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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, some members of the protein 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 the level of this phosphatase may be further augmented in the presence of platelets. We demonstrate that tumor-platelet interaction promotes GI tumor cell proliferation. Additionally, using know-down/-out models we show that LMWPTP expression in cancer cells contributes to a more efficient interaction with platelets and drives platelet-induced proliferation. These data are the first to demonstrate that phosphatases play a positive role in the tumor-promoting activities of platelets, with LMWPTP emerging as a key player promoting oncogenic phenotypic changes in tumor cells.

1. Introduction

Over the last few years, an important role for platelets in cancer progression has emerged. Platelets can interact with cancer cells, either via direct contact, or via released factors such as growth factors or extracellular vesicles [1–3]. The ensuing bi-directional interaction can have several tumor-promoting effects. With regards to cancer cells, 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 them to escape immunosurveillance through shielding of tumor antigens and platelet-induced shedding of immune cell ligands from the tumor cell surface [4]. Secondly, owing to their growth factor content, platelets can directly stimulate cancer growth [5,6]. For instance, co-culture of ovarian cancer cells with platelets increases tumor cell proliferation via binding of Transforming Growth Factor β (TGFβ) released by platelets to its receptor present on tumor cells [7]. Thirdly, a role for platelets in metastasis and cancer angiogenesis has thus far been demonstrated for breast, ovarian and prostate cancers, through either direct or indirect interactions [6,8,9]. Thus, it is clear that tumor cells may derive beneficial effects from their interaction with platelets. Conversely, platelets are also affected by this bidirectional interaction. The term 'tumor-educated platelets' has been coined to denote the molecular changes present in platelets from cancer patients [10], and tumor cells may cause Tumor Cell-Induced Platelet Aggregation (TCIPA) which is thought to play a role in the increased venous

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Table 1
Antibodies used in the study, their origin and catalog number.

Antibody	Company	Catalog number
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
CD42b	Abcam	ab183345
Anti-rabbit IRDye 800CW	Odyssey	926–32211
Anti-mouse IRDye 680RD	Odyssey	926-68070

thromboembolism (VTE) risk seen in cancer patients [11].

While the exact molecular mechanisms governing platelet-tumor cell interactions are still unclear, the receptors contributing to this interaction are relatively well described. For example, platelet receptors associated with agonist signaling, such as PARs, GPVI, integrins [12], as well as P-Selectin, play a role in cancer cell-platelet interaction [2,3]. The downstream signaling effects induced by these interactions are less well described. Nevertheless, an essential role for lipid and protein kinases in oncogenic signaling in tumor cells is evident [13], with many of these, predominantly protein tyrosine kinases, now being investigated as targets for treatment [14]. Over the past decade it has become clear that their enzymatic counterparts, the phosphatases, also contribute to tumor progression [15]. However, 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 [16–18], where it contributes to tumor cell invasiveness and chemotherapy resistance [19,20]. In addition, we showed that LMWPTP expression follows a stepwise increase through different levels of dysplasia in colorectal cancer (CRC) [18]. 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 positive 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 provide a novel avenue of investigation for treatment of gastrointestinal cancers.

2. Material and methods

2.1. Antibodies and reagents

Antibodies were purchased from Santa Cruz (USA), Cell Signaling Technology (USA) or SignalWay (USA). For details, see Table 1. Other reagents and materials were purchased from Sigma Aldrich (USA), Santa Cruz (USA), Merck (USA), Millipore (USA), Lonza (USA and Switzerland), Life Technologies (The Netherlands), Promega (USA), Qiagen (Germany), Dako (Belgium), Abcam (UK), Becton Dickinson (BD, USA), Chrono-Log Corp., (USA), ThermoFischer Scientific (USA), Merck chemicals BV (Germany), LI-COR Biosciences (USA), Nano3D Biosciences (USA), Greiner Bio-One (Brazil). Equipements from BioRad (USA), Etaluma Inc. (USA), LI-COR Biosciences (USA), ThermoFischer Scientific (USA), Leica (Germany).

2.2. Cell culture

CRC cell (HCT116) was routinely grown in McCoy 5A culture medium (Lonza). GES-1, HCT116 shScramble, HCT116 shLMWPTP, Caco-2 shScramble, Caco-2 shLMWPTP, HT29 wild type (HT29 WT) and HT29 LMWPTP knock-out (HT29 LMWPTP KO) were routinely grown in Dulbecco's Modified Eagles Medium (DMEM, Lonza), 23132/87, Kato III wild type (KatoIII WT) and Kato III LMWPTP knock-out (KatoIII KO) cells were routinely grown in Roswell Park Memorial Institute medium (RPMI1640, Lonza). All cell lines were supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich), and 1% 100 U/mL penicillin, 100 μg/mL streptomycin (Pen-Strep, Life Technologies) 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 (Brazil). HCT116 and Caco-2 (shScramble and shLMWPTP) cells were generated and described by Hoekstra [18]. HT29 WT, HT29 LMWPTP KO, KatoIII WT and KatoIII LMWPTP KO 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 48 h, 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. For more details about cell line information, see Supplementary Tables S1 and S2.

2.3. Patient information

Blood was obtained at diagnosis from three patients suffering from colorectal cancer, 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). All cancer patients were gender- and age-matched to a healthy control.

2.4. Immunohistochemistry

Immunohistochemistry was performed as described before [18]. Briefly, 5 μm 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 min. Endogenous peroxidases were blocked by immersing the slides for 10 min in 3% H_2O_2 in phosphate buffered saline (PBS, Lonza). Next, slides were blocked by incubation in PBS containing 10% goat serum in for 1 h at RT. Primary antibody Acp1 α/β (1:100) or CD42b (1:50) was added 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, Belgium) was used as secondary antibody. The slide scoring was based on Allred score taking the sum of intensity of staining (scored 0 to 5) and proportion of positively stained cells (scored from 0 to 3) [18,22].

