Plenary Paper

Check for updates

blood

PLATELETS AND THROMBOPOIESIS

Platelet microparticles infiltrating solid tumors transfer miRNAs that suppress tumor growth

James V. Michael,[1,2,*] Jeremy G. T. Wurtzel,[1,2,*] Guang Fen Mao,[2] A. Koneti Rao,[2,3] Mikhail A. Kolpakov,[4,5] Abdelkarim Sabri,[4,5] Nicholas E. Hoffman,[6,7] Sudarsan Rajan,[6,7] Dhanendra Tomar,[6,7] Muniswamy Madesh,[6,7] Marvin T. Nieman,[8] Johnny Yu,[9,10] Leonard C. Edelstein,[9,10] Jesse W. Rowley,[11,12] Andrew S. Weyrich,[11-13] and Lawrence E. Goldfinger[1,2,14]

[1]Department of Anatomy & Cell Biology, [2]The Sol Sherry Thrombosis Research Center, [3]Department of Medicine, [4]Department of Physiology, [5]Cardiovascular Research Center, [6]Department of Biochemistry, and [7]Center for Translational Medicine, Lewis Katz School of Medicine, Temple University, Philadelphia, PA; [8]Department of Pharmacology, Case Western Reserve University, Cleveland, OH; [9]Cardeza Foundation for Hematologic Research and [10]Department of Medicine, Sidney Kimmel Medical School, Thomas Jefferson University, Philadelphia, PA; [11]Department of Internal Medicine, [12]Program in Molecular Medicine, and [13]Division of Microbiology and Immunology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT; and [14]Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

Key Points

- Platelet MPs infiltrate solid tumors and transfer platelet-derived miRNAs to tumor cells within solid tumors in vivo.
- Transfer of platelet miRNAs to tumor cells results in downregulation of tumor cell genes and inhibition of solid tumor growth.

Platelet-derived microparticles (PMPs) are associated with enhancement of metastasis and poor cancer outcomes. Circulating PMPs transfer platelet microRNAs (miRNAs) to vascular cells. Solid tumor vasculature is highly permeable, allowing the possibility of PMP–tumor cell interaction. Here, we show that PMPs infiltrate solid tumors in humans and mice and transfer platelet-derived RNA, including miRNAs, to tumor cells in vivo and in vitro, resulting in tumor cell apoptosis. MiR-24 was a major species in this transfer. PMP transfusion inhibited growth of both lung and colon carcinoma ectopic tumors, whereas blockade of miR-24 in tumor cells accelerated tumor growth in vivo, and prevented tumor growth inhibition by PMPs. Conversely, *Par4*-deleted mice, which had reduced circulating microparticles (MPs), supported accelerated tumor growth which was halted by PMP transfusion. PMP targeting was associated with tumor cell apoptosis in vivo. We identified direct RNA targets of platelet-derived miR-24 in tumor cells, which included mitochondrial *mt-Nd2*, and *Snora75*, a noncoding small nucleolar RNA. These RNAs were suppressed in PMP-treated tumor cells, resulting in mitochondrial dysfunction and growth inhibition, in an miR-24–dependent manner. Thus, platelet-derived miRNAs transfer in vivo to tumor cells in solid tumors via infiltrating MPs, regulate tumor cell gene expression, and modulate tumor progression. These findings provide novel insight into mechanisms of horizontal RNA transfer and add multiple layers to the regulatory roles of miRNAs and PMPs in tumor progression. Plasma MP-mediated transfer of regulatory RNAs and modulation of gene expression may be a common feature with important outcomes in contexts of enhanced vascular permeability. (*Blood*. 2017;130(5):567-580)

Introduction

Platelets have been associated with tumor progression and metastatic dissemination through platelet–tumor cell (TC) interactions.[1-7] Platelet-TC interactions may contribute to tumor progression in several ways, including enhancing cancer-related coagulation and providing a TC "shroud" to shield them from the immune system.[8] The presence of cancer increases platelet production, which has been associated with poorer outcomes in multiple cancers.[9,10] Platelets can stimulate proliferation of human and murine cancer cells in a manner that does not require platelet-tumor contact.[11] However, the platelet-cancer axis still remains unsolved and is an area of active investigation.

Platelets and other cells release microparticles (MPs) into the plasma in response to receptor agonists and shear stress.[12] At least 45% of plasma-borne MPs are platelet-derived MPs (PMPs).[13,14] PMP release increases in individuals bearing solid tumors, but roles of PMPs in cancer progression are incompletely understood.[15,16] PMPs are enriched in platelet microRNAs (miRNAs), a small cohort of which are present at high copy number, accounting for the bulk of plasma miRNAs.[17-21] Purified PMPs are able to transfer at least some miRNA content to cells following coincubation in vitro, and regulate gene expression.[22-24] Several miRNAs enriched in PMPs, including miR-27a, miR-24, miR-155, miR-195, let-7a/b, and miR-223, target both tumor suppressor genes and oncogenes (in multiple cancer types), have been identified as diagnostic and prognostic markers of malignancy, and have been implicated in therapy resistance.[25-46] Tumor neovasculature

Submitted 17 November 2016; accepted 8 May 2017. Prepublished online as *Blood* First Edition paper, 12 May 2017; DOI 10.1182/blood-2016-11-751099.

*J.V.M. and J.G.T.W. contributed equally to this work.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2017 by The American Society of Hematology

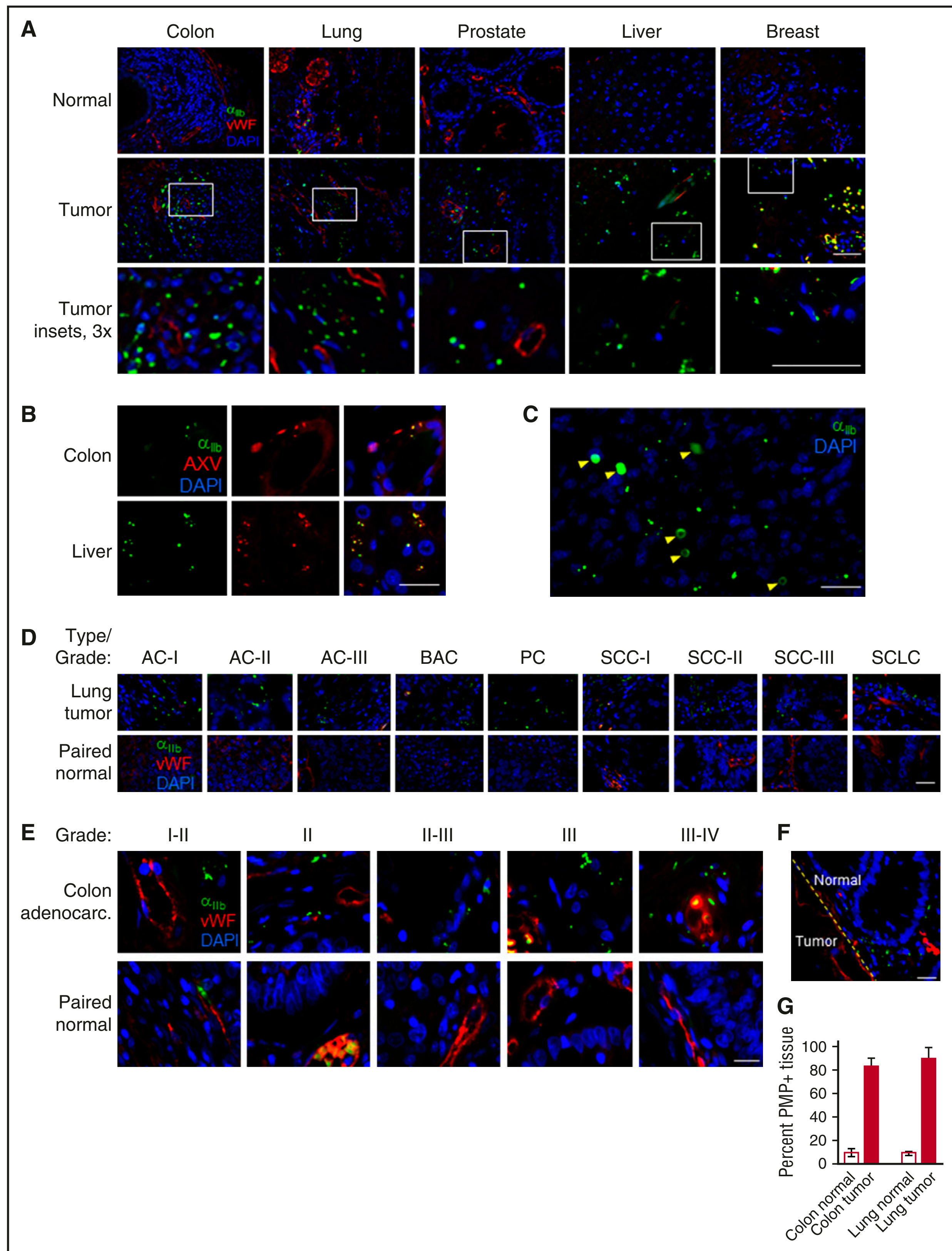

Figure 1. PMP infiltration in solid tumors in human patients. (A) Tissue microarray slides containing 5-μm sections from the indicated human tumors and uninvolved adjacent tissue ("Normal") were stained with the indicated antibodies and 4′,6-diamidino-2-phenylindole (DAPI). Colon, grade I-II colon carcinoma; lung, grade II lung squamous cell carcinoma; prostate, grade II prostate adenocarcinoma; liver, grade II-III hepatocellular carcinoma; breast, grade II-III invasive ductal carcinoma. α_{IIb} integrin, green; VWF, red; DAPI, blue. Bottom row, center area insets, original magnification ×3. Bars, 50 μm (n = 4). (B) Representative images from panel A, showing counterstain with fluorescein isothiocyanate (FITC)-Annexin V (AXV; shown as red). α_{IIb} integrin, green; DAPI, blue. Merged images with DAPI shown to the right; α_{IIb} integrin/Annexin V overlap appears as yellow. VWF staining was omitted from the merged images for clarity. (C) A section of human lung adenocarcinoma, grade II was incubated with 10^3 freshly isolated murine platelets for 15 minutes before being fixed and stained as indicated. Yellow arrowheads indicate ectopic intact platelets. (D) Representative images

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

Table 1. Presence of extravascular PMPs scored in graded lung carcinoma and colon adenocarcinoma, and adjacent uninvolved tissues

	Lung carcinomas									Colon adenocarcinomas				
Type and grade	AC I	AC II	AC III	BAC	PC	SCC I	SCC II	SCC III	SCLC	AC I-II	AC II	AC II-III	AC III	AC III-IV
Tumor	2/2	2/2	6/6	2/2	4/4	2/2	1/2	7/8	3/4	5/6	5/5	3/4	10/10	3/6
Uninvolved paired normal tissue	0/2	1/2	0/6	0/2	1/4	0/2	1/2	0/8	0/4	1/6	1/6	0/6	0/6	1/6

Stained slides from Figure 1C-D were scored for intratumoral PMPs by observers blinded to the sample type, and shown as a ratio of PMP$^+$ to total. Samples were counted as negative if no PMPs were observed in 5 separate fields of 500 μm^2.

is highly permeable, which we predicted might allow circulating MPs direct access to tumor cells. In this study, we investigated PMP infiltration in solid tumors, transfer of platelet-derived miRNAs to tumor cells, and cellular and physiological effects.

