Platelet microparticles infiltrating solid tumors transfer miRNAs that suppress tumor growth.

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**Key points**

Platelet microparticles 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.

**Abstract**

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, 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 non-coding small nucleolar RNA. These RNAs were suppressed in PMP-treated tumor cells, resulting in mitochondrial dysfunction and growth inhibition, in a miR-24-dependent manner. Thus, platelet-derived miRNAs transfer *in vivo* to tumor cells in solid tumors via infiltrating microparticles, regulate tumor cell gene expression, and modulate tumor progression. These findings shed novel insight into mechanisms of horizontal RNA transfer and add multiple layers to the regulatory roles of miRNAs and PMPs in tumor progression. Plasma microparticle-mediated transfer of regulatory RNAs and modulation of gene expression may be a common feature with important outcomes in contexts of enhanced vascular permeability.
Introduction

Platelets have been associated with tumor progression and metastatic dissemination through platelet-tumor cell (TC) interactions\textsuperscript{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\textsuperscript{8}. The presence of cancer increases platelet production, which has been associated with poorer outcomes in multiple cancers\textsuperscript{9,10}. Platelets can stimulate proliferation of human and murine cancer cells in a manner that does not require platelet-tumor contact\textsuperscript{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\textsuperscript{12}. At least 45\% of plasma-borne MPs are platelet-derived microparticles (PMPs)\textsuperscript{13,14}. PMP release increases in individuals bearing solid tumors, but roles of PMPs in cancer progression are incompletely understood\textsuperscript{15,16}. PMPs are enriched in platelet miRNAs, a small cohort of which are present at high copy number, accounting for the bulk of plasma miRNAs\textsuperscript{17-21}. Purified PMPs are able to transfer at least some miRNA content to cells following co-incubation \textit{in vitro}, and regulate gene expression\textsuperscript{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, and have been identified as diagnostic and prognostic markers of malignancy, and implicated in therapy resistance\textsuperscript{25-46}. Tumor neovasculature is highly permeable, which we predicted might allow circulating microparticles 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

1 x 10^6 cells/200 μL of Hank’s Balanced Salt Solution suspension was injected subcutaneously into the shaved flank of 8-week-old mice. Thrombocytopenia was induced by intraperitoneal 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 h. In some cases mice were injected i.p. with 100 mg/kg 4TU in 20% dimethyl sulfoxide (DMSO)/80% corn oil, or vehicle. Tumor volume was calculated as described^47. Tumors were resected from euthanized mice, cleaned of fat, skin and connective tissue for further processing.

miRNA:mRNA target adduct formation and screening

Cells suspended in 500 μL fractionation buffer (20 mM Tris-Cl pH 7.4, 100 mM NaCl, 2 mM MgOAc, 5 mM KCl, protease inhibitor cocktail [Roche, Indianapolis, IN, USA]) were dounce homogenized followed by sonication. Lysates were treated with 1 U/μl RNase T1 (Fisher, Pittsburgh, PA, USA) for 15 min at 22°C, followed by RNase inhibition with 10 mM MnCl2. T4 PNK minus 1 U/μl + 100 mM ATP, 5 mM DTT was added, 40 min at 10°C. RNA ligation was carried out with 0.2 U/μl T4 RNA ligase, 16 h at 4°C. RNA was extracted in TRIzol and resuspended in DEPC-treated H2O, followed 1 μl PNK and no ATP for 40 min at 10°C, and poly(dA) tailing and 1st strand cDNA synthesis with the NCode kit (see Supplemental Methods)^48. cDNA libraries were subjected to Taq 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 E. coli for ampicillin selection, colony propagation, plasmid DNA minipreps (Qiagen, Valencia, CA, USA) and sequencing.
Results

Platelet microparticles infiltrate solid tumors.