2.5. Oncomine and GEOdata analysis

The Oncomine and GEOdata analysis was performed as described before [18]. Expression profiles from publicly available Oncomine [23]

were used to assess *ACP1* mRNA expression in colorectal and gastric and esophageal cancer, while GEO databases were searched for additional databases not already represented in Oncomine. GEOdata analysis searching was based on raw RNA data available with RNA discrimination, and search on *ACP1* gene on each file. Information on *ACP1* expression in esophageal cancer was available in 2 additional arrays. GEO Dataset Record GSE26886 (transcript 201629_s_at, 201630_s_at, 215227_x_at) based on the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) was used to compare 21 esophageal adenocarcinoma and 19 normal esophageal epithelia [24]; GEO Dataset Record GSE161533 (transcript 201629_s_at, 201630_s_at, 215227_x_at) based on the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) was used to compare 28 esophageal squamous cell carcinoma and 28 normal esophageal squamous epithelia matched from same patients. Oncomine searching was based on: gene: *ACP1*; analysis type: esophageal adenocarcinoma vs normal analysis; esophageal squamous cell carcinoma vs normal analysis; concept type: over-expression (Oncomine concepts). Information on *ACP1* expression in gastric cancer was available in 1 additional arrays. GEO Dataset Record GSE2685 (transcript U25849_at) based on the GeneChip HuGeneFL array (Affymetrix) to compare 22 gastric cancer and 8 normal tissue samples [25]. Oncomine searching was based on: gene: *ACP1*; analysis type: gastric cancer vs normal analysis; concept type: over-expression (Oncomine concepts). For colorectal cancer GEO Dataset Record GSE24514 (transcript 201630_s_at) based on the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) was used to compare 34 colorectal cancer and 15 normal tissue samples [26]. GEO Dataset Record GSE21510 (transcript 201630_s_at, 201629_s_at, 215227_x_at) based on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix) to compare 123 CRC and 25 normal tissue samples [27]. Oncomine searching was based on: gene: *ACP1*; analysis type: colorectal cancer vs normal analysis; concept type: over-expression (Oncomine concepts). For platelet analysis, GEO Dataset Record GSE68086 (transcript ENSG00000143727) based on the Illumina HiSeq 2500 expression beadchip (Illumina, Inc.) was used to compare 45 healthy donors and 44 CRC platelets [10]. For correlation analysis, the interactome LMWPTP (*ACP1*) and kinases (Src, p38, FAK, paxillin, S6K, cofilin, AKT) by String-DB [28]. The correlation performs pair-wise gene expression correlation analysis for given sets of TCGA [29] from tumor and normal tissue expression data, using Pearson method. The parameters set-up were as x-axis: input *ACP1*, y-axis: input *SRC* or *PTK2*, TCGA Tumor/TCGA Normal: ESCA (Esophageal Carcinoma), STAD (Gastric Carcinoma), COAD (Colorectal Carcinoma). The R^2 is the coefficient of correlation and P (*p*-value) is the significance of correlation.

2.6. 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 [21]. Briefly, whole blood was collected from healthy, drug-free volunteers into 3.2% sodium citrate tubes (BD). Whole blood was centrifuged at 1500 rpm, 10 min, 22 °C, and PRP was collected. The remaining blood was centrifuged at 2500 rpm, 10 min, 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.

2.7. Platelet-cancer cells interaction assays

Platelet isolation was performed as described before [21]. 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 (Sigma-Aldrich). 500 μL of PRP were incubated with tumor cells (1.5×10^4 cells/test) following the protocol described before [30] with some modifications as usage of NaCl 0.9% instead of PBS at 37 °C for 5 min. Subsequently, the agonist collagen (2 μg/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 [31]. Quality controls of platelets were assessed by aggregation response at the beginning and end of experiments.

2.8. Co-culture

Gastric cells (GES-1 and 23132/87) and CRC cells (Caco-2 shScramble, Caco-2 shLMWPTP, HT29 WT, HT29 LMWPTP KD) were plated at 4×10^4 cells/cm^2 at 24-well plate for 24 h. After that, cells were washed with PBS, and the following conditions were applied: control (without platelets and without FBS), 5% PRP (platelet stimulation) or 10% FBS (growth factor positive control). After 24 h, microscopy analyses were made for CRC cells, as described in [21]. Briefly, microscopic images obtained by EVOS XL Core Cell Imaging System (ThermoFischer Scientific), using 10× 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.

2.9. Western blot assay

Western blot was performed as described before [32]. In short, cells were plated at a density of 4×10^4 cells/cm^2 for GES-1, 23132/87, KatoIII WT, KatoIII LMWPTP KO, HT29 WT and HT29 LMWPTP KD. After 24 h, 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 min. Cell extracts were resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membranes (Merck chemicals BV). Membranes were blocked in 50% odyssey blocking buffer (LI-COR Biosciences) 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) for 1 h. Detection was performed using Odyssey reader and analyzed using the manufacturer's software. For antibodies used, see Table 1. All densitometry was normalized using the internal control and the fold-change was calculated. Briefly, the densitometry (arbitrary units) from LMWPTP measured by Image Studio Acquisition Software (LI-COR Biosciences), was normalized by the densitometry intensity of the respectively internal control. After, the fold-change between the samples were compared based on this normalization, and the respectively graphics are shown.

2.10. Colony formation assay

Cells (GC and CRC) were plated at 2.0×10^3 cells/well in 6-well plates. After 24 h, 250 μL 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 min. 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: 2000 μm) - (2×, Nikon, Japan) and the colonies were analyzed using ImageJ software (NIH, USA).