Methods

Tumor allografts and immune-induced thrombocytopenia

A suspension of 1 × 10^6 cells/200 μL of Hanks balanced salt solution was injected subcutaneously into the shaved flank of 8-week-old mice. Thrombocytopenia was induced by intraperitoneal (i.p.) injection of 50 mg/kg rat-anti-mouse CD41 antibodies. Platelet count was assessed by Hemavet analysis, and was <20% of starting counts after 24 hours. In some cases, mice were injected i.p. with 100 mg/kg 4-thiouracil (4TU) in 20% dimethyl sulfoxide/80% corn oil, or vehicle. Tumor volume was calculated as described.[47] Tumors were resected from euthanized mice, and cleaned of fat, skin, and connective tissue for further processing.

miRNA:messenger RNA target adduct formation and screening

Cells suspended in 500 μL of fractionation buffer (20 mM Tris-Cl pH 7.4,100 mM NaCl, 2 mM MgOAc, 5 mM KCl, protease inhibitor cocktail [Roche, Indianapolis, IN]) were dounce-homogenized followed by sonication. Lysates were treated with 1 U/μL RNase T1 (Fisher, Pittsburgh, PA) for 15 minutes at 22°C, followed by RNase inhibition with 10 mM $MnCl_2$. T4 polynucleotide kinase (PNK) minus 1 U/μL plus 100 mM adenosine triphosphate (ATP), 5 mM dithiothreitol was added (40 minutes at 10°C). RNA ligation was carried out with 0.2 U/μL T4 RNA ligase (16 hours at 4°C). RNA was extracted in TRIzol and resuspended in diethyl pyrocarbonate–treated H_2O, followed by 1 μL of PNK and no ATP for 40 minutes at 10°C, and poly(dA) tailing and first-strand complementary DNA (cDNA) synthesis with the NCode kit (see supplemental Methods, available on the *Blood* Web site).[48] cDNA libraries were subjected to *Taq* polymerase chain reaction (PCR) using miR-specific 5′ oligonucleotides and universal poly(dT) 3′ primers. Products were subcloned by direct ligation of the reaction mixture into the pCR2.1 TA vector (Invitrogen), and ligation reactions were transformed into DH5α *Escherichia coli* for ampicillin selection, colony propagation, plasmid DNA minipreps (Qiagen, Valencia, CA), and sequencing.

Results

Platelet MPs infiltrate solid tumors

Circulating PMPs harbor miRNAs and can transfer platelet-derived miRNAs to endothelium and leukocytes.[22,23,49] Because tumor blood vessels are highly permeable due to endothelial dysfunction and poor pericyte coverage,[50] and PMP release correlates with solid tumor growth and metastasis,[4,16,51] we considered whether TCs in solid tumors are targets of PMPs. We observed PMP infiltration, indicated by antibodies to α_{IIb} integrin (CD41), a platelet/megakaryocyte-specific receptor and a PMP marker,[52] in the extravascular tumor environment as indicated by von Willebrand factor (VWF) staining for blood vessels, in grade II/III solid tumors derived from human patients, but not in adjacent normal tissue, in multiple cancer types (Figure 1A). The puncta ranged in diameter from ~100 to 1000 nm, the diameter range of PMPs,[53,54] and were Annexin V$^+$ (Figure 1B), indicating phosphatidylserine exposure on the outer leaflet, a characteristic of MPs and apoptotic cells. Most, but not all, Annexin V$^+$ puncta in the tumor sections also contained α_{IIb} integrin, consistent with PMPs being the major MP fraction in the infiltrates (Figure 1B). Examination of tissue sections spiked with freshly isolated platelets and stained with α_{IIb} integrin antibodies confirmed that the platelet-derived intratumoral material consisted of platelet fragments smaller than intact platelets (Figure 1C). PMP tumor infiltration was observed across tumor grades in lung and colon cancer subtypes, but extravascular PMPs were not observed in paired, uninvolved normal tissues except for a few cases (Figure 1D-G; Table 1). In these latter cases, PMP infiltration was only evident in normal tissue adjacent to the tumor, suggesting that infiltration reflected a specific effect of proximity to the tumor microenvironment (Figure 1F).

To gain mechanistic insight, we used an ectopic solid tumor allograft model in mice using Lewis lung carcinoma (LLC) cells injected as a bolus subcutaneously into mouse flanks.[55] PMPs were observed infiltrating LLC-allografted tumors in mice (Figure 2A), similar to human tumors (Figure 1). CD63, a marker for both PMPs and exosomes,[13] overlapped with α_{IIb} integrin in PMP-like structures (CD63-only exosomes were also evident), confirming that the α_{IIb} integrin-positive structures represent PMPs and not α_{IIb} integrin expression in TCs, which has been observed in metastatic melanoma and some cancer cell lines (Figure 2B).[56-59] Many TCs isolated from resected tumors at 21 days were decorated with PMPs (62.8% ± 3.2% of TCs; n = 5; >250 cells per experiment), indicating association of infiltrating PMPs with the TCs (Figure 2C). PMPs were also associated with green fluorescent protein–positive (GFP$^+$) TCs isolated from resected tumors from NIH3T3 cells transformed with GFP-HRAS (G12V)[47,60] (supplemental Figure 1A). Fewer PMPs appeared associated with TCs derived from 7-day tumors (supplemental Figure 1B), suggesting that robust neovascularization precludes PMP extravasation in solid tumors. TCs extracted at day 21, 24 hours after immune-induced thrombocytopenia, showed substantially reduced PMPs (3.1% ± 2.2% of TCs), indicating that the MPs were derived from

Figure 1 (continued) from human lung cancer array with paired uninvolved tissue, stained as in panel A. (E) Representative images from human colon cancer array with paired uninvolved tissue. Note that some α_{IIb} integrin-positive platelets can be seen within VWF-labeled blood vessels. (F) Representative image of colon adenocarcinoma, grade III, including adjacent normal tissue, showing PMP infiltration in the uninvolved tissue adjacent to the tumor border (indicated with a dotted line). Bars (B-F), 25 μm. (G) Percentage of PMP$^+$ tissues from total assayed tissues for colon adenocarcinomas and lung cancers, and adjacent uninvolved tissue, shown ± standard error of the mean (SEM) (n = 3). Colon, $P < .01$; lung, $P < .004$. AC, adenocarcinoma; BAC, bronchioalveolar carcinoma; PC, papillary carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

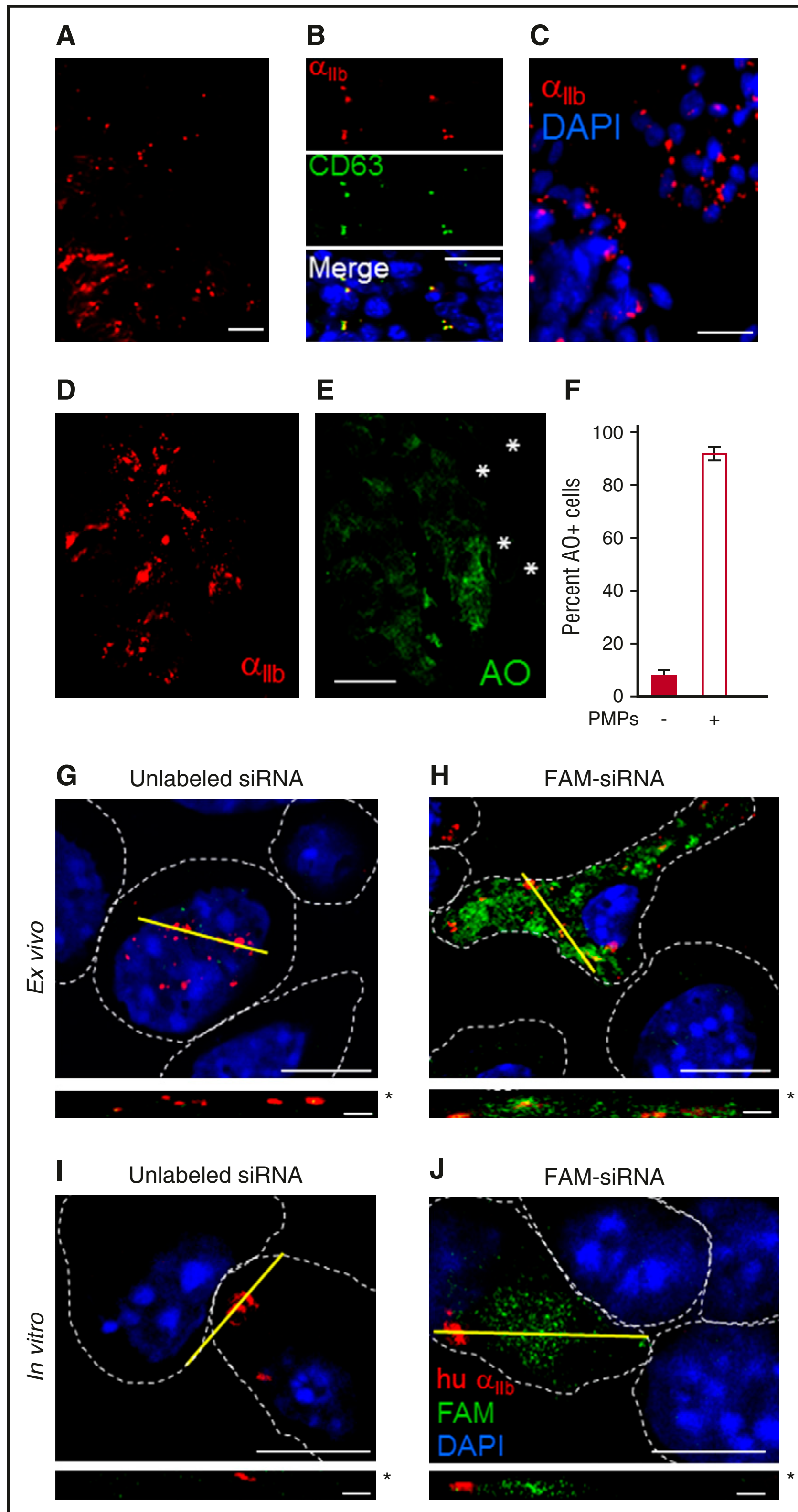

Figure 2. PMP infiltration in solid tumor allografts and RNA transfer in mice. (A) Immunohistochemistry (IHC) in a 5-μm section from LLC tumor allograft, 21 days. α_{IIb} integrin, red. (B) IHC in a 5-μm section from LLC tumor allograft, 21 days. α_{IIb} integrin, red (top); CD63, green (middle); DAPI, blue (shown in merged image, bottom). α_{IIb} integrin/CD63 overlap appears in some areas in the merged image as yellow. (C) Tumor cells from a resected LLC tumor at 21 days, isolated as described in supplemental Methods. α_{IIb} integrin, red; DAPI, blue. (D-E) Tumor cells from resected LLC allograft 24 hours after transfusion of AO-labeled platelets. (D) α_{IIb} integrin, red; (E) AO, green. Bars (A-E), 10 μm. *PMP$^-$ cells. (F) Percentage of cells with cytosolic AO staining, shown ± SEM (n = 3, >100 cells each). (G) Human platelets were transfected with unlabeled siRNA and transfused into mice bearing 20-day LLC tumors. After 24 hours, tumors were resected, digested, cleared of vascular cells with α-CD31 beads, and tumor cells were captured on fibronectin-coated coverslips. (H) Tumor cells ex vivo as in panel G, from mice transfused with human platelets transfected with FAM-siRNA. (I) LLC cells, treated in vitro with PMPs derived from human platelets 48 hours after platelet transfection with unlabeled siRNA. (J) Cells treated as in panel I with PMPs derived from human platelets transfected with FAM-siRNA. (H-J) Human α_{IIb} integrin, red; FAM, green; DAPI, blue. Dashed white lines indicate cell borders as determined from accompanying brightfield images (not shown). Yellow lines indicate x–y plane of z-section. Corresponding z-sections are shown below, minus DAPI stain. Bars (G-J), 7.5 μm; z-stack bars, 1 μm. Asterisks in panels G-J denote apical side of z-section.

