H-J**). To investigate whether LMWPTP might directly drive platelet-induced tumor cell proliferation, we knocked out LMWPTP in the gastric cell line KatoIII and investigated their colony formation potential. KatoIII cells form bigger colonies when expressing LMWPTP (**Figure 3K**). More importantly, however, LMWPTP-expressing KatoIII cells form bigger colonies in the presence of platelets as compared to LMWPTP KD cells, and the increase in colony size induced by the presence of platelets was no longer present upon knockdown of LMWPTP (**Figure 3K-L**). Together, these data suggest that LMWPTP in gastric cancer cells increases their interaction with platelets, which is associated with cancer cell proliferation signaling.

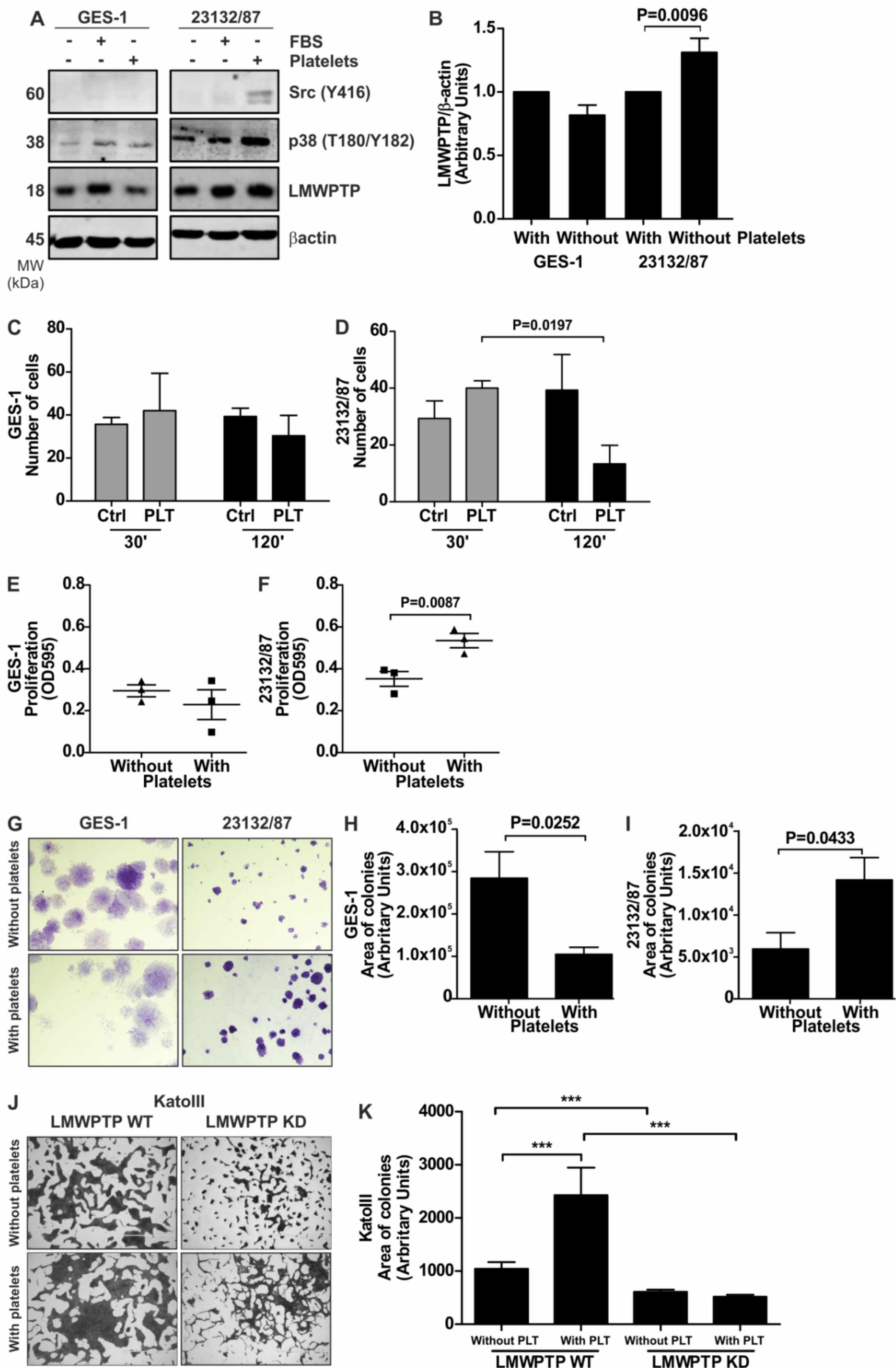


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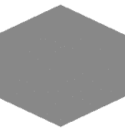
Figure 3. Platelets stimulate LMWPTP expression and proliferation in gastric cancer cells. (A-B) Kinase activation (Src and p38), and LMWPTP expression in normal and cancer gastric cell line in the presence or absence of platelets. Densitometry analysis for LMWPTP expression is represented in (B). β-actin was used as loading control. **(D-E)** Adhesion assay of GES-1 (D) and 23132/87 (E) cells in absence and presence of platelets. **(F-G)** MTT assay of GES-1 (F) and 23132/87 (G) cells in the absence or presence of platelets. **(H-J)** Colony formation of GES-1 and 23132/87 cells in the absence or presence of platelets. The size of GES-1 (I) and 23132/87 (J) colonies are presented. **(F)** Representative microscopic images of colony formation. **(K-L)** Colony formation of KatoIII wild type and KatoIII LMWPTP KD cells in the absence or presence of platelets. The size (L) of KatoIII wild type and KatoIII LMWPTP KD colonies are presented. Dots indicate individual experiments, and mean ± SEM is shown.

3.3. LMWPTP is overexpressed in colorectal cancer and supports cell migration and proliferation in presence of platelets

Our previous data suggested that LMWPTP is increased in colorectal cancer [14]. Here, we sought to validate and these findings in a new cohort of cases. A comparison of the 33 datasets present in Oncomine for colorectal cancer, showed an upregulation of *ACP1* in cancerous tissues as compared to normal colonic mucosa (p=0.001) (**Figure S1B**), which was confirmed by an additional GEO dataset comparing 34 colorectal cancer and 15 normal tissue samples [21] (**Figure 4A**). Employing immunohistochemistry, we further validated a significantly increased protein expression of LMWPTP in CRC tissues compared to their normal counterpart (**Figure 4B**). We subsequently investigated to what extent LMWPTP expression is modulated by platelets in colorectal cancer cell line models. As for GC cells, we observed that co-culture of CRC cells with platelets causes a distinct growth pattern (**Figure 4D, F**), which is accompanied by a significantly enhanced protein expression level of LMWPTP in HCT116 cells (**Figure 4D-E, H-I**) as well as HT29 cell models (**Figure 4F-G, J-K**), grown under 2D or 3D conditions, respectively.

Next, we aimed to determine whether LMWPTP also plays a role in platelet-mediated oncogenic potential of colorectal cancer cells. To this end, we employed shRNA to reduce LMWPTP expression in HCT116 cells (**Figure 5A**). Partial knock down of LMWPTP cells (18%) resulted in a ~30% reduction of the number of colonies (**Figure 5B,D**), confirming a role for LMWPTP in inherent tumorigenesis. The number of colonies was enhanced in the presence of platelets, but this was no longer the case when LMWPTP expression was reduced (**Figure 5D**). The size of the colonies, while not affected in control cells, was reduced in LMWPTP knock-down cells in the presence of platelets (**Figure 5D**), implying that LMWPTP is required to maintain colony size in the presence of platelets. To confirm the role of LMWPTP in platelet-induced cell growth in a separate cell model we investigated HT29

cell with genetic knock out of LMWPTP. Upon co-culture with platelets, a significantly higher colony size was seen upon in the presence of LMWPTP **(Figure 5A-B)**, while colony number *per se* was not affected. As an increase in tumor cell proliferation may be effected by growth factors released by platelets, we next sought to investigate to what extent LMWPTP contributes to direct interaction between platelets and tumor cells. To this end, we investigated platelet aggregation, which may be promoted by tumor cells, but requires physical interaction between these two cells, as tumor cell-conditioned medium does not elicit the same effect [25]. We showed that knock down of LMWPTP in a third CRC cell model (Caco2, **Figure 5I**) significantly reduces platelet aggregation in the presence of tumor cells as determined by microscopy and aggregometry (**Figure 5J,K**), which was confirmed in the HT29 knock-out model by microscopy (**Figure 5L**). Together, these data demonstrate that in CRC as well as upper GI cancer, LMWPTP upregulated in cancer cells may be further enhanced by platelet interaction with tumor cells, and that LMWPTP contributes significantly to platelet-tumor interaction and tumor proliferation.



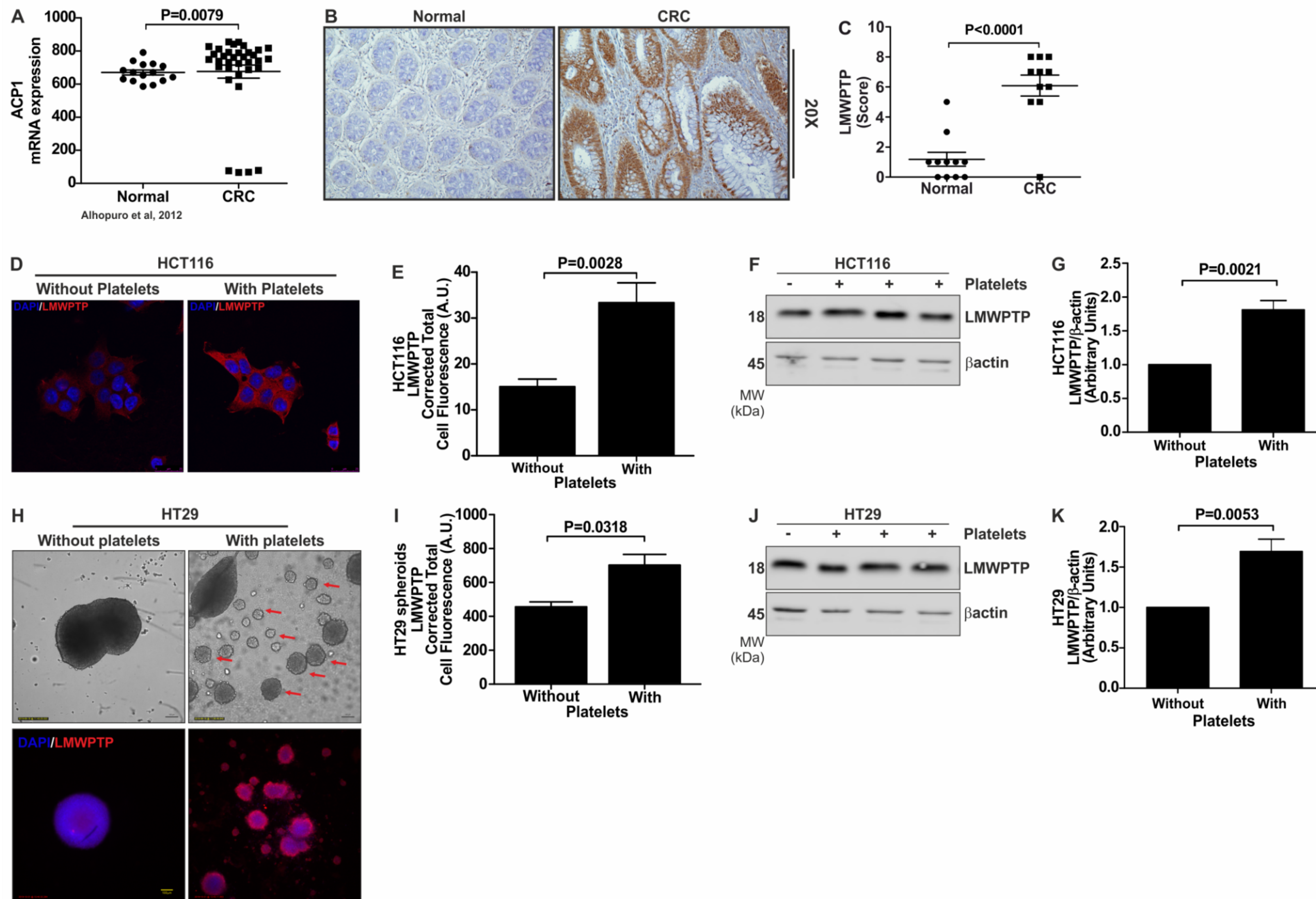


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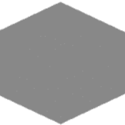
Figure 4. Platelets support CRC cell migration and proliferation in the context of LMWPTP. (A) GEOdata analysis of [21] indicates reduced *ACP1* mRNA expression in colorectal cancer tissues. **(B-C)** LMWPTP expression was determined by immunohistochemistry in CRC tumors. Normal colorectal specimens (n=11) and adenocarcinoma (n=11) were compared. Representative samples (20X) and quantifications are shown. The intensity and proportion of the staining were scored and analysis was performed comparing control and carcinoma groups. **(D-E)** Fluorescence microscopy comparing LMWPTP intensity (quantification in E) in HCT116 cells after co-culture with platelets. Nuclei are stained in blue (DAPI) and LMWPTP in red at 100X magnification. (F-G) LMWPTP expression in HCT116 as determined by Western blot analysis (densitometry analysis – G). β-actin was used as loading control. (H-I) 3D growth of HT29 cells in the presence of platelets leads to altered growth pattern and increased LMWPTP expression (quantification in I) as determined by fluorescence microscopy staining. Nuclei are stained in blue (DAPI) and LMWPTP in red at 10X magnification. (**J-K**) LMWPTP expression in HT29 as determined by Western blot analysis (densitometry analysis – K). β-actin was used as loading control. Legend: dots indicate individual experiments, and mean +/- SEM is shown.



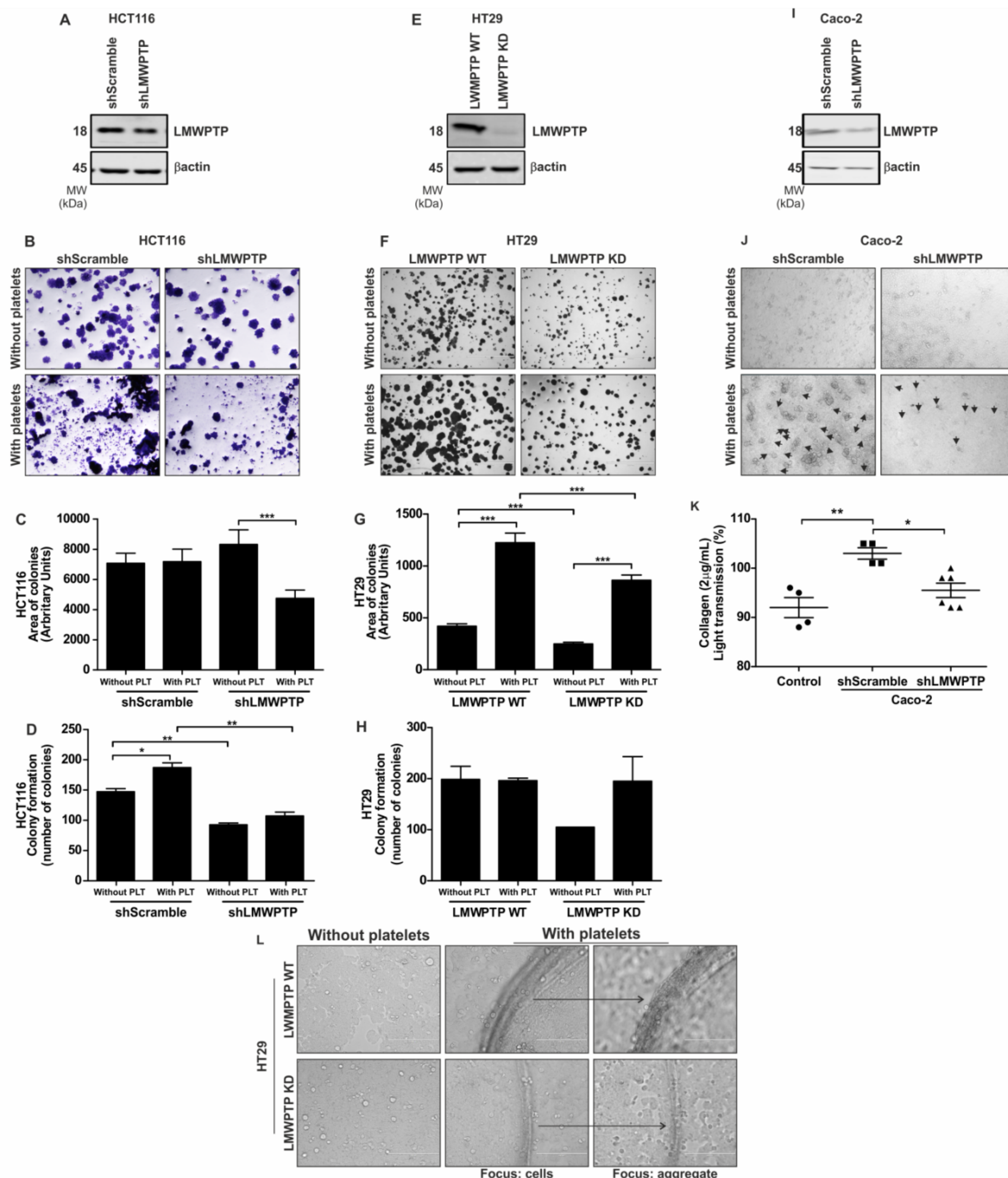


Figure 5. LMWPTP expression in CRC cells affects their interaction with platelets and promoted proliferation. (A) LMWPTP expression in HCT116 cells treated with either shScramble or shLMWPTP indicates an 18% reduction of LMWPTP expression in shLMWPTP cells. β-actin served as loading control. **(B-D)** Colony formation assay of shScramble or shLMWPTP HCT116 cells in the absence or presence of platelets indicated a reduced number of colonies in the absence of LMWPTP as well as a loss in platelet-induced increase thereof. Quantification of the size (C) and number (D) of colonies in shScramble and shLMWPTP cultured in the absence or presence of platelets are shown. (**E**) LMWPTP knock-out in HT29 cells. β-actin served as loading control. **(F-H)** Colony formation assay of wild type (WT) and LMWPTP knockout (KD) HT29 cells in the absence or presence of platelets indicated a reduced platelet-induced colony size increase in the absence of LMWPTP. Quantification of the size (G) and number (H) of colonies in WT and LMWPTP KD cells cultured in the absence or presence of platelets are shown. **(I)** LMWPTP expression in Caco-2

cells treated with either shScramble or shLMWPTP indicates a 44% reduction of LMWPTP expression in shLMWPTP cells. β-actin served as loading control. **(J)** Light microscopy images of Caco-2 cells showing reduced platelet aggregates upon shLMWPTP. **(K)** Aggregometry analysis of platelet aggregation shows that cancer cell-induced increase in platelet aggregation is reduced upon shLMWPTP in Caco-2 cells. **(L)** Light microscopy images of HT29 cells showing reduced platelet aggregates upon KD of LMWPTP. Representative pictures are shown at 10x magnification and the aggregate is highlighted by black arrow.

4. Discussion

Despite improvements in alimentary tract cancer detection and treatment, prognosis of these cancers remains abysmal with a 5-year survival rate of around 30% and 12% for GC and CRC, respectively [28,29]. While over the last decades, our knowledge regarding kinase signaling in cancer cells has expanded, the role of tyrosine phosphatase signaling in cancer remains poorly understood [11]. These enzymes are commonly regarded to be tumor suppressors, as their primary function of de-phosphorylating proteins and lipids is generally thought to inactivate signaling pathways. However, we have previously shown that dephosphorylation of the inhibitory site of the kinase Src by the phosphatase PTP1B can contribute to activation of oncogenic signaling [30]. Scr is also a target for LMWPTP, and activation of Src signaling upon activation of LMWPTP has been demonstrated in leukemia cells [15]. Thus removal inhibitory phosphorylation patterns by phosphatases may account for the enhanced phosphorylation of downstream oncogenic targets, as seen in the current study as well as others upon knock down of LMWPTP [11,24,31-34].

With this new knowledge, protein tyrosine phosphatases are now emerging as potential cancer biomarkers and targets for treatment [16]. Here, we show that LMWPTP is overexpressed in gastric and esophageal cancer, as well as CRC, suggesting that upregulation of phosphatase expression is a common feature amongst intestinal cancers and opening up the tantalizing possibility of a common target for treatment of these diseases. Using several different cell models, we demonstrate that platelets significantly enhance tumor cell proliferation and that this process is at least partially dependent on LMWPTP expression in tumor cells. Interestingly, co-culture of tumor cells with platelets further increases their expression of LWMPTP in this study. With LMWPTP directly conferring several tumorigenic properties [11,14], it is tempting to speculate that upon extravasation of tumor cells to the blood stream and their subsequent interaction with platelets, a further platelet-mediated upregulation of LWMPTP in part mediates the platelet-induced proliferative advantage. Indeed, our data show that tumor cell-expressed LMWPTP directly affects association of tumor cells with platelets,

which is in line with data showing that Integrin β3 on the surface of platelets can promote phosphatidylinositol 3-OH kinase (PI3K) signaling and proliferation of hemangioendothelioma cells [35]. However, platelets also produce substantial amounts of growth factors, and it is conceivable that these also contribute to LMWPTP expression and proliferation of tumor cells *in situ*. Indeed, supernatant obtained from stimulated platelets was able to stimulate breast cancer cells by activating the pro-survival kinases phosphatidylinositol 3-OH kinase (PI3K) and protein kinase C [10].

In summary, we demonstrate that LMWPTP expression in intestinal cancers takes part in the crosstalk between platelets and cancer cells, with platelets significantly enhancing GI cancer cell proliferation. Future research will have to extend these findings to additional cell line and pre-clinical models, to investigate to what extent LMWPTP may affect other platelet-mediated oncogenic properties and provide a target for treatment of GI cancers.

Author Contributions: Conceptualization, G.M.F. and C.V.F.-H.; Methodology, A.V.S.F., P.F.S.O., S.S.A., M.P.M. de M., C.V.F.-H., and G.M.F.; Formal Analysis, A.V.S.F., C.V.F.H. and G.M.F.; Investigation, A.V.S.F., M.M., P.F.S.O, B.Y.; Data Curation, A.V.S.F., C.V.F.-H. and G.M.F.; Writing—Original Draft Preparation, A.V.S.F.; Writing—Review & Editing, M.P.M. de M., M.M., P.F.S.O., S.S.A., M.P.M.M., M.P.P., C.V.F.-H., G.M.F.; Supervision, M.P.P., C.V.F.-H., M.P.M. de M., G.M.F.; Project Administration, A.V.S.F., M.P.P., C.V.F.-H., G.M.F.; Funding Acquisition, A.V.S.F., G.M.F. and C.V.F.-H.; All authors read and approved the final manuscript.

