Direct Signaling between Platelets and Cancer Cells Induces an Epithelial-Mesenchymal-Like Transition and Promotes Metastasis

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Direct Signaling between Platelets and Cancer Cells Induces an Epithelial-Mesenchymal-Like Transition and Promotes Metastasis

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SUMMARY

Interactions of cancer cells with the primary tumor microenvironment are important determinants of cancer progression toward metastasis but it is unknown whether additional prometastatic signals are provided during the intravascular transit to the site of metastasis. Here, we show that platelet-tumor cell interactions are sufficient to prime tumor cells for subsequent metastasis. Platelet-derived TGFβ and direct platelet-tumor cell contacts synergistically activate the TGFβ/Smad and NF-κB pathways in cancer cells, resulting in their transition to an invasive mesenchymal-like phenotype and enhanced metastasis in vivo. Inhibition of NF-κB signaling in cancer cells or ablation of TGFβ1 expression solely in platelets protects against lung metastasis in vivo. Thus, cancer cells rely on platelet-derived signals outside of the primary tumor for efficient metastasis.

INTRODUCTION

The dynamic crosstalk between tumors and their microenvironment is increasingly recognized as a key regulator of malignant progression. Tumor cells are known to secrete several cytokines, which activate stromal fibroblasts and induce the recruitment of immune cells to the tumor. In turn, signals provided by the local microenvironment promote the ability of tumor cells to invade and metastasize (Joyce and Pollard, 2009). In primary carcinomas, secreted growth factors and cytokines contributed by stromal cells are key in inducing epithelial-mesenchymal transition (EMT), a transient and reversible process that promotes cell motility, invasion, and dissemination of cancer cells out of the tumor microenvironment (Scheel et al., 2007; Thiery, 2002). Subsequently, tumor cells travel through the bloodstream before arresting and extravasating in a new microenvironment (secondary site). Some current models of tumor progression propose that the metastatic potential of tumor cells is entirely shaped at the primary tumor site, with few or no signaling events occurring during the intravascular transit of tumor cells. Considering that multiple growth factors and cytokines are released in the bloodstream, cancer cells may sense additional signaling cues outside of the primary microenvironment. However, it is unclear whether circulating cancer cells require additional instructive signals for effective metastasis, either while in the circulation or on arrival at the secondary site.

Among the multitude of different signaling molecules found in the blood, transforming growth factor-beta (TGFβ) is known to promote metastasis by enhancing EMT and invasiveness in primary carcinomas (Oft et al., 1998). Furthermore, inhibition of the ability of tumor cells to respond to TGFβ (by overexpression of dominant-negative TGFβ receptor II) reduces intravasation and metastatic seeding in the lungs (Biswas et al., 2007; Padua et al., 2008; Siegel et al., 2003) as well as the development of bone metastases (Yin et al., 1999). In particular, upon dissemination to the bones, tumor cells activate osteoclasts to degrade the bone matrix and release the stored TGFβ, which in turn leads to enhanced tumor cell malignancy (Kang et al., 2005). However,
in the case of metastasis to other tissues such as the lungs, the source of TGFβ bioavailable to tumor cells at the site of metastatic seeding remains unknown.

Platelets contain a plethora of growth factors and cytokines, including high concentrations of TGFβ (Assadian et al., 1983). Thus, platelet-derived factors could potentially be involved in the promotion of a metastatic phenotype. Consistent with a role of platelets in metastasis, defective platelet function or reduced platelet counts have been associated with decreased metastasis formation in various transgenic mouse models (Bakewell et al., 2003; Camerer et al., 2004; Gasic et al., 1968; Kim et al., 1998). The prometastatic effects of platelets have so far been attributed to their ability to promote adhesion or to their capacity to prevent cell death in the circulation by forming a physical shield around tumor cells. This shield protects tumors cells from natural killer cell-mediated lysis (Nieswandt et al., 1999; Palumbo et al., 2005), limits their exposure to shear stress, and promotes their adhesion to the endothelium (Erpenbeck and Schön, 2010; Gay and Felding-Habermann, 2011; Im et al., 2004; Jain et al., 2007; Karpatin et al., 1988; Sierko and Wojtukiewicz, 2007). In addition, it is possible that platelets provide instructive signals that affect tumor cell behavior and metastatic potential.

In this study, we have tested whether platelets can provide a signaling platform for cancer cells outside of the primary tumor in the context of metastasis.

RESULTS

Platelets Prime Tumor Cells For Metastasis

To investigate whether platelets can have a direct impact on tumor cell behavior, we tested whether platelets could prime tumor cells for metastasis. Colon carcinoma cells (MC38GFP; isolated from a grade III carcinoma chemically induced in a C57BL/6 mouse) (Corbett et al., 1975) or breast carcinoma cells (Ep5 [EpRas]; spontaneously immortalized mouse mammary epithelial cell line transformed by the v-Ha-Ras oncogene) (Oft et al., 1996) were coincubated with purified platelets for 40 hr in vitro. Platelets were then washed away, and tumor cells (substantially devoid of any platelets; see Figure S1A available online) were injected into the tail veins of mice. Pretreating either MC38GFP or Ep5 cells with platelets led to a marked increase in the number of metastatic foci in the lungs 14 days after tail-vein injection (Figure 1A). The increase in metastasis was presumably due to the enhanced capacity of MC38GFP and Ep5 cells to seed the lungs as demonstrated by increased numbers of cells present after 48 hr (Figure 1B). These results indicate that a transient interaction between tumor cells and platelets in vitro is sufficient to increase tumor cell metastatic seeding.