blood platelets (supplemental Figure 1C). Many of the PMPs were attached to TC surfaces, and α_{IIb} integrin could also be seen internalized in HRAS-containing recycling endosomes, suggesting PMP cargo internalization (supplemental Figure 1D).[61,62] In some cells, α_{IIb} integrin was broadly distributed at the plasma membrane, indicating redistribution of PMP-derived proteins in the TCs, either as a result of plasma membrane fusion, transport of internalized protein to the target cell membrane, or expression of platelet-derived messenger RNA (mRNA) (supplemental Figure 1E). Thus, platelet MPs can undergo extravasation beyond the blood circulation in solid tumors and associate with tumor cells.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

PMPs transfer platelet RNA, including miRNAs, to tumor cells in solid tumors in vivo and in vitro

We considered whether infiltrating PMPs could deliver RNA cargo to TCs. We transfused platelets, derived from wild-type (WT) mice and labeled with acridine orange (AO) DNA/RNA vital dye, into LLC tumor-bearing mice. PMP-associated TCs from resected tumors showed AO cytosolic fluorescence, indicating the presence of platelet-derived RNA in the PMP-targeted cell cytosol. TCs with no PMPs showed only background fluorescence (Figure 2D-F).

In addition to DNA/RNA, AO can also label platelet granules.[63] To confirm that transferred platelet-derived material included RNA, and to investigate platelet miRNAs, we transfected human platelets with fluorophore 6-carboxyfluorescein (FAM)–labeled or unlabeled short inhibitory RNA (siRNA) as an miRNA mimic[64] (supplemental Figure 2A-B) and transfused transfected platelets into tumor-bearing mice. Ex vivo TCs, which harbored PMPs, showed cytosolic FAM$^+$ fluorescence in cells from FAM$^+$-siRNA-transfused mice, but we did not observe FAM$^+$ cells from control mice (Figure 2G-H).

To study PMP RNA transfer further, we used human platelets, from which we could more easily derive substantial quantities of PMPs. We stimulated ex vivo human platelets to release PMPs (supplemental Figure 3A), and incubated collected PMPs with LLCs in culture.[23] After 1 hour of PMP exposure, most LLC cells were decorated with α_{IIb} integrin puncta which resembled the intratumoral PMPs, and these puncta were removed by trypsin, suggesting a protein-mediated anchorage to the target cell surface (supplemental Figure 3B). Similar to TCs ex vivo, LLC cells exposed to PMPs from control-transfected platelets showed no FAM fluorescence (Figure 2I), whereas LLC cells exposed to PMPs from platelets transfected with FAM-siRNA showed cytosolic FAM$^+$ fluorescence distinct from the α_{IIb} integrin structures, selectively in PMP-targeted cells (Figure 2J). Thus, PMPs can deliver miRNA to TCs in vitro, consistent with previous reports in other systems.[23,24] Together, these data demonstrate that platelet-derived siRNA (miRNA mimic) transfers horizontally to tumor cells in solid tumors via infiltrating PMPs, and transferred siRNA is not sequestered in PMPs but is distributed in the target cell cytosol.

Platelet-derived miRNAs transferred to tumor cells in solid tumors

We sought to characterize platelet-derived miRNAs transferred to TCs via MPs. Following in vitro PMP exposure, we extracted RNA from PMP-stripped TCs. PCR for previously identified abundant miRNAs in PMPs, many of which are involved in tumor progression,[17,19,21,65-67] indicated enrichment of several miRNAs in LLCs following PMP exposure (Figure 3A). We generated LLC cells stably expressing E2-Crimson fluorophore to allow ex vivo TC isolation from resected tumors by single-cell sorting. Several miRNAs were upregulated in TCs in vivo compared with cells in culture as indicated by conventional PCR (Figure 3B) and quantitative reverse transcription PCR (qRT-PCR [qPCR]; Figure 3C), including miR-27a, miR-24, miR-25, miR-191, miR-9, and let-7a. Let-7a has been reported to constitute as much as 48% of the total platelet miRNA content.[46] The moderate fold increase in let-7a and other miRNAs in TCs may reflect high endogenous expression levels of these miRNAs. However, miR-24 and miR-27a were evident only in PMP-treated LLC cells but not untreated cells in vitro and in ex vivo TCs by conventional PCR (Figure 3A-B). These miRNAs were each elevated more than ninefold ex vivo compared with cells in culture (Figure 3C), indicating that these miRNAs had been either expressed selectively in tumors or transferred to TCs in vivo.

To determine whether the additional miRNAs in TCs ex vivo were platelet-derived, we crossed CA-*loxP* (>)-GFPstop-*loxP*(>)-*Uprt* C57Bl/6 mice with *Pf4*-Cre C57Bl/6 mice to generate CA>*Uprt*/*Pf4*-Cre double heterozygotes for Cre-induced expression of *Toxoplasma gondii* uracil phosphoribosyltransferase (UPRT) selectively in megakaryocytes/platelets (Figure 3D). This enzyme is required for incorporation of 4TU, a cell-permeable, nonnative uracil variant, into nascent mammalian RNA; thus, only megakaryocytes can produce thio-RNA in these mice upon 4TU exposure, yielding platelets selectively harboring thio-RNA which can be isolated by thiol-biotinylation and avidin chromatography (Figure 3D).[68] We seeded LLC-E2-Crimson cell tumors in the flanks of CA>*Uprt*/*Pf4*-Cre heterozygotes or *Pf4*-Cre controls, followed by 4TU i.p. injection, TC collection, and biotin/avidin isolation of platelet-derived RNA in PMP-stripped TCs (Figure 3D). Both miR-24 and miR-223 were observed as thio-RNA extracted from isolated TC RNA from *Pf4*-Cre/CA>*Uprt* mice, but not from TCs from *Pf4*-Cre mice, demonstrating in vivo transfer of these platelet-derived miRNAs to TCs (Figure 3E). qPCR from thio-RNA samples revealed significant increases over control in each of the screened miRNAs, with the greatest increase observed in miR-24 (Figure 3F). 4TU-RNA in TCs was platelet-derived and not from PMP-mediated transfer of the UPRT enzyme from platelets to tumor cells, as hemagglutinin (HA)-UPRT was expressed selectively in platelets in *Pf4*-Cre/CA>*Uprt* mice, but we did not detect HA-UPRT in PMPs nor in ex vivo TCs in these mice or in control mice (supplemental Figure 4); thus, UPRT-driven RNA labeling was restricted to megakaryocytes/platelets in this system. Together, these data demonstrate that platelet-derived miRNAs transfer to TCs in solid tumors in vivo, and miR-24 was a major species in the transfer. Attachment of platelet-derived miRNAs to TC surfaces could not be ruled out in these experiments; however, TCs can internalize platelet-derived miRNAs in vivo (Figure 2).

PMPs induce tumor cell apoptosis in an miR-24–dependent manner

We considered what effects PMPs may have on TC proliferation. PMP exposure in vitro blunted proliferation in a dose-dependent manner in LLCs (Figure 4A), as well as MC-38 colon carcinoma cells (Figure 4B), indicating broad tumor type specificity in PMP-induced TC growth suppression. Growth inhibition by PMPs was reversed by transfection with antagomiR-24[69] (Figure 4C-D), indicating that miR-24 is a major driver of growth inhibition by PMP exposure in these carcinoma cells. We observed a time lag in growth inhibition (Figure 4C-D), which may be due in part to the time required for miRNA downregulation of target RNAs and degradation of residual proteins. Mir-24–dependent TC growth inhibition correlated with expression of cleaved caspase-3, a marker for apoptosis, in both TC lines (Figure 4E-F), whereas we did not observe significant effects of PMPs on cell cycle progression (supplemental Figure 5), indicating that the reduction in cell number by PMP exposure was associated with induction of TC apoptosis. Association of PMPs with caspase-3 and apoptosis more broadly has been observed in other systems and various disease states.[70,71]

PMPs inhibit tumor growth in an miR-24–dependent manner

We tested tumor growth effects of PMPs in vivo. Daily transfusion of freshly generated human PMPs into mice bearing LLC or MC-38 tumors, beginning at day 8, markedly inhibited tumor growth, which was abrogated by transfection with antagomiR-24 prior to allograft implantation (Figure 4E-F). These results indicate that miR-24 in the transfused PMPs was partially responsible for tumor growth inhibition, which we would predict as miR-24 is conserved between mouse and human, and therefore these homologs have identical seed target