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Conflicts of Interest: the authors declare no conflicts of interests.

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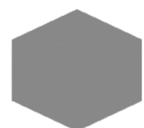


Supplemental Figures:

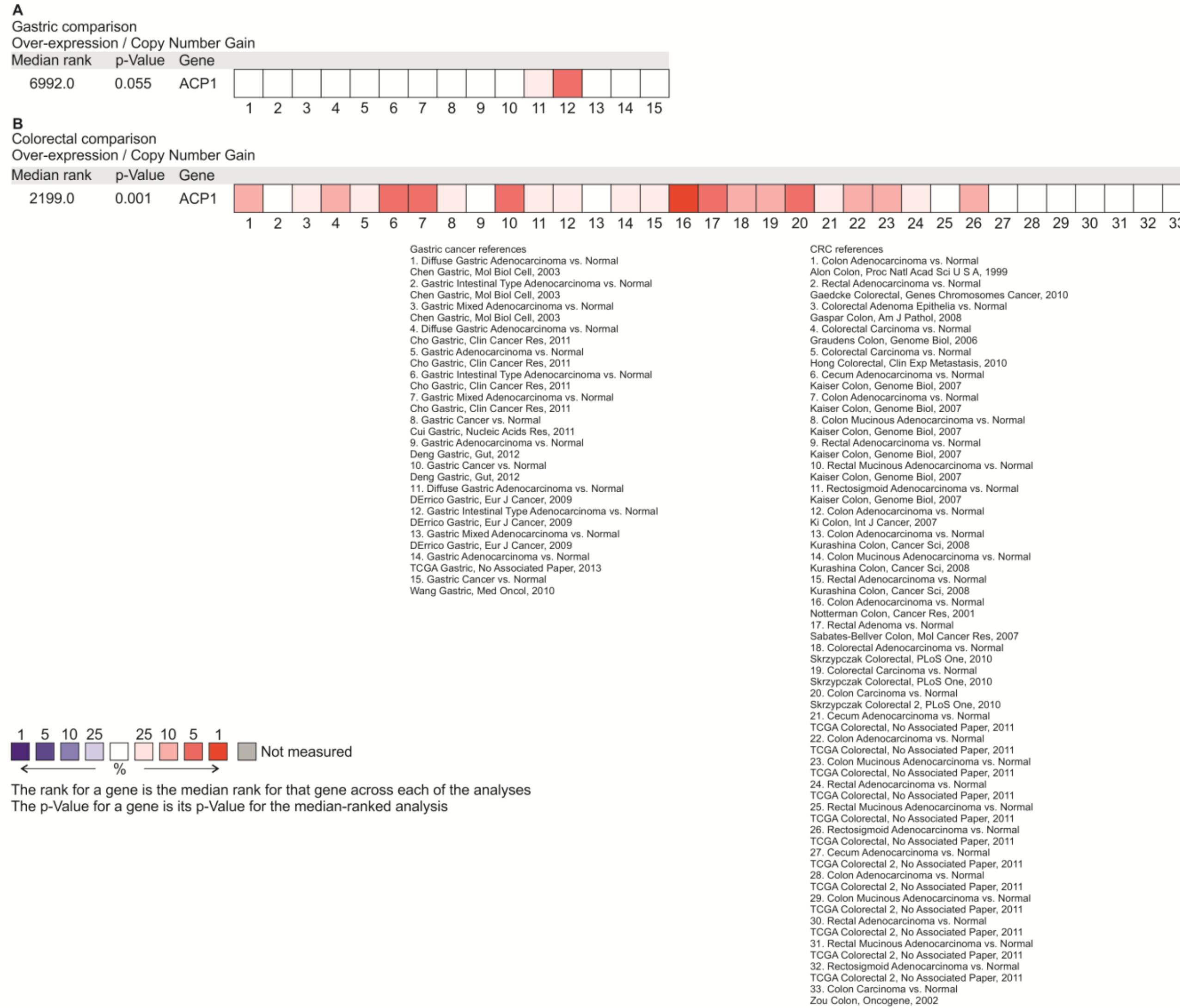


Figure S1. Oncomine analysis for gastric cancer and CRC. (A) Gastric cancer tissues showed a borderline significant increase in *ACP1* expression as compared to normal gastric mucosa in 15 datasets available. (B) A comparison of datasets present in Oncomine for colorectal cancer, an update of our previous investigation of public repositories [14], confirmed an upregulation of ACP1 in cancerous tissues as compared to normal colonic mucosa across 33 studies.

A Esophageal Squamous Cell Carcinoma comparison
Over-expression / Copy Number Gain

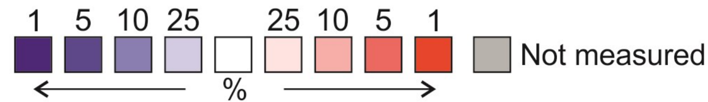
Median rank	p-Value	Gene
1922.0	0.001	ACP1

1 2 3

B Esophageal Adenocarcinoma comparison
Over-expression / Copy Number Gain

Median rank	p-Value	Gene
4206.0	0.174	ACP1

1 2 3



The rank for a gene is the median rank for that gene across each of the analyses
The p-Value for a gene is its p-Value for the median-ranked analysis

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Esophageal Adenocarcinoma references
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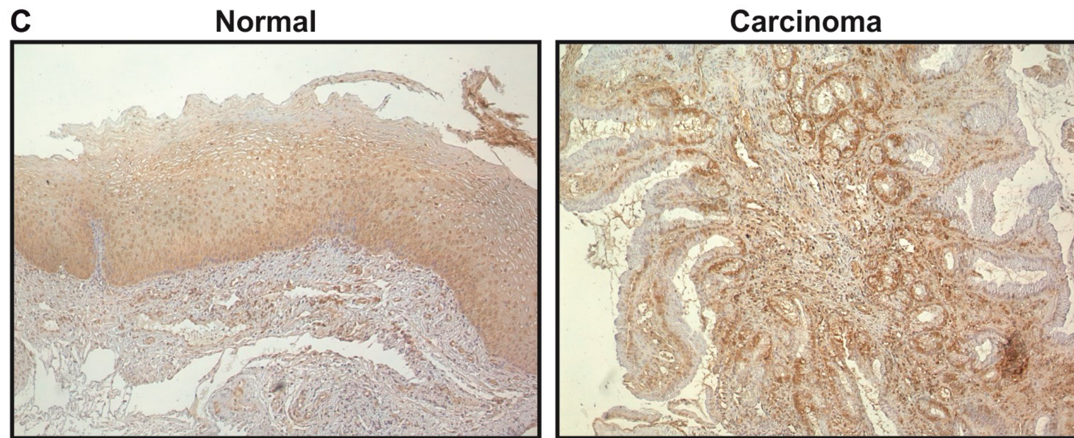

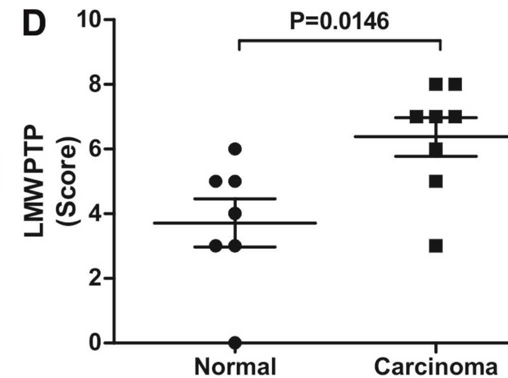

Figure S2. LMWPTP expression is increased in esophageal cancer. (A-B) Oncomine analysis from Esophageal Squamous Cell Carcinoma (A) and Esophageal Adenocarcinoma (B). (C-D) Normal esophageal (n=7) was compared to esophageal carcinoma (n=8) and representative samples (10X) as well as quantifications are shown. The intensity and proportion of the staining were scored and control and carcinoma groups were compared (D). Legend: dots indicate individual experiments, and mean +/- SEM is shown.

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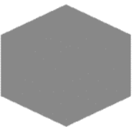
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Chapter 7

The role of phospho-tyrosine signaling in platelet biology and hemostasis

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Abstract

Platelets are small enucleated cell fragments specialized in the control of hemostasis, but also playing a role in angiogenesis, inflammation and immunity. This plasticity demands a broad range of physiological processes. Platelet functions are mediated through a variety of receptors, the concerted action of which must be tightly regulated, in order to allow specific and timely responses to different stimuli. Protein phosphorylation is one of the main key regulatory mechanisms by which extracellular signals are conveyed. Despite the importance of platelets in health and disease, the molecular pathways underlying the activation of these cells are still under investigation. Here, we review current literature on signaling platelet biology and in particular emphasize the newly emerging role of phosphatases in these processes.

Keywords

Platelet function; platelet biology; kinases; phosphatases; platelet signaling.

1. Introduction

Platelets are derived from megakaryocytes in a complex differentiation process called thrombopoiese, in which shedding of cytoplasmic extensions generates small cellular fragments lacking genomic DNA [1]. The human body has in the order of 10^{12} circulating platelets. Their average life span is 7-10 days, after which they are removed from the circulation and cleared by splenic or hepatic macrophages and hepatocytes. With a size of around 2-3µm diameter, a discoid shape, and a minimal displacement volume coupled with a highly active cytoskeleton, platelets are well suited to vascular circulation. As the first responders to tissue damage, platelets play an important role in hemostasis. But their functions extend beyond this. As a supplier of angiogenic and non-angiogenic factors, they induce blood vessel formation. They play a pivotal role in wound healing and regenerative processes contributing to tissue repair, amongst others by transporting soluble factors that contribute to the cell recruitment and phenotype transition required for tissue regeneration. In addition, a novel role for platelets in immunity is now emerging. Having retained several of the functions associated with their myeloid origin, including their granulocytic characteristics, platelets have the ability to internalize environmental factors such pathogens through phagocytosis [2]. Binding of bacteria via toll like receptors may facilitate the recruitment and subsequent uptake of bacteria by granulocytes [3]. Platelets also contribute to adaptive immunity, by activating dendritic cells and enhancing their antigen presentation in the context of major histocompatibility complex (MHC)II molecules to T or B cells [4], while MHCI molecules taken up from serum can be used to present antigens to T-cells [5]. Thus, platelets are versatile cells which play an important role in human physiology. While a protagonist in hemostasis, excessive platelet activation is also a major cause of morbidity and mortality in western societies. It is therefore not surprising that platelets have become an extensively investigated biological cell type. Nevertheless, precisely how platelets become activated under physiological and pathophysiological conditions are still under investigation.

Similar to the megakaryocytes from which they hail, platelets contain proteins and RNA transcripts, with up to 3,000–6,000 mRNA transcripts thus far identified. Thus, despite the fact that platelets do not contain a nucleus and are hence incapable of newly transcribing mRNA molecules, they do retain the capacity to synthetize their own functional proteins from the mRNAs inherited from their parental cell. Alternative splicing and signal-dependent translation of mRNAs may give rise to new proteins. Furthermore, platelets contain proteasomes and ubiquitin complexes, which together with the presence of platelet-derived extracellular ERp57, a thiol isomerase enzyme, contributes to protein modifications.

However, one of the most important post-translational protein modifications, allowing platelets to fulfil their highly dynamic and diverse functions [6,7], comes from protein phosphorylation. Reversible phosphorylation of proteins, catalyzed by phosphorylating kinases and dephosphorylating phosphatases, plays a role in almost every cellular function of eukaryotic cells. Contact of platelets with extracellular ligands causes a fast and transient burst in kinase activity and a substantial augmentation of intracellular phosphorylation. However, although much is known about the kinases present in platelets, comparatively little is known about phosphatases in the context of platelet function. While even in the early 90's platelets were recognized to contain at least 6 independent phosphatases with activity towards actin-related proteins including myosin light chain, these remained unnamed and were speculated to merely reduce platelet activation [8]. Subsequent progresses on platelet studies have shown that many phosphatases are present in platelets, and that their role is not always so straightforward. Therefore, in this review, we will discuss the recent advancements in our knowledge regarding the phosphatases governing platelet plasticity in response to different stimuli.

2. Phosphorylation in platelets

Cellular communication at the micro- and macro-environment depends on cellular adhesion molecules as well as the release of cytokines, chemokines and growth factors. Information delivered to the target cell through the interaction between these molecules and a ligand-dependent receptor at the cellular membrane results in intracellular signaling, ensuring amplification of the received information and execution of a specific cellular response. Activation or inhibition of a protein during signal transduction is often determined by their phosphorylation status. Zahedi and colleagues investigated the phosphoproteome from healthy human platelets, and demonstrated that almost 280 proteins were phosphorylated on different sites, with 55% of these proteins related to signaling pathways and cytoskeletal dynamics [9]. Thus, phospho-signaling in platelets is evidently important, and a better understanding of these signaling events may provide insight into platelet biology in health as well as pathological conditions (Figure 1). Protein phosphorylation is restricted to only a few amino acid residues, and according to their specificity, function and structure, kinases and phosphatases are classified as being tyrosine, serine/threonine or dual specificity enzymes. Over 500 protein kinases are encoded by the human kinome, many of which are expressed in platelets. Although full functional characterization is mainly limited to a few main signaling pathways, our knowledge of these pathways is relatively well advanced [10-12]. In contrast,

the human genome contains far fewer phosphatase genes, with a total of 107 protein tyrosine phosphatase (PTP) and ~30 serine/threonine phosphatases thus far identified [13]. Sixteen of these have been identified in platelets using a specialized PTP-proteomics approach [14] with several others identified by conventional proteomics, making a total tally to date of 10 receptor-like and 10 non-receptor PTPs present in platelets [15]. Over the past few years, more and more studies have revealed that PTPs are relevant modulators of platelet function, although the underlying mechanisms are still poorly understood. It is of interest to note that *pan* tyrosine phosphatase activity is increased in platelets in response to collagen stimulation, suggesting their role in adhesive processes (Figure 2). However, in contrast to collagen, thrombin stimulation reduces total phosphatase activity (Figure 2). Thus, specific regulation of phosphatase activity exists in platelets. Below, we summarize the ligand-receptor-signaling events involved in hemostasis and review what is currently known regarding the best described of these phosphatases in platelet activity.

3. Platelet receptors and their ligands

In order to perform their functions, platelets carry an array of membrane receptors allowing them to respond to various extracellular ligands. Of the extended family of integrins, platelets carry six: αIIβ1 (CD49b/CD29), αvβ1 (CD49e/CD29), α6β1 (CD49f/CD29), αLβ2 (CD11a/CD18), αIIbβ3 (CD41/CD61), and αvβ3 (CD51/CD41) [16,17]. Of these, arguable the best studied are αIIbβ3 [16] - (the most abundant platelet receptor) recognized by fibrinogen, von Willebrand factor (vWF) and the extracellular matrix components fibronectin and vitronectin, and the collagen receptor αIIβ1 (otherwise known as glycoprotein GPIa/IIa). Collagen is additionally recognized by the platelet receptor GPVI. The second most abundant platelet receptor is the glycoprotein receptor complex GP1b-IX-V, which primarily binds to vWF, but also recognizes thrombin, P-selectin, and clotting factors XI and XII [18]. Thrombin is also able to stimulate platelets via the protease activated receptor (PAR)1 and PAR4. It is of interest to note that platelets carry many components capable of enhancing their own activation. Amongst many others, the fibrin precursor fibrinogen, GPVI, αIIbβ3, P-selectin, and bioactive molecules such vWF, vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), coagulation factor V and anti-thrombin are contained within α-granules [19,20]. Dense-granules, lysosome-related organelle with a cargo profile that is largely dominated by smaller molecules, contain serotonin, ADP/ATP and Ca^{2+} [21]. Exocytosis of these granules can be triggered by specific platelet agonists and enhance levels of already present membrane phospholipids, glycoproteins and integrins, or cause novel

expression of granule-specific markers. Many of these membrane components play an essential role in platelet adhesion and aggregation, known as primary hemostasis, and their release can therefore further platelet activation cascades. Furthermore, as platelets themselves carry receptors for many of the vesicle components, including ADP receptors $P2Y_1$, $P2Y_{12}$ and the ATP receptor $P2X_1$, release of these factors allows autocrine activation [22].

4. Hemostasis

As mentioned, the primary role of platelets is to form a platelet plug in response to vascular injury. The hemodynamic force of the blood flow ensures the proximity of platelets to the vessel wall, allowing a quick response to such tissue damage. The process of thrombus formation is complex and depends on rheological circumstances. Under high shear conditions, the first step towards thrombus formation is the deceleration of platelets through binding of their GPIb-V-IX receptor complex to vWf captured onto collagen fibrils exposed at the sub-endothelial matrix lining of the vessel upon injury [23,24]. This transient interaction is stabilized by subsequent binding of αIIβ1 to collagen and αIIbβ3 binding to vWf/fibrinogen, fully anchoring platelets to the vessel wall [16,24,25]. After formation of a monolayer of platelets attached to the vessel wall, platelet-platelet interactions, i.e. platelet aggregation, is required for growth of the thrombus. These homotypic platelet interactions are mediated by GPIb-V-IX and αIIbβ3 binding to their ligands. Subsequent adhesion-dependent signals, including GPVI binding to collagen, triggers activation of the platelets [26]. This is further extended by locally generated or secreted factors like thrombin, ADP, epinephrine and thromboxane A2 (TxA2), amplifying the activation/aggregation process. At this stage, the platelet becomes fully activated and changes its morphology from a discoid shape to a flattened shape with lamellipodia (Figure 1A) – [27,28]. The ensuing increased platelet surface area may provide a scaffold for other aggregating platelets, although whether this actually contributes to the aggregation process is unclear [29]. In the last steps of platelet activation, intracellular signaling cascades lead to conformational changes in integrin αIIbβ3, giving it an enhanced affinity for its ligands. This subsequently allows fibrinogen to act as a bridging molecule, tethering platelets together [30,31]. The inside-out signaling required to initiate these conformational changes appears to be mediated largely through thrombin-mediated signaling via PAR1, and depends on actin-binding intermediator proteins such as talin, kindlins and the ezrin-radixin-moesin (ERM) family of proteins [32]. In addition to forming a platelet plug during primary hemostasis, platelets contribute to secondary hemostasis (coagulation cascade) mainly through their degranulation products. Released

coagulation Factors V, XI, and XIII, pro-thrombin and fibrinogen all feed into the coagulation cascade, a series of catalytic steps involving the cleaving of pro-thrombin to thrombin and culminating in the cleaving of fibrinogen by thrombin to form adhesive monomeric fibrin fibrils. Polymerization of these fibrils by activated factor XIII further constructs stable bridges between platelets [33]. Thus, many interlinked processes initiated and maintained by platelets are required for blocking the blood flow from damaged vessels.

5. Signaling during hemostasis

G-protein coupled receptor signaling

Extracellular signals are relayed primarily via two types of signaling receptors; tyrosine kinase-associated receptors and G-protein coupled receptors (GPCR). Examples of GPCR on platelets are the thrombin and ADP receptors. Attached to these serpentine receptors are heterotrimeric G-proteins, Gα, Gβ and Gγ, of which in particular the type of Gα subunit coupled to the receptor determines its signaling outcome. Four main Gα proteins can be distinguished, Gαq, Gαs, Gαi and $G\alpha_{12/13}$. Binding of a Gαq-coupled receptor leads to activation of phospholipase C, which in turn cleaves the membrane bound inositol lipid phosphatidylinositol biphosphate (PIP2) to diacylglycerol (DAG) and inositol 3-phosphate (IP3) – [34]. Subsequent binding of IP3 receptors on the endoplasmic reticulum leads to release of intracellular Ca^{2+} stores, while DAG binds and activates the serine/threonine Protein Kinase C (PKC), together leading to further intracellular signal transduction and cellular responses. In platelets, Gαq receptors include the receptors for thrombin [35], TxA2, and the P_2Y_1 ADP receptor, epinephrine activates Gαs. It was already early recognized that thrombin-mediated platelet aggregation relies on signaling via PLC, PKC (including PKC-α, -β, -δ, and –θ isoforms) as well as activation of tyrosine- and serine/threonine phosphatases [36]. While the exact phosphatases remained to be elucidated at that time, later studies demonstrated that activation of diverse PKC isoforms inhibits the myosin light chain phosphatase (MLCP), thereby stimulating the formation of actin stress fibers required for platelet secretion [37]. Modulation of actin cytoskeletal rearrangement in response to thrombin is also mediated by dephosphorylation of cofilin, in a signaling scheme that is dependent on Ca^{2+} and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin, but independent of PKC [38].

Gαs and Gαi proteins act by stimulating or inhibiting the enzyme adenylate cyclase (AC), respectively. AC activity triggers the formation of second messenger cyclic-AMP (cAMP) resulting in activation of cAMP-dependent Protein Kinase A (PKA), a potent inhibitor of

platelet function [39]. While prostaglandins and adenosines increase cAMP levels via Gαs, the thrombin receptors PAR1, as well as the ADP receptor $P2Y_{12}$, signal through Gαi (as well as Gq), thereby reducing activation of the serine/threonine PKA kinase [40]. Phosphotyrosine profiling showed that total cellular tyrosine phosphorylation was increased upon stimulation with ATP, which was near completely abolished by concurrent treatment of cells with PKA-activating prostacyclin analogue iloprost [41]. This suggests that kinase activity does not indiscriminately result in enhancement of platelet functions, as is often thought. Indeed phosphorylation of $GP1b\beta^{Ser166}$ by PKA can reduce its binding to vWF under flow conditions [42]. Large scale phosphoproteomics confirmed that inhibition of platelet functions through stimulation with prostacyclin (activating cAMP/PKA signaling) is indeed accompanied by upregulation of phosphorylation of several kinases, including $GRP2^{Ser587}$, Src^{Tyr530}, $VASP^{Ser157}$, $VASP^{Ser239}$, $GSK3\alpha^{Ser21}$, $GSK3\beta^{Ser9}$, $Zyxin^{Ser142/143}$, Filamin-$A^{Ser2152}$, $LASP^{Ser14}$. However, many of these phosphorylations reduce activity of the associated protein, which may in part account for the inhibition of platelet functioning [43,44]. In addition, phosphorylation of 3 phosphatases, namely $PTPRJ^{Ser1311}$ (increased), $CTDSPL^{Ser32}$ (decreased), and $PTPN12S^{er332}$ (decreased) were affected by prostacyclin stimulation. To what extent these contribute to inhibition of platelet signaling in response to Gαs signaling remains to be established (for more details on PTPRJ, see below) – [39]. Furthermore, in contrast to the thrombin-Gq-activated MLCP inhibition described above, prostaglandin-activated PKA enhances MLCP activity, thereby inhibiting the actin cytoskeleton rearrangements required for platelet shape changes [45]. On the other hand however, thrombin signaling through Gαi does cause phosphorylation and activation of the Src homology region 2 domain-containing phosphatase-1 SHP1), as well as its translocation to the cytoskeleton (see below for further discussion of SHP1 in platelets) – [46]. Thus, a complex series of activating and inactivating phosphorylation of both kinases and phosphatases contribute to PKC/PKA-mediated modulation of platelet functions. It should be noted that different isoforms of PKA also exist, which may localize to different cellular compartments and play different roles in platelet activation [47] suggesting that PKA signaling in platelets is complex and multifunctional [40]. Lastly, signaling via $G\alpha_{12/13}$ occurs via PAR4 and the TxA2 receptors, and activates cytoskeletal rearrangement and platelet shape changes [48]. Recently, Pyk2 (a member of the focal adhesion kinase [FAK] family) was described to be a direct target of PAR-4-mediated $G\alpha_{12/13}$ activity, regulating platelet aggregation and dense granule release [49]. Again, signaling via MLCP appears also to be affected by Pyk2 signaling through this G-protein [49].

