

# 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 TGF $\beta$  and direct platelet-tumor cell contacts synergistically activate the TGF $\beta$ /Smad and NF- $\kappa$ B pathways in cancer cells, resulting in their transition to an invasive mesenchymal-like phenotype and enhanced metastasis in vivo. Inhibition of NF- $\kappa$ B signaling in cancer cells or ablation of TGF $\beta$ 1 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 (TGF $\beta$ ) 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 TGF $\beta$  (by overexpression of dominant-negative TGF $\beta$  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 TGF $\beta$ , which in turn leads to enhanced tumor cell malignancy (Kang et al., 2005). However,

## Significance

The host-to-tumor signaling events governing cancer metastasis are poorly understood. We explored the possibility that the metastatic potential of tumor cells is modulated during their transit through the bloodstream. Our findings indicate that direct contact with platelets primes tumor cells for metastasis, and induces an epithelial-mesenchymal-like transition via synergistic activation of both the TGF $\beta$  and NF- $\kappa$ B pathways. Specific ablation of platelet-derived TGF $\beta$  or NF- $\kappa$ B signaling in cancer cells prevents metastasis. Globally, our study reveals that the metastatic potential of tumor cells continues to evolve outside the primary tumor site, in response to tumor-host interactions in the bloodstream. Platelet-tumor cell interactions and the signaling pathways that they trigger are therefore crucial determinants of cancer metastasis and potential targets for anti-metastatic therapies.

in the case of metastasis to other tissues such as the lungs, the source of TGF $\beta$  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 TGF $\beta$  (Assoian 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 tumor 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; Karparkin 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*),

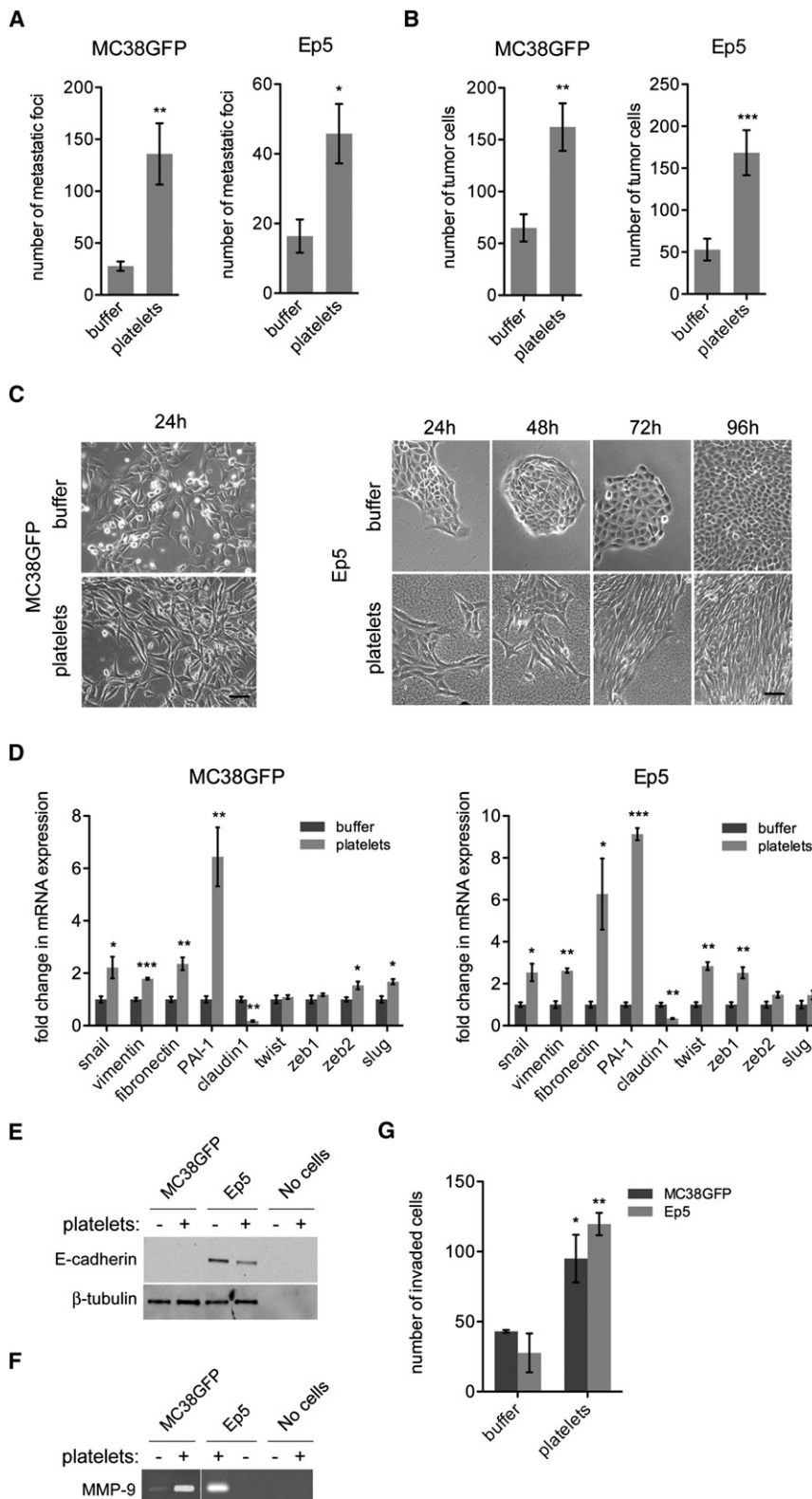
*vimentin* (*Vim*), *fibronectin* (*Fn1*), and *plasminogen activator inhibitor-1* (*PAI-1*; *Serpine1*) was consistently upregulated, whereas the epithelial marker *claudin 1* (*Cldn1*) was downregulated in platelet-treated cells (Figure 1D). E-cadherin protein levels were also reduced in platelet-treated 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 TGF $\beta$ /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 platelets 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*), *Fn1*, *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 TGF $\beta$ -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 TGF $\beta$  promotes EMT in many cancer cell lines, including Ep5 cells (Derynck and Akhurst, 2007; Maschler et al., 2005; Oft et al., 1996), we investigated whether platelet-derived TGF $\beta$  could activate TGF $\beta$ /Smad signaling in tumor cells. We found increased levels of active and latent TGF $\beta$ 1 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



**Figure 1. Pretreatment of Tumor Cells with Platelets Promotes Lung Metastasis by Increasing Tumor Cell Seeding and Inducing an EMT-Like Invasive Phenotype**

(A) Numbers of metastatic foci at the surface of lungs (two largest lobes) 14 days after tail-vein injection of MC38GFP cells or Ep5 cells stably expressing ZsGreen (Ep5-ZsGreen) pretreated with buffer (vehicle) or platelets for 40 hr (n = 5–12). (B) Numbers of tumor cells at the surface of lungs 48 hr after tail-vein injection of MC38GFP or Ep5-ZsGreen cells pretreated with buffer or platelets for 40 hr (n = 6–11).