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

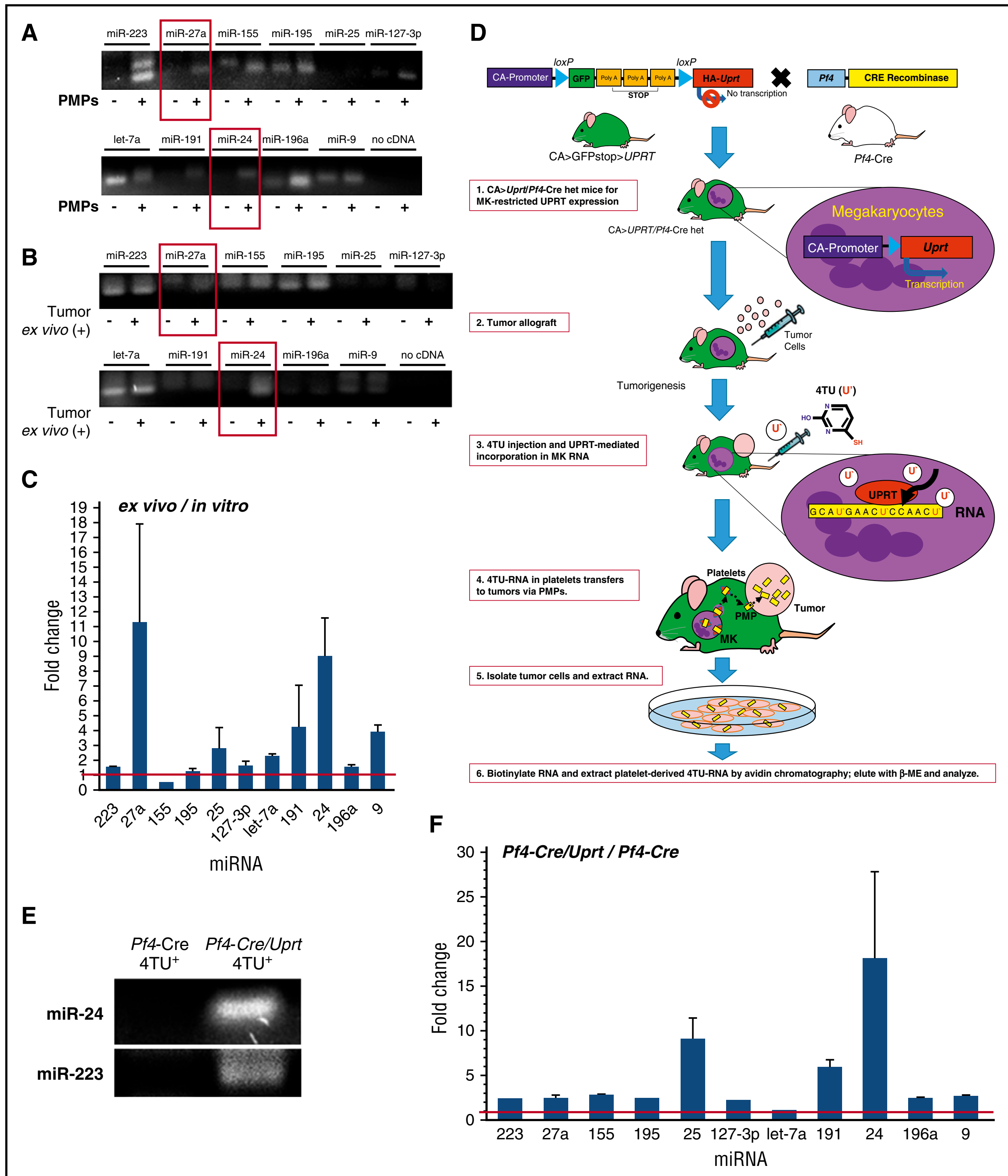

Figure 3. PMP transfer of platelet miRNAs to tumor cells in solid tumors. (A) Total RNA extracted from LLC cells posttrypsinization, untreated (−) or exposed to PMPs for 16 hours (+), was subjected to poly(dA) tailing, cDNA synthesis, and PCR with the indicated miRNAs as 5′ forward primers, and poly(dT) universal 3′ reverse primers. (B) Total RNA extracted from tumor cells isolated from resected LLC tumors, or from LLCs maintained in culture, was subjected to poly(dA) tailing, cDNA synthesis, and PCR with the indicated miRNAs as 5′ forward primers, and poly(dT) universal 3′ reverse primers. −, LLCs maintained in culture; +, LLCs ex vivo from resected tumors. The red boxes indicate undetectable levels of miR-27a and miR-24 in LLC cells maintained in culture, compared with a band corresponding to each miRNA from LLC cells treated with PMPs (A) or from resected tumors (B). "no cDNA" samples used miR-24 oligonucleotides. (C) qRT-PCR using 5′ forward primers matching indicated miRNAs paired with poly(dT) universal 3′ reverse primers on cDNA from poly(A)-tailed RNA from LLC cells isolated from resected tumors, fold change over expression in LLCs in culture, shown ± SEM. miRNA primers were for 5p arms unless otherwise indicated. $P < .05$ for each (n = 4). Red line denotes parity. (D) *Pf4*-Cre/*Uprt* mice and 4TU RNA labeling, biotinylation, and isolation. (1) CA>GFPstop>*Uprt* mice and *Pf4*-Cre mice crossing to generate CA>*Uprt*/*Pf4*-Cre heterozygotes, which express UPRT selectively in megakaryocytes (MKs) (> and blue triangle, *loxP* site). (2) Tumor seeding in the het mice and (3) 4TU (U′) injection for selective incorporation in MK RNA. (4) 4TU-RNA transfers from the MK platelet progeny to tumors via PMPs. (5) Tumor resection and tumor cell isolation by fluorescence-activated cell sorting (FACS), followed by RNA extraction. (6) Platelet-derived 4TU-RNA labeling with N-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (HPDP-biotin) added to the total tumor cell RNA, and isolation by affinity chromatography with avidin beads for further analysis. (E) PCR using miR-24 or miR-223 forward and poly(dT) reverse primers on avidin bead eluates from biotinylated RNA from tumor cells from 21-day tumors in 4TU-treated *Pf4*-Cre$^{+/-}$ (*Pf4*-Cre) and CA>HA-*Uprt*$^{+/-}$*Pf4*-Cre$^{+/-}$ (*Pf4*-Cre/*Uprt*) mice. (F) qRT-PCR from panel E, showing fold change ± SEM in tumor cells extracted from *Pf4*-Cre/*Uprt* vs *Pf4*-Cre mice. Red line denotes parity. Let-7a fold change = 1.2 ± 0.02. $P < .03$ for each (n = 8). β-ME, β-mercaptoethanol.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

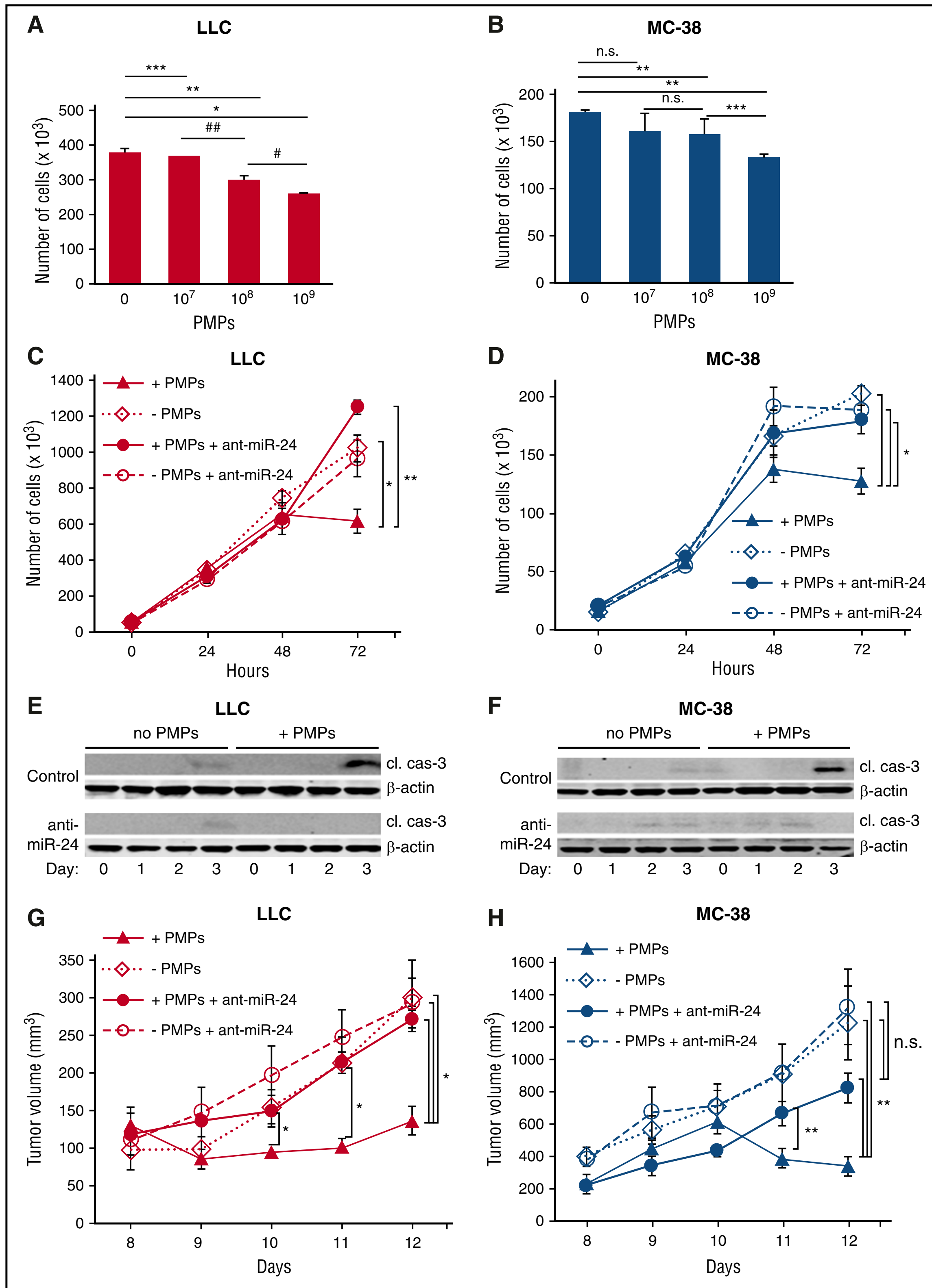

Figure 4. PMPs induce tumor cell apoptosis and inhibit tumor growth via miR-24. (A) The indicated number of PMPs collected from freshly isolated human platelets was coincubated with 5×10^4 LLC cells every 24 hours. Cells were counted at 60 hours (n = 8). (B) MC-38 cells, treated as assessed as in panel A (n = 3). (A-B) *$P < .0007$; **$P < .001$; ***$P < .05$; #$P < .02$; ##$P < .01$. (C) A total of 5×10^4 LLC cells were transfected and treated every 24 hours with 10^9 freshly isolated PMPs as indicated, and counted daily (n = 5). (D) MC-38 cells transfected, treated, and analyzed as in panel C. (C-D): *$P < .03$; **$P < .04$ (n = 3). (E) LLC cells treated as in panel C were harvested and lysates were processed for western blotting with antibodies to cleaved caspase-3 (cl. cas-3) and β-actin. (F) Lysates of MC-38 cells treated as in panel D were processed for western blotting as in panel E (n = 5) for panels E-F. (G) A total of 1×10^6 LLCs were transfected as indicated, and after 18 hours were seeded as allografts by bolus injection into the flanks of WT mice. Beginning at day 8, 1×10^{10} PMPs freshly isolated from human platelets were counted and transfused daily by tail vein injection. Tumor volumes were measured daily with calipers (n = 6). *$P < .02$. (H) MC-38 cells were transfected and implanted, followed by PMP transfusion, and tumor growth was monitored as in panel G. **$P < .003$ (n = 6). All plots, shown ± SEM. n.s., not significant.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

sequences and thus, with identical binding affinities, putatively can target the same RNAs. Transfused PMPs infiltrated tumors and appeared broadly distributed in the intratumoral environment (supplemental Figure 6A), but neither endogenous nor exogenous PMPs were detected in nontumor tissue (supplemental Figure 6B). Thus, PMPs are tumor suppressive both in vitro and in vivo via effects of transferred miRNAs including miR-24.

PMP depletion in Par4-null mice prevents PMP-mediated tumor growth inhibition in vivo via induction of apoptosis in PMP-targeted tumor cells

To investigate further the specific role of PMPs in tumor growth inhibition, we evaluated MPs and tumor growth in mice deleted for the Par4 thrombin receptor, which has been shown to be the major driver of PMP generation.[72] Plasma MPs were reduced by ~50% in *Par4* knockout (KO) mice compared with WT (Figure 5A), whereas total circulating platelet counts were unaltered (results not shown). In accordance with an inhibitory role for PMPs, solid tumor growth was accelerated in *Par4* KO mice compared with WT (Figure 5B-C). Daily transfusion of PMPs was sufficient to halt tumor growth in both WT and *Par4* KO mice (Figure 5C), indicating that PMPs are the principal effectors of tumor growth inhibition in this model. Tumor infiltration of endogenous PMPs was reduced in *Par4* KO mice compared with WT, consistent with depletion of PMPs in those mice (Figure 5D), and PMP transfusion restored PMP infiltration in tumors (Figure 5E). PMP-associated TCs showed expression of cleaved caspase-3, whereas adjacent, nontargeted cells did not (Figure 5D-E); thus, PMP targeting is associated with TC apoptosis in vivo.