Circulating platelet-derived microparticles (PMPs) harbor microRNAs (miRNAs) and can transfer platelet-derived miRNAs to endothelium and leukocytes\textsuperscript{22,23,49}. Because tumor blood vessels are highly permeable due to endothelial dysfunction and poor pericyte coverage\textsuperscript{50}, and PMP release correlates with solid tumor growth and metastasis\textsuperscript{4,16,51}, we considered whether tumor cells (TCs) in solid tumors are targets of PMPs. We observed PMP infiltration, indicated by antibodies to $\alpha_{\text{IIb}}$ integrin (CD41) - a platelet/megakaryocyte-specific receptor and a PMP marker\textsuperscript{52} – in the extravascular tumor environment as indicated by von Willebrand Factor 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 – 1000 nm, the diameter range of PMPs\textsuperscript{53,54}, and were Annexin V-positive (Figure 1B), indicating phosphatidylserine exposure on the outer leaflet, a characteristic of MPs and apoptotic cells. Most but not all Annexin V-positive puncta in the tumor sections also contained $\alpha_{\text{IIb}}$ 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 $\alpha_{\text{IIb}}$ 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 employed an ectopic solid tumor allograft model in mice using Lewis lung carcinoma (LLC) cells injected as a bolus s.c. into mouse flanks\textsuperscript{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\textsuperscript{13}, overlapped with $\alpha_{\text{IIb}}$ 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 d 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 GFP-positive 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 d tumors (supplemental Figure 1B), suggesting that robust neovascularization precludes PMP extravasation in solid tumors. TCs extracted at day 21, 24 h after immune-induced thrombocytopenia, showed substantially reduced PMPs (3.1% ± 2.2 of TCs), indicating that the MPs were derived from 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 mRNA (supplemental Figure 1E). Thus, platelet microparticles can undergo extravasation beyond the blood circulation in solid tumors and associate with tumor cells.

**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 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 (FAM)-labeled or unlabeled siRNA as a miRNA mimic\textsuperscript{64} (supplemental Figure 2A-B) and transfused transfected platelets into tumor-bearing mice. \textit{Ex vivo} TCs, which harbored PMPs showed cytosolic FAM\textsuperscript{+} fluorescence in cells from FAM\textsuperscript{+}-siRNA-transfused mice, but we did not observe FAM\textsuperscript{+} cells from control mice (Figure 2G-H).

To study PMP RNA transfer further, we utilized human platelets, from which we could more easily derive substantial quantities of PMPs. We stimulated \textit{ex vivo} human platelets to release PMPs (supplemental Figure 3A), and incubated collected PMPs with LLCs in culture\textsuperscript{23}. After 1 h PMP exposure, most LLC cells were decorated with \(\alpha_{\text{IIb}}\) 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 \textit{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\textsuperscript{+} fluorescence distinct from the \(\alpha_{\text{IIb}}\) integrin structures, selectively in PMP-targeted cells (Figure 2J). Thus, PMPs can deliver miRNA to TCs \textit{in vitro}, consistent with previous reports in other systems\textsuperscript{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.

\textbf{Platelet-derived miRNAs transferred to tumor cells in solid tumors.}

We sought to characterize platelet-derived miRNAs transferred to TCs via microparticles. Following \textit{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\textsuperscript{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 \textit{ex vivo} TC isolation from resected tumors by single cell sorting. Several miRNAs were upregulated in TCs \textit{in vivo} compared to cells in culture as indicated by conventional PCR (Figure 3B) and quantitative RT-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\(^\text{46}\). 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 \textit{in vitro} and in \textit{ex vivo} TCs by conventional PCR (Figure 3A-B). These miRNAs were each elevated > 9-fold \textit{ex vivo} compared to cells in culture (Figure 3C), indicating that these miRNAs had been either expressed selectively in tumors, or transferred to TCs \textit{in vivo}.