(C) Phase-contrast micrographs of MC38GFP or Ep5 cells treated with buffer or platelets for the times indicated. Scale bar represents 50  $\mu$ m.

(D) Relative fold change in mRNA expression in MC38GFP or Ep5 cells treated with buffer or platelets for 40 hr (n = 3). Values are normalized to *Gapdh* expression.

(E) Detection of E-cadherin protein levels by immunoblotting of lysates of MC38GFP or Ep5 cells treated as in (D). Amounts of platelets equal to those used to treat cells were also loaded as control (no cells).  $\beta$ -tubulin was used as loading control.

(F) Zymography for MMP-9 in the conditioned medium of MC38GFP or Ep5 cells treated as in (D). Amounts of platelets equal to those used to treat cells were also loaded as control (no cells).

(G) MC38GFP and Ep5 cells were added at the top of transwells coated with Matrigel and treated with buffer or platelets. The total number of cells that invaded to the bottom of the transwell was counted after 48 hr (n = 3).

For (A), (B), (D), and (G) bars represent the mean  $\pm$  SEM. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 were determined by Student's *t* test. See also Figure S1.

controls, demonstrating that interaction with platelets induces TGF $\beta$ /Smad signaling in tumor cells. We next investigated whether platelet-induced tumor cell invasion is dependent on TGF $\beta$  signaling. Interestingly, adding a TGF $\beta$ RI inhibitor (SB431542) or a TGF $\beta$ 1 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 TGF $\beta$  induces a prometastatic invasive phenotype in tumor cells via activation of the TGF $\beta$ /Smad signaling pathway.

### Platelet-Derived TGF $\beta$ 1 Is Necessary for Metastasis In Vivo

To test directly the specific contribution of platelet-derived TGF $\beta$ 1 to metastasis

platelets also showed increased phosphorylation of the TGF $\beta$  signaling effector Smad2 (Figure 2D) and Smad-binding element (SBE)-dependent transcription (Figure 2E) compared with

in vivo, *Pf4-cre* mice (Tiedt et al., 2007) were crossed with TGF $\beta$ 1<sup>fl/fl</sup> mice (Li et al., 2007) to generate mice lacking TGF $\beta$ 1 specifically in their megakaryocytes and platelets. *Pf4-cre*;

*TGFβ1<sup>fl/fl</sup>* mice had normal bleeding times and platelet counts (Table S3), showing that platelet hemostatic functions are not impaired in these mice. ELISA for TGFβ1 showed that platelets from *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice contained <1% of the amount of TGFβ1 present in platelets from wild-type (WT) mice (Figure 3A), confirming that *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice could be used to study the role of platelet-derived TGFβ1 in metastasis. Furthermore, the concentration of TGFβ1 was significantly lower in platelet-rich plasma from *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice compared with plasma from WT mice (Figure 3A), suggesting that platelets are a major source of TGFβ1 in the circulation.

We next tested whether platelets lacking TGFβ1 could induce Smad signaling in vitro and found a decrease in SBE-dependent luciferase reporter activity (Figure 3B) and MMP-9 secretion by Ep5 cells, in comparison with treatment with WT platelets (Figure 3C). Thus, although other members of the TGFβ family might be secreted by platelets, these results suggest that platelet-derived TGFβ1 is key for the platelet-induced activation of Smad signaling in cancer cells.

To test the role of platelet-derived TGFβ1 during metastasis in vivo, MC38GFP cells were injected into *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* or WT mice via the tail vein. Fourteen days after injection, the numbers of metastases present in the lungs of *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice were greatly reduced compared with those in littermate controls (WT and *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/+</sup>* mice) (Figures 3D and 3E). Importantly, cells pretreated with platelets from WT mice and injected into *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice also formed significantly fewer metastases than platelet-treated cells injected into WT mice (Figure 3F). Conversely, pretreating tumor cells with TGFβ1-deficient platelets led to the formation of significantly fewer metastases in WT mice, in comparison to cells pretreated with platelets from WT mice (Figure 3F; see Figure S1A for micrographs of injected cells). Thus, whereas a platelet pretreatment primes tumor cells for metastasis in WT mice in a TGFβ1-dependent manner, the presence of platelet-derived TGFβ1 in the host bloodstream is also required for efficient metastasis.

We next investigated the role of platelet-derived TGFβ1 in the early steps in the seeding of metastases. Whereas equivalent numbers of cells were found in the lungs 3 hr after injection for both WT and *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice (Figure 3G), significantly lower numbers of cells remained in the lungs after 21 hr or 48 hr in *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice (Figure 3G). Closer inspection by confocal microscopy and 3D-rendering of lungs revealed that, at 3 hr postinjection, tumor cells were mainly present within the blood vessels (Figures 3H and 3I) in association with platelets (Figure 3J), whereas after 48 hr they were found mainly outside of the blood vessels in both WT and *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice (Figures 3H and 3I). Interestingly, at the intermediate time point of 21 hr, tumor cells were found both in the intravascular and extravascular compartments, and a smaller proportion of cells were localized extravascularly in the lungs of *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice compared with WT, suggesting that tumor cell extravasation is impaired in *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice (Figure 3H).