Treatment of Tumor Cells with Platelets Induces an Invasive Mesenchymal-Like Phenotype

We next sought to define the molecular mechanisms induced by platelet-tumor cell interactions that could mediate the increased metastatic capacity of tumor cells. In tissue culture, both MC38GFP and Ep5 tumor cell lines underwent morphological changes reminiscent of an epithelial-mesenchymal transition (EMT) when treated with platelets for 24 hr (Figure 1C). Analysis of mesenchymal markers and transcription factors involved in EMT revealed that the mRNA expression of snail (Snai1), vimentin (Vim), fibronectin (Fn1), and plasminogen activator inhibitor-1 (PAI-1; Serpine1) was consistently upregulated, whereas the epithelial marker claudin 1 (Clcn1) was downregulated in platelet-treated cells (Figure 1D). E-cadherin protein levels were also reduced in platelet-treated Ep5 cells in comparison with controls (Figure 1E; Figure S1B), whereas N-cadherin was relocalized from cell-cell junctions to the cytoplasm (Figure S1B). In addition, zymography revealed increased matrix metalloproteinase-9 (MMP-9) secretion following exposure to platelets (Figure 1F), suggesting a higher capacity to degrade the extracellular matrix (ECM) and to invade the surrounding environment. In agreement with these findings, the human breast epithelial cell lines MCF10A and HMLER also displayed a more mesenchymal morphology upon exposure to platelets (Figure S1C), together with increased expression of EMT-associated genes and MMP-9 secretion (Figures S1D and S1E). Thus, platelets induce EMT-like features also in epithelial cells of human origin.

To test directly whether EMT-like morphological and molecular changes promote an invasive behavior, we seeded platelet-treated MC38GFP and Ep5 cells on Matrigel-coated transwells and detected increased invasion in comparison with untreated cells (Figure 1G). Altogether these results show that platelets promote the adoption of a more mesenchymal and invasive phenotype by tumor cells.

Platelets Promote Activation of the TGFβ/Smad Pathway in Tumor Cells

To gain better insight into the signaling pathways involved in platelet-to-tumor cell communication, we defined the platelet-induced gene expression signature by microarray analysis. For this purpose, gene expression profiles of Ep5 cells treated for 24 hr with platelets or buffer alone (untreated) were compared. Any contribution of platelet mRNA was excluded from the platelet-induced gene signature by performing a microarray with platelets alone (see Experimental Procedures and Figure 2A). Only 21 mRNAs were contributed at significant levels by the plateletes and were excluded from subsequent analyses. Among the most highly upregulated genes in tumor cells, we found several genes known to play prominent roles in EMT, ECM remodeling, and the promotion of metastasis (e.g., Mmp9, Serpine1 (PAI-1), Fnn1, Jag1, Vegfc, Vim, Edn1, Vegfa, Ctgf) (Tables S1 and S2). Bioinformatic analysis using GeneGo canonical pathway maps (Figure 2B) and gene set enrichment analysis (GSEA) (Table 1) confirmed that platelets strongly activate EMT-related genes and revealed TGFβ-dependent pathways as being the most significantly upregulated following platelet treatment (Figure 2B). Interestingly, GSEA analyses further revealed that previously defined gene signatures associated with cancer stem cells, poor prognosis and metastasis are also enriched in platelet-treated cells, suggesting that platelets induce an overall more aggressive phenotype in tumor cells (Table 1).

Considering that a prolonged exposure to TGFβ promotes EMT in many cancer cell lines, including Ep5 cells (Derynick and Akhurst, 2007; Maschler et al., 2005; Oft et al., 1996), we investigated whether platelet-derived TGFβ could activate TGFβ/Smad signaling in tumor cells. We found increased levels of active and latent TGFβ1 in the medium derived from the coculture of tumor cells and platelets, after the platelets were removed (Figure 2C). Ep5 and MC38GFP cells treated with
Platelets also showed increased phosphorylation of the TGFβ signaling effector Smad2 (Figure 2D) and Smad-binding element (SBE)-dependent transcription (Figure 2E) compared with controls, demonstrating that interaction with platelets induces TGFβ/Smad signaling in tumor cells. We next investigated whether platelet-induced tumor cell invasion is dependent on TGFβ signaling. Interestingly, adding a TGFβRI inhibitor (SB431542) or a TGFβ1 blocking antibody abolished platelet-induced cell invasion (Figure 2F). Treatment with SB431542 also inhibited the upregulation of EMT markers induced by platelets in Ep5 and MC38GFP cells (Figure 2G). Thus, platelet-derived TGFβ induces a prometastatic invasive phenotype in tumor cells via activation of the TGFβ/Smad signaling pathway.