To determine whether the additional miRNAs in TCs \textit{ex vivo} were platelet-derived, we crossed CA-\textit{loxP}(>)-GFP\textit{stop}-\textit{loxP}(>)-\textit{Uprt}\textit{C57Bl/6} mice with \textit{Pf4-Cre C57Bl/6} mice, to generate CA>\textit{Uprt}/\textit{Pf4-Cre} double heterozygotes for Cre-induced expression of \textit{T. gondii} uracil phosphoribosyltransferase (UPRT) selectively in megakaryocytes/platelets (Figure 3D). This enzyme is required for incorporation of 4-thiouracil (4TU), a cell-permeable, non-native 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)\(^\text{68}\). We seeded LLC-E2-Crimson cell tumors in the flanks of CA>\textit{Uprt}/\textit{Pf4-Cre} heterozygotes or \textit{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 \textit{Pf4-Cre/CA}\textgreater;\textit{Uprt} mice, but not from TCs from \textit{Pf4-Cre} mice, demonstrating \textit{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 UPRT enzyme from platelets to tumor cells, as HA-UPRT was expressed selectively in platelets in \textit{Pf4-Cre/CA}\textgreater;\textit{Uprt} mice, but we did not detect HA-UPRT in PMPs nor in \textit{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 \textit{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 a 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 (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 down-regulation 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.

**PMPs inhibit tumor growth in a 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 d 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 homologues have identical seed target 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 non-tumor tissue.
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 microparticles and tumor growth in mice deleted for the Par4 thrombin receptor, which has been shown to be the major driver of PMP generation. Plasma MPs were reduced by ~50% in Par4 knockout (KO) mice compared to 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 to 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 to 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, non-targeted 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, 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 non-coding 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 (RISC) 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 GAPDH as a housekeeping control, which is valid in this case as GAPDH is recruited into RISC and modulated by siRNAs or miRNAs, but known miRNAs targeting GAPDH have not been detected in platelets or PMPs64,74-76. PMP exposure led to increases in mt-Nd2 and Snora75 RNAs in Ago2-containing complexes compared to untreated cells (Figure 6C), indicating enhanced recruitment of these RNA targets to RISC by PMP exposure. These results were not due to transfer of pre-formed 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 down-regulation 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 down-regulation 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 nucleoli77; these data indicate that miR-24 is also a mitomiR78. This multi-organelle localization is consistent with the ability of miR-24 to target and down-regulate RNAs in each compartment.

Mt-Nd2 is a component of the NADH:ubiquinone oxidoreductase (complex I), responsible for mitochondrial oxidative phosphorylation and ROS production79. We measured mitochondrial membrane potential in PMP-treated and untreated cells. Nuclear localization of the membrane potential indicator, 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 III80. 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 tumors, with inhibitory effects on tumor progression by transfer of platelet miRNAs and down-regulation 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 (e.g., mitochondrial mRNAs) and other non-coding RNAs 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. 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 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 GP1b (potentially in complex with vWF), P-selectin, phosphatidylserine receptors on the tumor cell surface, or a combination of these and other interactions.

Interactions between microparticles and cells have been investigated in many contexts, these interactions yield a broad range of outcomes. Endothelium and blood cells are exposed to PMPs, and effects are just beginning to be explored. PMPs have been shown to transfer miRNAs to cells when co-incubated in vitro, and to modulate target cell gene expression. For example, PMP incubation with A549 lung carcinoma cells
in culture enhanced invasiveness due to increased miR-223 and suppression of anti-invasive genes. 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 to 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 non-canonical RNAs – a mitochondrial mRNA lacking a 3’-UTR, and a non-coding small nucleolar RNA (snoRNA), representing regulation of one non-coding RNA 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. Whether non-canonical miRNA response elements are biologically relevant remains controversial, as recent studies indicate that non-canonical sites may not contribute substantially to gene suppression. However, the miR-24 targets we identified were found through an unbiased screen following direct ligation of miRNAs to their targets within RISC. These RNAs were enriched in Ago2-containing complexes following PMP exposure, indicating recruitment to RISC 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. 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. 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.

Our results demonstrate tumor suppressive roles at earlier stages, via growth suppressive effects through down-regulation of TC genes and induction of tumor cell apoptosis. Platelet miRNA transfer may also modulate other aspects of tumor biology, such as multi-drug resistance, which is known to be regulated by MPs. 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 contexts of endothelial barrier dysfunction resulting in increased vascular permeability. Relationships of platelets and microparticles with vascular leak have recently been described in cardiovascular disease, ischemia and post-ischemic tissue repair, sepsis, wound healing, and diabetes, 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.