Because another gene (*stumpy*) was also targeted in the *TGFβ1<sup>fl/fl</sup>* mice (Li et al., 2007; Town et al., 2008), we generated *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/-</sup>* and *TGFβ1<sup>fl/-</sup>* mice, with one null allele of *TGFβ1*, which allows expression of one WT allele of *stumpy* in these mice. The numbers of metastases observed in lungs of

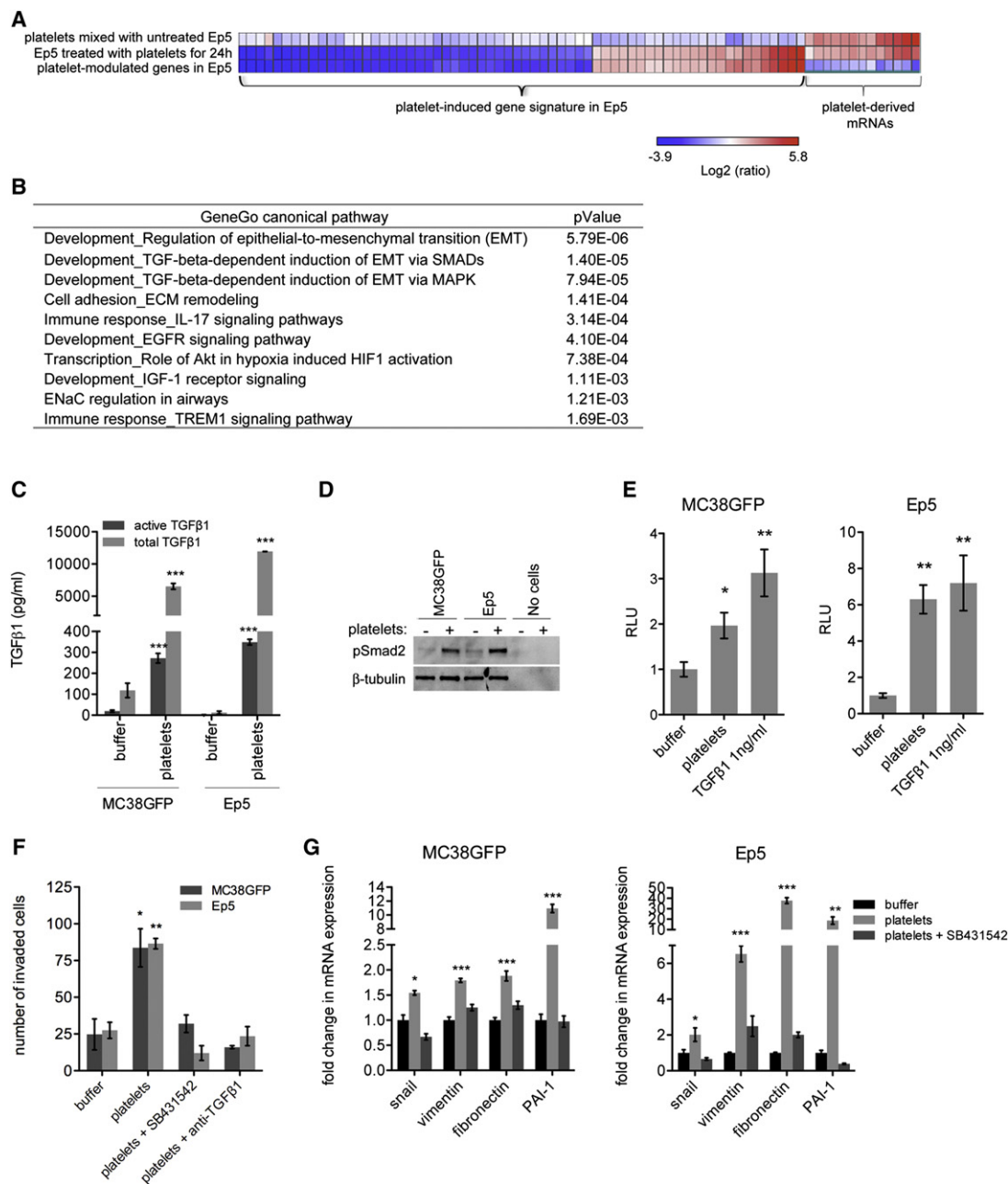
*TGFβ1<sup>fl/-</sup>* mice after 14 days were similar to those obtained for WT or *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/+</sup>* mice, whereas *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/-</sup>* mice had reduced numbers of metastases (Figure S2). These results show that the reduction in metastasis is not due to the deletion of *stumpy*, but attributable to the lack of TGFβ1. Altogether, our data reveal that platelet-derived TGFβ1 plays an important role in promoting metastatic seeding in the lungs possibly by inducing extravasation and invasion into the lung parenchyma.

### Platelet-Derived TGFβ1 and Platelet-Bound Factors Cooperate to Promote Metastasis

Considering that TGFβ1 is a secreted factor released by platelets upon activation (Assoian 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 and S3G). However, treatment with the platelet releasate resulted in only partial gene expression changes in comparison with treatment with either platelets or the pellet fraction (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 TGFβ alone, but also require additional platelet-bound factors. These data also suggest that these platelet-bound factors synergize with TGFβ 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  $\pm$  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).  $\beta$ -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 1 ng/ml TGFβ1 (positive control) ( $n = 5-6$ ). Each bar represents the mean  $\pm$  SEM, and \* $p < 0.05$ , \*\* $p < 0.01$  versus buffer were determined by one-way ANOVA followed by Tukey's posttest.

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

| Gene Sets  | NES    | Nominal p Value | FDR    |
|--|--------|-----------------|--------|
| <b>EMT signatures</b>                              |        |                 |        |
| BLICK_EMT-SIG_UP                                   | 1.993  | <0.001          | <0.001 |
| TAUBE_EMT_UP                                       | 1.328  | 0.060           | 0.060  |
| TAUBE_EMT_DN                                       | −2.081 | <0.001          | <0.001 |
| ONDER_CDH1_TARGETS_2_UP                            | 1.526  | <0.001          | 0.044  |
| ONDER_CDH1_TARGETS_2_DN                            | −1.832 | <0.001          | 0.006  |
| <b>TGFβ Signatures</b>                             |        |                 |        |
| GIAMPIERI_TGFB_UP                                  | 1.992  | <0.001          | <0.001 |
| GIAMPIERI_TGFB_DN                                  | −2.576 | <0.001          | <0.001 |
| VALCOURT_TGFB_UP                                   | 1.813  | <0.001          | 0.005  |
| VALCOURT_TGFB_DN                                   | −1.926 | <0.001          | <0.001 |
| <b>Cancer stem cell signatures</b>                 |        |                 |        |
| CREIGHTON_CSC_UP                                   | 1.675  | <0.001          | <0.001 |
| CREIGHTON_CSC_DN                                   | −1.778 | <0.001          | <0.001 |
| <b>Tumor progression and metastasis signatures</b> |        |                 |        |
| VANTVEER_BREAST_CANCER_POOR_PROGNOSIS              | 1.953  | <0.001          | <0.001 |
| JAEGER_METASTASIS_UP                               | 1.875  | <0.001          | 0.003  |