**Platelet-Derived TGFβ1 Is Necessary for Metastasis In Vivo**

To test directly the specific contribution of platelet-derived TGFβ1 to metastasis in vivo, P4H-cre mice (Tiedt et al., 2007) were crossed with TGFβ1fl/fl mice (Li et al., 2007) to generate mice lacking TGFβ1 specifically in their megakaryocytes and platelets. P4H-cre+;
Platelet-Derived TGFβ1 and Platelet-Bound Factors Cooperate to Promote Metastasis

Considering that TGFβ1 is a secreted factor released by platelets upon activation (Assion and Sporn, 1986), we next asked whether exposure to the releasate from activated platelets would be sufficient to prime tumor cells for metastasis in vivo. Platelets were therefore activated with thrombin and the releasate was separated from the exhausted platelets by centrifugation (neither thrombin nor its inhibitor hirudin affected tumor cell behavior in our system) (Figures S3A–S3C). Higher concentrations of active TGFβ1 were measured in the conditioned medium of tumor cells treated with the releasate than with the pellet from activated platelets, whereas similar amounts of total TGFβ1 were found in the conditioned medium of tumor cells treated with either fraction (Figure 4A). Furthermore, similar levels of Smad2 phosphorylation (Figure 4B) and SBE-based TGFβ reporter activity were induced in tumor cells incubated with the releasate or the pellet (Figure 4C), demonstrating that the concentration of TGFβ1 present in either fraction is sufficient to induce Smad signaling to comparable levels.

However, despite the higher concentrations of TGFβ1 present in the conditioned medium of tumor cells treated with the platelet releasate (Figure 4A), increased numbers of lung metastases were observed only when tumor cells were preincubated with the pellet fraction but not with the releasate from activated platelets (Figure 4D). Additionally, tumor cell retention in the lungs was increased 48 hr after tail-vein injection when cells were pretreated with platelets or the pellet fraction but not after treatment with the releasate (Figure 4E). Consistent with these results, cells adopted a more mesenchymal morphology (Figure 4F) and showed higher expression of prometastatic genes (Figure 4G; Figure S3D), increased secretion of MMP-9 (Figure 4H), and increased invasion (Figure S3E) upon incubation with the platelet pellet fraction but not with the platelet releasate. Furthermore, microarray analysis of pellet- or releasate-treated Ep5 cells revealed that treatment with the pellet fraction induced gene expression changes very similar to those observed upon platelet treatment (Figures S3F–S3I).

Gene expression signatures associated with EMT and tumor progression were also robustly enriched in the pellet-treated cells but not in the releasate-treated cells (Table S4). Thus, platelet-induced effects on EMT, invasion, and metastasis are not mediated by secreted TGFβ1 alone, but also require additional platelet-bound factors. These data also suggest that these platelet-bound factors synergize with TGFβ1 to enhance metastasis.
Figure 2. Platelet-Induced Gene Expression Signature Reveals Increased Expression of Prometastatic Genes and Activation of the TGFβ Pathway in Tumor Cells

(A) Heat map of genes regulated by >4-fold (p < 0.05) in Ep5 cells treated with platelets in comparison with untreated Ep5 cells (line 2). Line 1 and line 2 show Log2 ratios of gene expression compared to untreated Ep5 cells. mRNAs present in platelets (line 1) were removed from the list of genes modulated upon platelet treatment of Ep5 cells (line 2) to generate a platelet-induced gene signature (line 3), which is listed in Table S2.

(B) Canonical signaling pathways most significantly associated with the list of genes differentially expressed by Ep5 cells upon platelet treatment (platelet-induced gene signature; Table S1; threshold = 2-fold, up and downregulated genes considered, p < 0.05) as determined with GeneGo canonical pathway maps. The 10 pathways with the lowest p values are shown.

(C) Concentration of active and total TGFβ1 in conditioned medium from MC38GFP or Ep5 cells treated with buffer or platelets for 40 hr. The conditioned medium was collected, centrifuged to remove platelets, and the presence of TGFβ1 in the supernatant measured by ELISA. Each bar represents the mean ± SEM of n = 2–6. ***p < 0.001 as determined by Student’s t test.

(D) Detection of phospho-Smad2 protein levels by immunoblotting of Ep5 or MC38GFP cells treated as in (C). Amounts of platelets equal to those used to treat cells were also loaded as control (no cells). β-tubulin is used as loading control.

(E) Relative luciferase activity (RLU) in MC38GFP or Ep5 cells stably expressing a luciferase reporter under the control of an SBE promoter and treated for 40 hr with buffer, platelets or 1ng/ml TGFβ1 (positive control) (n = 5–6). Each bar represents the mean ± SEM, and *p < 0.05, **p < 0.01 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.
from either WT or 
(Figure 5A). Furthermore, adding TGF 
signaling activity than achievable with TGF 
activity, and were therefore used to dissect the specific contri-

bution of TGF 
activity, based on the promoter region of the TGF 
we used the MLEC cell line stably expressing a luciferase 
reporter to define the molecular pathway(s) induced 
with TGFβ signaling, and to promote metastatic gene expression and behavior. Using a set of lucif-
erase reporter assays for several pathways involved in cancer, we screened for their activation in cancer cells in response to interaction with platelets. Interestingly, coincubation with platelets increased activation of the JNK and NF-κB pathways in Ep5 
cells (Figure 6A). Whereas the JNK pathway seemed to be activated by the releasate, activation of the NF-κB pathway was increased only when cells were incubated with either platelets or the pellet fraction from activated platelets (Figure S4A). Ep5 
cells stably expressing an NF-κB luciferase reporter also displayed increased luciferase activity upon treatment with platelets or activated platelet pellets but not if treated with the releasate (Figure 6B). Furthermore, secretion of MCP-1, a known NF-κB-related 
pathway, was increased in the supernatant of Ep5 and MC38GFP tumor cells treated with platelets or the pellet fraction, further showing that platelet-bound factors activate the NF-κB pathway in tumor cells (Figure S4B). Notably, many of the genes found to be highly upregulated in the platelet-induced gene signature, such as Ccl2 (MCP-1), Mmp9, Vegfc, Tnc, Serpine1, Jag1, and Ncam1 have been previously reported as NF-κB target genes (Figure 2A; Table S1). Furthermore, GSEA analysis revealed that NF-κB-related 

**Table 1. Gene Set Enrichment Analysis for Ep5 Cells Treated with Platelets**

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Enrichment of gene sets from the literature (see Supplemental Information for references). Positive NES indicates enrichment in platelet-treated Ep5 
cells; negative NES indicates enrichment in untreated Ep5 cells. FDR, false discovery rate; NES normalized enrichment score.