2.11. MTT assay

Cells were plated at 4.0×10^4 cells/cm^2 (GES-1, KatoIII WT and

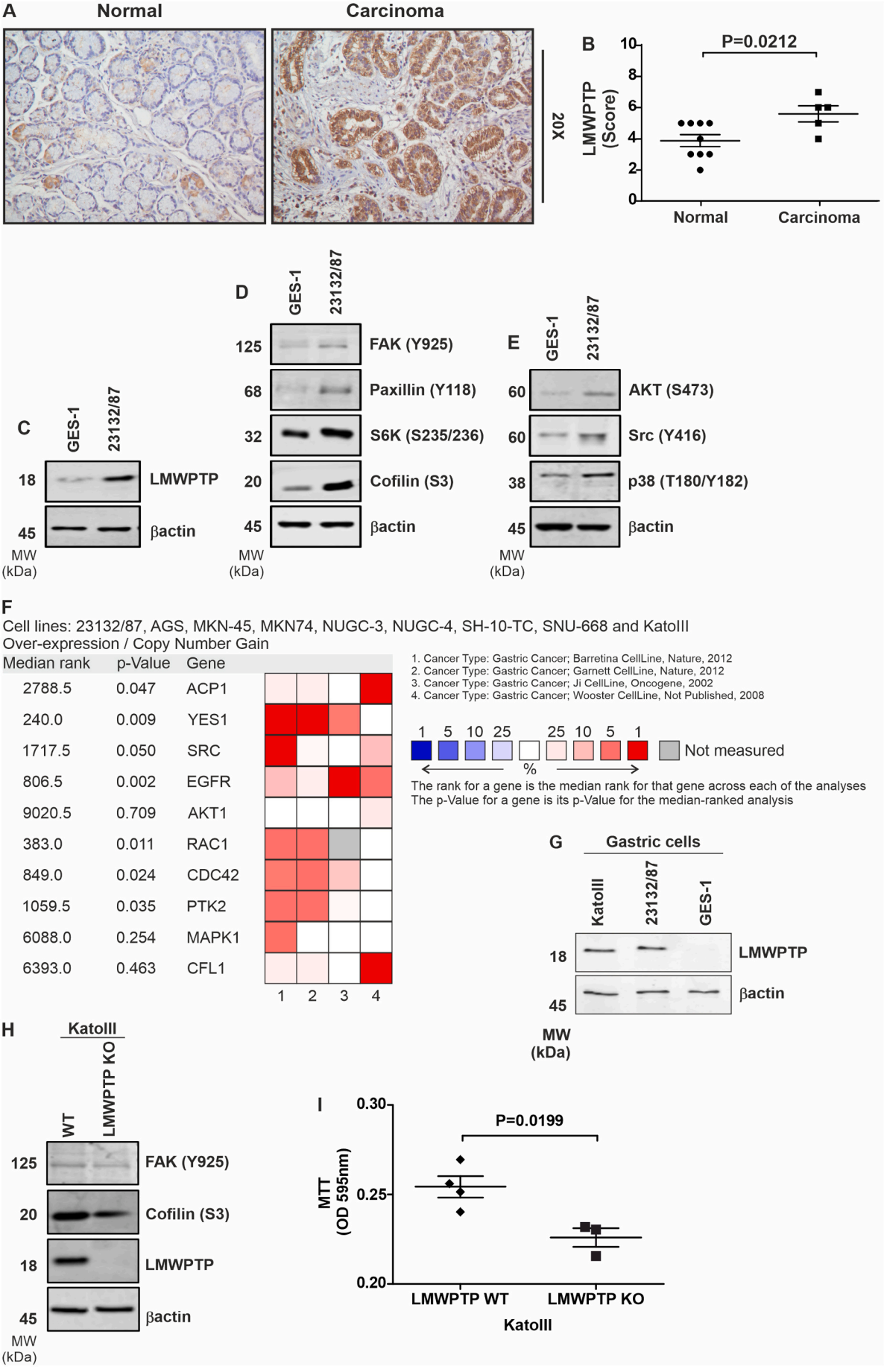

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Fig. 1. LMWPTP contributes to proliferative and cytoskeletal signaling in gastric cancer cells. (A–B) Normal gastric epithelium ($n = 9$) was compared to gastric carcinoma ($n = 5$) and representative samples (20×) as well as quantifications are shown. 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. (C) 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. (D–E) Phosphorylation of signaling molecules related to cytoskeletal remodeling (D) and survival (E) were investigated in normal and cancer gastric cell lines. β-actin served as loading control. (F) Oncomine [23] analysis of 23132/87, AGS, MKN-45, MKN74, NUGC-3, NUGC-4, SH-10-TC, SNU-668 and KatoIII, all gastric cancer cell lines [39]. (G) LMWPTP expression in the gastric cells (KatoIII, 23132/87 and non-transformed GES-1 cell line) was determined by Western blot analysis. β-actin was used as loading control. (H) Phosphorylation status of kinases comparing KatoIII wild type (WT) and LMWPTP knockout (KO) cells. (I) Proliferation rate using MTT assay comparing KatoIII WT and LMWPTP KO cells. Dots indicate individual experiments and mean ± SEM is shown. Abbreviation KO: knock-out, WT: wild type.

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

2.12. Adhesion assay

Cells in serum-free medium were allowed to adhere to plates for 30 and 120 min 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).

2.13. Immunofluorescence confocal microscopy

HCT116 were cultured under density 4.5 × 10^4 cells/well – 500 μL on glass coverslips for 24 h. Subsequently, cells were incubated with platelets (5%) in medium without FBS, and medium with FBS (control), and cultured for another 24 h. HCT116 and HT29 cells were grown in 3D cultured based on Souza et al. [33]. Cells were seeded in 6-wells microplates and grown in a 2D model for 24 h after which they were statically incubated for 24 h with 60 μL of NanoShuttle (Nano3D Biosciences) at a proportion of 2 μL/1 × 10^4 cells. After 24 h 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 1 × 10^4 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) to induce spheroid formation. After 24 h, the magnetic drive was removed. Images were taken by Luma Scope microscope (Etaluma Inc.) in a 10× magnification after 120 h of culturing. Next, the protocol followed as described here [34] with some modifications. Cells were fixed with 4% PFA for 10 min, washed with PBS, permeabilized with Triton X-100 (0.1%) and blocked with 3% BSA for 1 h. 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) 488 Mouse secondary antibody at 1:500 dilution for 1 h. Coverslips were subjected to a standard staining with DAPI-Invitrogen (Thermo Fisher Scientific) at 1:1000 dilution and it were mounted onto glass slides. Images were acquired on a LEICA TCS SP5 II confocal microscope (Leica, Germany) at Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP) - using 100× objectives (scale bar: 25 μm). Images' format 1024 × 1024. Images were analyzed using ImageJ software (NIH, USA).