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

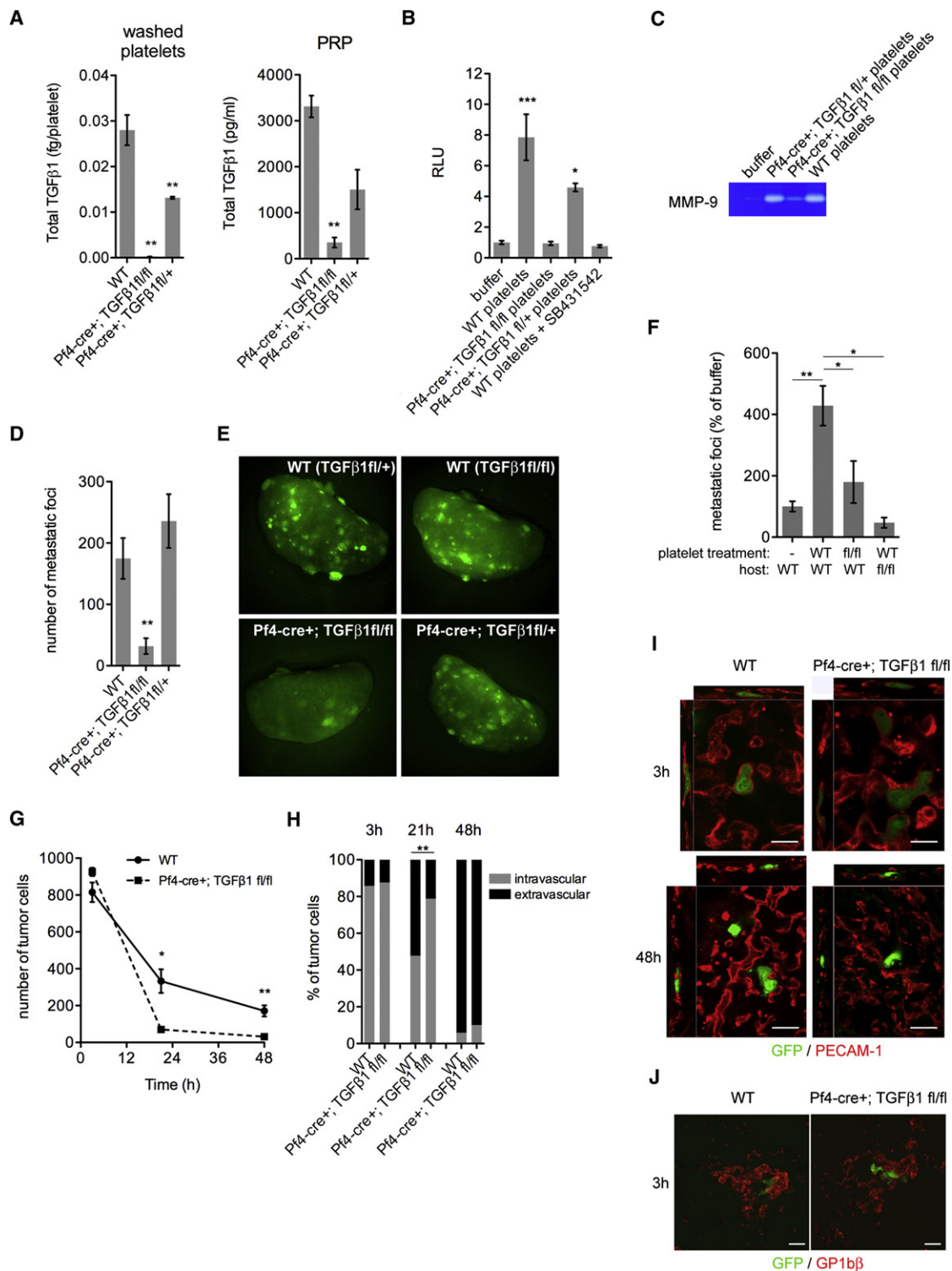
To test further whether platelets synergize with TGFβ signaling, we used the MLEC cell line stably expressing a luciferase reporter based on the promoter region of the TGFβ target gene *PAI-1* (Abe et al., 1994). These cells provide a specific readout for exogenous TGFβ bioavailability and signaling 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 *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* mice resulted in a synergistic 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 prometastatic 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, cocubation 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 (6 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, \*\*p < 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<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* or *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/+</sup>* 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<sup>+</sup>; TGFβ1<sup>fl/fl</sup>*, or *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/+</sup>* 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<sup>+</sup>; TGFβ1<sup>fl/fl</sup>* or *Pf4-cre<sup>+</sup>; TGFβ1<sup>fl/+</sup>* 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 TGF $\beta$ 1-deficient platelets or with WT platelets together with the TGF $\beta$ RI inhibitor SB431542 still led to activation of the NF- $\kappa$ B pathway reporter (Figure 6C), ruling out the possibility that activation of the NF- $\kappa$ B pathway is TGF $\beta$ 1-dependent in this context.

To study whether NF- $\kappa$ B signaling contributes to the prometastatic phenotype observed upon platelet contact, we established Ep5 cells stably expressing an I $\kappa$ B $\alpha$  mutant (I $\kappa$ B $\alpha$ S32A/S36A) that cannot be phosphorylated or degraded and therefore irreversibly sequesters NF- $\kappa$ B in the cytoplasm, and inhibits its function (I $\kappa$ B $\alpha$  super-repressor; Ep5-I $\kappa$ BSR) (Brown et al., 1995). In contrast to Ep5 expressing a control vector (Ep5-vector), Ep5-I $\kappa$ BSR cells failed to activate an NF- $\kappa$ B-dependent luciferase reporter and did not express increased levels of MCP-1 following platelet treatment, demonstrating that the NF- $\kappa$ B 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-I $\kappa$ BSR cells (Figure 6F). Similarly, retention of Ep5-I $\kappa$ BSR cells in the lungs after 48 hr was not enhanced by a pretreatment with platelets, showing that the NF- $\kappa$ B pathway is necessary for the prometastatic effects of platelets on tumor cells (Figure 6G). Furthermore, Ep5-I $\kappa$ BSR 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-I $\kappa$ BSR cells following platelet treatment. The inhibition of platelet prometastatic effects was not due to an impairment of TGF $\beta$ /Smad signaling pathway by the I $\kappa$ BSR construct because Smad2 phosphorylation levels were intact in Ep5-I $\kappa$ BSR 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- $\kappa$ B pathway (Figures S4D and S4E). JSH-23 treatment also abolished the synergy observed on *PAI-1* reporter activity between TGF $\beta$ 1 and platelets (Figure S4F), but had no effect on SBE-reporter activity (Figure S4G). This finding further demonstrates that platelets activate NF- $\kappa$ B independently of TGF $\beta$ /Smad signaling, and cooperate with TGF $\beta$ 1 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- $\kappa$ B and TGF $\beta$  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 blood-stream 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 immunosurveillance, 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 TGF $\beta$ , PDGF, VEGF and angiopoietin are abundant in platelets (Erpenbeck and Schön, 2010; Sierko and Wojtukiewicz, 2007) and may therefore

(D and E) Numbers of metastatic foci at the surface of lungs (two largest lobes) 14 days after tail-vein injection of MC38GFP cells in WT, *Pf4-cre<sup>+</sup>*; TGF $\beta$ 1<sup>fl/fl</sup> or *Pf4-cre<sup>+</sup>*; TGF $\beta$ 1<sup>fl/+</sup> mice (n = 7–9), and representative pictures of lungs (E). (D) Each bar represents the mean  $\pm$  SEM, and \*\*p < 0.01 versus WT was determined by one-way ANOVA followed by Tukey's posttest.