**TGFβ1 and Direct Platelet-Tumor Cell Contact**

**Synergize to Promote Prometastatic Gene Expression**

To test further whether platelets synergize with TGFβ signaling, we used the MLEC cell line stably expressing a luciferase reporter based on the promoter region of the TGFβ target gene PAI-1 (Abe et al., 1994). These cells provide a specific readout for exogenous TGFβ bioavailability and signaling activity, and were therefore used to dissect the specific contribution of TGFβ signaling to the effects observed upon platelet treatment. Interestingly, we found that platelets induced higher luciferase activity than achievable with TGFβ1 treatment alone (Figure 5A). Furthermore, adding TGFβ1 together with platelets from either WT or P4-cre” /TGFβ1fl/fl mice resulted in a syner-
gistic activation of the PAI-1 reporter that was completely blocked by adding either a TGFβRI inhibitor (SB431542) or an anti-TGFβ1 blocking antibody (Figure 5B). Moreover, separation of platelets (either resting or activated with thrombin) from the cancer cells by a semipermeable membrane blocked the synergistic activation of the PAI-1 promoter (Figure 5C), inhibited the secretion of MMP-9 (Figure 5D), and abolished the upregulation of prometastatic genes in Ep5 and MC38GFP cells (Figure 5E). Taken together, these results show that platelet-derived TGFβ1 is necessary to induce the expression of several prometastatic genes and to promote metastasis in vivo but that, in addition, platelets provide other prometa-
static signals that synergize with TGFβ signaling upon direct platelet-tumor cell contact.

**The NF-κB Signaling Pathway Is Activated by Platelets in a Contact-Dependent Manner and Cooperates with TGFβ Signaling to Enhance Metastasis**

We next sought to define the molecular pathway(s) induced upon platelet contact that cooperate with TGFβ to induce prometastatic gene expression and behavior. Using a set of luciferase reporter assays for several pathways involved in cancer, we screened for their activation in cancer cells in response to interaction with platelets. Interestingly, coincubation with platelets increased activation of the JNK and NF-κB pathways in Ep5 
cells (Figure 6A). Whereas the JNK pathway seemed to be activated by the releasate, activation of the NF-κB pathway was increased only when cells were incubated with either platelets or the pellet fraction from activated platelets (Figure S4A). Ep5 
cells stably expressing an NF-κB luciferase reporter also displayed increased luciferase activity upon treatment with platelets or activated platelet pellets but not if treated with the releasate (Figure 6B). Furthermore, secretion of MCP-1, a known target of the NF-κB pathway, was increased in the supernatant of Ep5 and MC38GFP tumor cells treated with platelets or the pellet fraction, further showing that platelet-bound factors activate the NF-κB pathway in tumor cells (Figure S4B). Notably, many of the genes found to be highly upregulated in the platelet-induced gene signature, such as Ccl2 (MCP-1), Mmp9, Vegfc, Tnc, Serpine1, Jag1, and Ncam1 have been previously reported as NF-κB target genes (Figure 2A; Table S1). Furthermore, GSEA analysis revealed that NF-κB-related 

(F) MC38GFP and Ep5 cells were added at the top of transwells coated with Matrigel and treated with buffer, platelets, platelets + SB431542 (10 μM) or platelets + TGFβ1 blocking antibody (8 μg/ml). The total numbers of cells that invaded to the bottom of the transwell were counted after 48 hr (n = 3). Each bar represents the mean ± SEM, and “p < 0.05 or 0.01 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.

(G) Relative fold change in mRNA expression in MC38GFP or Ep5 cells treated with buffer, or platelets +/- SB431542 (10 μM) for 40 hr (n = 3). Values are normalized to Gapdh expression. Each bar represents the mean ± SEM, and “p < 0.05, “p < 0.01, “p < 0.001 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.
Figure 3. Platelet-Derived TGFβ1 Promotes Lung Metastasis
(A) Concentration of TGFβ1 in washed platelets and platelet-rich plasma (PRP) from WT, Pf4-cre−; TGFβ1fl/fl or Pf4-cre−; TGFβ1f/f mice measured by ELISA (n = 2). Each bar represents the mean ± SEM, and **p < 0.01 versus WT were determined by one-way ANOVA followed by Tukey’s posttest.

(B) Relative luciferase activity (RLU) in Ep5 cells stably expressing a luciferase reporter under the control of the SBE promoter and treated with buffer, platelets from WT, Pf4-cre−; TGFβ1fl/fl, or Pf4-cre−; TGFβ1f/f mice, or with WT platelets + SB431542 (10 μM) for 20 hr (n = 3). Each bar represents the mean ± SEM, and *p < 0.05, ***p < 0.001 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.