Tyrosine kinase (associated) receptor signaling

The main tyrosine kinase-associated receptors involved in platelet activation are GPIb/IX/V and GPVI. Neither of these contain intrinsic kinase activity; instead, they rely on a family of protein-tyrosine kinases (PTKs) that are either associated with or in close proximity to their cytoplasmic tails, to transmit signals. For GPVI, this is provided by coupling with Fcγ-receptors, which have an intracellular domain containing an immunoreceptor tyrosine activation motif (ITAM). Upon ligand binding, ITAM motifs become phosphorylated by the Src family of kinases (SFK, including Src, Syk, Fyn, Lck, Btk, Fgr, Lyn, Yes), which results in tyrosine phosphorylation of the membrane adaptor protein LAT (linker of activation of T-cells (LAT), which in turn activates multiple signal transduction pathways including PLCγ and the lipid kinase phosphatidyl-inositol 3-OH kinase (PI3K) – [50,51]. For GPIb/IX/V, the associated tyrosine kinase appear to be less well known, and signaling could be either via FcγRII, directly through Src, or via PI3K [52]. It has also been suggested that FcγRII-ITAM signaling contributes to outside-in signaling of integrins, with in particular Fyn and Syk contributing to phosphorylation of αIIbβ3 integrin, leading to PLCγ activity [48]. Other tyrosine kinase receptors present on the surface of human platelets include thrombopoietin receptor (or c-MPL), TAM family (Tyro3, Axl, and Mer), ephrins and IGF-1 receptor. The contribution of each of receptor and its signal in thrombus formation or its bleeding risk, when absent, is under active study, but their involved intracellular signaling, are broadly speaking the same as for the tyrosine kinase-associated receptors [53].

Downstream signaling of tyrosine kinase (associated) receptors is complex, and in platelets appears to depend to a large extent on SFK activity (for review see [11]). vWF interaction with GPIb-V-IX leads to activation of SFK as well as Phospholipase A2, initiating platelet activation by changing its cytoskeletal arrangement [11,50]. Collagen stimulation of the receptors GPIIb/IIIa, GPIb, GPIIb/IIIa, GPVI and FcγR also results in SFK activation [50]. Furthermore, in platelets activated by thrombin, Ca^{2+}-mediated activation of the SFK results in Pyk activation [54], and Src and FAK proteins have described as master regulators of cytoskeletal remodeling in response to thrombin, showing that GPCR signaling also converges on tyrosine phosphorylation signaling [55]. Activation of SFK in platelets leads to activation of other intracellular kinases, including PI3K/protein kinase A (Akt). For instance, GPIa/IIa and GPIIb/IIIa activate SFK and PLC signaling, which coordinates PI3K/Akt activation [10,56]. PI3K was associated with platelet aggregation in response to ADP stimulation [57], but is also activated by thrombin [55]. Downstream of Src-PI3K signaling, Akt phosphorylation was confirmed to play an important role in collagen- and ADP-induced

platelet aggregation [58]. In addition to Src and PI3K signaling, members of MAPK family (ERK and p38) have generally been considered positive modulators of platelet function. Binding of vWF or thrombin to GPIb-IX-V results in activation of integrin αIIbβ3, an example of inside-out-signaling, and this process depends on both PKC and ERK activities [59]. Activated ERK is described to play an important role in platelet aggregation, morphological changes and clot retraction [60]. p38 is also known to be activated by Src phosphorylation during collagen- and thrombin-stimuli. The principal mechanism coordinated by p38 is cytoskeleton remodeling and Ca^{2+} release, and consequently, amplification of platelet aggregation [61].

The general kinase receptor-induced pathways described here are common among many different cell types, and phosphatases modulating these pathways have been identified in several cellular settings. Below, we describe in more detail what is known about these phosphatases in phosphate signaling in platelets.

6. The role of phosphatases in hemostasis

ITIMs, GPCRs and SHP

Platelet activation via ITAM motif-coupled receptors is counteracted to large extent by immunoreceptor tyrosine-based inhibitory domain (ITIM)-containing receptors. The best described of these in platelets are PECAM-1, which mainly inhibits collagen-mediated activation the GPVI-FcRγ-chain complex, and G6b-B, which reduces signaling from GPVI-FcRγ-as well as CLEC-2, the hemi-ITAM-containing receptor for podoplanin. In addition to an ITIM motif, PECAM-1 and G6b-B contain an immunoreceptor tyrosine-based switch motif (ITSM) – [62,63]. Phosphorylation of both ITAM and ITIM/ITSM by SFKs creates docking sites for SH2-domain containing proteins. But while for ITAM this results in recruitment of protein-tyrosine kinase Syk and downstream activation of (tyrosine) kinase signaling and Ca^{2+} release, ITIM/ITSMs recruit the SH2-domain containing phosphatases SHP1 (i.e. PTP1C, PTPN6) and SHP2 (i.e. PTP1D, PTP2C, PTPN11), which is associated with inhibition of platelet function [56,64]. SHP2 may directly dephosphorylate the ITAM receptor itself (as was shown for CLEC-2 in the case of G6b-B), but also may target the kinase Syk [62,64,65]. Additionally, it has been suggested that PECAM-1 itself may be a target (although the role for this in platelets is unclear) and that recruitment of PI3K to SHP-1 prevents its activation by molecules associated with LAT (although this appears to be independent of its phosphatase activity) – [66,67].

The role of SHP1 and SHP2 has also been investigated in non-ITIM-mediated signaling in platelets. As already alluded to above, GPCR signaling activates kinase signaling and phosphorylation patterns which are also subject to phosphatase regulation. One study showed that PKC-mediated phosphorylation of $SHP1^{Ser591}$ in response to thrombin-PAR1/4 stimulation inhibits SHP1 activity, which subsequently increases phosphorylation of its substrate, the guanine-nucleotide exchange factor Vav, a regulator of cytoskeletal rearrangement [68]. Indeed, overall, the general consensus appears to favor a negative modulatory role for SHP phosphatase activity in platelet adhesion [69]. However, several studies indicate that the role of SHP phosphatases may be more complicated. $SHP1^{Tyr536}$ and $SHP1^{Tyr564}$ phosphorylation in response to thrombin were shown to activate SHP-1 enzymatic activity [70], independently from integrin and PKC signaling [46] (Li et al, 1995). As the Scr kinase is a direct substrate of SHP1, this would paradoxically dampen thrombin-mediated responses. However, some studies showed that this may be a late event during thrombin signaling (upon 45 minutes stimulation), and therefore a means to prevent over-activation of thrombus formation [71]. Furthermore, by targeting the negative tyrosine regulatory site of Src kinase, SHP1 activity may actually enhance Src activity and platelet function [72]. Additionally, it was demonstrated that Gq-mediated signaling causes rapid (within seconds) degradation of a protein complex which keeps resting platelets inactivated, and this process is dependent on phosphorylation and activation of SHP1 [73]. Overall, both positive and negative roles for SHP-1 have been described in platelets and the exact role for SHP-1 in platelet function therefore, remains elusive [73,74]. Experiments in knock-out mice have demonstrated that SHP-1 deficiency leads to reduced platelet spreading, while SHP-2 deficiency increases platelet spreading on fibrinogen, demonstrating opposite effects of these phosphatases on at least some platelet functions. Both gain-of-function and loss-of-function of SHP-2 are found in humans: gain-of function mutations as seen in patients with Noonan syndrome (often associated with bleeding) cause reduced collagen-induced (but not thrombin or ristocetin-induced) platelet aggregation, while loss-of-function of SHP2 in patients with Noonan syndrome with multiple lentigines show normal aggregation, but increased collagen-adherence under shear stress [75]. Thus, overall, SHP2 activity appears mostly to reduce the hemostatic process in humans.

Positive modulation of SFK via CD148 and PTP1B

One of the first transmembrane PTPs identified in platelets is the receptor-like protein-tyrosine phosphatase (PTP) CD148, also known as PTPRJ, which is expressed in all

hematopoietic cells. It is known to dephosphorylate PLCγ1 and LAT, thereby inactivating signaling through the T-cell receptor CD3 in T-cells, while dephosphorylation of SFKs contributes to neutrophil migration [76,77]. It is tempting to speculate that it may contribute to the immunological functions of platelets as well. While one of the main substrates of this phosphatase is the platelet derived growth factor receptors (PDGFR), in platelets this phosphatase is best known for its ability to modulate activity of the SFK [78]. Knockdown of CD148 in mouse models was shown to increase Lyn and Src phosphorylation at their inhibitory sites, while reducing phosphorylation in their activation loops. In turn, this resulted in impaired platelet spreading on fibrinogen, suggesting a requirement of this phosphatase in integrin αIIbβ3 signaling. GPVI-dependent platelet aggregation and ATP secretion were also dependent on CD148 activity. Thrombin-induced aggregation and ATP secretion as well as CLEC-2-mediated platelet activation were only partly affected by CD148 knock-down, while ADP-induced responses were not affected at all, showing selective requirement of this phosphatase by specific platelet agonists [79]. In humans, it has recently been shown that loss of function mutations in CD148 cause a form of hereditary thrombocytopenia, with patients showing reduced pro-platelet formation as well as reduced GPVI-mediated Src activation and platelet responses [80]. Thus, the general consensus is that CD148 contributes to platelet activation by releasing inhibitory phosphorylation on SFK. However, a recent study suggests that CD148 may also reduce phosphorylation of SFK at its activation loop. However, this is likely required to limit platelet over-activation, and indeed over-activation of SFK signaling may provide a negative feedback loop [81]. While CD148 was shown to be essential for platelet activity, full aggregate formation also requires the phosphatase PTP1B, another positive modulator of SFK [82].

Expression of the non-transmembrane PTP1B (also known as PTPN1) was already described decades ago. This phosphatase can become activated upon proteolytic cleavage by calpain in response to αIIbβ3 integrin stimulation [83]. This in turn can reduce the FcyRIIa-mediated phosphorylation of LAT, resulting in cytoskeletal rearrangement and irreversible platelet aggregation [84]. This suggests that PTP1B activity is required for full platelet responses, and indeed, PTP1B deficient platelets fail to form full aggregates on collagen [79]. Upon fibrinogen stimulation of platelets, PTP1B is recruited to interact with αIIbβ3 and promotes Src kinase activation by dephosphorylation of its inhibitory site [79,85]. This results in αIIbβ3 outside-in signaling and adhesion of platelets to fibrinogen. These functions are very similar as those reported for CD148, but while CD148 appears to regulate SFK more generally, PTP1B involvement is more specific to αIIbβ3 integrin signaling [78,86]. It should be noted

however, that calpain knockout mice were described to exhibit increased platelet PTP1B levels as well as activity, which was associated with reduced thrombin-induced platelet aggregation, showing that PTP1B signaling is perhaps more complex [87]. Crosstalk between PTP1B and the serine/threonine phosphatase PP2A has also been shown. While PTP1B-mediated Src activation is required for αIIbβ3-mediated platelet adhesion, PP2A inhibits this process, adding a layer of regulation and complexity [88]. Indeed, serine phosphatases may contribute to tyrosine phosphorylation patterns in platelets [89]. For instance, αIIbβ3-activated PP2A inhibits ERK signaling in response to ATP [90]. Thus, not all phosphatases are alike and it is their concerted actions that decide platelet fate.

LMWPTP is activated upon collagen-dependent signaling

The presence of Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) was first described in platelets by Mancini and colleagues as late as 2007 [91]. They showed that while this phosphatase is less abundant in platelets as compared to PTP1B, it is nevertheless functional in causing dephosphorylation of the FcγRII receptor ITAM motif, as well as LAT, causing inactivation of Syk and PLCγ and inhibition of platelet function. Interestingly, despite the fact that Scr kinases have been shown to be an important target for LMWPTP in other cell types [92,93], pp60src phosphorylation in response thrombin, convulxin or FcγRIIA clustering did not appear to be affected by LMWPTP in this study. On the other hand, another study showed that collagen induced LMWPTP, PTP1B and Src kinase activity, and that inhibition of this phosphatase activity was associated with reduced Src activity and inhibition of platelet aggregation [94]. In addition, LAT is also a substrate for the kinases Syk and ZAP70, both of which are activated by LMWPTP [95,96]. Thus, the end result of LMWPTP activity in platelets may be activation rather than inactivation of platelet signaling, depending on the platelet agonist. While LMWPTP may play an important role at platelet activation, the molecular contribution of this phosphatase remains poorly understood.

Other tyrosine phosphatases

Very limited information is available concerning the other phosphatases expressed in platelets. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a well-known tumor suppressor, which, when down-regulated or mutated, is correlated to cancer development and obesity. In platelets, a function for PTEN was described by Weng and colleagues (2010) showing that platelets deficient for PTEN demonstrate increased collagen-induced aggregation and ATP secretion, as a result of enhanced activation of the PI3K-Akt

pathway [97]. Thus, knock-down of PTEN reduced bleeding times in mice [97]. In contrast, knock-down of the protein tyrosine phosphatase PTPN7 (or hematopoietic protein tyrosine phosphatase (HePTP)), which acts as a negative modulator of ERK, regulating GPCR-signaling rather than GPVI signaling, only modestly affected bleeding times [98]. ERK is also targeted by the Vaccinia H1-related (VHR) phosphatase, or dual-specificity phosphatase (DUSP) 3. In platelets, DUSP3 knockdown or inhibition causes a decrease of platelet aggregation efficiency [99]. Thus, conflicting results are obtained with knockdown of phosphatases despite partial overlap in their signaling activity.

7. Conclusions

Platelets play an important role in many physiological processes, including hemostasis, wound healing and immunity. Impaired platelet function may contribute to several diseases, with over-activation of platelets having been related to, for instance, cardiovascular diseases and their complications. Thus, the elucidation of the molecular mechanism governing platelet functionality in health and disease may improve the treatment of such diseases. The role of phosphatases in these processes is slowly gaining attention, but their roles are as complex as the multitude of signaling events regulating platelet functions themselves. Both positive and negative modulatory roles for these enzymes have been described, and it is clear that selectivity towards agonists exists for these proteins in platelets. However, differences in agonists used, read-outs for platelet reactivity and methods of studying phosphatase activity and their downstream targets hamper interpretation of the limited experimental evidence. Thus the role of phosphatases in platelet biology warrants further investigation. A better understanding of these phosphatases, the way they themselves are regulated and the pathways they modulate, may contribute to the discovery of new potential pharmacological targets.

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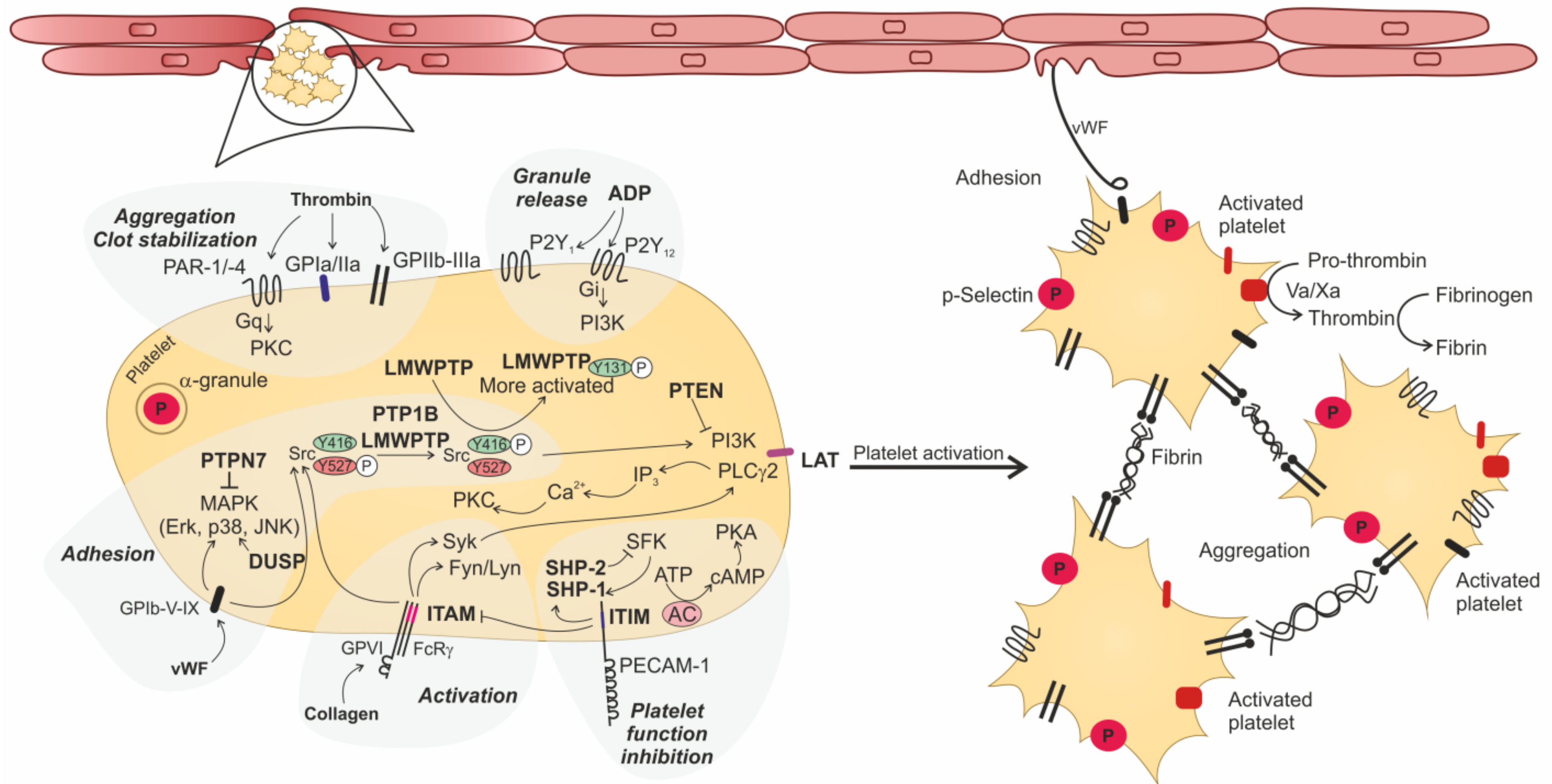

Figure 1. Platelet function. Platelet activation is modulated by the interaction of several agonists with specific receptors. After the activation through diverse stimuli, platelets change their shape to spread and expose adhesion molecules, which supports adherence and triggers granule release, further amplifying the aggregation process. Following the platelet activation, platelets releases pro-inflammatory factors for wound healing, tissue regenerations and angiogenesis by transporting and delivering factors. The pivotal signaling pathways associated to platelet activation are described in (A) coordinated to contribution for platelet function (B).

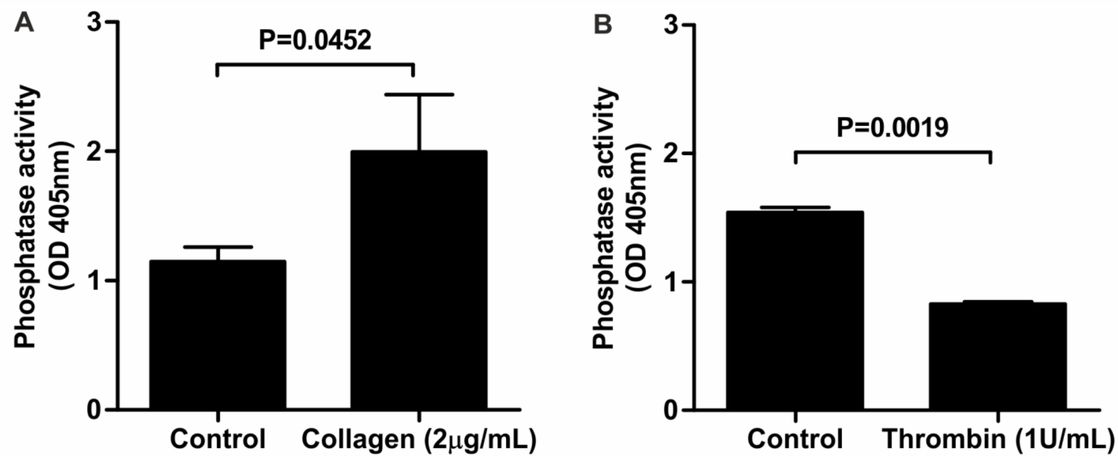

Figure 2. Total tyrosine phosphatase activity assay in stimulated platelets. After signed informed consent was obtained (Ethical committee Project NL66029.078.18 approved by Erasmus MC medical and ethical committee), venous blood from healthy donors (n=3) (all of them drug-free) was collected into conical plastic tubes containing 3.8% trisodium citrate 1:10 (v/v). Whole blood was centrifuged at 1500 rpm, 10 min, 22°C, and Platelet-Rich Plasma (PRP) was collected. NaCl (0.9%) was used to wash the platelets. Washed platelets were incubated with a final concentration of 2µg/mL Collagen or 1U/mL Thrombin for 10 minutes. All experiments were performed using 200-300 x 10^3platelets/µL. Platelets were lysed with 100 µL of lysis buffer (20 mM HEPES, pH7.4 with 2.5 mM $MgCl_2$, 0.1 mM EDTA) on ice for 2 h. After clarifying by centrifugation, protein-normalized platelets extracts were incubated in acetate buffer and the enzyme activity was assessed as followed: reaction medium (100 µL) contained 100 mM acetate buffer, 5 mM p-nitrophenyl phosphate (pNPP). After 60 min, at 37°C and under agitation (600rpm) the reaction was stopped by adding 100 µL 1M NaOH. The absorbance was measured at 405 nm (spectrophotometer - BioRad, California, USA). Statistical analysis was performed using t-student (paired, 95% confidence intervals, one-tailed) using GraphPad (GraphPad Inc, version 5.0, California, USA).