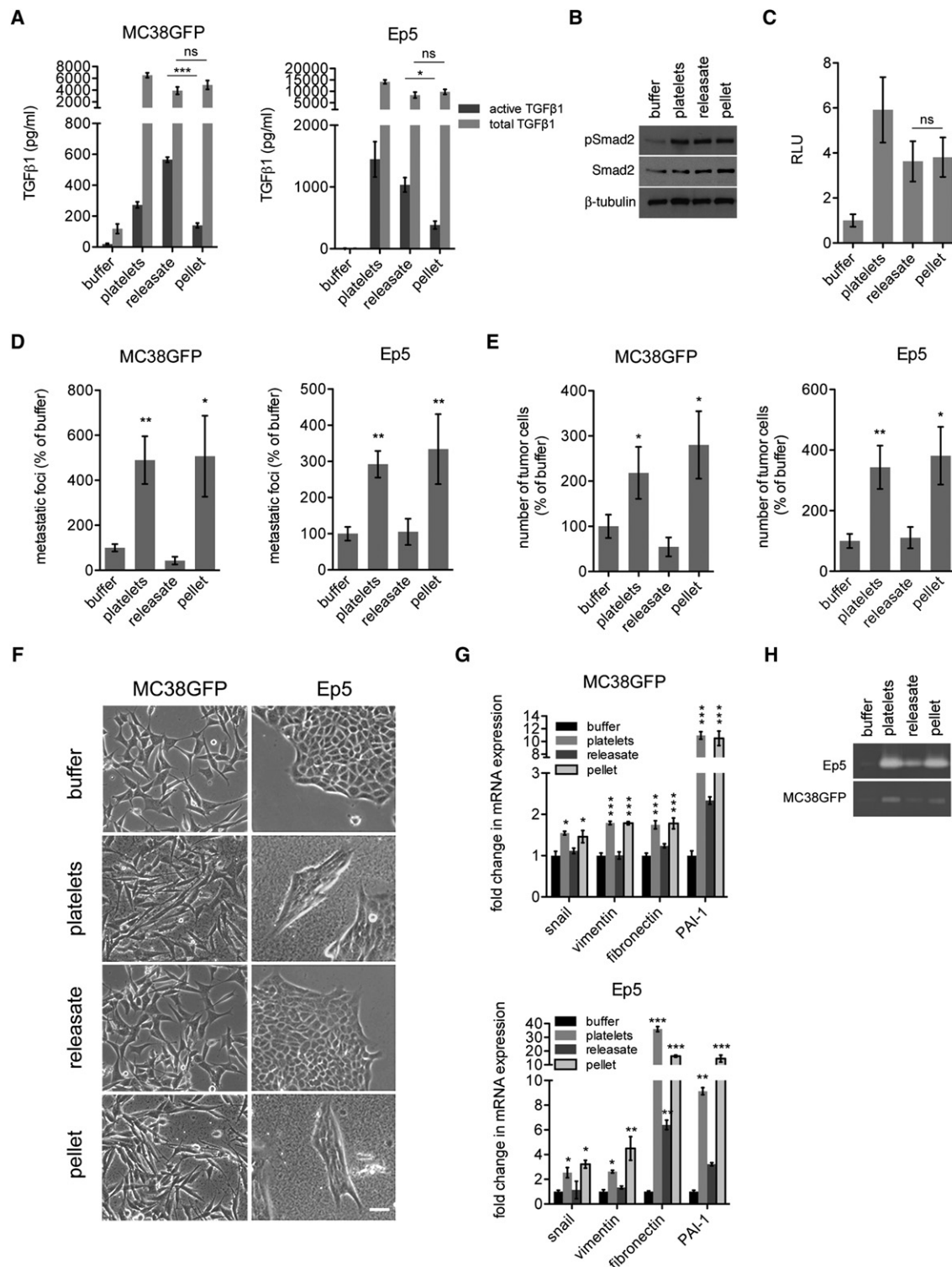
(F) Numbers of metastatic foci at the surface of lungs (two largest lobes) 14 days after tail-vein injection of MC38GFP cells pretreated with buffer (–), platelets from WT mice (WT) or platelets from *Pf4-cre<sup>+</sup>*; TGF $\beta$ 1<sup>fl/fl</sup> (fl/fl) and injected into WT or *Pf4-cre<sup>+</sup>*; TGF $\beta$ 1<sup>fl/fl</sup> mice (n = 9–14). Each bar represents the mean  $\pm$  SEM, and \*p < 0.05, \*\*p < 0.01 were determined by one-way ANOVA followed by Tukey's posttest.

(G) Numbers of tumor cells at the surface of lungs 3 hr, 21 hr, and 48 hr after tail-vein injection of MC38GFP cells in WT or *Pf4-cre<sup>+</sup>*; TGF $\beta$ 1<sup>fl/fl</sup> mice. Each point represents the mean  $\pm$  SEM number of cells/view field (3 $\times$ ) (n = 3–14). \*p < 0.05, \*\*p < 0.01 were determined by Student's t test.

(H) Percentage of intravascular and extravascular MC38GFP cells in lungs of WT or *Pf4-cre<sup>+</sup>*; TGF $\beta$ 1<sup>fl/fl</sup> mice 3 hr, 21 hr, and 48 hr after tail-vein injection of tumor cells (n = 16–46 cells). \*\*p < 0.01 as determined by Fisher's exact test.

(I and J) Confocal microscopy of lungs of WT or *Pf4-cre<sup>+</sup>*; TGF $\beta$ 1<sup>fl/fl</sup> mice 3 hr and 48 hr after tail-vein injection of tumor cells for MC38GFP cells (green) and either blood vessels (I; PECAM-1 staining; red) or platelets (J; GP1b $\beta$  staining; red. Note platelet aggregates surrounding tumor cells.). Scale bar represents 50  $\mu$ m. See also Figure S2 and Table S3.