Identification of tumor cell RNA targets of PMP-derived miR-24

To identify TC RNA targets of PMP-derived miR-24, we modified a method recently described,[48] in which single-strand chimeras derived from truncated miRNA:target RNA hybrid pairs are generated in cell lysate fractions (Figure 6A). We focused on miR-24 targets, as miR-24 was a major species transferred from PMPs and a major contributor to growth inhibitory effects of PMPs. Human and murine miR-24-1-5p and -2-5p sequences are identical. We performed miRNA:mRNA ligation reactions on lysates from LLC cells with or without PMP exposure, followed by PCR with miR-24 5′ and universal 3′ primers, and direct cloning and sequencing of the PCR products. Whereas some insert sequences were either too short or contained misalignments, preventing unambiguous identification of the miRNA-ligated target RNAs (supplemental Table 1), several of the miR-24 adduct inserts could be unambiguously assigned. A 21-nt insert corresponded exclusively to *Snora75*, an H/ACA box small nucleolar RNA (snoRNA), and another independent clone contained a 96-nt segment also corresponding exclusively to *Snora75*. Hence, some miR-24 adducts included a noncoding RNA (ncRNA). A 281-nt insert sequence in another clone corresponded exclusively and entirely to a segment of mitochondrial *mt-Nd2* mRNA, suggesting targeting of the coding region of a mitochondrial gene by miR-24 (Figure 6B; supplemental Figure 7).[73]

To evaluate *mt-Nd2* mRNA and *Snora75* regulation by miRNAs in RNA-induced silencing complexes (RISCs) in PMP-exposed cells, we extracted RNA from Ago2 immunoprecipitate (IP) fractions in untreated and PMP-treated TCs, and analyzed relative enrichment by qPCR. As before, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping control, which is valid in this case as GAPDH is recruited into RISCs and modulated by siRNAs or miRNAs, but known miRNAs targeting GAPDH have not been detected in platelets or PMPs.[64,74-76] PMP exposure led to increases in *mt-Nd2* and *Snora75* RNAs in Ago2-containing complexes compared with untreated cells (Figure 6C), indicating enhanced recruitment of these RNA targets to RISCs by PMP exposure. These results were not due to transfer of preformed miRNA:target RNA complexes via PMPs, as evidenced by lack of human *mt-Nd2* and *Snora75* RNAs in induced PMPs as well as in human PMP-treated TCs. In contrast, miR-24 was evident in platelets, PMPs, and PMP-treated TCs, further supporting direct transfer of this miRNA from platelets to TCs via PMPs (supplemental Figure 8).

RNA levels of *Snora75* and *mt-Nd2* were substantially suppressed in both LLC and MC-38 cells following PMP exposure (Figure 6D-E), indicating broad tumor type specificity in PMP-mediated gene suppression. AntagomiR-24 rescued *mt-Nd2* and *Snora75* downregulation by PMP exposure (Figure 6D-E). Interestingly, antagomiR-24 had no effect on expression of these RNAs in untreated LLCs, but led to *mt-Nd2* upregulation in MC-38 cells both with and without PMPs (Figure 6D-E). These results suggest that *mt-Nd2* steady-state expression is modulated by endogenous miR-24 in MC-38 cells to a greater extent than in LLC cells, and addition of exogenous PMP-derived miR-24 enhances gene suppression in both cell lines. Mt-Nd2 protein expression was also decreased by PMPs in both TC lines, and these decreases were prevented by antagomiR-24 (Figure 6F-G). Thus, PMP exposure in these TCs induces downregulation of *mt-Nd2* and *Snora75* via miR-24.

PMP-derived miR-24 localizes to mitochondria and inhibits tumor cell mitochondrial function

MiR-24 was broadly distributed in multiple compartments in PMP-exposed cells, including nucleus, nucleoli, mitochondria, and mitochondria-depleted cytoplasm (Figure 7A). MiR-24 was recently observed in nucleoli[77]; these data indicate that miR-24 is also a mitomiR.[78] This multiorganelle localization is consistent with the ability of miR-24 to target and downregulate RNAs in each compartment.

Mt-Nd2 is a component of the nicotinamide adenine dinucleotide: ubiquinone oxidoreductase (complex I), responsible for mitochondrial oxidative phosphorylation and reactive oxygen species production.[79] We measured mitochondrial membrane potential in PMP-treated and untreated cells. Nuclear localization of the membrane potential indicator, tetramethylrhodamine, methyl ester (TMRM), was significantly increased by PMP treatment (Figure 7B-C left panels), indicating mitochondrial depolarization following PMP exposure. This could be due to inactivation of complex I and shunt to complex III.[80] In support of this, ATP generation was strongly inhibited by PMP treatment (Figure 7B-C right panels). AntagomiR-24 abrogated these effects of PMP exposure (Figure 7B-C). AntagomiR-24 also led to mitochondrial depolarization in the absence of PMPs in MC-38 cells (Figure 7C), correlating with moderate mt-Nd2 suppression by PMPs in these cells, and increased *mt-Nd2* by antagomiR-24 alone (Figure 6). However, ATP production was not increased over baseline levels in this case. Platelet-derived mitochondria were not transferred via PMPs to TCs, as we did not detect Mitotracker Red–labeled platelet mitochondria in TCs after PMP generation and exposure (supplemental Figure 9), and as noted previously, human *mt-Nd2* RNA, which is contained within mitochondria, was also not transferred from platelets to PMPs or TCs (supplemental Figure 8). Together, these data demonstrate that miR-24, transferred from PMPs to tumor cells, causes mitochondrial dysfunction and tumor growth inhibition.

Discussion

Our findings establish a previously unappreciated mode of indirect intercellular communication between platelets and tumor cells in solid

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

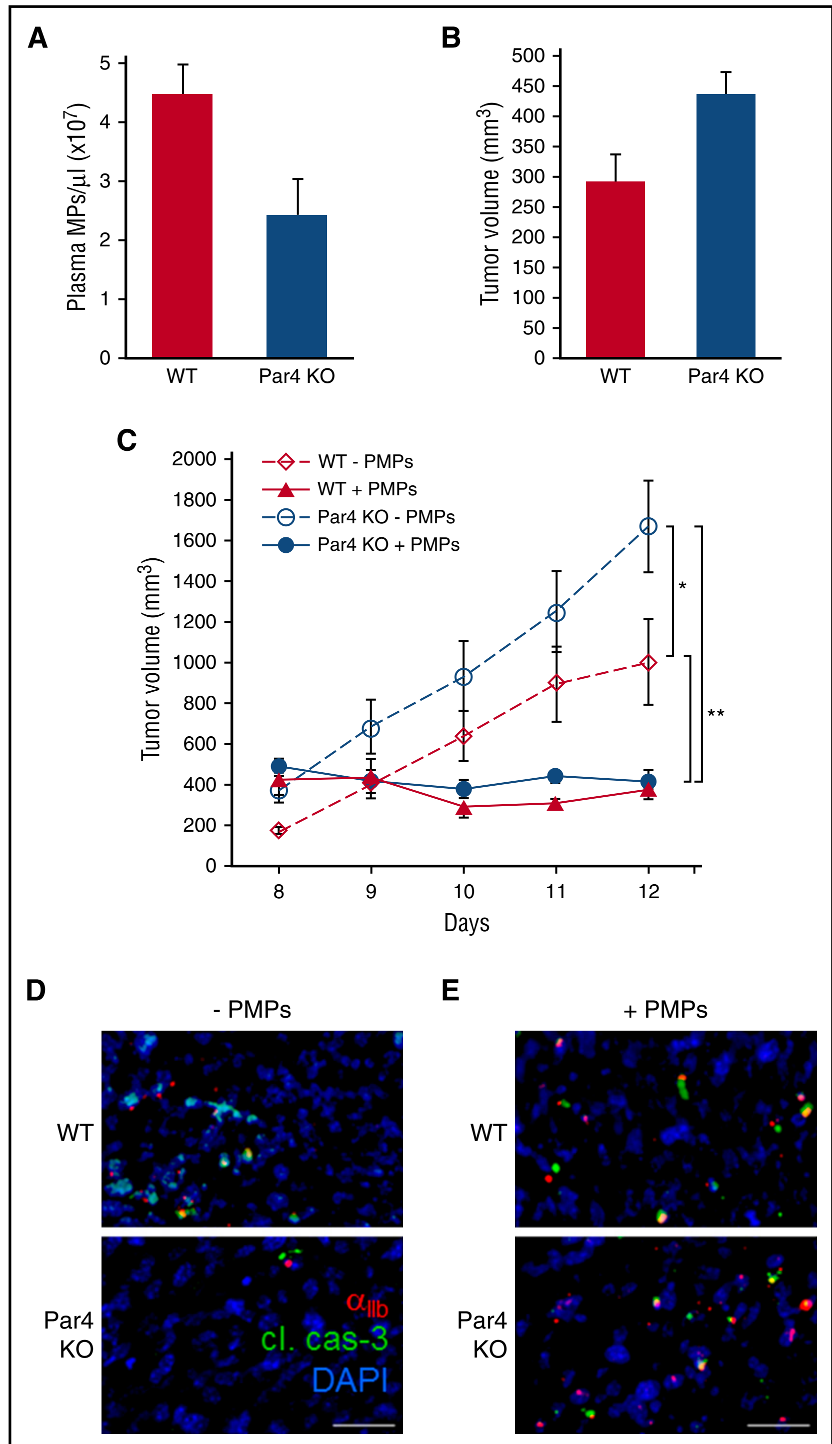

Figure 5. Plasma MPs and tumor growth in *Par4* KO mice. (A) Plasma MPs from WT and Par4 KO mice were analyzed by nanoparticle tracking, and are shown ± SEM (n = 6); $P < .03$. (B) A total of 1×10^6 LLC cells were seeded in the flanks of WT and *Par4* KO mice, and tumor volumes were measured 8 days after seeding, shown ± SEM (n = 6); $P < .03$. (C) LLC tumors were seeded as in panel B, and 1×10^{10} freshly isolated PMPs were transfused in the tail vein every 24 hours beginning at day 8 as indicated. Tumor volumes were measured daily, and are shown ± SEM (n = 6); $^*P < .03$; $^{**}P < .002$. (D) Tumors from panel C were resected, fixed, and processed for IHC with antibodies to murine CD41 (α_{IIb} integrin, red) to label endogenous PMPs, and cleaved caspase-3 (green); DAPI stain is shown in blue. (E) Tumor sections from panel C stained with antibodies to human CD41 (α_{IIb} integrin, red) to label transfused PMPs, cleaved caspase-3 (cl. cas-3, green), and DAPI (blue). Bars, 15 μm.

tumors, with inhibitory effects on tumor progression by transfer of platelet miRNAs and downregulation of tumor cell gene expression. PMP infiltration, transfer of platelet-derived miRNAs to TCs, and gene-regulatory and growth-suppressive effects, together extend the reach and capabilities of platelets to affect cancer progression beyond the intravascular space. Our findings further support broad specificities in miRNA targeting, including mRNA coding regions (eg, mitochondrial mRNAs) and other ncRNAs as viable targets for effective suppression. Our findings also implicate PMPs and their cargo more broadly, as important contributors to functional outcomes in pathological conditions of increased vascular permeability.