(C) Zymography for MMP-9 in the conditioned medium from Ep5 cells treated with buffer, platelets from WT, Pf4-cre−; TGFβ1fl/fl or Pf4-cre−; TGFβ1f/f mice for 40 hr.
gene signatures are enriched in platelet- or platelet pellet-treated Ep5 cells, but not in platelet releasate-treated cells (Table S4). Importantly, treatment of tumor cells with either TGFβ1-deficient platelets or with WT platelets together with the TGFβRII inhibitor SB431542 still led to activation of the NF-κB pathway reporter (Figure 6C), ruling out the possibility that activation of the NF-κB pathway is TGFβ1-dependent in this context.

To study whether NF-κB signaling contributes to the prometastatic phenotype observed upon platelet contact, we established Ep5 cells stably expressing an IkBα mutant (IkBαS32A/S36A) that cannot be phosphorylated or degraded and therefore irreversibly sequesters NF-κB in the cytoplasm, and inhibits its function (IkBα super-repressor; Ep5-IkBαSR) (Brown et al., 1995). In contrast to Ep5 expressing a control vector (Ep5-vector), Ep5-IkBαSR cells failed to activate an NF-κB-dependent luciferase reporter and did not express increased levels of MCP-1 following platelet treatment, demonstrating that the NF-κB signaling is blocked in these cells (Figures 6D and 6E). Importantly, pretreatment of Ep5-vector cells with platelets prior to tail-vein injection led to increased numbers of metastases, whereas it failed to enhance metastasis of Ep5-IkBαSR cells (Figure 6F). Similarly, retention of Ep5-IkBαSR cells in the lungs after 48 hr was not enhanced by a pretreatment with platelets, showing that the NF-κB pathway is necessary for the prometastatic effects of platelets on tumor cells (Figure 6G). Furthermore, Ep5-IkBαSR cells did not acquire a mesenchymal morphology (Figure 6H) and did not display increased invasion (Figure 6I) in response to treatment with platelets. These results correlated with lower levels of MMP-9 secretion (Figure 6J) and lower vimentin and fibronectin gene expression (Figure 6K) by Ep5-IkBαSR cells following platelet treatment. The inhibition of platelet prometastatic effects was not due to an impairment of TGFβ/Smad signaling pathway by the IkBαSR construct because Smad2 phosphorylation levels were intact in Ep5-IkBαSR cells (Figure S4C). In addition, inhibition of MMP-9 secretion and vimentin protein expression induced upon platelet treatment were also observed when Ep5 cells were treated with JSH-23, a pharmacological inhibitor of the NF-κB pathway (Figures S4D and S4E). JSH-23 treatment also abrogated the synergy observed on PAIL-1 reporter activity between TGFβ1 and platelets (Figure S4F), but had no effect on SBE-reporter activity (Figure S4G). This finding further demonstrates that platelets activate NF-κB independently of TGFβ/Smad signaling, and cooperate with TGFβ1 to induce an EMT-like transformation in cancer cells. Thus, the ability of platelets to prime tumor cells for metastasis depends on the synergistic interaction between the NF-κB and TGFβ signaling pathways, which is triggered by direct platelet-tumor cell contact (Figure 7).

**DISCUSSION**

Signals provided by the primary tumor microenvironment are important modulators of the capacity of tumor cells to invade, access the vasculature, and metastasize (Joyce and Pollard, 2009; Nguyen et al., 2009). However, the metastatic potential of tumor cells may be further defined in response to signals provided during their intravascular transit. Here, we have tested this hypothesis and show that platelets present in the bloodstream actively signal to tumor cells to promote their metastatic potential outside of the primary microenvironment. This effect is independent of any direct contribution of platelets to immune-surveillance, adhesion, or physical shielding functions, as tumor cells can be primed for metastasis by a transient exposure to purified platelets in vitro. Mechanistically, a transient contact between platelets and tumor cells is sufficient to induce a prometastatic gene expression signature, induce an EMT-like transformation and invasive behavior in vitro, and promote metastatic seeding in the lungs in vivo (Figure 7). Considering that tumor cells would normally interact with platelets once in the bloodstream, these results suggest that tumor cells could gain a more mesenchymal phenotype and increased metastatic capacities after leaving the primary tumor microenvironment. This implies that cells that have intravasated without losing their epithelial properties either via leaky blood vessels (Carmeliet and Jain, 2000; Mazzone et al., 2009) or via collective invasion mechanisms (Friedl and Gilmour, 2009) could acquire a mesenchymal phenotype during their transit in the vasculature. In support of this idea, circulating tumor cells have been found to express epithelial markers (EpCAM, cytokeratins), suggesting that EMT is not absolutely required to access the blood flow (Nagrath et al., 2007). Thus, interactions with platelets may be particularly important in mediating extravasation of circulating epithelial tumor cells, and to maintain or further enhance the extravasation potential of circulating mesenchymal tumor cells. In this respect, it would be interesting to define the impact of platelets on gene expression and metastatic potential of circulating tumor cells from cancer patients.

Several signaling molecules, including TGFβ1, PDGF, VEGF and angiopoietin are abundant in platelets (Erpenbeck and Schön, 2010; Sierko and Wojtukiewicz, 2007) and may therefore...
Figure 4. Platelet-Derived TGFβ1 and Platelet-Bound Factors Cooperate to Promote Metastasis

(A) Concentration of TGFβ1 measured by ELISA in the conditioned medium of MC38GFP or Ep5 cells incubated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) for 40 hr (n = 4–6). Each bar represents the mean ± SEM, and ns (p > 0.05), *p < 0.05, ***p < 0.001 were determined by one-way ANOVA followed by Tukey’s posttest.