**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 $\beta$  signaling pathway, and that abrogating either TGF $\beta$  signaling in tumor cells or TGF $\beta$  expression by platelets is sufficient to inhibit metastasis and EMT. Although TGF $\beta$  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 $\beta$  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 $\beta$  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 $\beta$ 1 many-fold higher than most cell types (Assoian et al., 1983). Furthermore, the amount of TGF $\beta$ 1 produced by other cells and taken up by platelets seems minimal, as purified platelets from *Pf4-cre<sup>+</sup>; TGF $\beta$ 1<sup>fl/fl</sup>* mice contained <1% of the amount of TGF $\beta$ 1 present in platelets from WT mice.

Most importantly, we show that abrogation of TGF $\beta$ 1 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 $\beta$ 1 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 $\beta$ 1 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 $\beta$ 1 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 $\beta$ 1 have an enhanced capacity to seed the lungs, whereas cells that are continuously exposed to TGF $\beta$ 1 have decreased metastatic capacity due to the cytostatic effect of TGF $\beta$ 1 (Giampieri et al., 2009; Padua et al., 2008). In this respect, specific therapeutic inhibition of platelet-derived TGF $\beta$ 1 might result in the impairment of tumor cell extravasation at the metastatic site. Importantly, *Pf4-cre<sup>+</sup>; TGF $\beta$ 1<sup>fl/fl</sup>* mice maintain normal platelet counts and hemostatic functions, suggesting that pharmacological inhibition of platelet-derived TGF $\beta$  could inhibit metastasis without adverse effects on physiological hemostasis.

We also find that, although required for metastasis, activation of TGF $\beta$  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 $\beta$ 1) do not. In line with this result, a prolonged treatment with TGF $\beta$ 1—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; Maschler et al., 2005). Our data further support the existence of additional platelet-bound factors synergizing with TGF $\beta$ 1. First, platelets induce the TGF $\beta$ -responsive *PAI-1* reporter to levels higher than achievable with TGF $\beta$ 1 alone. Second, combining exogenous TGF $\beta$ 1 with platelets from either WT or *Pf4-cre<sup>+</sup>; TGF $\beta$ 1<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 $\beta$ 1 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- $\kappa$ B pathway, which is specifically triggered upon direct contact between tumor cells and platelets independently from TGF $\beta$  activity.

NF- $\kappa$ B 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- $\kappa$ B promotes osteolytic bone metastasis by inducing the proinflammatory cytokine GM-CSF (Park et al., 2007). Notably, the activation of NF- $\kappa$ B has also been proposed to be part of the mechanism allowing TGF $\beta$  signaling to switch from a cytostatic to a prometastatic signal (Neil and Schiemann, 2008). In support of this idea, we found that NF- $\kappa$ B activation potentiates TGF $\beta$ -induced prometastatic gene expression, and that NF- $\kappa$ B 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 $\beta$  via NF- $\kappa$ B activation.

In conclusion, we establish platelets as a crucial source of TGF $\beta$  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 $\beta$  and NF- $\kappa$ B pathways to potentially 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.

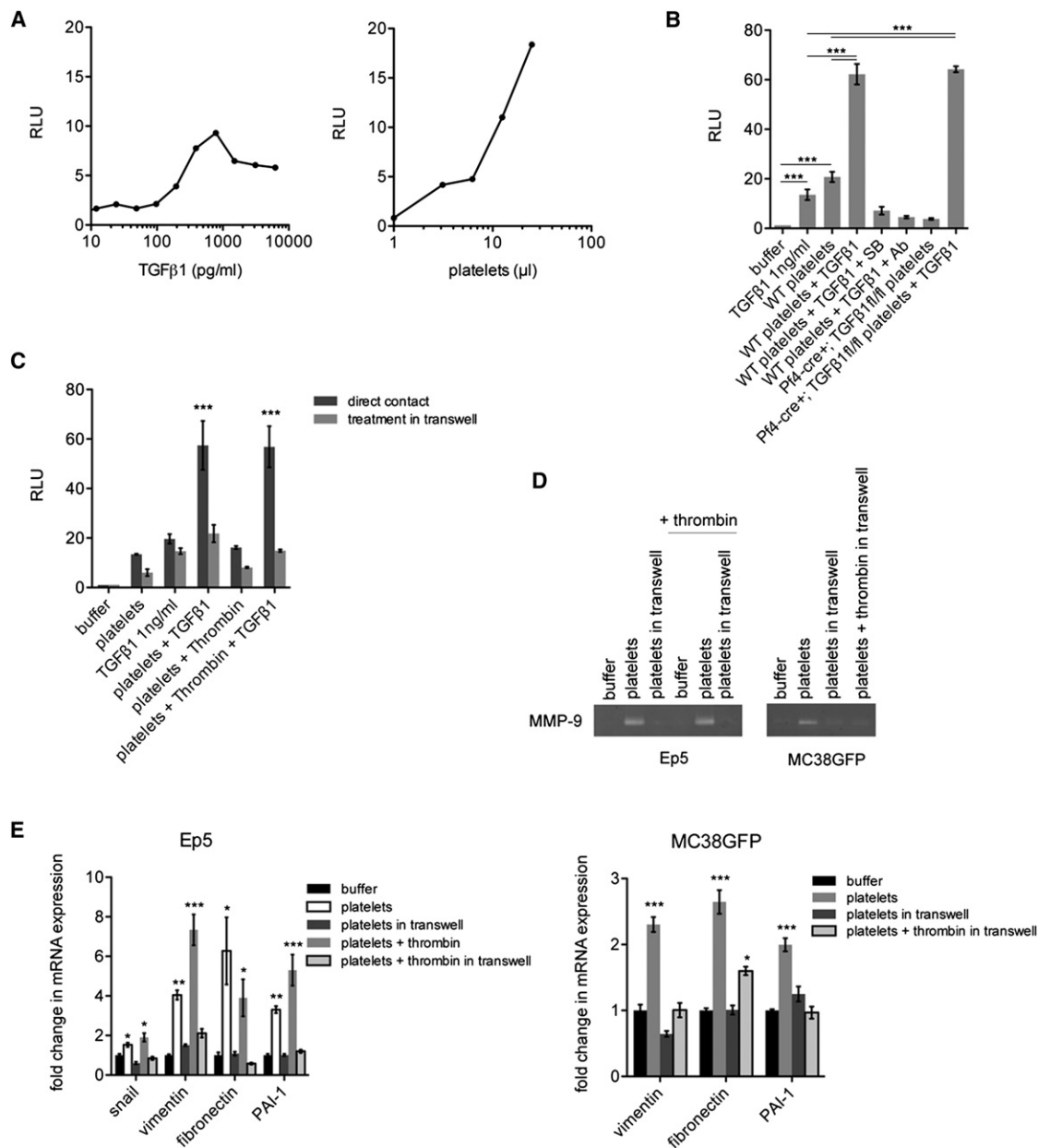
(D) Numbers of metastatic foci at the surface of lungs (two largest lobes) 14 days after tail-vein injection of MC38GFP or Ep5-ZsGreen cells pretreated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) for 40 hr ( $n = 5-17$ ). Each bar represents the mean  $\pm$  SEM, and \* $p < 0.05$ , \*\* $p < 0.01$  versus buffer were determined by one-way ANOVA followed by Tukey's posttest.