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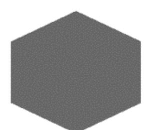

Chapter 8

Targeting Tyrosine Phosphatases by 3-Bromopyruvate Overcomes Hyperactivation of Platelets from Gastrointestinal Cancer Patients

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Abstract

Venous thromboembolism (VTE) is one of the most common causes of cancer related mortality. It has been speculated that hypercoagulation in cancer patients is triggered by direct or indirect contact of platelets with tumor cells, however the underlying molecular mechanisms involved are currently unknown. Unraveling these mechanisms may provide potential avenues for preventing platelet-tumor cell aggregation. Here, we investigated the role of protein tyrosine phosphatases in the functionality of platelets in both healthy individuals and patients with gastrointestinal cancer, and determined their use as a target to inhibit platelet hyperactivity. This is the first study to demonstrate that platelet agonists selectively activate low molecular weight protein tyrosine phosphatase (LMWPTP) and PTP1B, resulting in activation of Src, a tyrosine kinase known to contribute to several platelet functions. Furthermore, we demonstrate that these phosphatases are a target for 3-bromopyruvate (3-BP), a lactic acid analog currently investigated for its use in the treatment of various metabolic tumors. Our data indicate that 3-BP reduces Src activity, platelet aggregation, expression of platelet activation makers and platelet-tumor cell interaction. Thus, in addition to its anti-carcinogenic effects, 3-BP may also be effective in preventing platelet-tumor cell aggregationin cancer patients and therefore may reduce cancer mortality by limiting VTE in patients.

Keywords: platelet function; gastrointestinal cancer; venous thromboembolism; tyrosine phosphatases; LMWPTP; ACP1; PTP1B

1. Introduction

In 2018, over 18 million new cases of cancer were diagnosed worldwide. Despite improvements in cancer treatment, mortality rates are still high [1]. One of the most common comorbidities of cancer is thrombosis. Overall, 20% of cancer patients experience a thrombotic event, and in patients with gastrointestinal (GI) cancers the risk of developing a venous thromboembolism (VTE) is particularly high [2,3]. The association between cancer and thromboembolism events is termed Trousseau Syndrome, after Armand Trousseau [4], who first described the high occurrence of superficial migratory thrombophlebitis [5]. Because thrombosis is a common comorbidity in cancer, treatment strategies have been devised to include the use of anti-thrombotic drugs, such as low molecular weight heparin (LMWH), aspirin and warfarin, for co-adjuvant therapy in cancer treatment [6]. However, these drugs decrease the overall thrombus formation, and as a consequence, side effects pose a challenge [7]. Finding new compounds that combat VTE as well as primary cancer cells could mean a step forward in cancer treatment. In cancer patients, platelets appear to be more easily activated by agonists as compared to platelets from healthy individuals, and this hyperactivity may relate to VTE risk. However, despite the high impact of VTE in cancer morbidity, it is as yet unclear how platelet phenotype and inherent function are modulated in patients with cancer [8–11]. Thus, elucidating the molecular mechanisms related to cancer-associated VTE remains crucial.

Platelets contain a vast array of proteins, such as membrane proteins (e.g., glycoprotein IIb/IIIa integrins, P-Selectin, CD36), adhesive proteins (e.g., von Willebrand factor, fibrinogen, vitronectin), growth factors (PDGF, VEGF, EGF, TGF-B, and others) and clotting factors (V, IX, and XIII) [11]. Upon tissue damage, soluble von Willebrand factor binds to the exposed collagen and subsequently tethers platelets by binding to their glycoprotein Ib receptors (GPIbR) [12], thereby providing a scaffold for the generation of thrombin and formation of fibrin fibers. Coagulation in thrombosis and hemostasis is well described [13]. One important emerging regulator of collagen receptor and integrin-mediated platelet function is the Src family of kinases [14,15], although how these kinases themselves are regulated in platelets remains relatively unclear [16]. We and others have previously demonstrated that in hematopoietic and GI tumor cells, modulation of Src is dependent on protein tyrosine phosphatase activity, and inhibition of these phosphatases attenuates Src-dependent cancer cell growth and metastasis [17]. One potential modulator of this intracellular signaling pathway is the small molecule 3-bromopyruvate (3-BP), which is known to kill metabolically active tumor cells through inhibition of glycolysis. The use of 3-

BP for cancer treatment has been advocated [18,19]. Here, we investigate whether this compound may also hold promise for the prevention of platelet-tumor cell aggregation in cancer patients. We demonstrate for the first time that activity of Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) as well as Protein Tyrosine Phosphatase 1B (PTP1B) in platelets is selectively modulated by platelet agonists. We show that these phosphatases are a target for 3-BP, which also inhibits Src activity in platelets. Furthermore, 3-BP reduces collagen-induced aggregation and activation of platelets from both healthy controls and GI cancer patients, demonstrating the potential anti-thrombotic effect of this compound. Thus, 3-BP-like molecules may hold promise as an anti-tumor agent which simultaneously prevents platelet-tumor cell aggregation.

2. Material and methods

2.1. Antibodies and Reagents

Antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), Cell Signaling Technology (Danvers, MA, USA), SignalWay (College Park, , MD, USA). For details, see Supplemental File Table 1. Reagents were purchased from Sigma Aldrich, Santa Cruz, Merck Millipore, Chronolog. For details, see Supplemental File Table 2.

2.2. Cell Culture

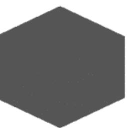
HT29, HCT116 and Caco-2 cells were obtained from ATCC (American Type Culture Collection, VA, USA) and routinely maintained in Dulbeco's Modified Eagles Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 100U/mL penicillin, 100mg/mL streptomycin (Life technologies, Bleiswijk, NL) and 10% Fetal Calf serum (FCS, Sigma-Aldrich, St. Louis, MO, USA) at 37°C under a 5% CO_2 humidified atmosphere. See Supplemental File Table 3 for characteristics of these lines.

2.3. Platelet Preparation

After signed informed consent was obtained (Ethical committee Project NL66029.078.18 approved by Erasmus MC medical and ethical committee), venous blood from healthy donors (n = 19) and gastrointestinal cancer patients (n = 3) was collected into conical plastic tubes containing 3.8% trisodium citrate 1:10 (v/v). Whole blood was centrifuged at 1500 rpm, 10 min, 22°C, and Platelet-Rich Plasma (PRP) was collected. For specific analysis NaCl (0.9%) was used to wash the platelets as previously described before [20]. All experiments were performed using 200–300×10^3 platelets/µL. Due to logistical restraints, not all experiments were performed on all donors. The number of times an experiment was performed is indicated in the figure legends.

2.4. Patient Information

Blood was obtained at diagnosis from three patients suffering from malignant esophageal neoplasia. The mean age was 73 ± 10 years, and two of them were male. Two patients used salbutamol, two patients took gastric pH modulators (Esomeprazol, Famotidine), and two patients used antidiabetics (hydrochlorothiazide). One patient took paracetamol, and one patient took metoclopramide as well as beclomethasone. None of these drugs were described to have an antiplatelet effect according to Chronolog (Chronolog Corp., city, PA, USA). All cancer patients were gender-matched to a healthy control.



2.5. Platelet Aggregation Assay by Light Transmission

A 500 µL aliquot of PRP was placed in an aggregometer cuvette and incubated at 37°C for 5 min in the presence or absence of compounds (100µM 3-bromopyruvate, 10µM CinnGEL, 100µM NSC87887). Subsequently, the agonist collagen (2µg/mL) was added to the samples. An aggregation curve was recorded for 10 min after the addition of agonist. Light transmission changes (an indicator of aggregation) were monitored with an aggregometer (Chrono-Log Corp.) under shear stress conditions by stirring at 1200 rpm following the method described before [21]. Quality controls of platelets were assessed by aggregation response at the beginning and end of experiments.

2.6. Platelet Activation Assay

Washed platelets were incubated with a final concentration of 100µM 3-bromopyruvate, 10µM CinnGEL, or 100µM NSC87887, for 60 min at room temperature, followed by stimulation of platelets with 2µg/mL Collagen or 1.25mg/mL Ristocetin for 10 minutes. After treatment, samples were incubated with antibodies CD41b (92800/040408 M1674); CD42b (65117/151106 M1729); CD62 (AK4) (304910-B239360 Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 15 min and data was acquired using a MACSQuant® Analyzer 10. Data analysis was performed with FlowJo, LLC v10 (Becton, Dickinson and Company, Franklin Lakes , NJ, USA).

2.7. Platelet-Cancer Cells Interaction Assays

For co-culture experiments, colorectal cancer cells (HCT116, Caco-2 and HT29) (1.0×10^4 cells/cm^2) were plated in 24-well plates for 24 h. After that, cells were washed with PBS, and PRP was added to each well for 6 h, together with either collagen (2µg/mL), 3-BP (100µM), or no agonists. After co-culture, the cells were either imaged by microscopy (Nikon), and the platelet-tumor cell aggregates were counted using a 10X magnification, at the well center quadrant, or platelets were harvested and analyzed by western blot as described before [22].

For aggregation assays in the presence of cancer cells, cancer cell lines were detached with trypsin-EDTA and washed several times with NaCl 0.9% to remove the excess of trypsin-EDTA. 500 uL of PRP was incubated with tumor cells (1.5×10^4 cells/test in NaCl 0.9%)—(protocol described before [23] with some modifications) at 37°C for 5 min in the presence or absence of 100µM 3-BP. Subsequently, the agonist collagen (2µg/mL) was added to the samples. An aggregation curve was recorded for 10 min after the addition of agonist. Light

transmission changes (an indicator of aggregation) were monitored with an aggregometer (Chrono-Log Corp.) following the method described before [21]. Quality controls of platelets were assessed by aggregation response at the beginning and end of experiments.

2.8. Western Blot

Two different platelet treatments were performed: (A) washed platelets (20,000,000-30,000,000) were incubated for 5 min in the absence or presence of compounds (final concentration: 100µM 3-BP, 10µM CinnGEL, 100µM NSC87887). Subsequently, platelet agonist collagen (2µg/mL) was added to the samples and after 10 min, the platelets were collected, washed and lysed as described below; (B) Platelets collected from co-cultures with colorectal cancer cells were washed with NaCl 0.9% and lysed in 2X concentrated Laemmli buffer (100mM Tris–HCl [pH 6.8], 200mM dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) and samples were boiled for 10 min. Cell extracts were resolved by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membranes (Merck chemicals BV, Darmstadt, Germany). Membranes were blocked in 50% odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) in TBS and incubated overnight at 4°C with a primary antibody. After washing in TBS-T (TBS with 0.5% Tween 20), membranes were incubated with IRDye antibodies (LI-COR Biosciences, Lincoln, NE, USA) for 1 h. Detection was performed using Odyssey reader and analyzed using the manufacturer's software. For the primary antibodies used, see Supplementary Table 1.

2.9. Immunoprecipitation Phosphatases

Platelets were treated with test compounds and subsequently stimulated with agonists as described above. Immunoprecipitation was performed for LMWPTP and PTP1B as described previously [24,25]. Briefly, cells were lysed with 100µL lysis buffer (20mM HEPES, pH7.4 with 2.5mM $MgCl_2$, 0.1 mM EDTA) on ice for 2 h. After clarifying by centrifugation and pre-clearing with uncoupled G-Sepharose beads (Thermo Fisher Scientific, Waltham, MA, USA), the platelet extracts were incubated overnight at 4°C under rotation with antibodies against LMWPTP (Acp1) or PTP1B. G-Sepharose beads were added to lysate and incubated for 3 h at 4°C. Samples were washed 3 times with acetate buffer (100mM pH5.5) before performing phosphatase activity assays.

2.10. Phosphatase Activity Assay

After immunoprecipitation (IP), the pellet was re-suspended in acetate buffer and the enzymatic activity was assessed as follows: reaction medium (100µL) containing 100mM acetate buffer, 5 mM p-nitrophenyl phosphate (PNPP) was added to the precipitated phosphatase. After 60 min at 37°C and under agitation (600 rpm) the reaction was stopped by adding 100 µL 1M NaOH. The absorbance was measured at 405 nm (spectrophotometer-BioRad, Hercules, CA, USA), and results are indicated as optical density measured normalized for bead controls (OD).

The effect of 3-BP and CinnGEL on LMWPTP activity was examined after 10 min of pre-incubation with LMWPTP immunopreciptated as described above. Subsequently, the substrate was added to the reaction medium.

The effect of 3-BP and CinnGEL on PTP1B activity was examined after 10 min of pre-incubation with recombinant PTP1B (0010896, lot 04529). Subsequently, the substrate was added to the reaction medium.

2.11. MTT Assay

MTT assay was performed as described before [26]. Briefly, platelets were seeded into a 96-wells plate for 3 h (total volume per well 180µL). 20µL of MTT (Sigma Aldrich) solution (5 mg/mL in PBS) was added to each well. After incubating for 4 h at 37°C, the plate was centrifuged 2500 rpm, 10 min, the MTT solution was removed and the formed formazan crystals were solubilized in 100µL of ethanol. The absorbance was measured at $\lambda = 585$ nm with a microplate reader (BioRad).

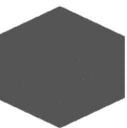
2.12. Statistical Analysis

The data is represented by means ± SEM. Statistical analysis was performed using t-student (paired, 95% confidence intervals, two tailed) using GraphPad (version 5.0, GraphPad Inc, city, California, USA).

3. Results

3.1. Protein Tyrosine Phosphatases Are Selectively Activated by Classic Platelet Agonists in Healthy Blood Donors

The role of kinases in platelet biology has received much more attention than phosphatases. We therefore set out to investigate the expression and activity of two tyrosine phosphatases known to be overexpressed in gastrointestinal cancer; LMWPTP and PTP1B. Our findings show that both of these phosphatases are expressed in human platelets (Figure 1A). Next, we investigated the activity of these phosphatases in the presence of either the physiological agonist collagen or the synthetic agonist ristocetin, both of which activate a robust platelet aggregation response (supplemental Figure S1). As demonstrated in Figure 1B, constitutive activity of LMWPTP was present in platelets, which could be further increased by stimulation of cells with collagen (2µg/mL), but not with ristocetin. In contrast, constitutive activity of PTP1B in platelets was lower, but drastically enhanced by treatment with either collagen or ristocetin (Figure 1C). These data suggest that PTP1B activity is a general response to platelet activation, whereas LMWPTP activity is dependent on the selective agonist used. As tyrosine phosphatase activity generally affects cellular protein phosphorylation levels, we next determined the phosphorylation status of several known targets of LMWPTP and PTP1B [24,25]. Constitutive phosphorylation of FAK, Integrinβ3 and p38 was present in platelets, but the most noticeable activation of signaling upon collagen stimulation was seen for the Src family kinases, as determined by their phosphorylation at tyrosine residue Y416 (Figure 1D). To confirm the importance of Src for platelet function, we performed aggregation assays in the presence of the selective Src family kinase inhibitor PP2. Interestingly, only collagen-stimulated aggregation was reduced in the presence of PP2 (Figure 1E, Figure S1-I), while ristocetin-induced aggregation was not (Figure 1F, Figure S1-I), suggesting that collagen activation of platelets in particular depends on Src signaling.



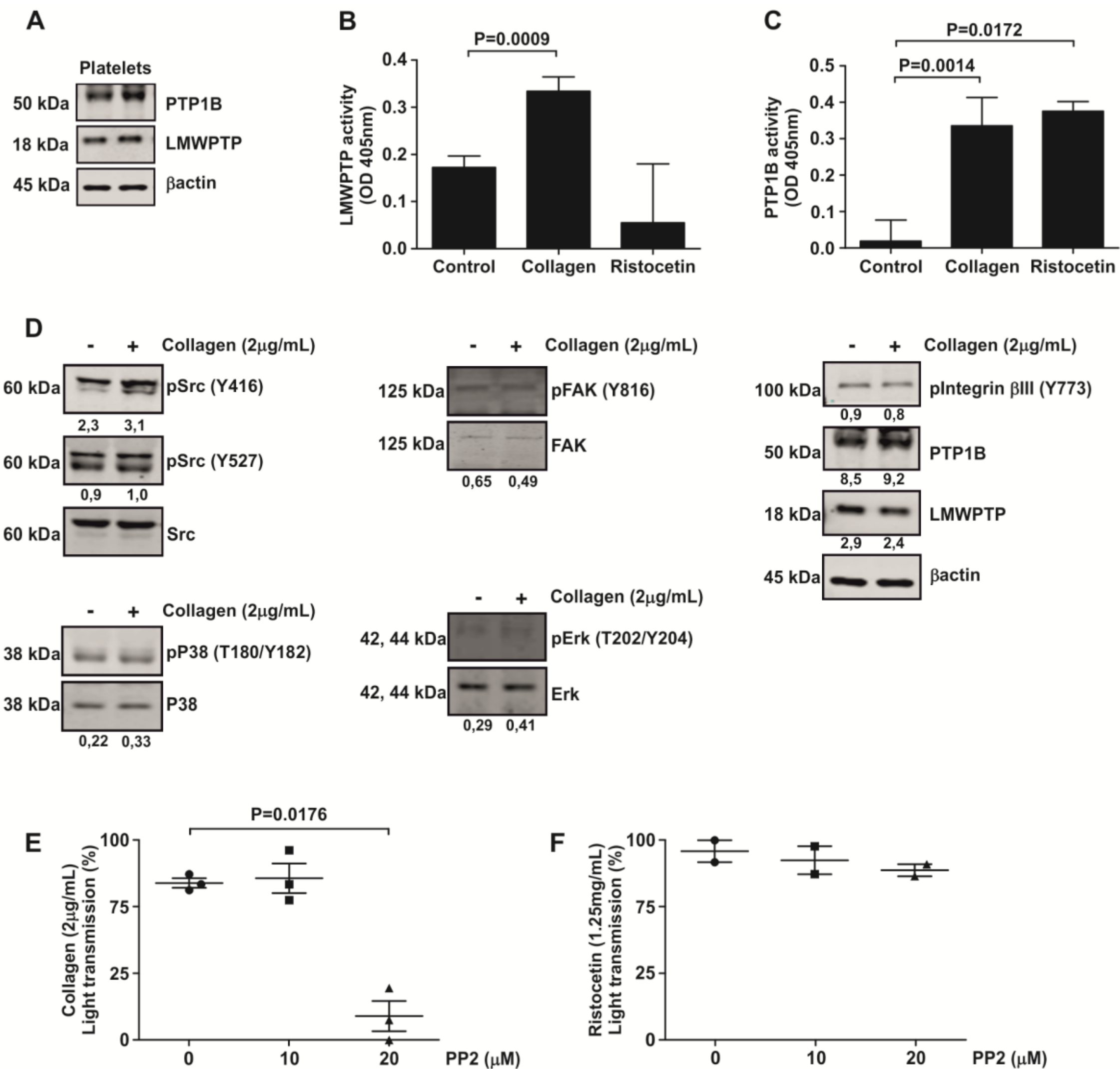


Figure 1. Platelets contain LMWPTP and PTP1B activity, which are selectively activated by platelet agonists. (A) Western blot analysis of platelets from two independent donors indicates protein expression of LMWPTP and PTP1B in these cells. (B, C) Platelets were stimulated with either collagen (2µg/mL) or ristocetin (1.25mg/mL) and LMWPTP (B) and PTP1B (C) were immunoprecipitated from the platelet lysates and subjected to phosphatase activity assay. Statistical analysis was performed using t-student (paired, 95% confidence intervals, two tailed) (n = 4). (D) Platelets were stimulated with collagen (2 µg/mL) and subjected to western blot analysis of the indicated (phospho-)proteins. β-actin served as a loading control. (-) Without collagen; (+) With collagen. The numbers under the blot indicate densitometry values corrected for loading controls. A representative blot of at least two independent experiments is shown. (E, F) Following pre-incubation with PP2 for 5 min, platelets were stimulated with either collagen (2µg/mL) (E) or ristocetin (1.25 mg/mL) (F) and the aggregation was measured for 10 min. Each data point corresponds to an individual single experiment, indicated by: Circle – 0µM; Square – 50µM and Triangle – 100µM condition.

3.2. Collagen-Induced Intracellular Signaling in Platelets from Healthy Donors is Inhibited by 3-BP

3-BP has been suggested as a promising antitumor agent by targeting a set of key metabolic enzymes, including kinases [27]. Therefore, we investigated the effect of this compound on cellular signaling in platelets. Interestingly, both collagen-induced LMWPTP activity and PTP1B activity were significantly reduced by 3-BP (Figure 2A). Furthermore, pretreatment of platelets with 3-BP drastically reduced both constitutive (Figure 2B) and collagen-stimulated levels (Figure 2C) of Src, FAK, and Integrinβ3 phosphorylation. However, the MAPK p38 was activated as demonstrated by an increase of the phosphorylation of T180 and Y182 residues (Figure 2C). Taken together, these data suggest that stimulation of platelets with collagen stimulates phosphatase activity and enhances Src activity, both of which are reduced by 3-BP treatment.