2.14. Statistical analysis

The data is represented by means ± SEM. Statistical analysis was performed using t-student (paired, 95% confidence intervals, two tailed) for Figs. 1(B,I), 2(B,C,D,E,F,H,I,), 3(B,K,L), Fig. Supplemental Figure S1 (B,D,G,H,J), Fig. Supplemental Figure S4(A,C) and One-way ANOVA with post-test corrected for multiple testing for Fig. 2(K) and 4(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., USA). Correlation analysis was performed for Fig. Supplemental Figure S2 using Pearson method. All experiments were performed a minimum of three independent times.

3. Results

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

Having previously established a role for LMWPTP in CRC [18], we first investigated whether these findings also extend to upper GI cancers. We analyzed LMWPTP by immunohistochemistry and detected an overexpression of this phosphatase in a small cohort of gastric cancer tissues as compared to normal gastric epithelium (Fig. 1A–B). To investigate whether enhanced *ACP1* levels are also found at mRNA level, data from public repositories explored. A comparison of the 15 datasets presents in Oncomine for GC showed a near significant increase in *ACP1* expression in gastric tumors as compared to normal gastric mucosa ($p = 0.055$, Supplemental Fig. S1A), which was confirmed in an additional GEO dataset [25] (Supplemental Fig. S1B). To verify whether these findings extend to other upper GI cancers, we investigated LMWPTP by immunohistochemistry in esophageal adenocarcinoma ($n = 8$) as compared to normal squamous epithelium ($n = 7$), again showing increased LMWPTP in tumor tissues (Supplementary Fig. S1C–D), which was confirmed for *ACP1* mRNA expression in Oncomine and GEO-datasets (Supplementary Fig. S1E–H). These results indicate that upregulation of *ACP1* expression is present in upper GI cancers.

Subsequently, we employed gastric cell lines as a model system to investigate the molecular contribution of LMWPTP to carcinogenesis. To this end, we compared the non-transformed cell line GES-1 to in the gastric cancer cell line 23132/87. Corresponding to the immunohistochemistry data, LMWPTP expression is enhanced in 23132/87 cells as compared to non-transformed cells (Fig. 1C). When investigating the phosphorylation pattern of several kinases related to cell proliferation and cytoskeletal remodeling, such Src, FAK and Cofilin, an enhanced activation of these kinases was observed in gastric cancer cells (Fig. 1D–E). These findings are consistent with literature indicating the importance of LMWPTP function for cytoskeletal remodeling and Src activation [19,35–38], and TGCA data showing enhanced expression of these genes in cancer, as well as their correlation with *ACP1* expression (Supplementary Fig. S2). To further validate these findings for the cell lines, we investigated Oncomine HPRD interaction sets comparing normal and cancer cells for these kinases. The set-up was based on information available for 23132/87, AGS, MKN-45, MKN74, NUGC-3, NUGC-4, SH-10-TC, SNU-668 and KatoIII cells, all of which have higher metastasis potential according to MetMap Explorer [38] (KatoIII was not included in the MetMap Explorer study). These data from public repositories confirm a higher expression in these cancer cell lines of LMWPTP, FAK, several Src family members, cytoskeletal regulators, as well as EGFR, which was already associated with CRC aggressive phenotype [18] (Fig. 1F–G). Thus, these data imply that LMWPTP overexpression is associated with stimulation of oncogenic signaling in gastric cancer cells. 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