(E) Numbers of tumor cells at the surface of lungs 48h after tail-vein injection of MC38GFP or Ep5-ZsGreen cells pretreated as in (D). Each bar represents the mean  $\pm$  SEM number of cells/view field ( $3\times$ ) ( $n = 5-13$ ). \* $p < 0.05$ , \*\* $p < 0.01$  versus buffer were determined by Student's  $t$  test.

(F) Phase-contrast micrographs of MC38GFP and Ep5 cells treated as in (A) for 24 hr. Scale bar represents 50  $\mu$ m.

(G) Relative fold change in mRNA expression in MC38GFP or Ep5 cells treated as in (A) for 40 hr. Values are normalized to *Gapdh* expression ( $n = 3$ ). Each bar represents the mean  $\pm$  SEM, and \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus buffer were determined by one-way ANOVA followed by Tukey's posttest.

(H) Zymography for MMP-9 in the conditioned medium from Ep5 or MC38GFP cells treated as in (A) for 40 hr. See also Figure S3 and Table S4.



**Figure 5. TGFβ1 and Direct Platelet-Tumor Cell Contact Synergize to Promote Prometastatic Gene Expression**

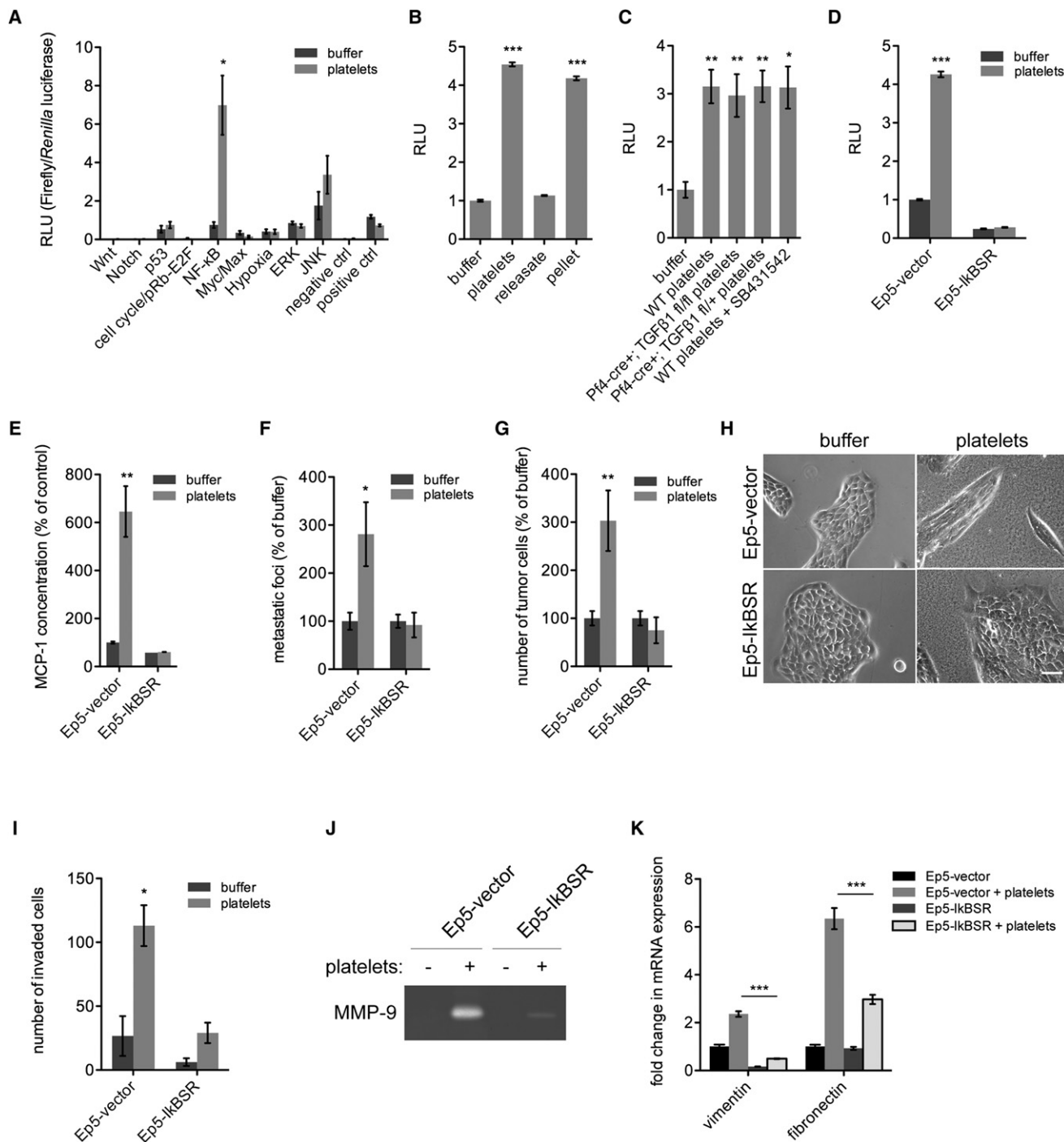
(A) Relative luciferase activity (RLU) in MLEC cells stably expressing a luciferase reporter under the control of a TGFβ responsive *PAI-1* promoter construct and treated with different concentrations of TGFβ1 (left) or platelets (right). Note that the y axis scale is the same for both panels and that 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<sup>+</sup>*; *TGFβ1<sup>fl/fl</sup>* 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.