We observed PMP infiltration in multiple solid tumor types, and at each tumor grade, but not in unaffected normal tissues. This difference likely reflects increased permeability of tumor neovasculature, which unlike mature blood vessels, is perforated with pores >>100 nm as a result of dysfunctional endothelial junctions, allowing for leak of blood-borne protein complexes and other macromolecular complexes into the intratumoral space.[50] This pore size is permissive for extravascular leak of PMPs, and it is likely that other cell-derived MPs as well as exosomes (30-100 nm diameter) invade the tumor microenvironment through these pores. PMP exposure due to vascular leak is therefore likely restricted to solid tumors, distinct from normal tissues, adding

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

B

Alignment	Genetic region	TA clone insert sequence: miRNA segment	TA clone insert sequence: Target / adduct segment	TA clone insert sequence: poly(A) segment
mt-Nd2	coding	GTGCCTACTGAGCTGATATCAGT	ACTATCATTTTAATACTAGCAATTACTTCTATTTTCATAGGGGCA TGAGGAGGACTTAACCAAACACAAATACGAAAAATTATAGCCTA TTCATCAATTGCCCACATAGGATGAATATTAGCAATTCTTCCTT ACAACCCATCCCTCACTCTACTCAACCTCATAATCTATATTATTC TTACAGCCCCTATATTCATAGCACTTATACTAAATAACTCTATAA CCATCAACTCAATCTCACTTCTATGAAATAAAACTCCAGCAATA CTAACTATAATCTC	AAAAAAAAAAAAAAAAAAAA
Snora75	ncRNA	GTGCCTACTGAGCTGATATCAGT	CGTGCGAATCCATAATCTATC	AAAAAAAAAAAAAAAAAAAA
Snora75	ncRNA	GTGCCTACTGAGCTGATATCAGT	AATAGATTATGGATTCGCACGAGAAGAAGAGAGAATTCACAGA ACTGGCACTTATCTTCTGTTTTTGCAGAAGTATATTTGGCTGTT GTGTGAGAC	AAAAAAAAAAAAAAAAAAAACA

Figure 6.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

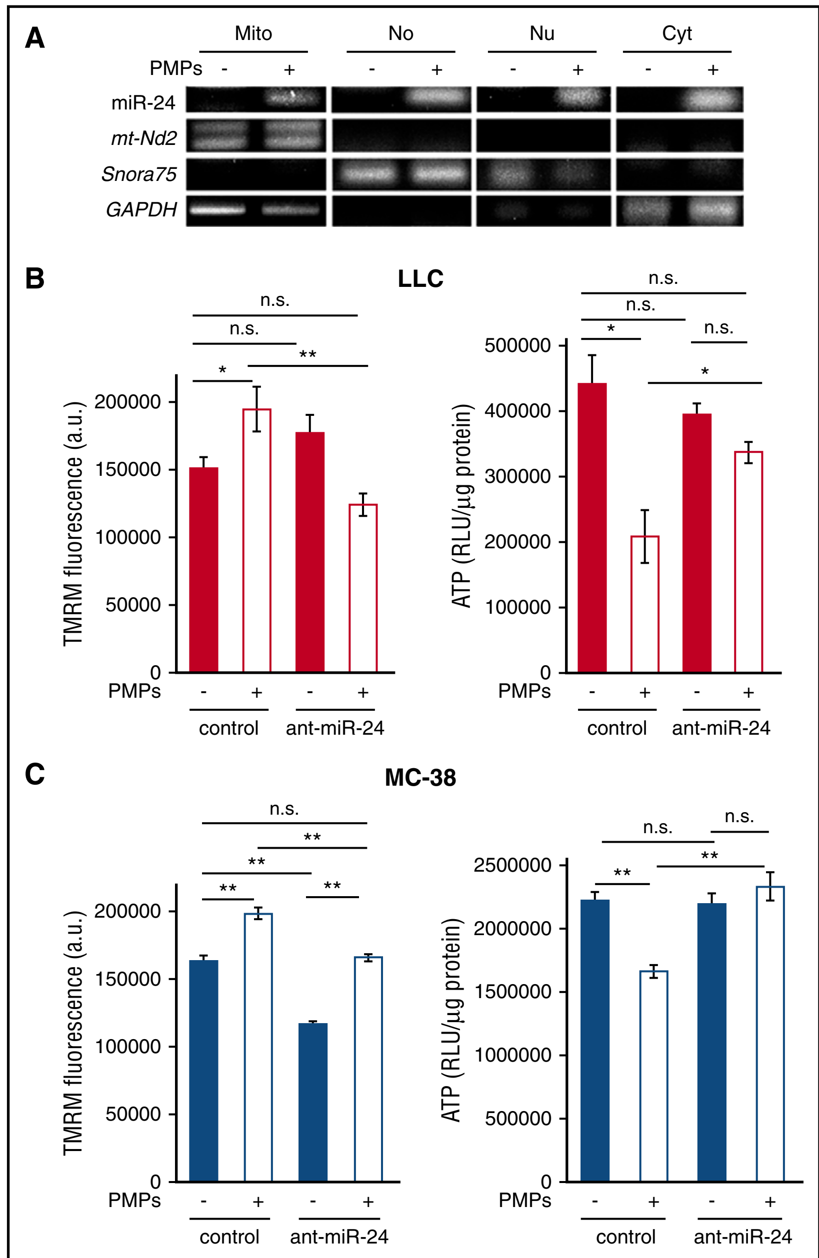

Figure 7. PMP-transferred miR-24 inhibits mitochondrial function in tumor cells. (A) cDNA from RNA isolated from mitochondria (Mito), nucleolar (No), nuclear (Nu), and post-mitochondria (Cyto) fractions of untreated and PMP-treated LLC cells (−/+) was subject to PCR for the indicated genes. (B) Mitochondrial membrane potential (TMRM, left) and ATP levels (right) were assessed in LLC cells ± PMPs and antagomiR-24 as indicated. (C) TMRM (left) and ATP (right) in MC-38 cells, treated as in panel B. $*P < .01$; $**P < .0001$ (n = 3). All histograms, shown ± SEM. RLU, relative luminescence unit.

PMPs and other microvesicles to the unique composition of the tumor microenvironment. Mechanisms of PMP attachment and internalization in tumor cells remain to be elucidated, but could involve interactions with multiple receptors such as glycoprotein 1b (potentially in complex with VWF),[81] P-selectin,[82] phosphatidylserine receptors on the tumor cell surface,[83] or a combination of these and other interactions.

Interactions between MPs and cells have been investigated in many contexts, these interactions yield a broad range of outcomes.[84,85] Endothelium and blood cells are exposed to PMPs, and effects are

Figure 6. RNA targets of PMP-derived miR-24. (A) Schematic for low-throughput miRNA target identification. PMP-treated cells were lysed posttrypsinization by suspension in hyposmotic buffer followed by sonication. Whole-cell extracts were treated with RNase T1, 3′ end blocking with PNK minus lacking 3′ phosphatase activity, followed by T4 RNA ligase. RNA extracted from these samples with TRIzol was tagged with poly(dA) tails and subjected to first-strand cDNA synthesis, followed by conventional PCR with *Taq* polymerase using miRNA-specific 5′ primers and poly(dT) 3′ primer, direct cloning of unsorted PCR products into the pCR2.1 (TA) vector, and transformation of the DNA ligation reactions into *E coli*. Colonies were selected and plasmid DNA preparations were analyzed by conventional sequencing. (B) miR-24:target RNA adduct clones. TA clones with inserts matching unique sequences are shown. The insert sequences are separated in the table into the apparent miRNA segment, the cognate target/adduct segment, and the poly(A) segment. (C) *mt-Nd2* and *Snora75* RNA enrichment in RISC complexes following tumor cell exposure to PMPs. Shown are qPCR ratios for *mt-Nd2* and *Snora75* RNA content in Ago2 immunoprecipitate (IP) fractions from LLC (left) or MC-38 cells (right) treated with PMPs, relative to IP fractions from untreated cells. $*P < .05$ (n = 3). (D) LLC cells were transfected with 25 µg of phosphorothioate, LNA 8-nt control or antagomiR-24 (ant-miR-24), 24 hours prior to PMP exposure. RNA was isolated from cells 16 hours after exposure to PMPs or blank media. qRT-PCR was performed using 100-bp PCR fragments of each transcript, and relative expression levels were quantified using GAPDH as a housekeeping gene control, normalized to target RNA expression in untreated cells, shown as 1. $*P < .001$; $**P < .05$ (n = 7). (E) MC-38 cells treated and analyzed as in panel D. $***P < .01$; $\#P < .02$ (n = 5). All histograms, shown ± SEM. (F-G) Western blotting with α-mt-Nd2 antibodies (Nd2) of lysates of cells treated with PMPs for up to 3 days. β-actin was used as a loading control. Densitometry results are shown for Nd2/β-actin ratios, ± SEM for 3 independent experiments. $*P < .05$, all others n.s.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

just beginning to be explored.[19,86] PMPs have been shown to transfer miRNAs to cells when coincubated in vitro, and to modulate target cell gene expression.[23,24] For example, PMP incubation with A549 lung carcinoma cells in culture enhanced invasiveness due to increased miR-223 and suppression of anti-invasive genes.[24] The present study expands on this background by demonstrating that PMP-cell interactions have functionally important roles in vivo, via miRNA transfer. Differences in the degree of miRNA increases ex vivo compared with in vitro may reflect upregulation by TCs during tumor progression, and/or internalization of exogenous miRNA from stromal sources other than PMPs. However, our results point to miR-24 as a major PMP-derived regulator of tumor growth in two cancer cell lines. Together, our data support growth inhibitory roles of PMP interactions with TCs in solid tumors, via direct transfer of platelet-derived miRNAs and modulation of TC gene expression, resulting in tumor cell apoptosis, thus representing a new inhibitory function of this interaction in tumor progression.

Targets of transferred miR-24 included noncanonical RNAs, a mitochondrial mRNA lacking a 3′-untranslated region (UTR), and a small nucleolar ncRNA (snoRNA), representing regulation of one ncRNA by another. MiRNAs can regulate many RNA classes, and can bind many types of target sites, not limited to 3′-UTRs and with variations in seed complementarity.[48,87-89] Whether noncanonical miRNA response elements are biologically relevant remains controversial, as recent studies indicate that noncanonical sites may not contribute substantially to gene suppression.[90] However, the miR-24 targets we identified were found through an unbiased screen following direct ligation of miRNAs to their targets within RISCs. These RNAs were enriched in Ago2-containing complexes following PMP exposure, indicating recruitment to RISCs and enhanced RISC targeting by PMP-derived miRNAs. Together these findings suggest flexibility in functional seed recognition in these cells. Alternatively, PMPs, like platelets, may be enriched for variant miRNA isoforms (isomiRs) with base-shifted seed sites, including isomiRs of miR-24 and other miRNAs.[46,73] Indeed, a 7-mer sequence in *mt-Nd2* (AGTAGGC, conserved in human and murine) corresponds to a seed response element for a putative isomiR of miR-24.[91] Nonetheless, the functional outcome of miRNA transfer from PMPs to TCs included suppression of multiple genes and inhibition of tumor growth.

The results of this study demonstrate new functions for platelets beyond the vascular space, and expanded roles in tumor progression, further complicating the platelet-cancer relationship. Platelets support cancer progression at several levels, particularly at late stages in primary tumors and in metastatic dissemination.[4,92] Our results demonstrate tumor-suppressive roles at earlier stages, via growth-suppressive effects through downregulation of TC genes and induction of tumor cell apoptosis. Platelet miRNA transfer may also modulate other aspects of tumor biology, such as multidrug resistance, which is known to be regulated by MPs.[93-96] The full cohort of platelet miRNAs is likely to modulate expression of multiple genes, potentially in a tumor type-specific manner, in TCs as well as in the tumor microenvironment. Thus, platelets contribute both positively and negatively to cancer progression through various modes and at multiple stages.