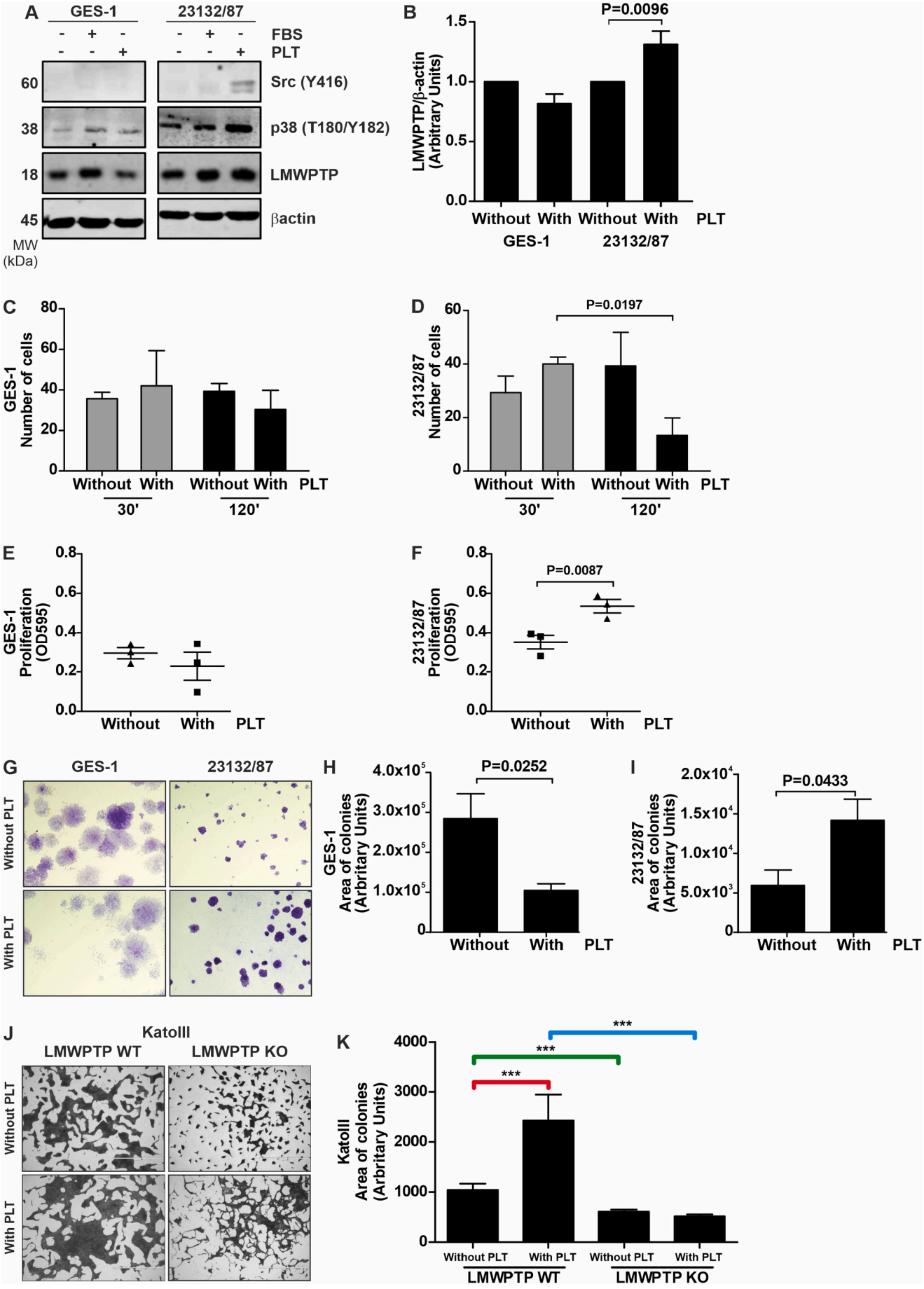

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Fig. 2. Platelets stimulate LMWPTP expression and proliferation in gastric cancer cells. (A–B) Kinase activation (Src and p38), and LMWPTP expression in normal and cancer gastric cell line in the presence or absence of platelets. Densitometry analysis for LMWPTP expression is represented in (B). β-actin was used as loading control. (C–D) Adhesion assay of GES-1 (C) and 23132/87 (D) cells in absence and presence of platelets. (E–F) MTT assay of GES-1 (E) and 23132/87 (F) cells in the absence or presence of platelets. (G–I) Colony formation of GES-1 and 23132/87 cells in the absence or presence of platelets. The size of GES-1 (H) and 23132/87 (I) colonies are presented. (G) Representative microscopic images of colony formation. (J–K) Colony formation of KatoIII wild type and KatoIII LMWPTP KD cells in the absence or presence of platelets. The size (K) of KatoIII wild type and KatoIII LMWPTP KD colonies are presented. Significance bars are colored to facilitate comparison with plots presented in Fig. 4. Dots indicate individual experiments and mean ± SEM is shown. Abbreviation PLT: platelets, KO: knock-out, WT: wild type.

KatoIII. A reduced phosphorylation of cytoskeletal proteins (Fig. 1H), as well as decreased proliferation (Fig. 1I), were observed upon knockout of LMWPTP in these gastric cancer cells.

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

Having established that LMWPTP is upregulated in gastric cancer and modulates GC signaling, we next investigated whether LMWPTP-mediated signaling affects GC cell interactions with platelets. To this end, 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 stimulated (Fig. 2A). Interestingly, co-culturing GC cells with platelets further increased their LMWPTP protein expression, something which was not observed in non-transformed cells (Fig. 2A, B). When detached gastric cells were incubated with platelets prior to plating, attachment of 23132/87 cells, but not GES-1 cells, was reduced (Fig. 2C, D), suggesting that direct interaction between gastric cells and platelets is more pronounced in tumor cells. Next, we assessed gastric cell viability in the presence or absence of platelets by MTT assays. While normal gastric cells (GES-1) were not affected by co-culture with platelets, the capacity of MTT reduction by GC cells (23132/87) was significantly increased in the presence of platelets (Fig. 2E, F), an indication that cancer cell proliferation was stimulated. 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 with platelets, non-transformed cells showed a decrease rather than increase in size of colonies (Fig. 2G–I). To investigate whether LMWPTP might directly drive platelet-induced tumor cell proliferation, KatoIII LMWPTP-KO cells were investigated for their colony formation potential. KatoIII cells form bigger colonies when expressing LMWPTP (Fig. 2J, K green significance bar). More importantly, however, LMWPTP-expressing KatoIII cells form bigger colonies in the presence of platelets as compared to LMWPTP-KO cells, and the increase in colony size induced by the presence of platelets was no longer present upon knockdown of LMWPTP (Fig. 2J, K, blue and red significance bars). Thus, these data imply that LMWPTP overexpression leads to stimulation of platelet-induced oncogenic signaling in gastric cancer cells.

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

Next, we sought to investigate to what extent LMWPTP overexpression contributing to tumor-platelet cell interaction is a general feature of GI tract cancers and also present in CRC. First, employing immunohistochemistry, we further confirmed a significantly increased protein expression of LMWPTP in a new set of CRC tissues compared to their normal counterpart (Fig. 3A–B). Additionally, we validated our previous findings by a comparison of the GEO dataset (3433 datasets present in Oncomine for CRC), showing an upregulation of *ACP1* in cancerous tissues as compared to normal colonic mucosa ($p = 0.001$) (Supplemental Fig. S1I), which was confirmed by an additional GEO dataset comparing 34 CRC and 15 normal tissue samples [27] (Supplemental Fig. S1J). We subsequently investigated to what extent LMWPTP expression is modulated by platelets in colorectal cancer cell line models with various levels of LMWPTP expression (Fig. 3C). As for GC cells, an enhanced LMWPTP expression and associated signaling is seen in CRC cells (Fig. 3D) with high metastatic potential according to MetMap [39]. Using two cell lines from the Oncomine panel (HCT116 and HT29), we showed that co-culture of CRC cells with platelets causes a significantly enhanced protein expression level of LMWPTP as demonstrated by Western blot and immunofluorescence (quantifications shown in Fig. 3K–L), which is accompanied by a distinct growth pattern, in both HCT116 cells (Fig. 3E–G) as well as HT29 cell models (Fig. 3H–J) grown under 2D and 3D conditions.