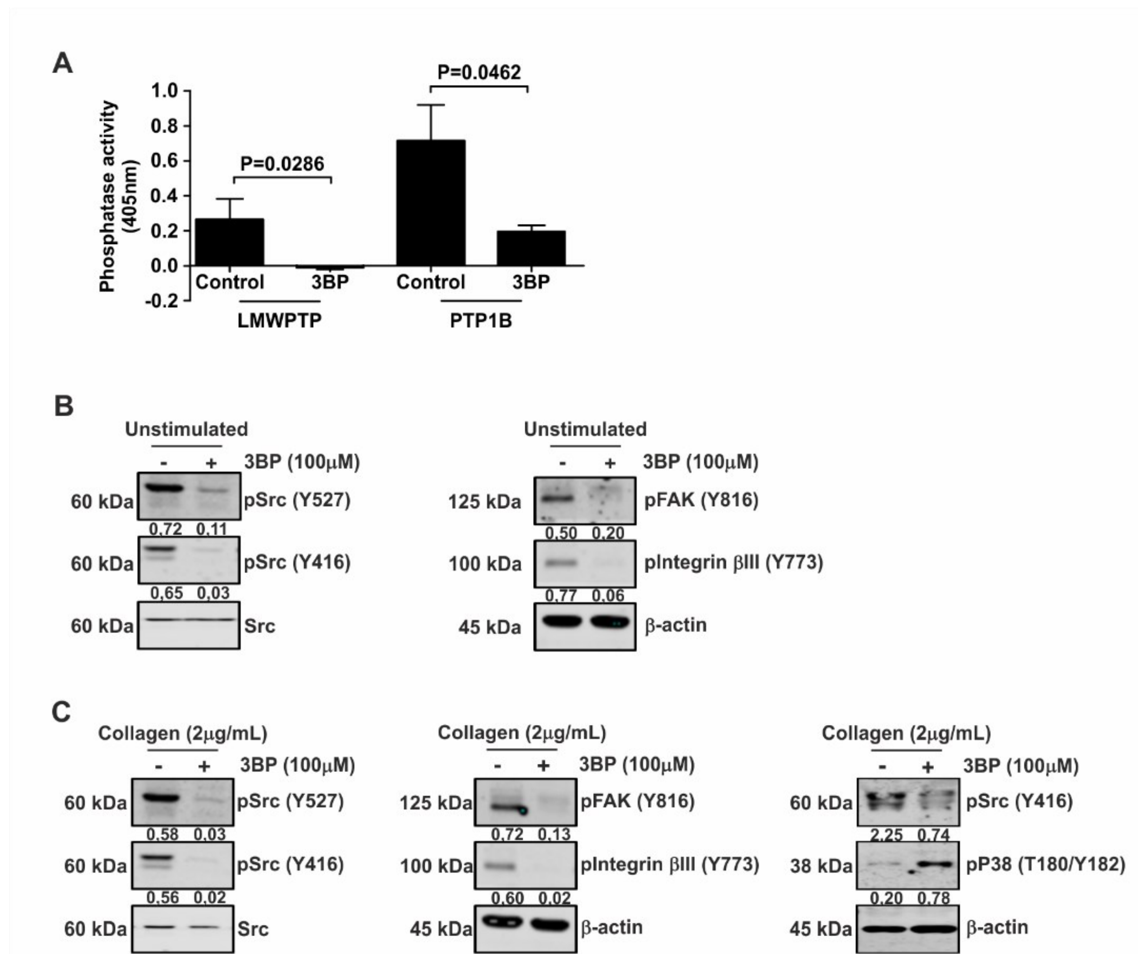


Figure 2. 3-BP inhibits intracellular signaling in platelets. (A) Platelets were stimulated with collagen (2μg/mL) and LMWPTP was immunoprecipitated. For PTP1B activity assay, active human recombinant protein was used. Following treatment with 3-BP, precipitates were subjected to phosphatase activity assays. Statistical analysis was performed using t-student (paired, 95% confidence intervals, two tailed) (n = 4). (B, C) Platelets were pretreated

with 3-BP for 30 min and left either unstimulated (B) or were treated with collagen (C). Western blot analysis was performed for the indicated proteins, with β-actin serving as loading control. The numbers under the blot indicate densitometry values that were corrected for loading controls. A representative blot of at least two independent experiments is shown.

3.3. 3-BP Abrogates Collagen-Induced Platelet Aggregation

Based on the inhibitory effect of 3-BP on both kinases and phosphatases in platelets, we set out to investigate the functionality of these cells in the presence of this compound. We first confirmed that 3-BP was not cytotoxic to platelets, by demonstrating that neither caspase-3 nor caspase-8 integrity, both of which are cleaved upon apoptosis induction [28,29], were changed upon 3-BP treatment (Figure 3A). Furthermore, expression levels of pro-apoptotic BAX and the anti-apoptotic Bcl-2 proteins (Figure 3B) were unaffected by 3-BP treatment of platelets.

The glycolytic pathway has been described as an important mediator of platelet function [30,31,32]. 3-BP is able to inhibit enzymes from this metabolic pathway [32]. This was confirmed by our finding that 3-BP reduces tetrazolium formation, known to be dependent on cellular glucose metabolism, by platelets (Figure 3C) [33].

In light of the involvement of Src in collagen-mediated platelet aggregation and the inhibitory effect of 3-BP on Scr signaling, we next investigated whether 3-BP could disturb platelet activation and aggregation-specific events. As shown in Figure 3D and Figure S1-II, 3-BP at a concentration of 100 μM was able to inhibit platelet aggregation induced by collagen, which binds to integrin α1β2 and glycoprotein GpVI on the platelet surface [34,35]. In contrast, platelet aggregation induced by ristocetin and mediated via vWF binding to Gp1b receptors [36], was not reduced by pretreatment of platelets with 3-BP (Figure 3E, Figure S1-II). Activation of platelets was accompanied by increased expression of Integrinβ3, vWF receptor and P-Selectin on the cell surface, and 3-BP treatment of platelets significantly reduced collagen-induced expression of both Integrinβ3 and vWF receptor on these cells as determined by FACS analysis (Figure 3F), while the expression of these receptors in the presence of ristocetin was not affected (Figure 3G). Thus, we conclude that 3-BP induces selective inactivation of platelets, without causing wide-scale apoptosis.

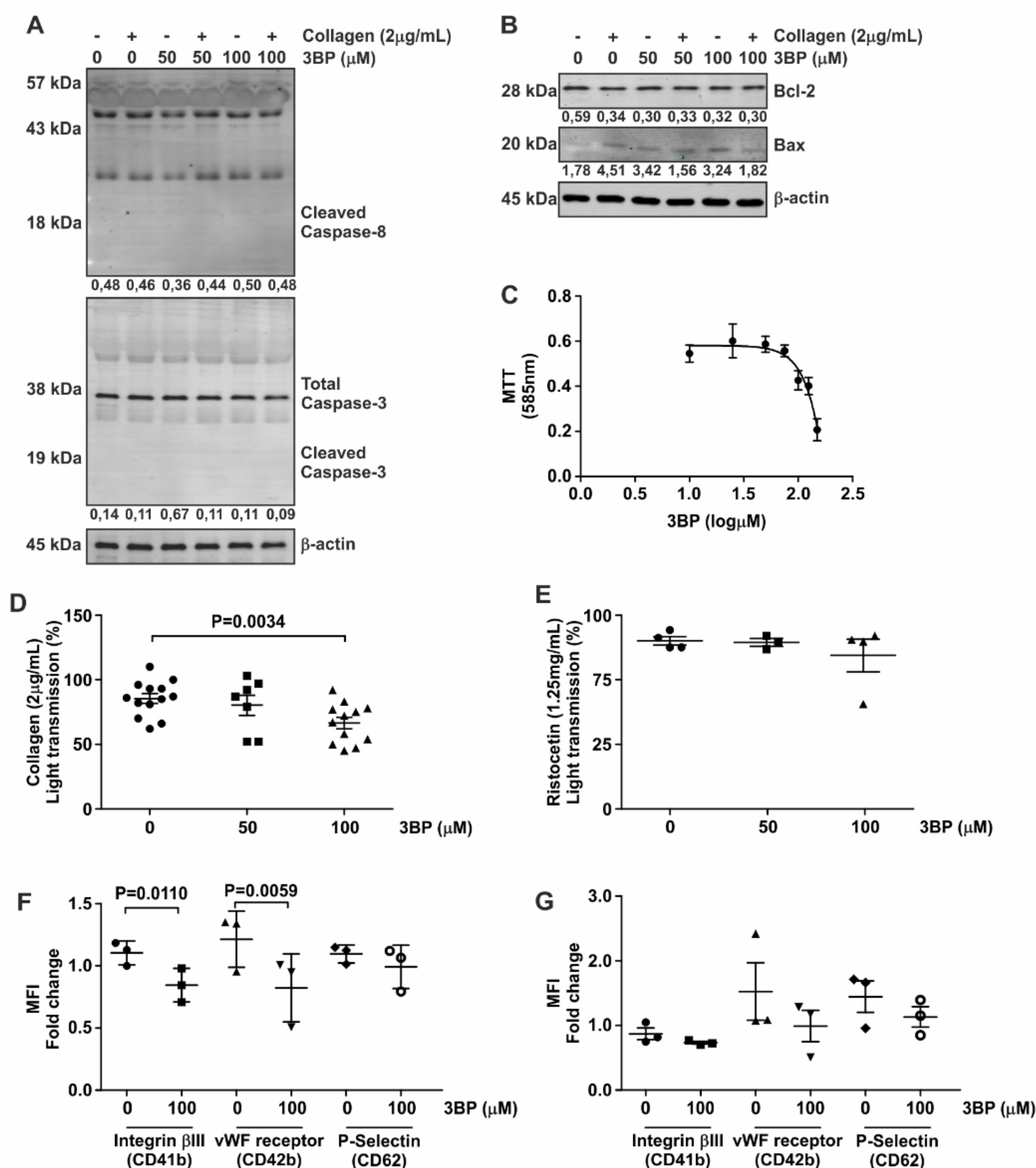


Figure 3. 3-BP decrease affects platelet metabolic activity and function. (A, B) Platelets were subjected to 50μM and 100μM 3-BP for the indicated time points, and expression of Bcl2 and BAX (A) as well as caspase cleaving (B) were determined by Western blot analysis. β-actin served as loading control. The numbers under the blot indicate densitometry values corrected for loading controls. A representative blot of at least two independent experiments is shown. (C) Isolated platelets were subjected to the indicated concentrations of 3-BP, and cell metabolic activity was followed by MTT assay (mean ± SEM shown). (D, E). Aggregation assay using 3-BP as inhibitor of platelet function. Following preincubation with 3-BP (5 min) platelets were stimulated with either collagen (2μg/mL) (D) or ristocetin (1.25mg/mL) (E) and the aggregation was measured for 10 min. Circle indicates 0μM; Square indicates 50μM and Triangle indicates 100μM condition. (F, G) Expression of platelet activation markers in the presence of 3-BP. Platelets were stimulated with either collagen

(2µg/mL) (F) or ristocetin (1.25mg/mL) (G) and stained using CD41b-FITC, CD42b-PE and CD62-APC antibodies to detect surface expression of vWF-receptor, Integrinβ3 and P-Selectin, respectively. Statistical analysis was performed using t-student (paired, 95% confidence intervals, two tailed). Each data point (special shapes) corresponds to an individual experiment and indicates the data as: Circle – Integrinβ3 without 3BP, Square – Integrinβ3 with 3BP, Up-triangle – vWF-receptor without 3BP, Down-triangle – vWF-receptor with 3BP, Diamond – P-Selectin without 3BP, Hollow circle – P-Selectin with 3BP.

3.4. Platelet function is Dependent on Specific Phosphatases

3-BP is not a specific inhibitor of PTPs. Therefore, to confirm the involvement of phosphatases in platelet activation, we employed the PTP1B inhibitor CinnGEL. As expected, CinnGEL specifically inhibits PTP1B (Figure 4A, upper panel), but not LMWPTP (Figure 4A, lower panel). Furthermore, inhibition of PTP1B was accompanied by a reduced activation of Src (Figure 4B). Investigation of platelet activation in the presence of CinnGEL demonstrated that platelet aggregation was significantly diminished upon PTP1B inhibition (Figure 4C). In addition, collagen-induced expression of platelet activation markers Integrinβ3, vWF receptor and P-Selectin were significantly reduced upon inhibition of PTP1B activity (Figure 4D). None of these platelet functions were affected by treatment with a selective inhibitor of the protein tyrosine phosphatase SHP1 (NSC87887) (Figure S1-III and Figure S2), demonstrating that specific protein tyrosine phosphatase activity is required for platelet functionality.

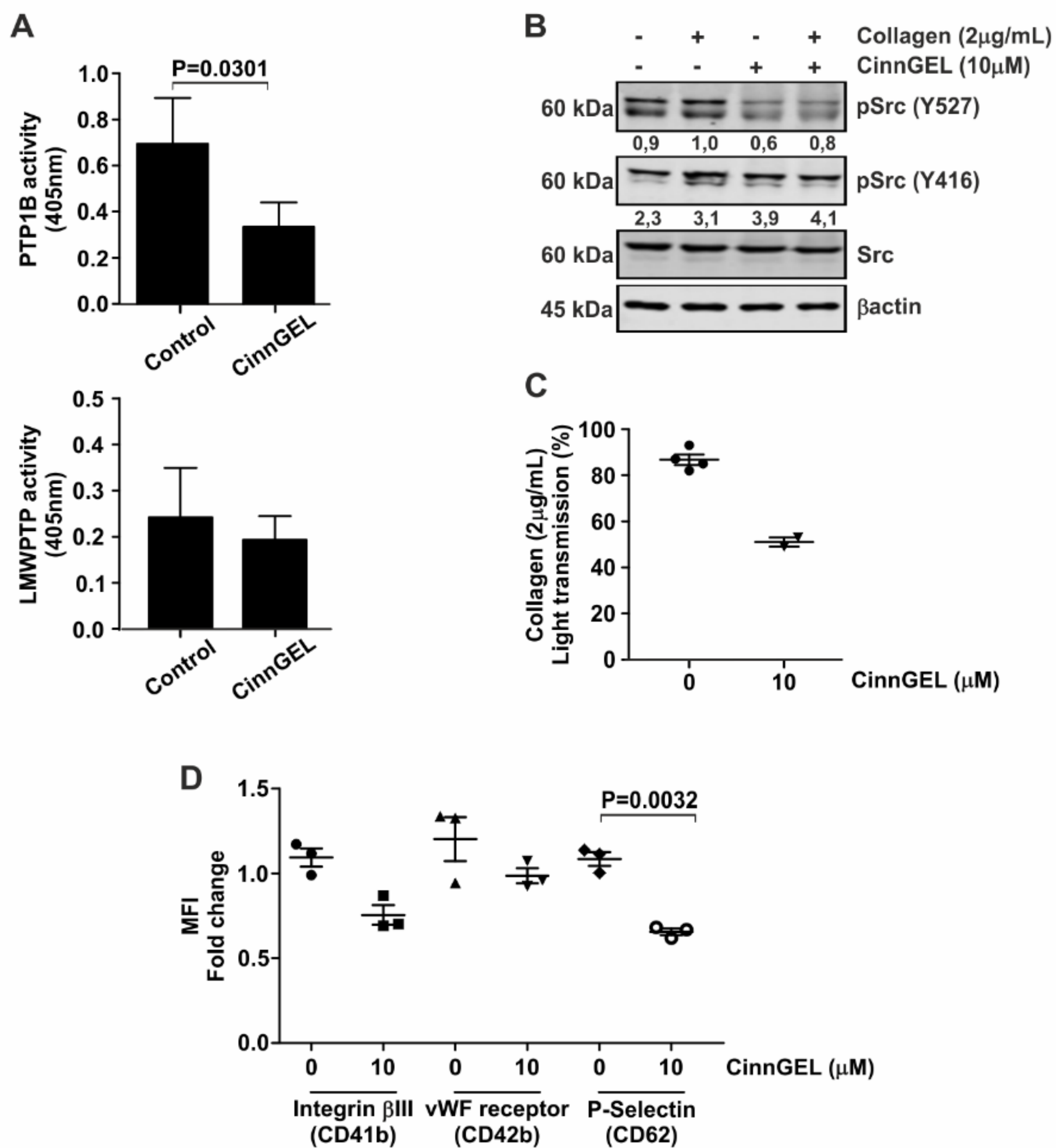


Figure 4. Platelet function is inhibited by selective PTP1B inhibition. (A) PTP1B activity assays were performed using active recombinant human protein. For LMWPTP activity assays, platelets were stimulated with collagen (2µg/mL) and LMWPTP was immunoprecipitated. Following treatment with CinnGEL (PTP1B inhibitor), recombinant proteins and precipitates were subjected to phosphatase activity assays. (B) Platelets were pretreated with CinnGEL or left untreated for 5 min. Cells were subsequently left either unstimulated or were treated with collagen. Western blot analysis was performed for the indicated proteins, with β-actin serving as loading control. The numbers under the blot indicate densitometry values corrected for loading controls. A representative blot of at least two independent experiments is shown. (C) Aggregation assays were performed for collagen-stimulated platelets after pretreatment with CinnGEL. (D) Cell surface expression of vWF-receptor (CD41-FITC), Integrinβ3 (CD42-PE) and P-Selectin (CD62-APC) was investigated on collagen-stimulated platelets that were pre-treated with CinnGEL. Each data point (special

shapes) corresponds to an individual experiment and indicates the data as: Circle – Integrinβ3 without 3BP, Square – Integrinβ3 with 3BP, Up-triangle – vWF-receptor without 3BP, Down-triangle – vWF-receptor with 3BP, Diamond – P-Selectin without 3BP, Hollow circle – P-Selectin with 3BP.

3.5. 3-BP Decreases the Capacity of Colorectal Cancer Cell Lines to Activate Platelets

Since the risk of VTE in cancer patients is in part mediated through platelet activation by tumor cells, we investigated the behavior of platelets using co-culture with colorectal cancer cells (HCT116, HT29, and Caco-2) as a model system. As shown in Figure 5A and Figure S1-IV, CRC cells were able to increase collagen-dependent platelet aggregation. Furthermore, treatment of co-cultures with 3-BP significantly reduced both platelet aggregation as determined by lumi-aggrogometry (Figure 5A, Figure S1-IV) and platelet aggregation as suggested by limited counting of aggregates using microscopy (Figure 5B and C). An increased presence of LMWPTP and PTP1B was observed in platelets co-cultured with either HCT116 or Caco-2 cells, with a concomitant upregulation of Integrinβ3 and Src phosphorylation. This effect was mediated through cell-cell contact, as conditioned medium from tumor cells did not elicit the same effect (Figure 5D). As a validation of our findings, we assessed the cell surface expression of platelet activation markers (vWF-receptor, integrin β3 and P-Selectin) in the presence of tumor cells, and showed a significant reduction of these markers upon treatment of co-cultures with 3-BP (Figure 5E–G). Taken together, these data demonstrate that platelet activity can be directly modulated through the physical contact with cancer cells, and show the potential of 3-BP to disturb this cancer cell-platelet interaction.

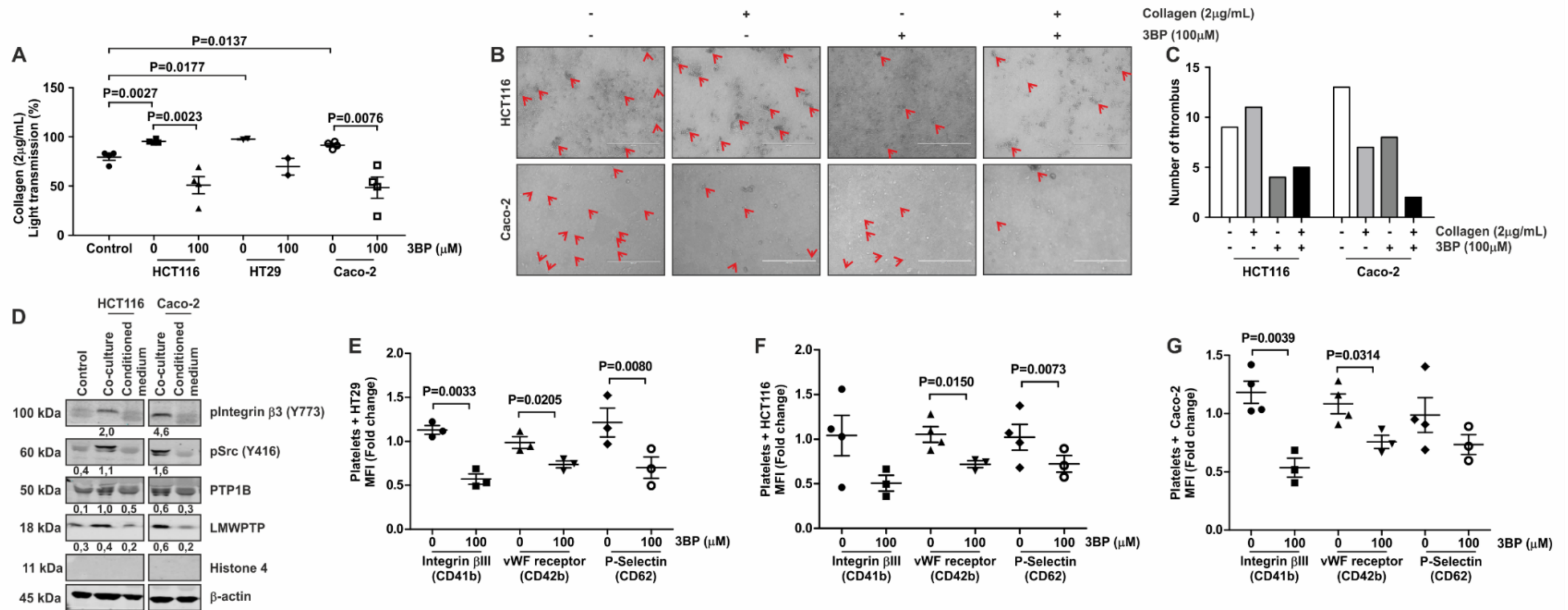


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Figure 5. Tumor-cell induced aggregation, thrombus formation and activation are decreased by 3-BP. (A) Platelets were pre-incubated with CRC cells (1.5×10^4 cells) and 3-BP (100µM) for 5 min, and subsequently stimulated using collagen (2µg/mL). Aggregation was measured for 10 min. Platelets stimulated with collagen only served as controls for the experiment. Each data point (special shapes) corresponds to an individual experiment and indicates the data for platelets marked with: Circle – only platelets, Square – with HCT116, Up-triangle – with HCT116 and 3BP, Down-triangle – with HT29, Diamond – with HT29 and 3BP, Hollow circle – with Caco-2, Hollow Square – with Caco-2 and 3BP. (B) Co-cultures of tumor cells and platelets were visualized by microscopy. Platelet aggregation as assessed by microscopy is indicated with arrows and quantified (C). (D) Platelets from co-culture with CRC cells (HCT116 and Caco-2) were lysed and loaded for western blot analysis of platelet activation markers and phosphatases. β-actin served as loading control. Densitometric values, corrected for loading control, are indicated. A representative blot of at least 2 independent experiments is shown. (E–G) Platelets co-cultured with HT29 (E), HCT116 (F) or Caco-2 (G) cells in the absence or presence of 3-BP were subsequently subjected to FACS analysis to determine cell surface expression of vWF-receptor (CD41-FITC), Integrinβ3 (CD42-PE) and P-Selectin (CD62-APC). Each data point (special shapes) corresponds to an individual experiment and indicates the data as: Circle – Integrinβ3 without 3BP, Square – Integrinβ3 with 3BP, Up-triangle – vWF-receptor without 3BP, Down-triangle – vWF-receptor with 3BP, Diamond – P-Selectin without 3BP, Hollow circle – P-Selectin with 3BP.