(B) Detection of phospho-Smad2 and total Smad2 protein levels by immunoblotting in Ep5 cells treated as in (A). β-tubulin was used as loading control.

(C) Relative luciferase activity (RLU) in Ep5 cells stably expressing a luciferase reporter under the control of the SBE promoter and treated as in (A) for 20 hr (n = 2). Each bar represents the mean ± SEM, and ns (p > 0.05) was determined by one-way ANOVA followed by Tukey’s posttest.
impact tumor cell behavior and induce EMT. Our results show that the prometastatic effects of platelets are in large part mediated via activation of the TGF-β signaling pathway, and that abrogating either TGF-β signaling in tumor cells or TGF-β expression by platelets is sufficient to inhibit metastasis and EMT. Although TGF-β1 has been implicated in the induction of a prometastatic phenotype in many contexts (Padua et al., 2008; Siegel et al., 2003), the relevant cellular source of TGF-β1 bioavailable to circulating tumor cells, particularly at the site of metastatic extravasation, was previously unclear.

Our results strongly indicate that platelets are an important source of bioavailable TGF-β1 for tumor cells in the circulation and at the site of extravasation. This finding is supported by the observation that platelets contain concentrations of TGF-β1 many-fold higher than most cell types (Assoian et al., 1983). Furthermore, the amount of TGF-β1 produced by other cells and taken up by platelets seems minimal, as purified platelets from PF4-cre<sup>b</sup>; TGF-β1<sup>fl/fl</sup> mice contained <1% of the amount of TGF-β1 present in platelets from WT mice.

Most importantly, we show that abrogation of TGF-β1 expression solely in platelets and megakaryocytes is sufficient to inhibit metastasis and prevent the seeding of tumor cells in the lungs. Furthermore, the presence of platelet-derived TGF-β1 in situ in the host bloodstream is crucial for metastasis, because pretreating tumor cells with platelets from WT mice fails to enhance metastasis formation in mice lacking TGF-β1 in their platelets. Because platelet-tumor cell interactions are transient and occur only within the first 24 hr (Läubli et al., 2006 and data not shown), we propose that platelets could provide a pulse of TGF-β1 to circulating tumor cells, which would allow them to gain a more invasive, mesenchymal-like phenotype and extravasate. Along these lines, previous studies have shown that tumor cells transiently exposed to TGF-β1 have an enhanced capacity to seed the lungs, whereas cells that are continuously exposed to TGF-β1 have decreased metastatic capacity due to the cytostatic effect of TGF-β1 (Giampieri et al., 2009; Padua et al., 2008). In this respect, specific therapeutic inhibition of platelet-derived TGF-β1 might result in the impairment of tumor cell extravasation at the metastatic site. Importantly, PF4-cre<sup>b</sup>; TGF-β1<sup>fl/fl</sup> mice maintain normal platelet counts and hemostatic functions, suggesting that pharmacological inhibition of platelet-derived TGF-β1 could inhibit metastasis without adverse effects on physiological hemostasis.

We also find that, although required for metastasis, activation of TGF-β1 signaling alone is unable to generate effects of the magnitude of those observed with platelets. Indeed, although tumor cells treated for 24 hr with platelets or with the pellet fraction from activated platelets undergo EMT, cells treated with the releasate of activated platelets (that contained a similar concentration of TGF-β1) do not. In line with this result, a prolonged treatment with TGF-β1—typically 1 week or longer—is needed to induce EMT in several epithelial cancer cell lines including Ep5 cells (Labelle et al., 2008; Mani et al., 2008; Mascherler et al., 2005). Our data further support the existence of additional platelet-bound factors synergizing with TGF-β1. First, platelets induce the TGF-β1-responsive PAI-1 reporter to levels higher than achievable with TGF-β1 alone. Second, combining exogenous TGF-β1 with platelets from either WT or PF4-cre<sup>b</sup>; TGF-β1<sup>fl/fl</sup> mice results in a synergistic activation of the PAI-1 reporter. Last, the synergistic effects on PAI-1 reporter activity as well as the induction of prometastatic genes are blocked if platelets are separated from tumor cells by a semipermeable membrane. Thus, our results clearly demonstrate that additional platelet-bound factors synergize with TGF-β1 to promote metastasis, and to induce a prometastatic EMT program in tumor cells.

In particular, we show that this synergy is dependent on the activation of the NF-κB pathway, which is specifically triggered upon direct contact between tumor cells and platelets independently from TGF-β1 activity. NF-κB regulates the expression of proinflammatory genes and has been associated with increased metastasis and EMT induction (Huber et al., 2004; Lin and Karin, 2007). For example, NF-κB promotes osteolytic bone metastasis by inducing the proinflammatory cytokine GM-CSF (Park et al., 2007). Notably, the activation of NF-κB has also been proposed to be part of the mechanism allowing TGF-β1 signaling to switch from a cytosstatic to a prometastatic signal (Neil and Schieman, 2008). In support of this idea, we found that NF-κB activation potentiates TGF-β1-induced prometastatic gene expression, and that NF-κB signaling is necessary for the induction of EMT and efficient metastatic seeding upon platelet-cancer cell interactions. Thus, platelet-tumor cell contacts during metastasis potentiate tumor cell transcriptional responses to TGF-β1 via NF-κB activation.