Next, we aimed to determine whether LMWPTP also plays a role in the platelet-mediated oncogenic potential of colorectal cancer cells. To this end, we performed genetic knock-out of *ACP1* in HT29 cells (Fig. 4A) and employed shRNA to reduce LMWPTP expression in HCT116 cells (Fig. 4E). As for gastric cells (KatoIII, Fig. 1H–I), co-culture with platelets induces a significant increase in colony size for HT29 cells (Fig. 4B–D, red significance bars), while a significant increase in colony number, though not colony size, was seen for HCT116 (Fig. 4F–H, red significance bars). While different growth patterns may account for this, both are indicative of platelets stimulating colony formation/proliferation. Knocking out LMPWTP significantly reduces colony size for HT29 cells, while partial knock down of this gene (18% ± 0.97%) resulted in a ~30% reduction of the number of colonies for HCT116 (see green significance bars), indicating the importance of LMWPTP for inherent colony growth of these tumor lines. Knocking down LMWPTP also reduced the colony size or number in the presence of platelets as compared to LMWPTP-competent co-cultures for HT29 and HCT116, respectively (blue line in Fig. 4C and H). Thus, these results indicate that, as for GC, CRC lines require LMWPTP for both inherent and platelet-induced oncogenic properties.

As an increase in tumor cell proliferation may be affected by growth factors released by platelets, we next sought to investigate to what extent LMWPTP contributes to direct interaction between platelets and CRC 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 [21]. We showed that knockdown of LMWPTP in a third CRC cell model (Caco-2, Fig. 4I) significantly reduces platelet aggregation in the presence of tumor cells as determined by microscopy and aggregometry (Fig. 4J, K), which was confirmed in the HT29 knock-out model by microscopy (Fig. 4L). Together, these data demonstrate that in CRC as well as upper GI cancer, overexpression of LMWPTP in cancer cells may be further enhanced by their interaction with platelets, and that LMWPTP contributes significantly to platelet-tumor interaction and tumor proliferation.

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% for GC, and 90% for CRC patients with non-metastatic tumor, while this number decreases to 11.7% for patients whose suffer from distant metastatic spread [40,41]. 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 [15]. 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, paradoxically, enhanced activity of specific