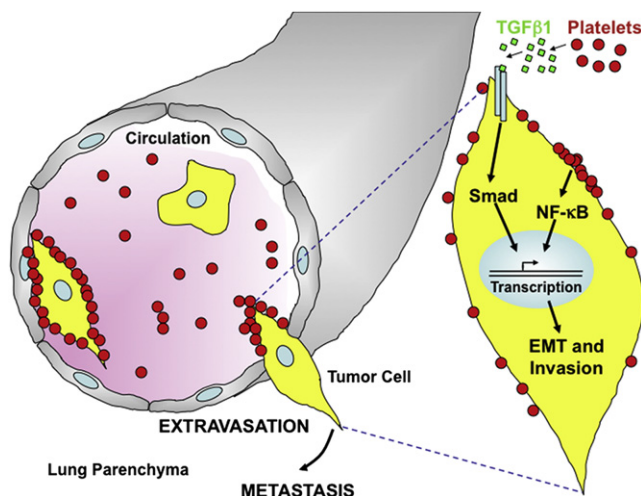
**Figure 6. The NF-κB Signaling Pathway Is Activated by Platelets in a Contact-Dependent Manner and Cooperates with TGFβ Signaling to Induce an EMT-Like Transition and Promote Metastasis**

(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  $\pm$  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  $\pm$  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<sup>+</sup>*; *TGFβ1<sup>fl/fl</sup>*, or *Pf4-cre<sup>+</sup>*; *TGFβ1<sup>fl/+</sup>* mice, or with WT platelets + SB431542 (10  $\mu$ M) for 20 hr ( $n = 5$ ). Each bar represents the mean  $\pm$  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 TGF $\beta$ 1 Synergize to Promote an EMT-Like Transition and Metastasis**

Platelets secrete TGF $\beta$ 1, which activates the TGF $\beta$ /Smad pathway in tumor cells. Upon direct platelet-tumor cell contact, the NF- $\kappa$ B pathway is also activated in tumor cells and synergizes with TGF $\beta$ /Smad signaling to induce a rapid EMT, enhance invasiveness and promote metastasis. Activation of neither the TGF $\beta$ /Smad nor the NF- $\kappa$ B pathway alone is sufficient to promote metastasis. Thus, platelet-tumor cell contact triggers a synergistic interaction between TGF $\beta$ /Smad and NF- $\kappa$ B 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 TGF $\beta$ 1 floxed allele (TGF $\beta$ 1<sup>fl/fl</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 TGF $\beta$ 1-deficient platelets, *Pf4-cre*<sup>+</sup>; TGF $\beta$ 1<sup>fl/+</sup> mice were bred with TGF $\beta$ 1<sup>fl/fl</sup> or TGF $\beta$ 1<sup>fl/+</sup> mice. TGF $\beta$ 1<sup>+/-</sup> mice (the null allele was obtained by *egfp* knockin, which disrupted TGF $\beta$ 1 without affecting *stumpy*) (Li et al., 2007) were crossed with *Pf4-cre*<sup>+</sup>; TGF $\beta$ 1<sup>fl/fl</sup> mice to obtain *Pf4-cre*<sup>+</sup>; TGF $\beta$ 1<sup>fl/-</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 IkB $\alpha$  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 (AGCCAGACA tandem repeats)-luciferase and NF- $\kappa$ B (GGGACTTTCC tandem repeats)-luciferase reporter constructs were generated by infecting cells with Signal 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  $\times$  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/ $\mu$ l and equivalent volumes of releasate and pellet fractions were added. Where indicated, cells were treated with 1 or 10 ng/ml of recombinant TGF $\beta$ 1 (R&D Systems), 10  $\mu$ M SB431542 (Sigma), or 6  $\mu$ g/ml anti-TGF $\beta$ 1 blocking antibody (R&D systems).

### In Vivo Metastasis Assays

For lung metastasis assays, cells treated with platelets for 40 hr were washed in PBS, and either trypsinized (Ep5) or lifted with 2mM EDTA in PBS (MC38GFP). Cells were then washed and centrifuged twice to remove platelets, and resuspended in HBSS at a constant number of cells for all mice in a given experiment (250,000 to 1,000,000 cells/injection). One hundred microliters of cell suspension were then injected via the tail vein of syngeneic mice. The numbers of single cells and metastatic foci were determined as described in Supplemental Experimental Procedures.

### 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  $\mu$ l Opti-MEM were plated in a 96-well Signal Finder Multi Pathway reporter Array plate as recommended by the manufacturer (SABiosciences) and incubated for 24 hr. The medium was

(D) Relative luciferase activity (RLU) in Ep5 cells stably expressing a NF- $\kappa$ B luciferase reporter and either a IkB super-repressor (Ep5-IkBSR) or a control vector (Ep5-vector) and treated with buffer or platelets for 20 hr ( $n = 4$ ). Each bar represents the mean  $\pm$  SEM, and \*\*\* $p < 0.001$  versus buffer was determined by two-way ANOVA followed by Bonferroni's posttest.

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

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

(G) Numbers of tumor cells at the surface of lungs 48 hr after tail-vein injection of Ep5-IkBSR and Ep5-vector cells pretreated with buffer or platelets for 40 hr. Each bar represents the mean  $\pm$  SEM of  $n = 4-7$ . \*\* $p < 0.01$  was determined by Student's  $t$  test.

(H) Phase-contrast micrographs of Ep5-IkBSR and Ep5-vector cells treated with buffer or platelets for 24 hr. Scale bar represents 50  $\mu$ m.

(I) Ep5-IkBSR and Ep5-vector cells were added at the top of transwells coated with Matrigel and treated with buffer or platelets. The total numbers of cells that invaded to the bottom of the transwell were counted after 48 hr ( $n = 2$ ). Each bar represents the mean  $\pm$  SEM, and \* $p < 0.05$  versus buffer was determined by two-way ANOVA followed by Bonferroni's posttest.

(J) Zymography for MMP-9 in the conditioned medium from Ep5-IkBSR and Ep5-vector cells treated with buffer or platelets for 40 hr.

(K) Relative fold change in mRNA expression in Ep5-IkBSR and Ep5-vector cells treated as in (J) ( $n = 3$ ). Values are normalized to *Gapdh* expression. Each bar represents the mean  $\pm$  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.

### TGF $\beta$ 1 ELISA

TGF $\beta$ 1 levels were detected in tissue-culture-conditioned medium (40 hr), washed platelets or platelet-rich plasma either by direct assay (active TGF $\beta$ 1), or following acid treatment to activate latent TGF $\beta$ 1 (total TGF $\beta$ 1) with the Quantikine TGF $\beta$ 1 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.

### ACCESSION 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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