3.6. Hyperactivity of Platelets from Gastrointestinal Cancer Patients Is Reduced upon Treatment with 3-BP

We performed a small proof-of-concept study to investigate the capacity of 3-BP to decrease platelet aggregation in blood samples from three patients with gastrointestinal cancer. It has previously been described that platelets from cancer patients are more sensitive to in vitro collagen stimulation as compared to healthy controls. While our group is too small to make claims regarding significance as interpersonal variation may exist, we did observe a similar trend (% of light transmission of 95.67 ± 3.167 for patients vs 59.67 ± 21.40 for controls, Figure 6A, Figure S1–V). However, within the same experimental set-up, ristocetin-triggered aggregation was less different between three patients and three controls, which may suggest that specific molecular dysfunctions of adhesion processes are present in these patients (82.83 ± 7.949 for patients vs. 61.33 ± 24.93 for controls). Accordingly, P-Selectin levels on platelets from cancer patients appeared to be higher as compared to control, although again, we only assessed few patients (Figure 6B). Interestingly, expression of LMWPTP, but not PTP1B, was enhanced in all three patients studied, as compared to the experimental control subjects (Figure 6C). Importantly, 3-BP significantly decreased collagen-stimulated platelet function (Figure 6D, Figure S1-VI). Together, these data suggest

that phosphatases are key players in platelet function and aggregation in cancer patients and may be targeted by 3-BP.

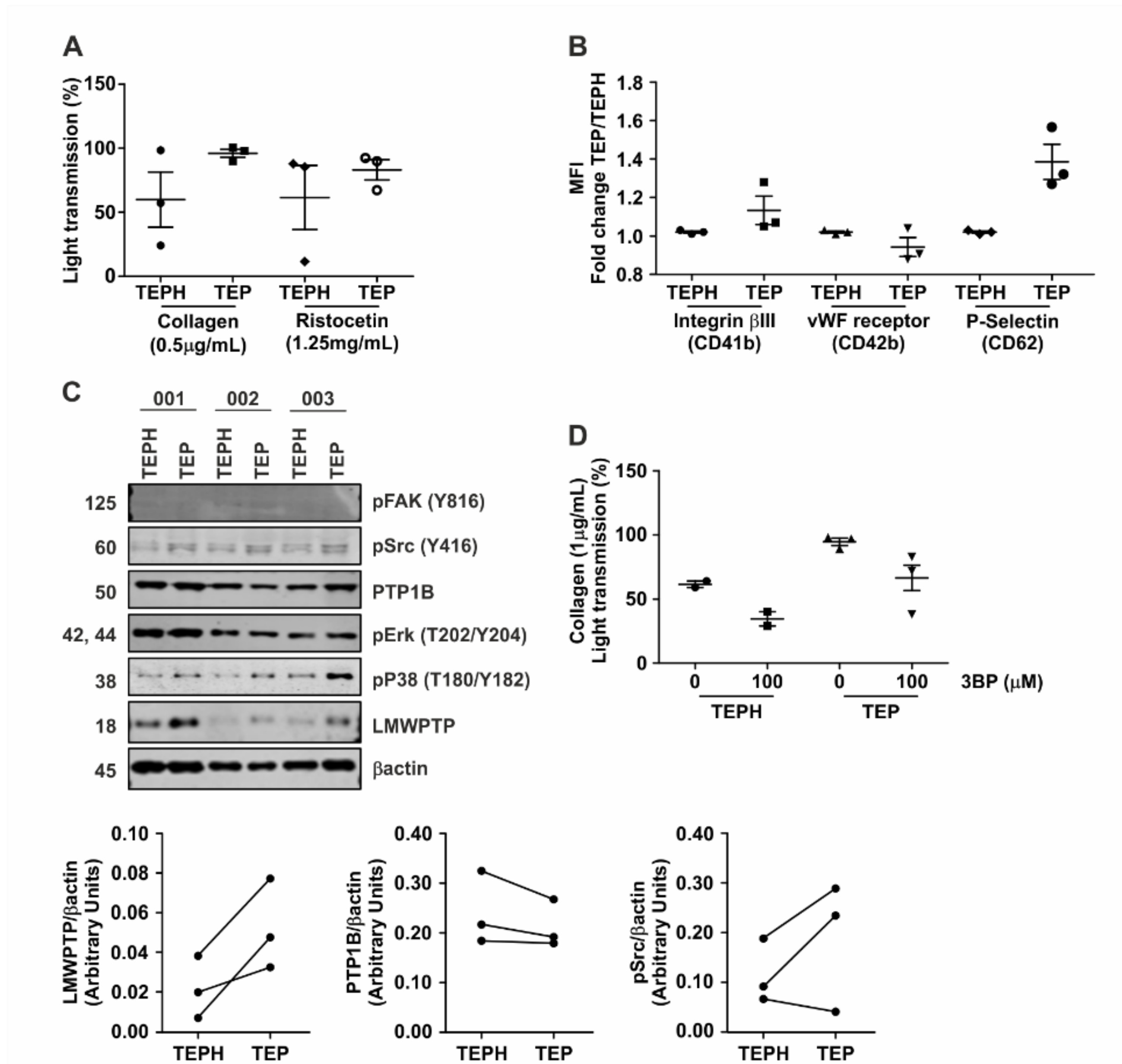


Figure 6. Platelets from patients with gastrointestinal cancer show hyperaggregation which can be inhibited by 3-BP. (A) Platelets from 3 gastrointestinal (GI) cancer patients (TEP) and 3 healthy controls (TEPH) were stimulated with either collagen (0.5μg/mL) or ristocetin (1.25mg/mL) and the aggregation was measured for 10 min. (B) Cell surface expression of vWF-receptor (CD41-FITC), Integrinβ3 (CD42-PE) and P-Selectin (CD62-APC) was investigated on platelets from GI cancer patients (TEP) and healthy controls (TEPH). (C) Platelets obtained from GI cancer patients and controls were lysed and subjected to western blot analysis of the indicated (phospho-)proteins. Densitometric analysis of LMWPTP, PTP1B and p-Src expression are shown. (D) Aggregation assay using 3-BP (100μM) as inhibitor of platelet function in 2 healthy controls and 3 GI cancer patients. 3-BP was incubated at pre-test step for 5 min, and after platelets were stimulated using collagen (1μg/mL).

4. Discussion

Cancer patients, in particular those suffering from gastrointestinal tumors, have a severely increased risk of developing VTE. Although the cause of this increased risk has not yet been fully elucidated, it has been described that tumor cells are capable of enhancing platelet aggregation in a process known as tumor cell-induced platelet aggregation (TCIPA) [37,38]. We investigated the molecular mechanisms associated with platelet hyper-aggregability. In the present study, we confirmed the importance of Src activity for collagen-induced platelet function, and demonstrated that both Src and Integrinβ3 activation are enhanced upon co-culture of platelets with cancer cells. Furthermore, we show here that phosphatases modulating Src activity, i.e., LMWPTP and PTP1B, are present and active in platelets, and that the levels of these phosphatases are enhanced upon co-culture with CRC cells. While it may seem surprising that inhibition of these phosphatases reduces platelet activation which relies so heavily on Src activity, both PTP1B and LMWPTP have previously been shown to be essential for Src activation, which is dependent on the balance between its phosphorylation sites [17,39].

In the present study, we observed differences in phosphatase activation upon stimulation of platelets with different agonists. Unlike collagen, ristocetin was not able to induce LMWPTP activity, suggesting that collagen-induced platelet activation relies more on LMWPTP activity than ristocetin-induced activity. Furthermore, in the cancer patients tested in this study, LMWPTP expression and collagen-induced aggregation, but not ristocetin-induced activity, were increased in comparison to healthy controls. Interestingly, increased LMWPTP mRNA profiles have been identified in platelets from colorectal, pancreatic, breast and hepatobiliary cancer patients [40] which, in light of our current data, suggests that this phosphatase may be related to tumor cell-induced platelet aggregation risk.

A growing body of evidence point towards an important role of PTPs in platelet biology [41,42]. Recently, it was shown that platelets contain the dual specificity phosphatase DUSP3, and that inhibition of this phosphatase reduces arterial thrombosis in mice [43,44]. A functional role for PTP1B has also been suggested in platelets [45,46]. Here, we show for the first time, that platelet LMWPTP activity is modulated by collagen, but not by ristocetin, and identified this phosphatase as a druggable target for platelet hyperactivity. Importantly, we found that 3-BP diminished the reactivity of platelets from healthy individuals as well as gastrointestinal cancer patients, at least in part through the inhibition of LMWPTP, PTP1B and Src kinaseds. However, the fact that ristocetin-induced aggregation and activation marker

expression was not inhibited by 3-BP treatment might suggest that, even though in vitro 3-BP is able to inhibit PTP1B activity, cellular LMWPTP is more affected by 3-BP (Figure 7).

Despite the great progress in the development of cancer treatment protocols, the treatment of several tumors is still a challenge, especially in metastatic cases. The use of antithrombotic treatment as a co-adjuvant strategy in cancer treatment was already suggested in 1982, in particular in its capacity to decrease metastasis [47]. Indeed, it has been described that antithrombotic therapy improves survival in patients with colorectal cancer [48]. We have previously shown that LMWPTP and PTP1B contribute to metastatic potential of (intestinal) cancer cells [24,25]. Here, we demonstrate the important role of these phosphatases in platelet biology. Thus, targeting these phosphatases through 3-BP and its derivatives may provide a potential strategy to reduce both VTE risk and tumor metastasis in GI cancer patients.

We acknowledge several limitations in this study. Platelet aggregation is a complex, multistep process, requiring many agonists at different time points of the process. Here, we limited our investigations to collagen and ristocetin-induced platelet functions, as previous studies have indicated that in particular collagen-induced platelet aggregation was increased in cancer patients in in vitro experiments [49], and that this correlates well to VTE risk scores [50]. However, in vivo, Tissue Factor may be a more relevant cancer-derived platelet agonist [51], and further studies will have to elucidate the role of phosphatases in the activation of platelets with this and other agonists. Furthermore, these in vitro experiments such as these do not take into account fibrinogen and the plethora of other factors encountered by platelets in vivo, and therefore care should be taken when trying to extrapolate these findings to an in vivo setting. Secondly, platelets are notoriously easily activated in in vitro experiments, and several different isolation and washing protocols to reduce unwanted activation have been published [52–54]. The protocol used here was taken from Jankowski et al, as this was the most compatible with downstream analysis of platelets for phosphatase activity assays. While we did not observe unwanted activation in our studies, it is conceivable that other wash protocols may have given slightly different results. Lastly, we only included 3 cancer patients in our proof-of-concept study, and larger studies using more patients and controls matched for age as well as gender, and more different agonists are warranted to further elucidate the role of phosphatases in these settings, and the potential of 3BP to affect platelet function in GI patients.

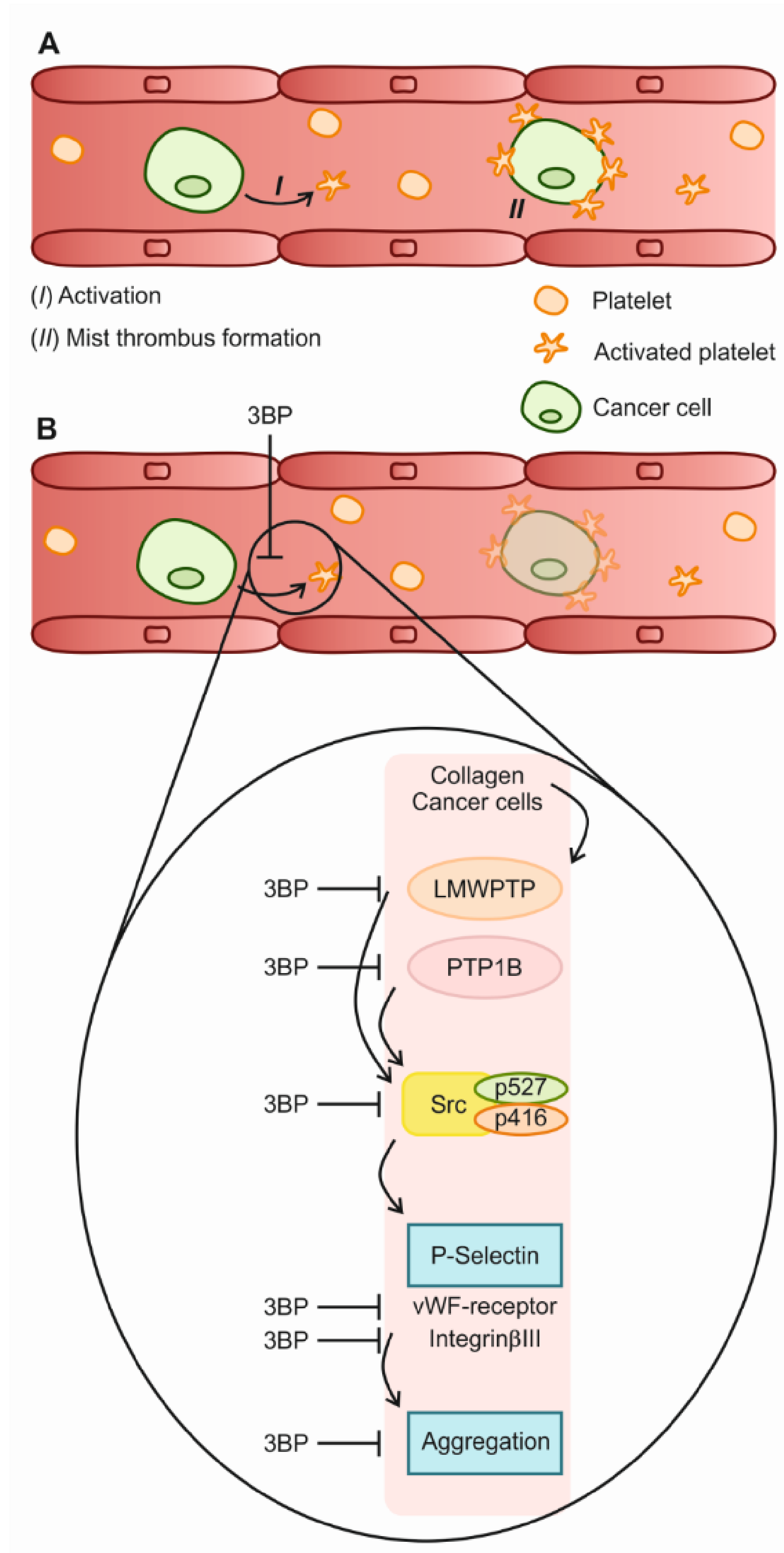
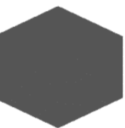
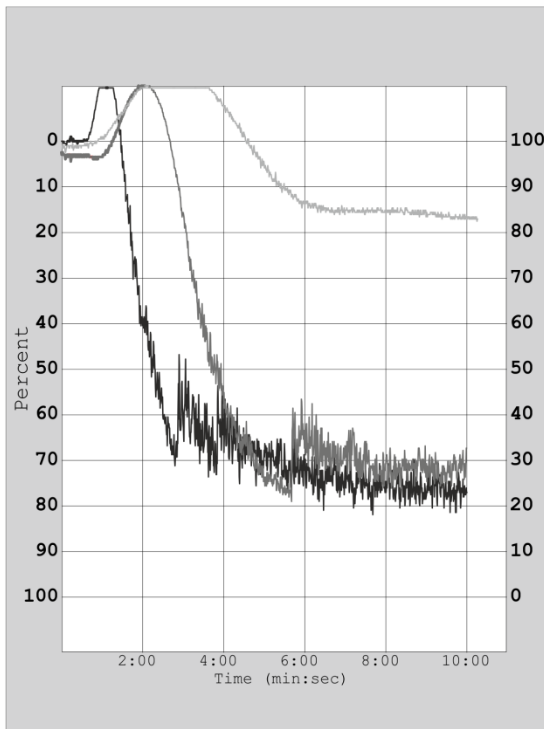


Figure 7. The 3-BP effect on platelet activation. (A) Platelets can be activated by collagen and tumor cells. (B) In the presence of 3-BP, the modulation of phosphatases plays contributes to a decreased platelet activity. This can culminate in a decreased aggregation, including cancer cell-platelet mist thrombi.

Supplemental Figure S1. Representative aggregation graphics from each experiment are combined in one file (pdf). I: Treatment of platelets with collagen/ristocetin in the absence or presence of the Src inhibitor PP2; II: Treatment of platelets with collagen/ristocetin in the absence or presence of 3-BP; Treatment of platelets with collagen in the absence or presence of the PTP1B inhibitor CinnGEL or the SHP inhibitor NSC87887; IV: Treatment of platelets with collagen in the absence or presence of tumor cells (Caco-2, HT29, HCT116) and 3-BP; V: Treatment of patient and control platelets with collagen/ristocetin; VI: Treatment of patient and control platelets with collagen/ristocetin in the absence or presence of 3-BP.



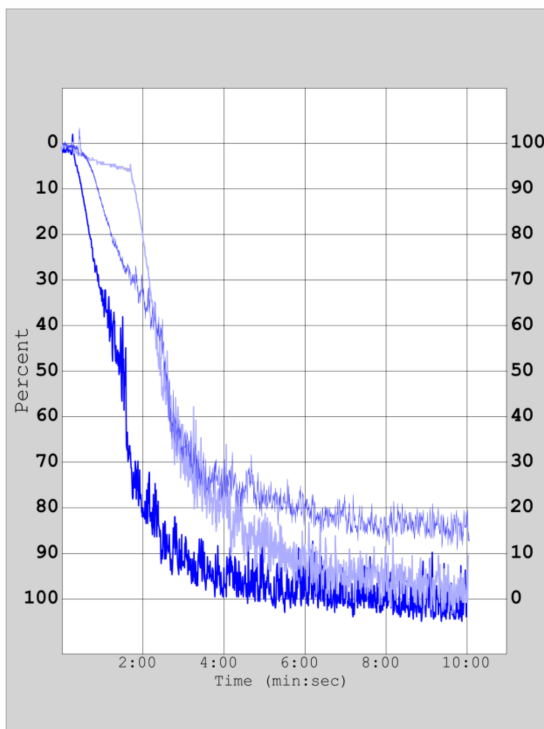
I



Collagen 2μg/mL

Collagen 2μg/mL + PP2 10μM

Collagen 2μg/mL + PP2 10μM

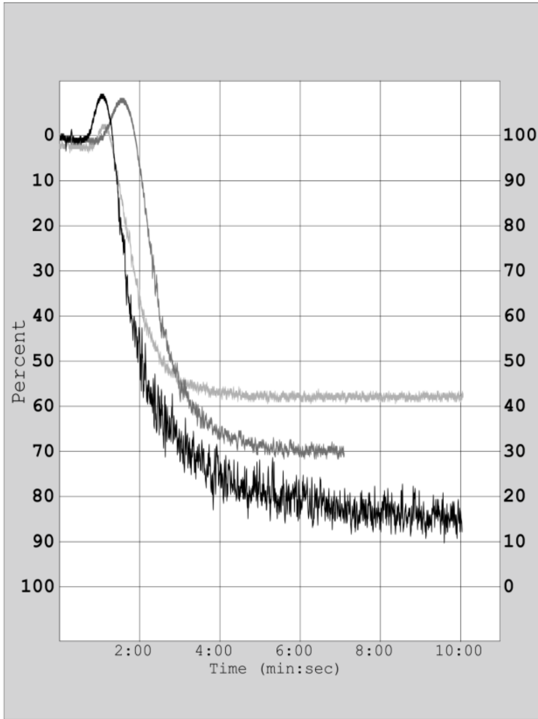


Ristocetin 1.25mg/mL

Ristocetin 1.25mg/mL + PP2 10μM

Ristocetin 1.25mg/mL + PP2 20μM

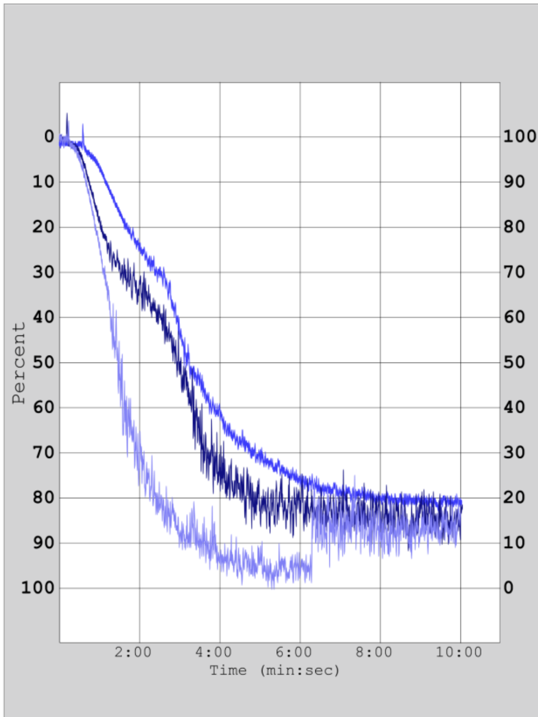
II



Collagen 2μg/mL

Collagen 2μg/mL + 3BP (50μM)

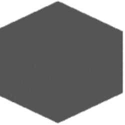
Collagen 2μg/mL + 3BP (100μM)

