Cancer Cell, *20*

Supplemental Information

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

Myriam Labelle, Shahinoor Begum, and Richard O. Hynes

Inventory of Supplemental Information

SUPPLEMENTAL INFORMATION

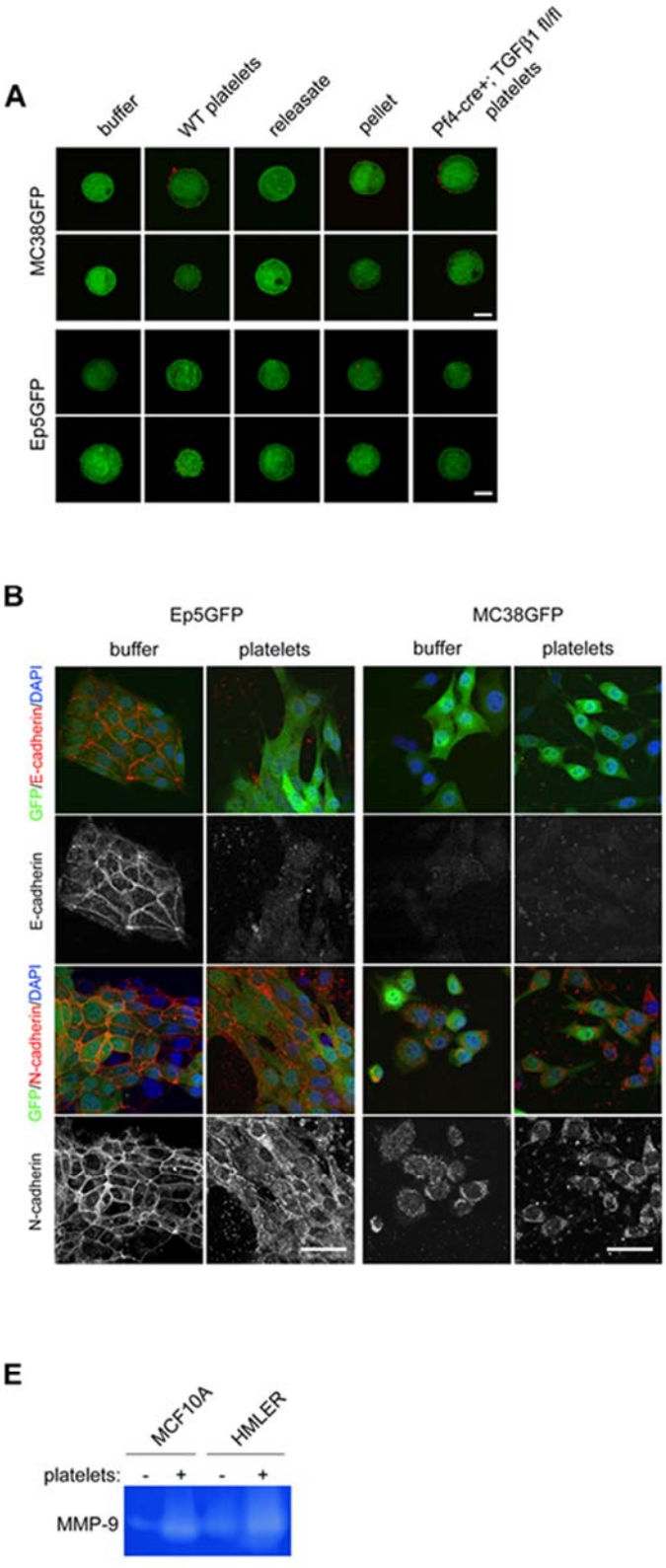

Figure S1, related to Figure 1. Pretreatment of Tumor Cells with Platelets Induces an EMT-Like Phenotype in Mouse and Human Cell Lines.