In conclusion, we establish platelets as a crucial source of TGF-β1 bioavailable to tumor cells in the vasculature and necessary for tumor cell extravasation and metastasis formation. Importantly, our study reveals that platelets are more than physical shields and actively signal to tumor cells via the TGF-β and NF-κB pathways to potently induce a prometastatic phenotype. We thus propose a model whereby the metastatic potential of tumor cells continues to evolve outside of the primary tumor site, in response to tumor-host interactions in the bloodstream and at the site of metastasis. In particular, we identify platelet-tumor cell interactions and the signaling pathways that they trigger as fundamental determinants of cancer metastasis that may provide the basis for developing effective anti-metastatic therapies.
Figure 5. TGFβ1 and Direct Platelet-Tumor Cell Contact Synergize to Promote Prometastatic Gene Expression

(A) Relative luciferase activity (RLU) in MLEC cells stably expressing a luciferase reporter under the control of a TGFβ1 responsive PAI-1 promoter construct and treated with different concentrations of TGFβ1 (left) or platelets (right). Note that the y axis scale is the same for both panels and that platelets give higher stimulation than achievable with TGFβ1 alone.

(B) Relative luciferase activity (RLU) in MLEC cells stably expressing a luciferase reporter under the control of a PAI-1 promoter construct treated with buffer, platelets from WT or Pf4-cre+; TGFβ1fl/fl mice, TGFβ1 (1 ng/ml) or with combinations of platelets + TGFβ1 (1 ng/ml), +/- SB431542 (SB; 10 μM) or +/- TGFβ1 blocking antibody (Ab; 6 μg/ml)(n = 3–16). Each bar represents the mean ± SEM, and ***p < 0.001 were determined by one-way ANOVA followed by Tukey’s posttest.

(C) Relative luciferase activity (RLU) in MLEC cells stably expressing a luciferase reporter under the control of a PAI-1 promoter construct treated with buffer, platelets, or thrombin-activated platelets +/- TGFβ1 (1 ng/ml) seeded either at the bottom (direct contact with tumor cells) or in the upper chamber of a transwell (0.4 μm pore size) to prevent direct contact between platelets and tumor cells (n = 2–3). Each bar represents the mean ± SEM, and ***p < 0.001 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.

(D) Zymography for MMP-9 in the conditioned medium from Ep5 or MC38GFP cells treated with buffer, platelets, or thrombin-activated platelets seeded either at the bottom or in the upper chamber of a transwell (0.4 μm pore size).

(E) Relative fold change in mRNA expression in Ep5 or MC38GFP cells treated as in (D) (n = 3). Values are normalized to Gapdh expression. Each bar represents the mean ± SEM, and *p < 0.05, **p < 0.01, ***p < 0.001 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.
Figure 6. The NF-κB Signaling Pathway Is Activated by Platelets in a Contact-Dependent Manner and Cooperates with TGFβ Signaling to Induce an EMT-Like Transition and Promote Metastasis

(A) Ep5 cells were transfected with pathway-specific firefly luciferase reporters and constitutively expressed control Renilla luciferase reporters. Twenty-four hours after transfection, cells were treated with buffer or platelets for 20 hr, and the relative luciferase activity (RLU) was measured. Firefly luciferase activity was normalized to Renilla luciferase activity. Each bar represents the mean ± SEM of n = 3. *p < 0.05 was determined by Student’s t test.

(B) Relative luciferase activity (RLU) in Ep5 cells stably expressing an NF-κB luciferase reporter and treated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) for 20 hr (n = 2). Each bar represents the mean ± SEM, and ***p < 0.001 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.

(C) Relative luciferase activity (RLU) in Ep5 cells stably expressing a NF-κB luciferase reporter and treated with buffer, platelets from WT, Pf4-cre+; TGFβ1fl/fl, or Pf4-cre+; TGFβ1fl/+ mice, or with WT platelets + SB431542 (10 μM) for 20 hr (n = 5). Each bar represents the mean ± SEM, and *p < 0.05, **p < 0.01 versus buffer were determined by one-way ANOVA followed by Tukey’s posttest.
Figure 7. Platelet-Tumor Cell Contact and Platelet-Derived TGFβ1 Synergize to Promote an EMT-Like Transition and Metastasis
Platelets secrete TGFβ1, which activates the TGFβ/Smad pathway in tumor cells. Upon direct platelet-tumor cell contact, the NF-κB pathway is also activated in tumor cells and synergizes with TGFβ1/Smad signaling to induce a rapid EMT, enhance invasiveness and promote metastasis. Activation of neither the TGFβ/Smad nor the NF-κB pathway alone is sufficient to promote metastasis. Thus, platelet-tumor cell contact triggers a synergistic interaction between TGFβ1/Smad and NF-κB pathways that is necessary for efficient metastasis. The metastatic potential of tumor cells therefore continues to evolve outside of the primary tumor site in response to platelet-to-tumor cell signaling.

**EXPERIMENTAL PROCEDURES**

**Mice**

Mice homozygous for the TGFβ1 floxed allele (TGFβ1<sup>f0/f0</sup>) obtained from R. Flavell (Li et al., 2007) on a C57BL/6 genetic background were crossed with Pf4-cre mice on a C57BL/6 background (obtained from S. Shattil) (Tiedt et al., 2007). To obtain mice with TGFβ1-deficient platelets, Pf4-cre<sup>−/−</sup>; TGFβ1<sup>f0/f0</sup> mice were bred with TGFβ1<sup>f0/f0</sup> or TGFβ1<sup>flox/flox</sup> mice. For genotyping primers, see Supplemental Experimental Procedures. All mice were housed and handled in accordance with approved Massachusetts Institute of Technology Division of Comparative Medicine protocols.