PMP extravasation may be a common consequence in the context of endothelial barrier dysfunction resulting in increased vascular permeability. Relationships of platelets and MPs with vascular leak have recently been described in cardiovascular disease,[97] ischemia and postischemic tissue repair,[98] sepsis,[99-101] wound healing,[102] and diabetes,[103-105] implicating PMPs and miRNA transfer more broadly beyond solid tumor progression as a potential regulator of physiological responses to vascular leak. Such effects merit further exploration.

Acknowledgments

The authors thank John Kostyak, Maria de la Fuente, and Ilya Serebriiskii for technical assistance, Satya Kunapuli for support, and the Kunapuli laboratory for discussions. The authors thank Erica Golemis for critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (National Heart, Lung, and Blood Institute R01HL109568 [A.K.R.]; National Heart, Lung, and Blood Institute R01HL128446 [A.S.]; National Institute of General Medical Sciences R01GM109882, National Heart, Lung, and Blood Institute R01HL086699, National Heart, Lung, and Blood Institute R01HL119306, and National Institute of General Medical Sciences 1S10RR027327 [M.M.]; National Institute of General Medical Sciences K01GM103806 [J.W.R.]; and National Heart, Lung, and Blood Institute U54HL112311 [A.S.W.]) and from the American Heart Association (14GRNT20460004 [J.Y. and L.C.E.] and 16GRNT27260319 [L.E.G.]).

Authorship

Contribution: L.E.G. conceptualized the study; A.K.R., M.M., J.W.R., M.T.N., L.C.E., A.S.W., and L.E.G. designed the study methodology; J.V.M., J.G.T.W., G.F.M., N.E.H., S.R., D.T., J.W.R., J.Y., and L.E.G. investigated; J.V.M., J.G.T.W., and L.E.G. wrote the original draft of the manuscript; J.V.M., J.G.T.W., M.T.N., A.S., A.K.R., J.W.R., A.S.W., and L.E.G. reviewed and edited the manuscript; A.K.R., M.M., J.W.R., A.S.W., J.Y., L.C.E., and L.E.G. acquired funding; M.T.N., M.A.K., and A.S. provided resources; and A.K.R., M.M., A.S., L.C.E., A.S.W., and L.E.G. supervised the study.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Lawrence E. Goldfinger, Department of Anatomy & Cell Biology and The Sol Sherry Thrombosis Research Center, Lewis Katz School of Medicine at Temple University, 3420 N Broad St, Philadelphia, PA 19140; e-mail: goldfinger@temple.edu.

References

1. Gasic GJ, Gasic TB, Stewart CC. Antimetastatic effects associated with platelet reduction. *Proc Natl Acad Sci USA*. 1968;61:46-52.
2. Camerer E, Qazi AA, Duong DN, Cornelissen I, Advincula R, Coughlin SR. Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood*. 2004;104(2):397-401.
3. Kim YJ, Borsig L, Varki NM, Varki A. P-selectin deficiency attenuates tumor growth and metastasis. *Proc Natl Acad Sci USA*. 1998;95(16):9325-9330.
4. Labelle M, Begum S, Hynes RO. Platelets guide the formation of early metastatic niches. *Proc Natl Acad Sci USA*. 2014;111(30):E3053-E3061.
5. Gay LJ, Felding-Habermann B. Platelets alter tumor cell attributes to propel metastasis: programming in transit. *Cancer Cell*. 2011;20(5):553-554.
6. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat Rev Cancer*. 2011;11(2):123-134.
7. Sabrkhany S, Griffioen AW, Oude Egbrink MG. The role of blood platelets in tumor angiogenesis. *Biochim Biophys Acta*. 2011;1815(2):189-196.
8. Bambace NM, Holmes CE. The platelet contribution to cancer progression. *J Thromb Haemost*. 2011;9(2):237-249.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

9. Stone RL, Nick AM, McNeish IA, et al. Paraneoplastic thrombocytosis in ovarian cancer [published correction appears in *N Engl J Med*. 2012;367(18):1768]. *N Engl J Med*. 2012;366(7): 610-618.
10. Buergy D, Wenz F, Groden C, Brockmann MA. Tumor-platelet interaction in solid tumors. *Int J Cancer*. 2012;130(12):2747-2760.
11. Cho MS, Bottsford-Miller J, Vasquez HG, et al. Platelets increase the proliferation of ovarian cancer cells. *Blood*. 2012;120(24):4869-4872.
12. Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol*. 1967; 13(3):269-288.
13. Horstman LL, Ahn YS. Platelet microparticles: a wide-angle perspective. *Crit Rev Oncol Hematol*. 1999;30(2):111-142.
14. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. *Cardiovasc Res*. 2003;59(2):277-287.
15. Rao AK, Rao DA. Platelets signal and tumors take off. *Blood*. 2012;120(24):4667-4668.
16. Varon D, Shai E. Role of platelet-derived microparticles in angiogenesis and tumor progression. *Discov Med*. 2009;8(43):237-241.
17. Hunter MP, Ismail N, Zhang X, et al. Detection of microRNA expression in human peripheral blood microvesicles [published correction appears in *PLoS One*. 2010;5(3)]. *PLoS One*. 2008;3(11): e3694.
18. Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. *Nat Struct Mol Biol*. 2009;16(9):961-966.
19. Diehl P, Fricke A, Sander L, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. *Cardiovasc Res*. 2012;93(4): 633-644.
20. Edelstein LC, McKenzie SE, Shaw C, Holinstat MA, Kunapuli SP, Bray PF. MicroRNAs in platelet production and activation. *J Thromb Haemost*. 2013;11(suppl 1):340-350.
21. Willeit P, Zampetaki A, Dudek K, et al. Circulating microRNAs as novel biomarkers for platelet activation. *Circ Res*. 2013;112(4): 595-600.
22. Risitano A, Beaulieu LM, Vitseva O, Freedman JE. Platelets and platelet-like particles mediate intercellular RNA transfer. *Blood*. 2012;119(26): 6288-6295.
23. Laffont B, Corduan A, Plé H, et al. Activated platelets can deliver mRNA regulatory Ago2•microRNA complexes to endothelial cells via microparticles. *Blood*. 2013;122(2):253-261.
24. Liang H, Yan X, Pan Y, et al. MicroRNA-223 delivered by platelet-derived microvesicles promotes lung cancer cell invasion via targeting tumor suppressor EPB41L3. *Mol Cancer*. 2015; 14(1):58.
25. Chen Z, Ma T, Huang C, Hu T, Li J. The pivotal role of microRNA-155 in the control of cancer. *J Cell Physiol*. 2014;229(5):545-550.
26. Haneklaus M, Gerlic M, O'Neill LA, Masters SL. miR-223: infection, inflammation and cancer. *J Intern Med*. 2013;274(3):215-226.
27. He JF, Luo YM, Wan XH, Jiang D. Biogenesis of MiRNA-195 and its role in biogenesis, the cell cycle, and apoptosis. *J Biochem Mol Toxicol*. 2011;25(6):404-408.
28. Jurkovicova D, Magyerkova M, Kulcsar L, et al. miR-155 as a diagnostic and prognostic marker in hematological and solid malignancies. *Neoplasma*. 2014;61(3):241-251.
29. Li XL, Jones MF, Subramanian M, Lal A. Mutant p53 exerts oncogenic effects through microRNAs and their target gene networks. *FEBS Lett*. 2014;588(16):2610-2615.
30. van Jaarsveld MT, Helleman J, Berns EM, Wiemer EA. MicroRNAs in ovarian cancer biology and therapy resistance. *Int J Biochem Cell Biol*. 2010;42(8):1282-1290.
31. Vimalraj S, Miranda PJ, Ramyakrishna B, Selvamurugan N. Regulation of breast cancer and bone metastasis by microRNAs. *Dis Markers*. 2013;35(5):369-387.
32. Gao Y, Liu Y, Du L, et al. Down-regulation of miR-24-3p in colorectal cancer is associated with malignant behavior. *Med Oncol*. 2015;32(1):362.
33. Listing H, Mardin WA, Wohlfromm S, Mees ST, Haier J. MiR-23a/-24-induced gene silencing results in mesothelial cell integration of pancreatic cancer. *Br J Cancer*. 2015;112(1): 131-139.
34. Pan B, Chen Y, Song H, Xu Y, Wang R, Chen L. Mir-24-3p downregulation contributes to VP16-DDP resistance in small-cell lung cancer by targeting ATG4A. *Oncotarget*. 2015;6(1): 317-331.
35. Goto Y, Kojima S, Nishikawa R, et al. The microRNA-23b/27b/24-1 cluster is a disease progression marker and tumor suppressor in prostate cancer. *Oncotarget*. 2014;5(17): 7748-7759.
36. Inoguchi S, Seki N, Chiyomaru T, et al. Tumour-suppressive microRNA-24-1 inhibits cancer cell proliferation through targeting FOXM1 in bladder cancer. *FEBS Lett*. 2014;588(17):3170-3179.
37. Sochor M, Basova P, Pesta M, et al. Oncogenic microRNAs: miR-155, miR-19a, miR-181b, and miR-24 enable monitoring of early breast cancer in serum. *BMC Cancer*. 2014;14:448.
38. Duan Y, Hu L, Liu B, et al. Tumor suppressor miR-24 restrains gastric cancer progression by downregulating RegIV. *Mol Cancer*. 2014;13: 127.
39. Ma Y, She XG, Ming YZ, Wan QQ. miR-24 promotes the proliferation and invasion of HCC cells by targeting SOX7. *Tumour Biol*. 2014; 35(11):10731-10736.
40. Martin EC, Elliott S, Rhodes LV, et al. Preferential star strand biogenesis of pre-miR-24-2 targets PKC-alpha and suppresses cell survival in MCF-7 breast cancer cells. *Mol Carcinog*. 2014;53(1):38-48.
41. Yin JY, Tang Q, Qian W, et al. Increased expression of miR-24 is associated with acute myeloid leukemia with t(8;21). *Int J Clin Exp Pathol*. 2014;7(11):8032-8038.
42. Giglio S, Cirombella R, Amodeo R, Portaro L, Lavra L, Vecchione A. MicroRNA miR-24 promotes cell proliferation by targeting the CDKs inhibitors p27Kip1 and p16INK4a. *J Cell Physiol*. 2013;228(10):2015-2023.
43. Du WW, Fang L, Li M, et al. MicroRNA miR-24 enhances tumor invasion and metastasis by targeting PTPN9 and PTPRF to promote EGF signaling. *J Cell Sci*. 2013;126(Pt 6):1440-1453.
44. Katoh M. Cardio-miRNAs and onco-miRNAs: circulating miRNA-based diagnostics for non-cancerous and cancerous diseases. *Front Cell Dev Biol*. 2014;2:61.
45. Mironova N, Patutina O, Brenner E, Kurilshikov A, Vlassov V, Zenkova M. MicroRNA drop in the bloodstream and microRNA boost in the tumour caused by treatment with ribonuclease A leads to an attenuation of tumour malignancy. *PLoS One*. 2013;8(12):e83482.
46. Plé H, Landry P, Benham A, Coarfa C, Gunaratne PH, Provost P. The repertoire and features of human platelet microRNAs. *PLoS One*. 2012;7(12):e50746.
47. Michael JV, Wurtzel JGT, Goldfinger LE. Regulation of H-Ras-driven MAPK signaling, transformation and tumorigenesis, but not PI3K signaling and tumor progression, by plasma membrane microdomains. *Oncogenesis*. 2016; 5(5):e228.
48. Grosswendt S, Filipchyk A, Manzano M, et al. Unambiguous identification of miRNA:target site interactions by different types of ligation reactions. *Mol Cell*. 2014;54(6):1042-1054.
49. Gidlof O, van der Brug M, Ohman J, et al. Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression. *Blood*. 2013;121(19):3908-3917.
50. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev*. 2005;15(1): 102-111.
51. Mezouar S, Mege D, Darbousset R, et al. Involvement of platelet-derived microparticles in tumor progression and thrombosis. *Semin Oncol*. 2014;41(3):346-358.
52. Horstman LL, Jy W, Jimenez JJ, Bidot C, Ahn YS. New horizons in the analysis of circulating cell-derived microparticles. *Keio J Med*. 2004; 53(4):210-230.
53. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. *Circ Res*. 2010;107(9):1047-1057.
54. Salanova B, Choi M, Rolle S, Wellner M, Luft FC, Kettritz R. Beta2-integrins and acquired glycoprotein IIb/IIIa (GPIIb/IIIa) receptors cooperate in NF-kappaB activation of human neutrophils. *J Biol Chem*. 2007;282(38): 27960-27969.
55. Lee S, Wurtzel JG, Singhal SS, Awasthi S, Goldfinger LE. RALBP1/RLIP76 depletion in mice suppresses tumor growth by inhibiting tumor neovascularization. *Cancer Res*. 2012; 72(20):5165-5173.
56. Rásó E, Tóvári J, Tóth K, et al. Ectopic alphaIIbbeta3 integrin signaling involves 12-lipoxygenase- and PKC-mediated serine phosphorylation events in melanoma cells. *Thromb Haemost*. 2001;85(6):1037-1042.
57. Puerschel WC, Gawaz M, Worret WI, Ring J. Immunoreactivity of glycoprotein IIb is present in metastasized but not in non-metastasized primary malignant melanoma. *Br J Dermatol*. 1996;135(6):883-887.
58. Timar J, Trikha M, Szekeres K, Bazaz R, Honn K. Expression and function of the high affinity alphaIIbbeta3 integrin in murine melanoma cells. *Clin Exp Metastasis*. 1998;16(5):437-445.
59. Trikha M, Raso E, Cai Y, et al. Role of alphaII(b) beta3 integrin in prostate cancer metastasis. *Prostate*. 1998;35(3):185-192.
60. Michael JV, Wurtzel JG, Goldfinger LE. Inhibition of galectin-1 sensitizes HRAS-driven tumor growth to rapamycin treatment. *Anticancer Res*. 2016;36(10):5053-5061.
61. Misaki R, Morimatsu M, Uemura T, et al. Palmitoylated Ras proteins traffic through recycling endosomes to the plasma membrane during exocytosis. *J Cell Biol*. 2010;191(1): 23-29.
62. Wurtzel JG, Kumar P, Goldfinger LE. Palmitoylation regulates vesicular trafficking of R-Ras to membrane ruffles and effects on ruffling and cell spreading. *Small GTPases*. 2012;3(3):139-153.
63. Popov EG, Mejlumian AG, Gavrilov IYu, Gabbasov ZA, Pozin EYa. Evaluation of the ability of intact platelets to accumulate acridine orange. *Experientia*. 1988;44(7):616-618.
64. Hong W, Kondkar AA, Nagalla S, et al. Transfection of human platelets with short interfering RNA. *Clin Transl Sci*. 2011;4(3): 180-182.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024