(A) Immunofluorescence staining for platelets (CD41;red) in cell suspension (prepared as for tail-vein injection) of MC38GFP or Ep5 cells stably expressing GFP. Two representative cells for each condition are shown. Note that very few platelets remain attached to tumor cells treated with platelets or the platelet pellet fraction from WT mice, or with platelets from *Pf4-cre*$^{+}$*;* *TGFβ1*$^{fl/fl}$ mice. Scale bar=10μm.

(B) Immunofluorescence stainings for E-cadherin and N-cadherin (red) in MC38GFP cells or Ep5 cells stably expressing GFP treated with buffer or platelets for 40h. Scale bar=50μm.

(C) Phase-contrast micrographs of MCF10A or HMLER cells treated with buffer or platelets for 24h. Scale bar=50μm.

(D) Relative fold change in mRNA expression in human breast epithelial MCF10A or HMLER human cells treated with buffer or platelets for 40h (n=3). Values are normalized to *GAPDH* expression. Bars represent the mean ± SEM. **p<0.01, ***p<0.001 were determined by Student's t-test.

(E) Zymography for MMP-9 in the conditioned medium of MCF10A or HMLER human cells treated as in (D).

Table S1, related to Figure 2. List of Genes Modulated by More than 2 Fold in Ep5 Cells upon Exposure to Platelets (p<0.05) (Provided as an Excel File)

Table S2, related to Figure 2. List of Genes Modulated by More than 4 fold in Ep5 Cells upon Exposure to Platelets (p<0.05)

Gene Symbol	Log2 fold change	P value
1700112C13Rik	2.098184	1.60E-08
4930572J05Rik	-3.766710667	2.01E-05
9330175M20Rik	-2.024273333	0.000366637
Aldh3a1	-3.059570667	1.60E-05
Ankrd22	-2.472888667	3.88E-06
Arg2	-2.04233	4.25E-05
Atp13a4	-3.085166	1.98E-08
Atp8b1	-2.33801	0.002798052
Bhlhe40	2.119186667	1.36E-08
Bhlhe41	-2.822264667	3.12E-05
C1qtnf1	2.86726	2.56E-10
Ccl2	3.415371333	0.000501074
Chst2	2.286672667	1.05E-08
Cldn1	-2.493964667	3.62E-08
Clu	2.452976667	1.82E-09
Ctsw	5.22753	3.26E-11
Cxcl5	-2.299436667	0.001381252
Edn1	2.325914667	1.57E-05
Eya2	-2.221118667	5.07E-07
Fermt1	2.084543333	9.63E-10
Fn1	2.147283333	4.66E-07
Gm1006	-3.230466	1.20E-07
Gm14203	-2.115369333	1.75E-09
Gpr146	-2.591415333	7.70E-05
Gstt1	-2.132094667	2.31E-06
Id2	-2.530128	0.000104486
Irs1	-2.202704	7.78E-07
Jag1	2.010252	2.09E-06
Klhl1	2.246299333	7.20E-06
Klk6	-3.232498667	3.42E-06
Lrrc26	-3.754262667	6.93E-08
Mcpt8	-2.434465333	2.93E-05
Mgat3	-2.665078667	0.000169619
Mmp3	-2.934538667	3.48E-06
Mmp9	5.549030667	4.86E-11
Mt2	3.137014	3.12E-05
Mxd1	-2.005537333	3.17E-09

Ncam1	3.060405333	2.89E-09
Npnt	-2.537079333	7.44E-06
Nt5e	2.16121	2.99E-05
Padi1	-2.58373	4.09E-07
Pdia5	2.178992	4.35E-05
Ppl	-2.346288	3.27E-05
Rhpn2	-2.334857333	3.05E-07
Rom1	-2.792601333	1.39E-06
Serpinb2	-2.026295333	0.003003047
Serpine1	4.16097	4.47E-09
Sgk2	-3.672803333	1.48E-05
Slc25a35	-2.232655333	0.001304895
Slc26a9	-2.669384667	9.74E-06
Slc44a3	-3.123658	7.31E-05
Styk1	-2.362784	4.20E-06
Tc2n	-2.454634	0.000866249
Tmem71	-2.043573333	0.000126913
Tns4	-2.508330667	7.11E-09
Vegfc	3.681836	1.39E-07
Vim	2.430557333	0.0143674
Wisp1	4.779488	9.59E-11