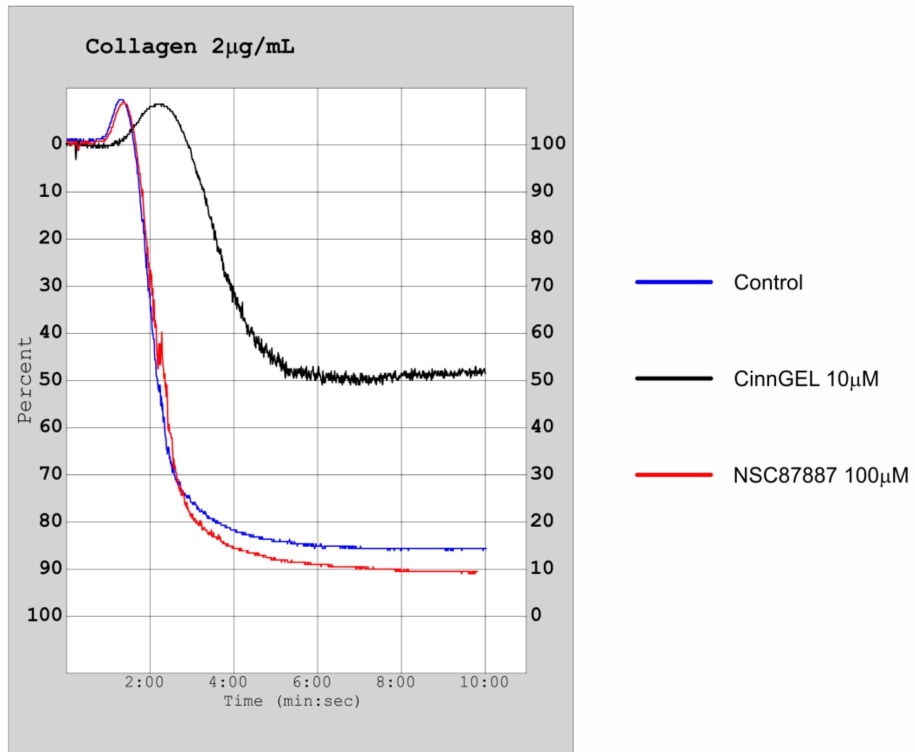


Ristocetin 1.25mg/mL

Ristocetin 1.25mg/mL + 3BP (50μM)

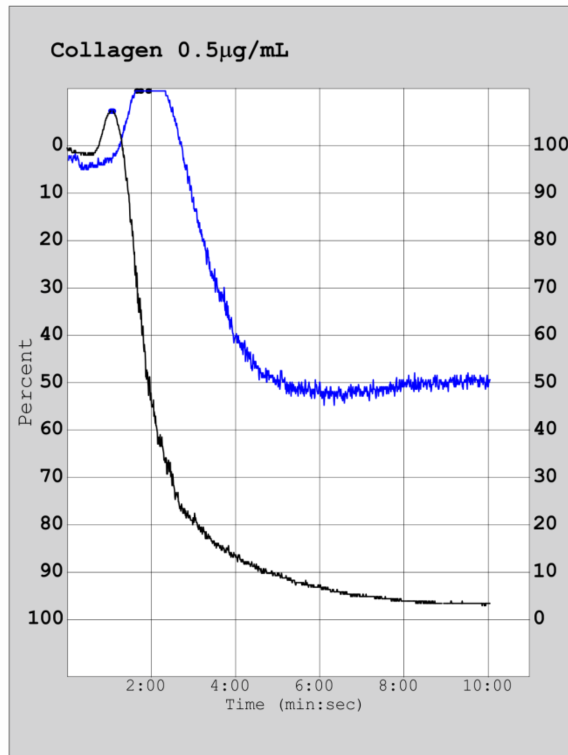
Ristocetin 1.25mg/mL + 3BP (100μM)

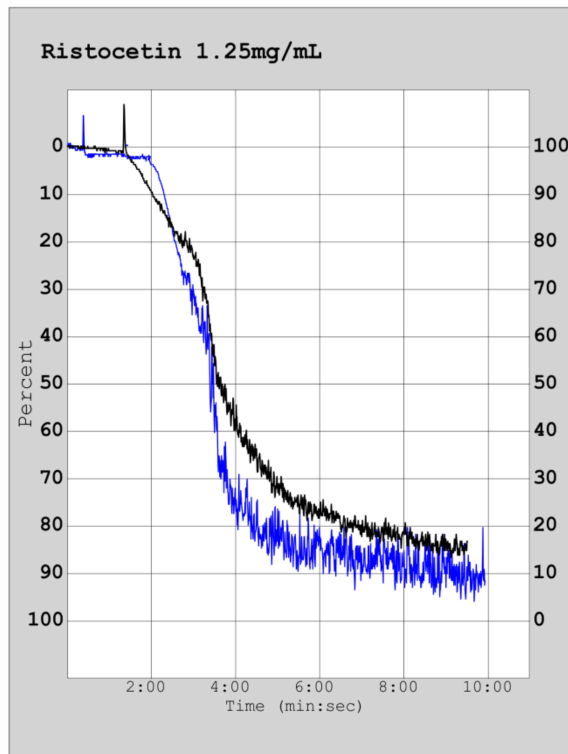


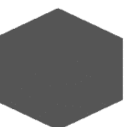


Collagen 2μg/mL
Percent
0
10
20
30
40
50
60
70
80
90
100
100
90
80
70
60
50
40
30
20
10
0
2:00
4:00
6:00
8:00
10:00
Time (min:sec)
Control
CinnGEL 10μM
NSC87887 100μM

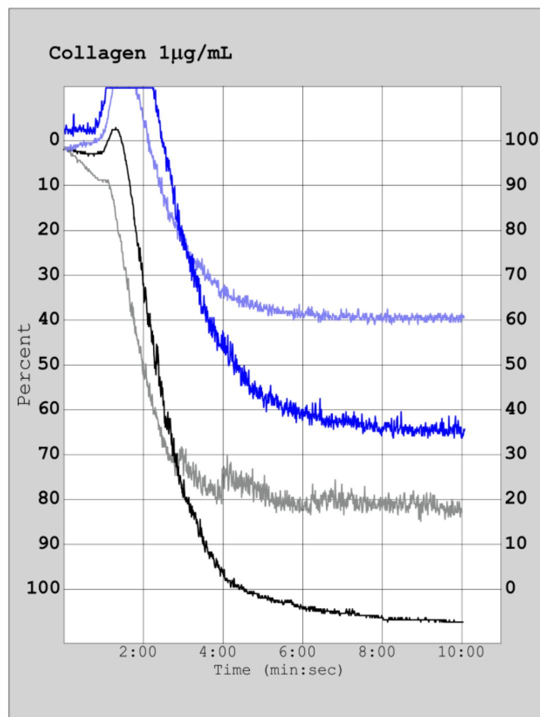
IV







V

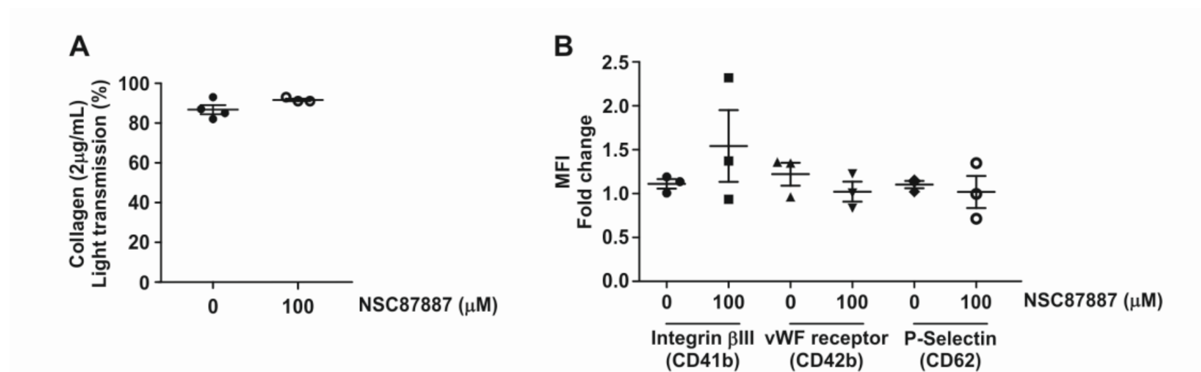


TEPH

TEPH + 3BP (100uM)

TEP

TEP + 3BP (100uM)



Supplemental Figure 2. Platelet function is not inhibited by selective SHP inhibitor. (A) Aggregation assays were performed for collagen-stimulated platelets after pretreatment with specific SHP inhibitor (NSC87887). (B) Cell surface expression of vWF-receptor (CD41-FITC), Integrinβ3 (CD42-PE) and P-Selectin (CD62-APC) was investigated on collagen-stimulated platelets that were pre-treated with specific SHP inhibitor (NSC87887).

Supplemental tables

Table 1. Western blot antibodies

Antibody	Company	Code
Acp1 α/β	Santa Cruz Biotechnologies	sc-100343
PTP1B	Santa Cruz Biotechnologies	sc-14021
βactin	Santa Cruz Biotechnologies	sc-47778
Phospho-Src family (Tyr416)	Cell Signaling	2101
Phospho-Src (Tyr572)	Cell Signaling	2105
Src	Cell Signaling	2123
Phospho-FAK (Tyr925)	SignalWay Antibodies	11123-2
FAK	Cell Signaling	3285S
Phospho-Integrin βIII (Tyr773)	SignalWay Antibodies	11060-1
Phospho-Erk (T202/Y204)	Cell Signaling	4370
Erk	Cell Signaling	4695 (137F5)
Phospho-p38 (T180/Y182)	Cell Signaling	4511
p38	Cell Signaling	9228
Caspase-3	Cell Signaling	9662
Cleaved Caspase-8	Cell Signaling	9496
Bax	Cell Signaling	2772
Bcl-2	Cell Signaling	2876

Table 2. Chemicals

Reagent	Company	Code
3-bromopyruvate	Sigma Aldrich	16490-10
CinnGel	Santa Cruz	205633
NSC87887	Merck Millipore	565851
Collagen	Chronolog	385
Ristocetin	Chronolog	396

Table 3. Cell line characteristics, as described in [1-9].

Cell line	HCT116	HT29	Caco-2
Patient	48-Year-old male	44-Year-old female	72-Year-old male
Organ	Colon ascendens	Colon	Colon
Disease	colorectal carcinoma	colorectal adenocarcinoma	colorectal adenocarcinoma
Stage	Dukes' D	Dukes' C	
Derived from	primary tumor	primary tumor	
MSI status	MSI	MSS	MSS
CIMP panel 1	+	+	+
CIMP panel 2	+	+	-
CIN	-	+	+
KRAS	G13D	WT	WT
BRAF	WT	V600E	WT
PIK3CA	H1047R	P449T	WT
PTEN	WT	WT	WT
TP53	WT	R273H	E204X

Abbreviations: CIN, chromosomal instability pathway; MSI, microsatellite instability; MSS, microsatellite stable; CIMP, CpG island methylator phenotype

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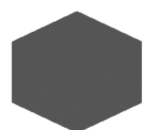
Author Contributions

Conceptualization, C.V.F.-H. and G.M.F.; Methodology, A.V.S.F., S.S.A., M.C.W.S., C.V.F.-H. and G.M.F.; Formal Analysis, A.V.S.F., S.S.A., C.V.F.H. and G.M.F.; Investigation, A.V.S.F., S.S.A., A.N.R.; Data Curation, A.V.S.F., C.V.F.-H. and G.M.F.; Writing—Original Draft Preparation, A.V.S.F.; Writing—Review & Editing, S.S.A., A.N.R., M.C.W.S., M.P.M.M., M.P.P., C.V.F.-H., G.M.F.; Supervision, M.P.P., C.V.F.-H., G.M.F.; Project Administration, A.V.S.F., M.P.P., C.V.F.-H., G.M.F.; Funding Acquisition, A.V.S.F. and C.V.F.-H. All authors read and approved the final manuscript.

Declaration of Interests

The authors declare no competing interests.



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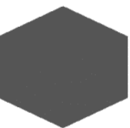

Chapter 9

Concluding remarks
Summary Discussion
Nederlandse Samenvatting
Acknowledgements
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Summary discussion

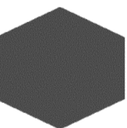
Phosphorylation is the most common post-translation modification and regulatory mechanism of proteins. Indeed, for many enzymes, phosphorylation leads to a reversible and fast modulation of enzymatic activity. Furthermore, protein stability, interactions, and subcellular localization are largely dependent on protein phosphorylation (Han et al, 2018; Torresano et al, 2020). As such, the phosphorylation system regulates cellular survival, proliferation, differentiation, metabolism, and cytoskeletal signaling (Noorolyai et al, 2019). The balance between kinases and phosphatases dictates the net cellular phosphor-signaling and has emerged as a potential target for overcoming many diseases, such as cancer, cardiovascular disease, and (auto)immune diseases (Kumar et al, 2007; Ferguson and Gray, 2018; Kenefick, 2019). The focus of this thesis was mainly on the role of this aspect of cellular signaling in cancer. While cancer treatment has improved over the last decades, so that overall cancer death rates are decreasing, this is not equally clear for all cancers. The 5-year survival of, for instance, prostate cancer has increased from 67.8% in 1990 to 98.6% in 2016. However, in particular, gastrointestinal cancers are still lethal. For instance, the 5-year survival rate for colorectal cancer improved only from 50% to 64%, gastric cancer from 15 to 30%, esophageal cancer from 5 to 19%, and pancreatic cancer from 2.5 to a dismal 8.2%. Thus, a better understanding of the molecular processes underlying carcinogenesis and finding novel targets for treatment is still warranted and phospho-signaling may provide such targets (Ritchie, 2019). Large-scale kinase activity assays are a valuable tool to discover such potential novel targets of treatment or to investigate why some treatments might fail.

Kinome profiling is a pan-kinase analysis tool to elucidate canonical and non-canonical pathways in cancer

A case in point was shown in **Chapter 2**, where we investigated the canonical and non-canonical pathways associated with Hedgehog-induced (Hh) signaling in normal mouse embryonic fibroblasts. Hh is important for organ formation during embryogenesis, and an adult, tissues seem in particular important for stem cell functions. Three Hh ligands are known in humans (Sonic Hh, Indian Hh, and Desert Hh), of which SHh is the best studied. All Hh members bind to the Patched receptor (Ptch), which under unbound conditions represses the activity of the G-protein coupled receptor Smoothened (Smo). Binding of Hh to Ptch releases this inhibition, allowing Smo to signal, resulting in activation of GLI transcription factors and transcription profiles favoring tumor cell migration, invasion, cell

cycle, and stem cell self-renewal. Besides the important effect during tissue development, dysregulated SHh signaling has been highlighted as a cancer hallmark in tumor growth and progression (Rimkus et al, 2016). Recurrence of cancer through remaining cancer stem cells remains a major problem in cancer treatment and targeting tumor cells as well as cancer stem cells with Hh inhibitors has been suggested (Sari et al, 2018). Cancer treatment to reduce activation of these transcription patterns center mostly around inhibition of Smo, which is supposed to completely switch off canonical SHh signaling. Nevertheless, cancer treatment with Smo inhibitors such as Vismodegib does not always have the efficacy which one might expect (Hansel et al, 2015). In part, this might be explained by the fact that patients who develop resistance to vismodigib may acquire mutations in Smo which renders it less susceptible to inhibitor binding (Atwood et al, 2015; Sharpe et al, 2015). However, we considered that it is also conceivable that alternative, non-canonical signaling of Hh may exist, which may account for resistance against Smo inhibitors, something which we investigated in Chapter 2. There, we showed that under SHh stimulation (Niyaz et al, 2019), kinome pathways associated with survival and cytoskeletal rearrangement were activated. However, upon either Smoothened knockdown or Vismodegib inhibitions of Smo, survival and cytoskeletal pathways were still activated, as were inflammatory and stemness pathways. These results demonstrate that non-canonical signaling based on kinase activity takes place downstream of Ptch, independent of Smo signaling. Interestingly, under SHh agonist (SAG) treatment, which triggers Smo activity independent of Ptch, survival pathways were not activated, while only cytoskeletal pathways were positively modulated. Taking all these findings together, independent Ptch-dependent and Smo-dependent signaling exist, but cytoskeletal remodeling pathways were activated under all treatment conditions tested. Perhaps this finding is not surprising, as cell migration, which is dependent on cytoskeletal remodeling, is important for homeostasis and regeneration, especially for building tissues and organs during embryonic development (Te Boekhorst and Friedl, 2016).

Perhaps the most notable finding of this study was that the SHh inhibitor treatment (vismodegib) was not able to negatively modulate survival signaling. Thus, despite the main effect of Smo inhibitors on canonical pathways, the associated receptor or down-stream kinases can be still activated and maintain signaling commonly related to cancer malignancy. Indeed, in the presence of vismodegib, SHh-induced Patched-dependent Smoothened-independent signaling kept cytoskeletal remodeling and Wnt pathways activated, potentially compromising the anti-cancer effect of this drug. For instance, cell motility activation is associated with metastasis, which in turn is the cause of 90% of cancer deaths (Chaffer and



Weinberg, 2015). Our results suggest that co-treatment with kinase inhibitors may provide benefit by targeting the non-canonical pathways activated by SHh. Since kinases have been highlighted to play a major role in cancer, including in therapeutic resistance, several chemotherapeutic drugs were already developed to target kinases, such as Erlotinib targeting EGFR, Trametinib targeting MEK1/2, Dasatinib targeting Src kinases (Bhullar et al, 2018). It would be of interest to see whether kinase inhibitors targeting non-canonical kinase signaling induced by SHh may be of benefit.

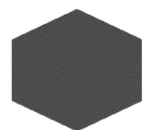
The data described in **Chapter 2** provide an example of how, using a pan-kinase analysis tool, canonical and non-canonical pathways associated with specific signals and/or ligands can be investigated, including potential targets for overcome cancer. Using the same pan-kinase analysis, in **Chapter 3**, we turned our attention to drug resistance and compared the kinome profile from two chronic myeloid leukemia cell lines: the chemotherapy-sensitive K562 cell line and its chemoresistant counterpart Lucena-1. Previous data using western blot analysis of individual proteins has shown that Src and BcrAbl were overexpressed/overactivated in Lucena-1, as well as, STAT signaling (Ferreira et al, 2012; Mencalha et al, 2014). In **Chapter 3**, we aimed for a broader view of signaling changes between these two lines. Interestingly, by employing kinome profiling, it was possible to track signaling differences between chemosensitive and chemoresistant hematological cancer cells to metabolic changes. Since Warburg in 1956 described the importance of mitochondria metabolism in cancer, alternate energy metabolism has been highlighted as a cancer hallmark (Warburg, 1956; Hanahan and Weinberg, 2011). The Warburg postulation describes how tumor cells show excessive glucose uptake, which is converted to lactate through aerobic glycolysis. In this case, the tumor glucose metabolism provides energy by glycolysis, even in the presence of sufficient oxygen, rather than oxidative-phosphorylation via mitochondria. While glycolysis is faster as compared to oxidative phosphorylation (Zheng et al, 2012), it also results in less ATP production, thus the exact benefit to the tumor cell remains debated (Liberti et al, 2016). Reasons may include the fact that tumor cells may be more often oxygen-deprived and have quicker growth rates. However, the pathway might also be deflected to biomolecule production, for instance, to nucleotides and reducing agents required for tumor growth (Schwartz et al, 2017). Our data indicate that drug-resistant Lucena-1 cells show a higher lactate production and lower mitochondria function. Furthermore, it was observed that Lucena-1 cells were less sensitive to treatment with oxidants. Changes in glucose metabolism have been highlighted as a cancer hallmark, the cancer cell addiction to glycolysis playing a

role in two important cell events: (i) crosstalk between pentose phosphate pathway predominance flux, in the end, to favor the synthesis of biomolecules; (ii) and lower mitochondria function, which directly affect the ROS production by OXPHOS (Amoedo et al, 2017). Several glycolysis-kinases over-activated in Lucena-1 are associated with chemoresistance: Lactate Dehydrogenase, Hexokinase, and mTOR (Zhao et al, 2013), suggesting that these processes may be functionally linked. Thus, kinome profiling in this case unexpectedly provided evidence for altered metabolic signaling and provides potential targets for interfering with this cancer hallmark.

Novel contributions of LMWPTP to cancer hallmarks

Interestingly, the chemoresistant Lucena-1 cell line has higher LMWPTP expression and Src kinase activity as compared to its K652 counterpart, which directly contributes to its drug resistance (Ferreira et al, 2012). Besides several contributions of kinases in cancer, a contribution of phosphatases to cancer progression, especially tyrosine phosphatases, has emerged during the last decades. Our research group has been investigating the PTPs as potential targets and biomarkers for gastrointestinal cancer (Hoekstra et al, 2015; Hoekstra et al, 2016) as well as other cancers (Ruela-de-Sousa et al, 2016; this thesis). A role for LMWPTP in tumor proliferation, migration, and patient survival is emerging. In **Chapter 3**, we demonstrate for the first time the association between LMWPTP expression and activation of glycolytic metabolism in cancer. As a proof-of-concept, using transient LWMPTP transfection (silencing or overexpression), it was observed that maintenance of a high glycolytic profile was dependent on higher expression of this phosphatase.

Based on this, in **Chapter 4**, we further explored the role of LMWPTP in the metabolic processes and resistance to environmental stresses in the chronic myeloid leukemia cells model. Autophagy is one mechanism of cell fate regulation based on the sequestering of molecules and their degradation. Autophagy plays a dual role in cancer. While it can: promoting cell survival by removing damaged organelles and proteins and promoting cell proliferation by drug neutralization and controlling metabolic stress, prolonged autophagy may lead to a 'point of no return' at which point ongoing cellular degradation switches to cell death (Yang et al, 2011; Lauzier et al, 2019; Li et al, 2020; Mulcahy Levy and Thorburn, 2020). Furthermore, low O_2 concentration is linked to a metabolic switch from oxidative metabolism to glycolysis (Ianniciello et al, 2017). Autophagy and ROS accumulation act as a differentiation promoter in CML affecting directly chemotherapeutic responsiveness (Colosetti et al, 2009; Carrett-Dias et al, 2016; Baquero et al, 2018). The gold-standard CML



treatment, imatinib, was described to induce autophagy as part of its mechanism of action (Ertmer et al, 2007). We showed that LMWPTP is also linked to autophagy regulation as a primary response under antioxidant exposure. Under basal conditions, autophagy was down-modulated in Lucena-1 cells compared to K562. However, under hydrogen peroxide exposure or when LMWPTP expression was reduced, the autophagy process is activated to control the excess of ROS, and it is conceivable that this also contributes to the drug resistance profile of this cell line. In cumulus cells, autophagy is also required to guarding against oxidative stress and it is dependent on DUSP1 expression (Fu et al, 2019).