65. Yu F, Jia X, Du F, et al. miR-155-deficient bone marrow promotes tumor metastasis. *Mol Cancer Res*. 2013;11(8):923-936.

66. Finnerty JR, Wang WX, Hébert SS, Wilfred BR, Mao G, Nelson PT. The miR-15/107 group of microRNA genes: evolutionary biology, cellular functions, and roles in human diseases. *J Mol Biol*. 2010;402(3):491-509.

67. Yang Y, Li M, Chang S, et al. MicroRNA-195 acts as a tumor suppressor by directly targeting Wnt3a in HepG2 hepatocellular carcinoma cells. *Mol Med Rep*. 2014;10(5):2643-2648.

68. Gay L, Miller MR, Ventura PB, et al. Mouse TU tagging: a chemical/genetic intersectional method for purifying cell type-specific nascent RNA. *Genes Dev*. 2013;27(1):98-115.

69. Obad S, dos Santos CO, Petri A, et al. Silencing of microRNA families by seed-targeting tiny LNAs. *Nat Genet*. 2011;43(4):371-378.

70. Cohen Z, Gonzales RF, Davis-Gorman GF, Copeland JG, McDonagh PF. Thrombin activity and platelet microparticle formation are increased in type 2 diabetic platelets: a potential correlation with caspase activation. *Thromb Res*. 2002;107(5):217-221.

71. Melki I, Tessandier N, Zufferey A, Boilard E. Platelet microvesicles in health and disease. *Platelets*. 2017;28(3):214-221.

72. Duvernay M, Young S, Gailani D, Schoenecker J, Hamm HE. Protease-activated receptor (PAR) 1 and PAR4 differentially regulate factor V expression from human platelets [published correction appears in *Mol Pharmacol*. 2013; 84(3):487]. *Mol Pharmacol*. 2013;83(4):781-792.

73. Telonis AG, Loher P, Jing Y, Londin E, Rigoutsos I. Beyond the one-locus-one-miRNA paradigm: microRNA isoforms enable deeper insights into breast cancer heterogeneity. *Nucleic Acids Res*. 2015;43(19):9158-9175.

74. Zhao H, Ardelt B, Ardelt W, Shogen K, Darzynkiewicz Z. The cytotoxic ribonuclease onconase targets RNA interference (siRNA). *Cell Cycle*. 2008;7(20):3258-3261.

75. Sikand K, Singh J, Ebron JS, Shukla GC. Housekeeping gene selection advisory: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin are targets of miR-644a. *PLoS One*. 2012;7(10):e47510.

76. Bray PF, McKenzie SE, Edelstein LC, et al. The complex transcriptional landscape of the anucleate human platelet. *BMC Genomics*. 2013;14:1.

77. Bai B, Liu H, Laiho M. Small RNA expression and deep sequencing analyses of the nucleolus reveal the presence of nucleolus-associated microRNAs. *FEBS Open Bio*. 2014;4:441-449.

78. Bandiera S, Matégot R, Girard M, Demongeot J, Henrion-Caude A. MitomiRs delineating the intracellular localization of microRNAs at mitochondria. *Free Radic Biol Med*. 2013;64: 12-19.

79. Gusdon AM, Votyakova TV, Mathews CE. mt-Nd2a suppresses reactive oxygen species production by mitochondrial complexes I and III. *J Biol Chem*. 2008;283(16):10690-10697.

80. Yao Z, Jones AW, Fassone E, et al. PGC-1β mediates adaptive chemoresistance associated with mitochondrial DNA mutations. *Oncogene*. 2013;32(20):2592-2600.

81. Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, Ruggeri ZM. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood*. 2006;107(9):3537-3545.

82. Schmidtke DW, Diamond SL. Direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow. *J Cell Biol*. 2000;149(3): 719-730.

83. Kim R, Emi M, Tanabe K. Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. *Cancer Biol Ther*. 2005;4(9): 924-933.

84. Nomura S, Ozaki Y, Ikeda Y. Function and role of microparticles in various clinical settings. *Thromb Res*. 2008;123(1):8-23.

85. Boon RA, Vickers KC. Intercellular transport of microRNAs. *Arterioscler Thromb Vasc Biol*. 2013;33(2):186-192.

86. Walsh TG, Metharom P, Berndt MC. The functional role of platelets in the regulation of angiogenesis. *Platelets*. 2015;26(3):199-211.

87. Lee EK, Gorospe M. Coding region: the neglected post-transcriptional code. *RNA Biol*. 2011;8(1):44-48.

88. Beitzinger M, Peters L, Zhu JY, Kremmer E, Meister G. Identification of human microRNA targets from isolated argonaute protein complexes. *RNA Biol*. 2007;4(2):76-84.

89. Fang Z, Rajewsky N. The impact of miRNA target sites in coding sequences and in 3'UTRs. *PLoS One*. 2011;6(3):e18067.

90. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *eLife*. 2015;4:4.

91. Liang T, Yu J, Liu C, Guo L. An exploration of evolution, maturation, expression and function relationships in mir-23 ~ 27 ~ 24 cluster. *PLoS One*. 2014;9(8):e106223.

92. Sharma D, Brummel-Ziedins KE, Bouchard BA, Holmes CE. Platelets in tumor progression: a host factor that offers multiple potential targets in the treatment of cancer. *J Cell Physiol*. 2014; 229(8):1005-1015.

93. Bebawy M, Combes V, Lee E, et al. Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells. *Leukemia*. 2009; 23(9):1643-1649.

94. Gong J, Jaiswal R, Dalla P, Luk F, Bebawy M. Microparticles in cancer: A review of recent developments and the potential for clinical application. *Semin Cell Dev Biol*. 2015;40:35-40.

95. Gong J, Jaiswal R, Mathys JM, Combes V, Grau GE, Bebawy M. Microparticles and their emerging role in cancer multidrug resistance. *Cancer Treat Rev*. 2012;38(3):226-234.

96. Jaiswal R, Raymond Grau GE, Bebawy M. Cellular communication via microparticles: role in transfer of multidrug resistance in cancer. *Future Oncol*. 2014;10(4):655-669.

97. Chistiakov DA, Orekhov AN, Bobryshev YV. Endothelial barrier and its abnormalities in cardiovascular disease. *Front Physiol*. 2015;6: 365.

98. Hayon Y, Shai E, Varon D, Leker RR. The role of platelets and their microparticles in rehabilitation of ischemic brain tissue. *CNS Neurol Disord Drug Targets*. 2012;11(7):921-925.

99. Opal SM, van der Poll T. Endothelial barrier dysfunction in septic shock. *J Intern Med*. 2015; 277(3):277-293.

100. Reid VL, Webster NR. Role of microparticles in sepsis. *Br J Anaesth*. 2012;109(4):503-513.

101. Meziani F, Delabranche X, Asfar P, Toti F. Bench-to-bedside review: circulating microparticles–a new player in sepsis? *Crit Care*. 2010;14(5):236.

102. Tetta C, Bruno S, Fonsato V, Deregibus MC, Camussi G. The role of microvesicles in tissue repair. *Organogenesis*. 2011;7(2):105-115.

103. Shin ES, Sorenson CM, Sheibani N. Diabetes and retinal vascular dysfunction. *J Ophthalmic Vis Res*. 2014;9(3):362-373.

104. Randriamboavonjy V, Fleming I. Platelet function and signaling in diabetes mellitus. *Curr Vasc Pharmacol*. 2012;10(5):532-538.

105. Vazzana N, Ranalli P, Cuccurullo C, Davì G. Diabetes mellitus and thrombosis. *Thromb Res*. 2012;129(3):371-377.

Downloaded from http://ashpublications.net/blood/article-pdf/130/5/567/1403991/blood751099.pdf by guest on 28 May 2024