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References


Tables

Table 1. Presence of extravascular PMPs scored in graded lung carcinoma and colon adenocarcinoma, and adjacent uninvolved tissues. 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-positive to total. Samples were counted as negative if no PMPs were observed in 5 separate fields of 500 \( \mu \text{m}^2 \). AC, adenocarcinoma; BAC, bronchioalveolar carcinoma; PC, papillary carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

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<th>Type and grade</th>
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Figure legends

Figure 1. PMP infiltration in solid tumors in human patients. (A) Tissue microarray slides containing 5 \( \mu \text{m} \) sections from the indicated human tumors and uninvolved adjacent tissue (“Normal”) were stained with the indicated antibodies and 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. \( \alpha_{\text{IIb}} \) integrin, green; von Willebrand Factor (vWF), red; DAPI, blue. 3x magnification center area insets shown in bottom row. Bars, 50 \( \mu \text{m} \). \( n = 4 \). (B) Representative images from (A),
showing counter-stain with FITC-Annexin V (AXV, shown as red). α\textsubscript{IIb} integrin, green; DAPI, blue. Merge images with DAPI shown to the right; α\textsubscript{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 min before being fixed and stained as indicated. Yellow arrowheads indicate ectopic intact platelets. (D) Representative images from human lung cancer array with paired uninvolved tissue, stained as in (A). AC, adenocarcinoma; BAC, bronchioalveolar carcinoma; PC, papillary carcinoma; SCC, squamous cell carcinoma; SCLC, small cell lung cancer. (E) Representative images from human colon cancer array with paired uninvolved tissue. Note that some α\textsubscript{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-positive tissues from total assayed tissues for colon adenocarcinomas and lung cancers, and adjacent uninvolved tissue, shown ± s.e.m. n = 3. colon, p < 0.01; lung, p < 0.004.

**Figure 2. PMP infiltration in solid tumor allografts and RNA transfer in mice.** (A) IHC in 5 μm section from Lewis lung carcinoma (LLC) tumor allograft, 21 d. α\textsubscript{IIb} integrin, red. (B) IHC in 5 μm section from LLC tumor allograft, 21 d. α\textsubscript{IIb} integrin, red (top); CD63, green (middle); DAPI, blue (shown in merged image, bottom). α\textsubscript{IIb} integrin/CD63 overlap appears in some areas in the merged image as yellow. (C) Tumor cells from a resected LLC tumor at 21 d, isolated as described in Supplemental Methods. α\textsubscript{IIb} integrin, red; DAPI, blue. (D-E) Tumor cells from resected LLC allograft 24 h after transfusion of acridine orange (AO)-labeled platelets. (D) α\textsubscript{IIb} integrin, red; (E) AO, green. Bars (A-E), 10 μm. *, PMP-negative cells. (F) Percentage of cells with cytosolic AO staining, shown ± s.e.m. n = 3, >100 cells each. (G) Human platelets were transfected with unlabeled siRNA and transfused into mice bearing 20 d LLC tumors. After 24 h, 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 (G), from mice transfused with human platelets transfected with FAM-siRNA. (I) LLC cells, treated *in vitro* with PMPs derived from human platelets 48 hr after platelet transfection with unlabeled siRNA. (J) Cells treated as in (I) with PMPs derived from human platelets transfected with FAM-siRNA. (H-K), 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 (G-J) denote apical side of z-section.

**Figure 3. PMP transfer of platelet miRNAs to tumor cells in solid tumors.** (A) Total RNA extracted from LLC cells post-trypsinization, untreated (-) or exposed to PMPs for 16 h (+), 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 Lewis lung carcinoma (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 to 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 + s.e.m. miRNA primers were for 5p arms unless otherwise indicated. $p < 0.05$ 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 platelet-derived microparticles (PMPs). (5) Tumor resection and tumor
cell isolation by FACS, followed by RNA extraction. (6) Platelet-derived 4TU-RNA labeling with HPDP-biotin added to the total tumor cell RNA, and isolation by affinity chromatography with avidin beads for further analysis. β-ME, β-mercaptoethanol. (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 d tumors in 4TU-treated Pf4-Cre+/− (Pf4-Cre) and CA>HA-Uptr+/− Pf4-Cre+/− (Pf4-Cre/Uptr) mice. (F) qRT-PCR from (E), showing fold change + s.e.m. in tumor cells extracted from Pf4-Cre/Uptr vs. Pf4-Cre mice. Red line denotes parity. Let-7a fold change = 1.2 ± 0.02. p < 0.03 for each. n = 8.