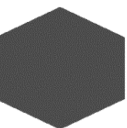
Thus, these data have demonstrated that LMWPTP plays a versatile role in cancer, contributing to many cancer hallmarks, including glycolysis and modulation of cellular stress pathways. A role for phosphatases in cancer is slowly being extended to several different enzymes and different processes (see Hoekstra et al, 2012; Ruela-de-Sousa et al, 2010). For instance, PTP1B also plays an important role in cancer hallmarks. The higher expression of PTP1B was associated with higher cancer cell migration in CRC, and to the invasion process in esophageal squamous cell carcinoma (Wang et al, 2012; Hoekstra et al, 2016). Furthermore, SHP-2 can also be a cancer predictor, and SHP-2 overexpression leads to cell migration (Han et al, 2015). Despite the different phosphatase contributions on several processes, most of them appear to play a role in cancer progression.

Tumor and platelets: reciprocal actions in the pre-metastatic niche

Including the work presented in **Chapters 3** and **4**, our laboratories have spent considerable effort trying to elucidate the effect of LMWPTP on cancer properties. However, cancer cells are not solitary - the tumor microenvironment provides support for cancer progression by supplying a metabolic network, including nutrients, extracellular matrix (ECM) components, and immune cells (Doglioni et al, 2019). While the role of individual ECM components on cancer progression is relatively well described (Henke et al, 2019), a new interactive player has emerged which deserves more attention: platelets. Platelets are the mandatory element for the thrombosis process; however, this anucleated blood component also participates in immunologic and inflammatory responses (Morrell et al, 2014; Koupenova et al, 2018). Normal platelet function might be disturbed under several conditions, leading to cardiovascular complications such as venous thromboembolism (VTE). The aging process has been highlighted as a physiological cause for platelet functionality changes. Cancer and cardiovascular comorbidities are age-associated diseases that directly affect platelet function. Indeed, enhanced risk of VTE is seen in the elderly, but also cancer patients. To what extent

VTE risk is related to underlying diseases and whether platelet function in itself is altered by the aging process was investigated in **Chapter 5**. We reviewed the current literature on platelet alterations in healthy aging, discussing their contribution to hemostasis and immunity. We conclude that while studies vary considerably in terms of methodology and age-groups studied, the overall picture emerging is that while platelet count is decreased in the elderly, their reactivity towards agonists and general activation pattern appears to be increased, and differences start to become more prominent in the 60-80 year age-group. Mechanistically, inflammatory processes taking place during aging (e.g. TNFα) appear to age megakaryocytes, inducing them to release hyper-activated platelets (Davizon-Castillo et al, 2019). Thus, finding safe and affordable ways to interfere with these inflammatory pathways may in the future perhaps contribute to healthy aging.

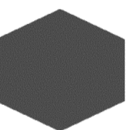
We go on to discuss the changes in platelets that may account for their association with cancer. Tumor cells induce several platelet modifications and can alter their intracellular content and function (Menter et al, 2014), which may contribute to increased VTE risk seen in cancer. Importantly, some studies have shown that this is independent of aging, suggesting that cancer itself is the primary trigger for VTE risk in this case (Heit, 2015). Conversely, platelets, and the growth factors they release may trigger tumor cell proliferation and metastasis, with extravasation of tumor cells presenting an additional risk to VTE development. In this direction, platelets are part of the tumor microenvironment and also might support cancer progression. Platelets can facilitate cancer cell proliferation and invasion. Platelets might improve vascularization, which directly provides nutrient and oxygen supply for cancer growth (Pietramaggiori et al, 2008). In **Chapter 6** we further investigate the interaction between tumor cells and platelets and investigate the molecular mechanisms involved. In particular, we wondered whether LMWPTP in tumor cells, which we had already shown to be important for several oncogenic properties, would also mediate the tumor-platelet interactions. In **Chapter 6**, we first demonstrate that LMWPTP is overexpressed in the stomach and esophageal cancer, as well as colorectal cancer, suggesting that this is a common defect seen in gastrointestinal cancer. When investigating kinase activation in gastric cancer cells with high LMWPTP expression compared to non-transformed gastric cells with lower LMWPTP expression levels, we observed higher Src and FAK activation in the stomach cancer cell line. These findings were supported by previous studies from our research group (Zambuzzi et al, 2008; Ferreira et al, 2012), showing the importance and consistency of the data. We went on to study the importance of platelets on tumor progression. While we observed no stimulation by platelets of normal cell line



proliferation, stomach tumor cells presented higher proliferation rates, as well as activation of Src and p38, in the presence of platelets. Similarly, CRC cell lines also showed enhanced proliferation in the presence of platelets. Using various knockdown models, we went on to demonstrate that LMWPTP plays an important contribution in this process: knockdown of LMWPTP reduced cancer cell interaction with platelets, as well as platelet-mediated proliferation effects. Taking this together, platelets and tumors directly improve their functions by establishing supporting networks: tumor cells might induce platelet hyper-activation, but also platelets, in turn, improve tumor growth and local migration by factors releasing, events which are dependent on LMWPTP expression. It is of interest to note that our data also indicated that LMWPTP itself was upregulated in tumor cells upon interaction with platelets, suggesting a positive feedback loop.

Having investigated the molecular signaling events in cancer cells contributing to platelet-mediated oncogenic stimulation, we next wondered to what extent signaling in platelets themselves might be contributing to this interaction. To this end, we first reviewed contemporary literature on signaling in platelets, with particular emphasis on the role of phosphatases in platelet activation. In **Chapter 7**, we show that while the presence of several phosphatases has been shown in platelets, their role in platelet functions remains relatively poorly understood, in particular in comparison to kinases. For this reason, we turned our attention to phosphatase signaling in platelets in **Chapter 8**. We demonstrated that under collagen stimulation, both LMWPTP and PTP1B were activated in platelets, while ristocetin only activated PTP1B. These data show the selectivity of phosphatase signaling under different conditions. Further, Src kinase was identified as an important player in platelet hyper-reactivity using collagen as an agonist. Using a phosphatase inhibitor, the pyruvate analog 3-bromopyruvate (3-BP), decreases platelet function on the molecular level, by phosphatase and Src kinase family inhibition, as well as on cellular level, by decreasing platelet surface activation markers, as Integrin βIII and P-Selectin expression. We next investigated the molecular mechanisms of platelet-tumor cell interactions and showed that cancer cells were able to enhance platelet function under collagen stimulation, suggesting the participation of kinases and phosphatases on this process. Indeed, either collagen or colorectal cancer (CRC) cells induced platelet aggregation and expression of activation markers, and this interaction was susceptible to 3-BP targeting. In a proof-of-concept experiment, platelets from cancer patients were shown to be more responsive to collagen activation compared to normal platelets, and 3-BP disturbed this platelet hyper-functionality. Of note, co-culture between platelets and cancer cells increased LMWPTP expression in platelets, and a higher LMWPTP

expression in patients' platelets was seen compared to healthy control platelets, confirming previous data that platelets may be 'educated' by cancer cells (Best et al, 2015). In summary, kinases and phosphatases, especially LMWPTP, play an important role in platelets in health and disease. In particular, in the cancer setting, LMWPTP appears to play a central role. This enzyme appears to be upregulated in both tumor cells and platelets in cancer, with these cell types further reciprocally increasing LMWPTP expression. With this enhanced expression subsequently activating both platelets and propagating (platelet-mediated) oncogenic properties in tumor cells, this phosphatase might present an interesting target to disrupt both VTE risk in cancer as well as cancer growth *per se*. Additional targets are needed, as targeting kinases in cancer may result in bleeding by affecting platelets (Tullemans et al, 2018). Overcoming platelet-hyperreactivity in cancer without provoking bleeding requires a careful approach.



Future perspectives

Protein tyrosine kinases and phosphatases have been investigated as major players on cancer hallmarks. Indeed, several cell biology events associated with proliferation and motility are associated with FAK/Src and LMWPTP function. With our studies indicating that non-canonical signaling and drug resistance are dependent on kinase signaling, making better use of available kinase inhibitors may provide a step forward in the treatment of cancer. On the other hand, we also demonstrate that a misbalance over-activating LMWPTP leads to improved cancer plasticity sustaining proliferative signaling, resisting cell death, deregulating cellular energetics, avoiding immune destruction, and activating invasion and metastasis. Investigation of the contribution of tyrosine phosphatases in other tissues and organs will likely emphasize their importance in diverse diseases. The next step may, therefore, be to focus on the development of better inhibitors of phosphatases. Kinases are not only more widely studies in disease states, the development of inhibitors for this class of enzymes is also facilitated by the fact that they all contain an ATP binding pocket and a relatively high selectivity of the catalytic domain for peptide substrates. Many kinase inhibitors thus are ATP analogs (Lazo et al, 2017). Unfortunately, the search for phosphatase inhibitors is limited by several factors, including lower selectivity and bioavailability (De Munter et al, 2013). Protein tyrosine phosphatases are characterized by the PTP motif: (V/H)CX5R (Stanford and Bottini, 2017), which makes it so complex to evaluate appropriate and selective inhibitors. Nevertheless, with more and more evidence suggesting that phosphatases play a stronger role in carcinogenesis than previously anticipated and current medicine still losing the battle against cancer, exploring this class of enzymes as druggable targets could present a step forward in the treatment of these as yet lethal and debilitating diseases.

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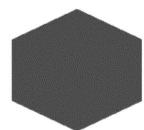

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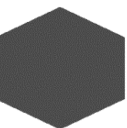
Nederlandse samenvatting

Eiwit fosforylering is een van de meest voorkomende post-translationele modificaties. Dit proces is essentieel voor het moduleren van enzymatische activiteit van eiwitten, hun stabiliteit, hun interacties met andere moleculen en hun sub-cellulaire lokalisatie. Als cruciaal onderdeel van het doorgeven van omgevingssignalen binnen in de cel, de zogenaamde signaal transductie, is fosforylering van eiwitten dan ook essentieel voor alle functies en processen die een cel uit moet voeren – van cel deling, overleving en migratie tot productie van voor het lichaam essentiële stoffen. Eiwit fosforylering in de cel wordt bepaald door de acties van twee verschillende types van enzymen – de kinasen, die in staat zijn een fosfaatgroep aan een eiwit te bevestigen, en de fosfatasen, die fosfaatgroepen van eiwitten kunnen verwijderen. Een onbalans tussen deze twee klassen van enzymen en de daarmee gepaard gaande verstoring van intracellulaire fosforyleringspatronen kan dan ook verregaande gevolgen hebben. Verschillende ziektebeelden zijn geassocieerd met verstoorde activatie van kinasen en fosfatasen. In dit proefschrift heb ik mij met name gericht op de verstoorde signaal transductie die kenmerkend is voor tumor cellen. De belangrijkste signaleringsroutes zoals deze nu bekend zijn in kanker worden beschreven in **Hoofdstuk 1**. Hoewel de behandeling van kanker in de afgelopen jaren wel verbeterd is, blijft deze ziekte nog steeds voor het overgrote deel ongeneselijk, en vaak zelfs dodelijk. Een beter inzicht in de verstoring van intracellulaire signaleringsroutes in de cel zou mogelijk kunnen bijdragen tot het identificeren van potentiële aangrijpingspunten voor nieuwe medicatie. Een van de methodes die hiervoor bij uitstek geschikt is, is het zogenaamde 'kinome-profiling', het op grote schaal bekijken van de activiteit van vele verschillende kinasen in een cel-lysaat. Met deze techniek zou theoretisch in 1 oogopslag kunnen worden gezien welke kinases een belangrijke rol vervullen in het oncogene proces, alsmede waarom tumor cellen in sommige gevallen niet reageren op behandeling met medicatie of resistent worden tegen zulke medicatie.

Een goed voorbeeld hiervan laten we zien in **Hoofdstuk 2**. In dit hoofdstuk onderzoeken we hoe het kan dat in sommige gevallen kankerbehandeling met de drug Vismodigib niet werkt. Vismodigib blokkeert signalering die aangezet wordt door de stof sonic Hedgehog (SHh). SHh is erg belangrijk voor het in stand houden van stamcellen, en speelt daarom een belangrijke rol in de embryogenese maar ook in volwassen weefsels die afhankelijk zijn van stamcellen voor hun vernieuwing. In verschillende tumoren is aangetoond dat SHh signalering overactief is, waardoor de gedachte is ontstaan dat remming van dit signaal mogelijk kan bijdragen aan de behandeling van deze kankers. Desondanks is remming van



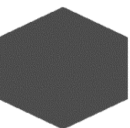
SHh signalering met onder andere Vismodigib toch niet altijd succesvol gebleken. Indien er geen SHh aanwezig is, wordt signalering actief geremd doordat de receptor Ptched (Ptch) de receptor smoothened (Smo) blokkeert. Binding van SHh aan Ptch heft deze remming op, waardoor Smo in staat is gen transcriptie patronen te activeren die leiden tot stamcel activatie. Deze cascade van reacties wordt gezien als de bekendste signalering via SHh. In **Hoofdstuk 2** laten wij echter zien dat SHh ook kinase signalering aanzet, en dat Ptch en Smo allebei verschillende kinase patronen activeren. Behandeling van cellen met Vismodigib, dat specifiek de bekende signalering via Smo blokkeert, remt niet alle kinase activatie patronen die door SHh in gang worden gezet. De kinase patronen die nog doorkomen zijn vaak ook betrokken bij kankerceldeling. Dit zou een mogelijke verklaring kunnen zijn voor het feit dat tumoren met over-activatie van SHh signalering niet altijd goed reageren op behandeling met vismodigib,

Een tweede voorbeeld waarin we laten zien dat kinome profiling behulpzaam kan zijn bij de opsporing van resistentiemechanismen van tumoren komt naar voren in **Hoofdstuk 3**. In dit hoofdstuk onderzochten we de verschillen in kinase activatie patronen tussen de drug-gevoelige leukemie cellijn K562, en een drug-resistente afgeleide cellijn hiervan, Lucena-1. Opmerkelijk genoeg vonden we een kinase patroon dat duidt op veranderingen in energiehuishouding in Lucena-1 cellen. Vervolgexperimenten lieten inderdaad zien dat mitochondriële functie in Lucena-1 cellen verstoord is, en dat deze cellen meer lactaat produceren. Dat wijst erop dat deze cellen in plaats van normale energieproductie via mitochondriële oxidatieve fosforylering zijn overgeschakeld op glycolyse, een sneller proces om energie te genereren. Een dergelijke omschakeling wordt vaker gezien in tumor cellen, en wordt ook wel het Warburg effect genoemd, naar de ontdekker hiervan. Dit is echter de eerste keer dat dit aangetoond wordt in de context van drug-resistentie, en de daadwerkelijke link tussen deze twee processen behoeft dan ook verdere aandacht.

Eerder heeft onze onderzoeksgroep laten zien dat Lucena-1 cellen een verhoogde expressie hebben van de fosfatase LMWPTP, en dat dit bijdraagt aan de drug resistente eigenschappen van deze cel lijn. Om te kijken of LMWPTP ook bijdraagt aan de switch van mitochondriële oxidatieve fosforylering naar glycolyse werd dit enzym uitgeschakeld in Lucena-1 cellen. Deze proeven toonden aan dat LMWPTP inderdaad gedeeltelijk rechtstreeks bijdraagt aan dit proces. Dit lijkt wellicht tegenstrijdig, wetende dat activatie van fosforyleringspatronen werden gezien middels kinome profiling. Echter fosforylering van eiwitten kan zowel activerende als inactiverende functies hebben, en het verwijderen van remmende

fosforyleringspatronen door fosfatasen, waaronder LMWPTP, kan derhalve bijdragen tot stimulering van kankerprocessen. Dit concept is echter relatief nieuw, en hoewel kinasen in het kankeronderzoek duidelijk verankerd zijn, wordt er nog relatief weinig gekeken naar de fosfatasen. In **Hoofdstuk 4** hebben wij getracht meer inzicht te verkrijgen in de rol van LMWPTP in cellulaire kankerprocessen, en laten ze zien dat deze een belangrijke rol speelt in hoe een tumor cel omgaat met de cellulaire stresscondities.

Hoewel een stimulerende rol voor LMWPTP in kankercellen langzamerhand duidelijk wordt (o.a. **Hoofdstuk 3** en **4** en eerder onderzoek uit onze groep heeft laten zien dat LMWPTP maligniteit van kankercellen versterkt), staan kankercellen niet op zichzelf in het menselijk lichaam. Ze zijn verankerd in een extracellulaire matrix en ontvangen veel signalen uit hun omgeving, van andere cellen (o.a. immuun cellen) alsmede signaleringsstoffen die worden vrijgegeven door andere cellen. Recentelijk is duidelijk geworden dat bloedplaatjes een interessante interactie kunnen hebben met tumor cellen. Bloedplaatjes zijn in staat om tumor cellen aan te zetten tot celdeling, en kunnen helpen bij de metastasering van tumor cellen via de bloedbaan. Omgekeerd kunnen tumor cellen bloedplaatjes activeren. Aangezien de belangrijkste fysiologische functie van bloedplaatjes is het helpen stollen van bloed bij een verwonding, kan tumorcel gemedieerde activering van bloedplaatjes leiden tot het ontstaan van bloedpropjes in de bloedbaan, en trombose is dan ook een veelvoorkomende serieuze complicatie van kanker. Trombose wordt echter ook vaker gezien op hogere leeftijd, en aangezien kanker ook een ouderdomsziekte is, is het mogelijk dat veroudering bijdraagt aan veranderingen in bloedplaatjes die vervolgens hun interactie met tumor cellen versterkt. In **Hoofdstuk 5** hebben we onderzocht of publicaties in de literatuur deze aannames staven. We concludeerden dat het verouderingsproces inderdaad resulteert in verhoogde mate van activatie van bloedplaatjes. Echter tumorcellen zelf hebben een direct activerend effect op bloedplaatjes dat onafhankelijk is van leeftijd. Inzicht in de moleculaire processen die bijdragen aan de interactie tussen bloedplaatjes en kankercellen kan mogelijk resulteren in de ontwikkeling van nieuwe therapieën ter bestrijding van zowel trombose in kankerpatiënten alsmede bloedplaatjes-gemedieerde kankerceldeling en metastasering. In **Hoofdstuk 6** hebben we daarom gekeken naar de signaaltransductie routes die worden aangezet in kankercellen door bloedplaatjes. Hierbij zagen we activatie van kinases, maar ook een op-regulatie van de fosfatase LMWPTP wanneer maag- of-darmkanker cellen werden gekweekt in de aanwezigheid van bloedplaatjes. Aangezien LMWPTP inderdaad verhoogd aanwezig is in tumoren van maag- en darm kankerpatiënten, lijkt dit ook klinisch relevant te zijn. In



celkweekexperimenten bleken bloedplaatjes in staat te zijn de celgroei van maag- en -darmkanker cellen te stimuleren, en dit effect werd significant verminderd indien we LMWPTP expressie genetisch verminderden in de tumorcellen.

Deze resultaten tonen duidelijk aan dat LMWPTP expressie in tumor cellen bijdraagt aan oncogene eigenschappen die door bloedplaatjes worden doorgegeven, maar zeggen niets over de signaaltransductie in de bloedplaatjes zelf. Om hier een beter inzicht in te krijgen hebben we in **Hoofdstuk 7** de literatuur onderzocht om inzicht te krijgen in wat er bekend was over signalering in bloedplaatjes. We concludeerden dat hoewel er veel informatie beschikbaar is over de kinasen die geactiveerd worden door de verschillende receptoren die bloedplaatjes bezitten, er maar relatief weinig bekend is over de rol van fosfatasen in activatie van bloedplaatjes. Om deze reden onderzochten we in **Hoofdstuk 8** of fosfatasen ook actief zijn in bloedplaatjes. We toonden voor het eerst aan dat LMWPTP, maar ook de fosfatase PTP1B, geactiveerd kunnen worden in bloedplaatjes onder invloed van verschillende stimuli. Een remmer van fosfatase activiteit, 3-bromopyuvate (3-BP), blokkeerde de functie van bloedplaatjes op moleculair niveau (remming van kinase activiteit) alsmede cellulair niveau (remming van activatie en aggregatie). We toonden aan dat incubatie van bloedplaatjes met tumor cellen resulteerde in een verhoogde mate van signalering en aggregatie van bloedplaatjes, en dat dit geremd kon worden met 3-BP. Opmerkelijk genoeg leek het incuberen van bloedplaatjes met tumor cellen ook te leiden tot een verhoging van LMWPTP in bloedplaatjes in vitro, iets wat we in een kleine steekproef ook bij kankerpatiënten zagen. Al met al lijkt de rol van LMWPTP in de interactie tussen tumor cellen en bloedplaatjes dus sterk verankerd te zijn in allebei de celtypes. Mogelijk kan remming van fosfatasen in de toekomst bijdragen aan vermindering van het risico op trombose, alsmede een betere bestrijding van tumoren.

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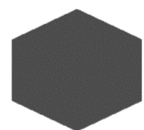

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- Mutagen Subject "Topics of DNA metabolism and genotoxicology". XII Mutagen-Brasil Congress. Jan/2016
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- Understanding Cancer Metastasis. Johns Hopkins University. On-line. Coursera. Jun/2017.
- Advanced Mass Spectrometry-based proteomics for Post-Translational Modifications (PTMs) Analysis. 46a. Annual Meeting of Brazilian Society for Biochemistry and Molecular Biology (SBBq). Jul/2017.
- A short course in epigenomics – Institute of Biology/University of Campinas. 12 hours. Jul/2019.

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- I Simpósio Iberoamericano de Investigação em Câncer. Sep/2013.
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  Brazilian Society for Biochemistry and Molecular Biology (SBBq)
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- 12th International Conference of Pharmaceutical Sciences. FCFRP – USP. 2019.
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  The European Association for Cancer Research

Poster presentations:

- **Faria AVS**, Tornatore TF, Ferreira CV, “Relationship between chemoresistant cells of chronic myeloid leukemia and protein kinases”, 2014. In: II Workshop do Programa de Pós-Graduação em Biologia Funcional e Molecular, Campinas – SP. Aug/2014.
- **Faria AVS**, Tornatore TF, Ferreira CV, “Biologia da resistência de células leucêmicas resistentes a quimioterápicos sob o aspecto de proteínas quinases”, 2014. In: XXII Congresso de Iniciação Científica da UNICAMP, Campinas – SP. Oct/2014.
- **Faria AVS**, Tornatore TF, Milani R, Queiroz KCS, Sampaio IH, Azoubel S, Fonseca EMB, Rocha-Brito KJP, Silveira LR, Peppelenbosch MP, Ferreira-Halder CV, “Oncophosphosignaling supports a glycolytic switch in drug resistance leucemia”, 2016. In: 2nd International Symposium on Inflammatory Diseases, Ribeirão Preto – SP. Jun/2016.
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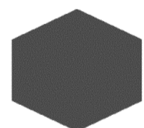

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