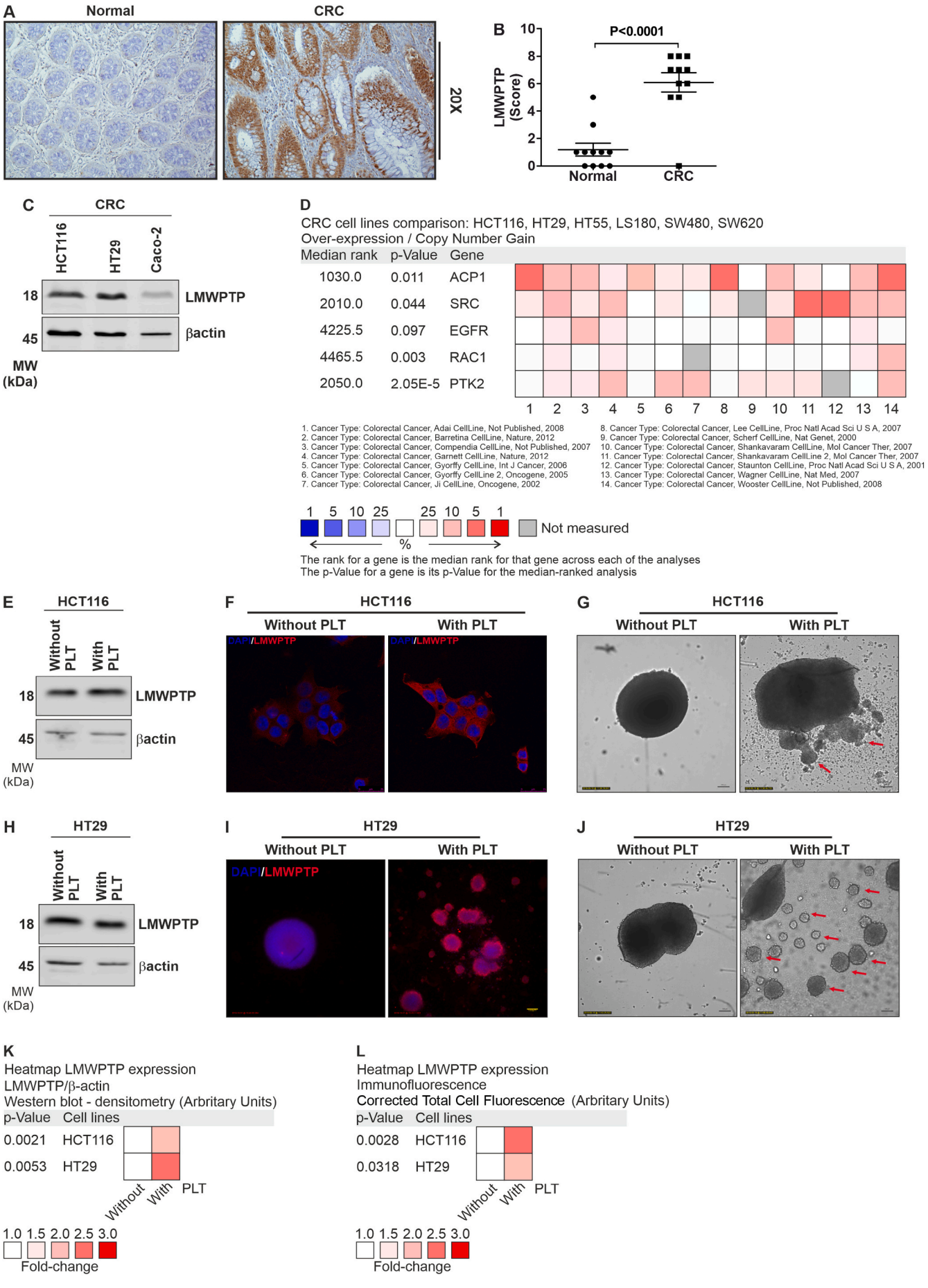

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Fig. 3. Platelets support CRC morphological changes in the context of LMWPTP. (A–B) LMWPTP expression was determined by immunohistochemistry in CRC tumors. Normal colorectal specimens ($n = 11$) and adenocarcinoma (n = 11) were compared. Representative samples (20×) and quantifications are shown. The intensity and proportion of the staining were scored and analysis was performed comparing control and carcinoma groups. (C) LMWPTP expression in the CRC cells (HCT116, HT29 and Caco-2) was determined by Western blot analysis. β-actin was used as loading control. (D) Oncomine analysis [23] of HCT116, HT29, HT55, LS180, SW480, SW620, all CRC cell lines with high metastatic potential [39]. (E) LMWPTP expression in HCT116 as determined by Western blot analysis (densitometry analysis shown in K). β-actin was used as loading control. (F) Fluorescence microscopy comparing LMWPTP intensity (quantification in L) in HCT116 cells after co-culture with platelets. Nuclei are stained in blue (DAPI) and LMWPTP in red at 100× magnification. (G) 3D growth of HCT116 cells in the presence of platelets leads to altered growth pattern. (H) LMWPTP expression in HT29 as determined by Western blot analysis (densitometry analysis in K). β-actin was used as loading control. (I–J) 3D growth of HT29 cells in the presence of platelets leads to altered growth pattern and increased LMWPTP expression (quantification in L) as determined by fluorescence microscopy staining. Nuclei are stained in blue (DAPI) and LMWPTP in red at 10× magnification. (K-L) LMWPTP expression heatmap (*p*-value and foldchange intensity) is represented for Western blot (K) and immunofluorescence (L). Legend: dots indicate individual experiments, and mean ± SEM is shown. Abbreviation PLT: platelets.

phosphatases can result in enhanced, rather than reduced, phosphorylation of several signaling moieties, in particular if their targets are repressive [15,18,35,42,43]. For instance, 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 [42]. Src is also a target for LMWPTP [35], and activation of Src signaling upon LMWPTP upregulation has been demonstrated in leukemia cells [19]. Thus, removal of 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 [18–20,34,36,37,44].

With this new knowledge, protein tyrosine phosphatases are now emerging as potential cancer biomarkers and targets for treatment [20]. Based on that, firstly 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 among intestinal cancers and opening up the tantalizing possibility of a common target for treatment of these diseases. Secondly, 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, which findings were supported by our previous data [19,35].

Next, we wondered whether LMWPTP in tumor cells would also mediate the interaction of tumor cells with platelets. Tumor cells induce several platelet modifications and can alter their intracellular content and function [45], which may contribute to increased VTE risk seen in cancer. Conversely, platelets, and the growth factors they release, may trigger tumor cell proliferation and survival, and facilitate cancer cell invasion, including in gastrointestinal cancer [46–48]. In vivo and ex vivo data suggest that platelets surrounding the tumor supports chemoresistance of breast and gastric tumors [48], is associated with poor overall survival in gastric cancer [50] and promotes metastasis of colorectal cancer [51]. Expression of the platelet marker protein CD42b is seen in FFPE sections of lower gastrointestinal tumors (Supplementary Fig. S3), although it remains to be investigated to what extent this is linked to LMWPTP in vivo. Using several different cell models, we demonstrate that platelets affect gastrointestinal tumor cell proliferation and that this process is at least partially dependent on LMWPTP expression in these tumor cells, at least in an in vitro setting. 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 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, with knockdown of LMWPTP reducing cancer cell interaction with platelets, as well as platelet-mediated proliferation effects.

Interestingly, co-culture of tumor cells with platelets further increases their expression of LWMPTP in this study. The exact mechanisms contributing to this process remain uninvestigated, but could include activation of transcriptional processes, prolonged LMWPTP protein stability, or direct exchange of cellular material between the two cell compartments. Intriguingly, LMWPTP (RNA and protein) is also overexpressed in platelets from CRC patients ([10] and Supplementary Fig. S4), and can be upregulated in vitro by exposure to tumor cells [21]. It has been shown that extracellular vesicles derived from cancer cells can modulate platelet content, and the reverse may also be possible [49,53]. Thus, while platelets and tumor cells clearly affect each other's signaling and function, it remains difficult to define the 'chicken and the egg' in this scenario [52,54,55]. With LMWPTP directly conferring several tumorigenic properties, 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 physical 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 [56], platelets also induced epithelial mesenchymal transition upon co-culture with CRC cells [57]. 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, as was shown for breast cancer cells, where PI3K activity and proliferation were enhanced by supernatant obtained from stimulated platelets [13].

Our study has several weaknesses. For in vitro analysis, we investigated tumor cell growth/proliferation, being one of the main tumor hallmarks. However, several other cancer properties, e.g. migration, chemoresistance, are of interest and remain to be investigated. Secondly, we did not show the importance of the LMWPTP-driven interaction between tumor cells and platelets in vivo, and thus cannot speculate on its clinical relevance. However, as a role for tumor expression of LMWPTP as well as platelet presence have been shown to affect clinical phenotype in cancer patients, based on our in vitro data, it is tempting to speculate on a role for LMWPTP at the hub of cancer-platelet interaction in vivo. Lastly, the role of LMWPTP in the reciprocal interaction between tumor cells and platelets is not easy to study when both cell types express these enzymes, and expression in one cell type might drive further expression in the other. While manipulation of LMWPTP in cancer cells in the current study shows the importance of LMWPTP for tumor cells, manipulation of LMWPTP in short-lived platelets is much more difficult, hampering investigation of the reverse relationship. Animal studies using platelet-specific knock-out of LMWPTP might be of use to answer these questions.

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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2021.166280.