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 co-incubated with 5x10⁴ LLC cells every 24 hr. Cells were counted at 60 hr. n = 8. (B) MC-38 cells, treated as assessed as in (A). n = 3. (A-B) *, p < 0.0007; **, p < 0.001; ***, p < 0.05. #, p < 0.02; ##, p < 0.01. (C) 5x10⁴ LLC cells were transfected and treated every 24 h with 10⁹ freshly isolated PMPs as indicated, and counted daily. n = 5. (D) MC-38 cells transfected, treated and analyzed as in (C). (C-D): *, p < 0.03; **, p < 0.04. n = 3. (E) LLC cells treated as in (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 (D) were processed for western blotting as in (E). n = 5 for (E-F). (G) 1x10⁶ LLC cells were transfected as indicated, and after 18 h were seeded as allografts by bolus injection into the flanks of WT mice. Beginning at d 8, 1x10¹⁰ 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 < 0.02. (H) MC-38 cells were transfected and implanted, followed by PMP transfusion, and tumor growth was monitored as in (G). **, p < 0.003. n = 6. All plots, shown ± s.e.m.

Figure 5. Plasma microparticles and tumor growth in Par4 knockout mice. (A) Plasma MPs from WT and Par4 knockout (KO) mice were analyzed by nanoparticle tracking, and are shown ± s.e.m. n = 6. p < 0.03. (B) 1x10⁶ LLC cells were seeded in the flanks of WT and Par4 knockout (KO) mice, and tumor volumes were
measured 8 d after seeding, shown ± s.e.m. \( n = 6 \). \( p < 0.03 \). (C) LLC tumors were seeded as in (B), and \( 1 \times 10^{10} \) freshly isolated PMPs were transfused in the tail vein every 24 hr beginning at day 8 as indicated. Tumor volumes were measured daily, and are shown ± s.e.m. \( n = 6 \). *, \( p < 0.03 \); **, \( p < 0.002 \). (D) Tumors from (C) were resected, fixed and processed for immunohistochemistry with antibodies to murine CD41 (\( \alpha_{\text{IIb}} \) integrin, red) to label endogenous PMPs, and cleaved caspase-3 (green); DAPI stain is shown in blue. (E) Tumor sections from (C) stained with antibodies to human CD41 (\( \alpha_{\text{IIb}} \) integrin, red) to label transfused PMPs, cleaved caspase-3 (cl. cas-3, green) and DAPI (blue). Bars, 15 \( \mu \)m.

**Figure 6. RNA targets of PMP-derived miR-24.** (A) Schematic for low-throughput miRNA target identification, modified from \(^{48}\). PMP-treated cells were lysed post-trypsinization by suspension in hyposmotic buffer followed by sonication. Whole cell extracts were treated with RNase T1, 3’ end blocking with polynucleotide kinase 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 1st 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. ncRNA, non-coding RNA. (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. *, \( p < 0.05 \). \( n = 3 \). (D) LLC cells were transfected with 25 \( \mu \)g phosphorothioate, LNA 8-nt control or antagomiR-24 (ant-miR-24), 24 hr prior to PMP exposure. RNA was isolated from cells 16 hr 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 < 0.001; **, p < 0.05. n = 7. (E) MC-38 cells treated and analyzed as in (D). ***, p < 0.01; #, p < 0.02. n = 5. All histograms, shown ± s.e.m. (F-G) Western blotting with α-mt-Nd2 antibodies (Nd2) of lysates of cells treated with PMPs for up to 3 d. β-actin was used as a loading control. Densitometry results are shown for Nd2/β-actin ratios, ± s.e.m. for 3 independent experiments. *, p < 0.05, all others n.s.

**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 (B). RLU, relative luminescence units. *, p < 0.01; **, p < 0.001. n = 3. All histograms, shown ± s.e.m.
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Platelet microparticles infiltrating solid tumors transfer miRNAs that suppress tumor growth.