**Generation of Cell Lines Stably Expressing ZsGreen, IkBSR, and Luciferase-Based Reporters**

Retroviral vectors coding for ZsGreen or IkBα super-repressor plus GFP (IkBSR) were transduced into Ep5 cells as described previously (Stern et al., 2008) and in Supplemental Experimental Procedures. Ep5 and MC38GFP cell lines stably expressing SBE (AGCCAGACAG tandem repeats)-luciferase and NF-κB (GGGACTTTCC tandem repeats)-luciferase reporter constructs were generated by infecting cells with Cignal Lenti Reporter vectors (SABiosciences) according to manufacturers’ instructions.

**Preparation of Platelets and Platelet Fractions**

Mouse blood was collected by cardiac puncture and washed platelets were prepared as described previously (Frenette et al., 1995; Hartwell et al., 1998). To prepare platelet fractions, platelets were activated with thrombin 0.5 U/ml for 15 min at 37°C. The pellet fraction was separated from the releasate (supernatant) by centrifugation at 2800 × g for 7 min.

**Treatment of Tumor Cells with Platelets**

Cells were seeded in DMEM 10% FCS and incubated overnight. Immediately prior to treatment the medium was changed for fresh DMEM. A total of 150,000 platelets/μl and equivalent volumes of releasate and pellet fractions were added. Where indicated, cells were treated with 1 or 10 ng/ml of recombinant TGFβ1 (R&D Systems), 10 μM SB431542 (Sigma), or 6 μg/ml anti-TGFβ1 blocking antibody (R&D systems).

**Microarray Analysis**

Total RNA was isolated from platelet-treated or untreated Ep5 cells (n = 5). In order to detect mRNAs contributed by platelets, RNA was also isolated from platelet lysates mixed with untreated Ep5 cells lysates immediately prior to RNA isolation (n = 3) (the concentration of RNA isolated from platelets alone was below detection limits and could therefore not be used for microarray analysis). cRNA was then synthesized and hybridized onto GeneChip Mouse Exon 1.0 ST Arrays (Affymetrix).

**Luciferase Assay**

A total of 15,000 Ep5 cells/50 μl Opti-MEM were plated in a 96-well Cignal Finder Multi Pathway reporter Array plate as recommended by the manufacturer (SABiosciences) and incubated for 24 hr. The medium was changed for fresh DMEM and treated with buffer or platelets for 20 hr (n = 4). Each bar represents the mean ± SEM, and ***p < 0.001 versus buffer was determined by two-way ANOVA followed by Bonferroni’s posttest.

**MCP-1 concentration in the conditioned medium from Ep5 cells stably expressing an IκBα super-repressor (Ep5-IkBα) or a control vector (Ep5-vector) and treated with buffer or platelets for 20 hr (n = 2). Each bar represents the mean ± SEM, and **p < 0.01 versus buffer was determined by two-way ANOVA followed by Bonferroni’s posttest.**

**Numbers of metastatic foci at the surface of lungs (two largest lobes) 14 days after tail-vein injection of Ep5-IkBRSR and Ep5-vector cells pretreated with buffer or platelets for 40 hr. Each bar represents the mean ± SEM of n = 5–7. *p < 0.05 was determined by Student’s t test.**

**Phase-contrast micrographs of Ep5-IkBRSR and Ep5-vector cells treated with buffer or platelets for 24 hr. Scale bar represents 50 μm.**

**Zymography for MMP-9 in the conditioned medium from Ep5-IkBRSR and Ep5-vector cells treated with buffer or platelets for 40 hr.**

**Relative fold change in mRNA expression in Ep5-IkBRSR and Ep5-vector cells treated as in (j) (n = 3). Values are normalized to Gapdh expression. Each bar represents the mean ± SEM, and **p < 0.001 were determined by one-way ANOVA followed by Tukey’s posttest. See also Figure S4.**
then changed and cells were treated with platelets or platelet fractions. The ratios of Firefly to Renilla Luciferase activities (relative light units [RLU]) were measured in cell lysates with the Dual Luciferase Reporter Assay System (Promega) 24 hr after treatment with platelets.

**TGFβ1 ELISA**

TGFβ1 levels were detected in tissue-culture-conditioned medium (40 hr), washed platelets or platelet-rich plasma either by direct assay (active TGFβ1), or following acid treatment to activate latent TGFβ1 (total TGFβ1) with the Quantikine TGFβ1 immunoassay kit (R&D Systems).

**MCP-1 Detection**

Concentration of MCP-1 in conditioned medium of Ep5 or MC38GFP cells was analyzed with CBA soluble Flex Set cytometric beads, following manufacturer's instructions (BD Biosciences).

**Statistics**

Statistical analyses were performed with the GraphPad Prism Software following guidelines found in Bremer and Doerge (2010) and in GraphPad Prism. Briefly, the student’s t test was used to compare means of two independent groups to each other, whereas one-way ANOVA followed by Tukey’s posttest was used to compare the means of more than two independent groups. Two-way ANOVA followed by Bonferroni’s posttest was used to compare the means of groups influenced by two independent factors.

**ACCESSORY NUMBERS**

Microarray data are deposited in Gene Expression Omnibus under accession number GSE27456.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.09.009.

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