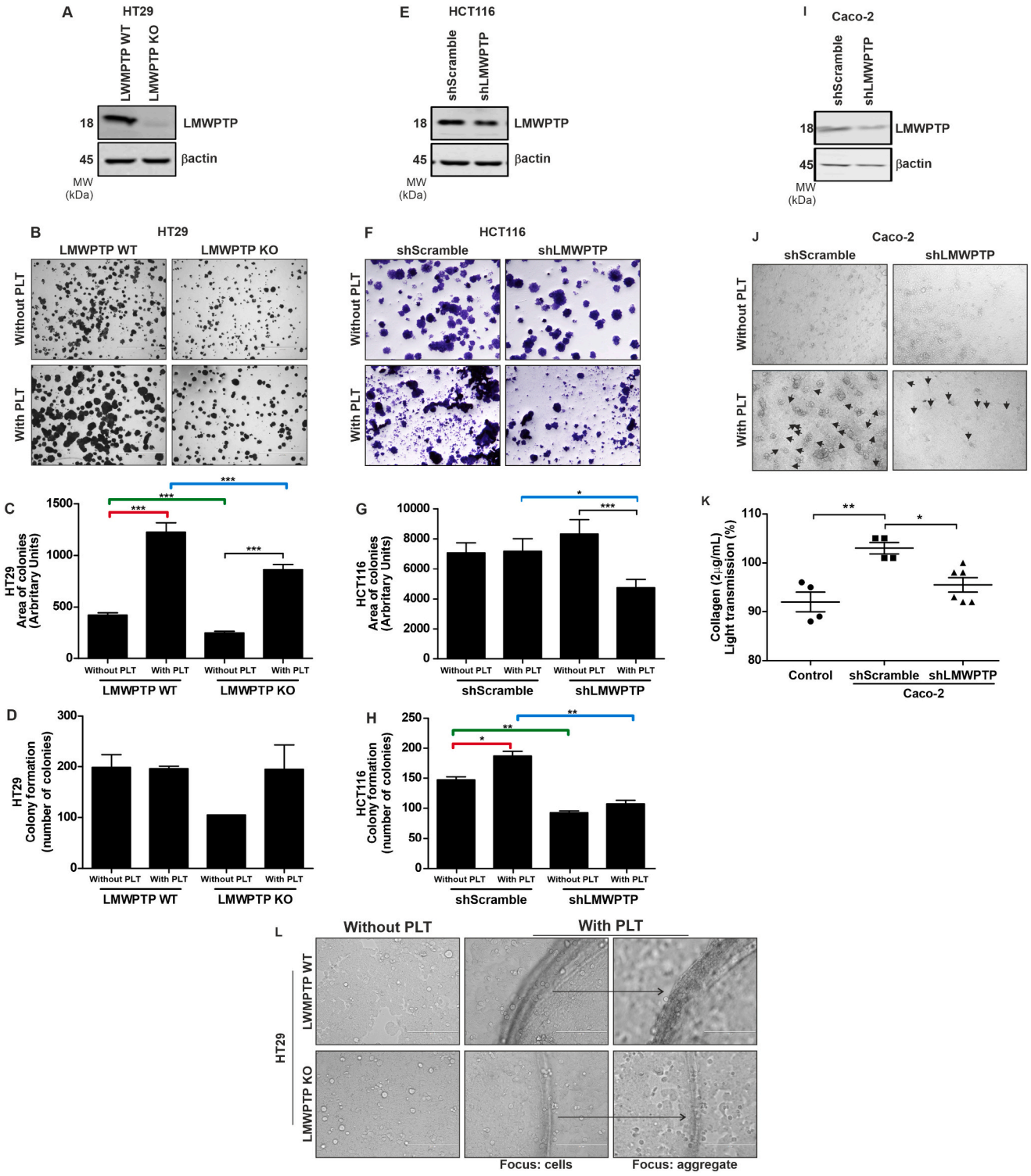

Fig. 4. LMWPTP expression in CRC cells affects their interaction with platelets and promoted proliferation. (A) LMWPTP knock-out in HT29 cells. β-actin served as loading control. (B–D) Colony formation assay of wild type (WT) and LMWPTP knockout (KO) HT29 cells in the absence or presence of platelets indicated a reduced platelet-induced colony size increase in the absence of LMWPTP. Quantification of the size (C) and number (D) of colonies in WT and LMWPTP KO cells cultured in the absence or presence of platelets are shown. (E) LMWPTP expression in HCT116 cells treated with either shScramble or shLMWPTP showing an 18% reduction of LMWPTP expression in shLMWPTP cells. β-actin served as loading control. (F-H) Colony formation assay of shScramble or shLMWPTP HCT116 cells in the absence or presence of platelets indicates a reduced number of colonies in the absence of LMWPTP as well as a loss in platelet-induced increase thereof. Quantification of the size (G) and number (H) of colonies in shScramble and shLMWPTP 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. Aggregates are indicated by arrowheads. (K) Aggregometry analysis of platelet aggregation shows that cancer cell-induced increase in platelet aggregation is reduced upon knockdown of LMWPTP in Caco-2 cells. (L) Light microscopy images of HT29 cells showing reduced platelet aggregates upon KO of LMWPTP. Representative pictures are shown at 10× magnification and the aggregates are highlighted by black arrow. Abbreviation PLT: platelets, KO: knock-out, WT: wild type.

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Ethics approval

MEC-2018-113 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.

CRediT authorship contribution statement

Alessandra V.S. Faria: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Bingting Yu:** Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Michiel Mommersteeg:** Investigation, Writing – review & editing. **Patrícia F. de Souza-Oliveira:** Methodology, Investigation, Writing – review & editing. **Sheila S. Andrade:** Methodology, Formal analysis, Writing – review & editing. **Manon C.W. Spaander:** Resources, Writing – review & editing. **Moniek P.M. de Maat:** Methodology, Writing – review & editing. **Maikel P. Peppelenbosch:** Supervision, Writing – review & editing, Project administration. **Carmen V. Ferreira-Halder:** Conceptualization, Methodology, Formal analysis, Data curation, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Gwenny M. Fuhler:** Conceptualization, Methodology, Formal analysis, Data curation, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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