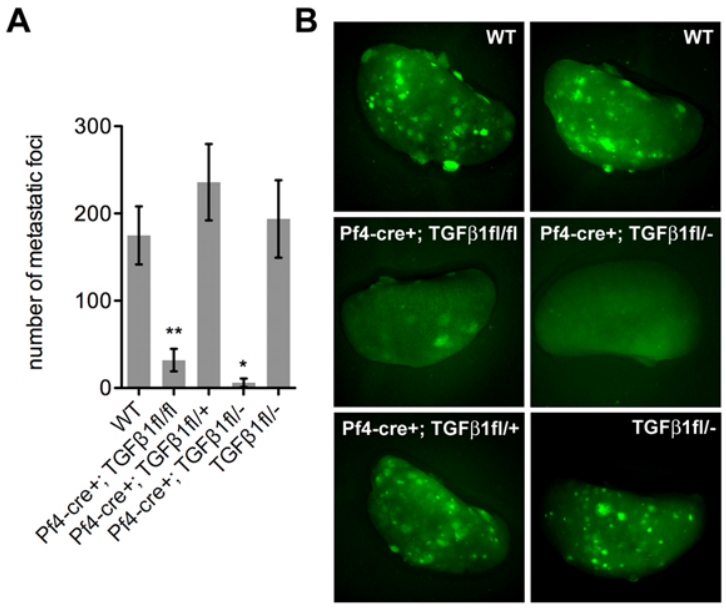

Figure S2, related to Figure 3. *Stumpy* Deletion Does Not Affect Metastasis

(A) Numbers of metastatic foci at the surface of lungs (2 largest lobes) 14 days after tail-vein injection of MC38GFP cells in wild-type (WT), *Pf4-cre*$^{+}$*; TGFβ1*$^{fl/fl}$, *Pf4-cre*$^{+}$*; TGFβ1*$^{fl/+}$ mice, *Pf4-cre*$^{+}$*; TGFβ1*$^{fl/-}$ mice or *TGFβ1*$^{fl/-}$ mice. Each bar represents the mean ± SEM of n=4-9. *p<0.05, **p<0.01 vs WT were determined by one-way ANOVA followed by Tuckey's post test.

(B) Micrographs of lungs 14 days after tail-vein injection of MC38GFP cells in wild-type (WT), *Pf4-cre*$^{+}$*; TGFβ1*$^{fl/fl}$, *Pf4-cre*$^{+}$*; TGFβ1*$^{fl/+}$ mice, *Pf4-cre*$^{+}$*; TGFβ1*$^{fl/-}$ mice, or *TGFβ1*$^{fl/-}$ mice.

Table S3, related to Figure 3. Bleeding Times and Platelet Counts

Genotype	Bleeding time (s)	Platelet count (10^6/μl)
WT	176 ± 27 (n=18)	1.04 ± 0.05 (n=5)
Pf4-cre$^+$*; TGFβ1*$^{fl/fl}$	172 ± 37 (n=6)	1.11 ± 0.16 (n=4)
Pf4-cre$^+$ *; TGFβ1*$^{fl/+}$	187 ± 44 (n=5)	1.12 ± 0.09 (n=5)
Pf4-cre$^+$ *; TGFβ1*$^{fl/-}$	149 ± 19 (n=5)	1.19 ± 0.10 (n=2)
TGFβ1$^{fl/-}$	180 ± 28 (n=7)	0.76 ± 0.16 (n=4)

Bleeding times and platelet counts ± SEM

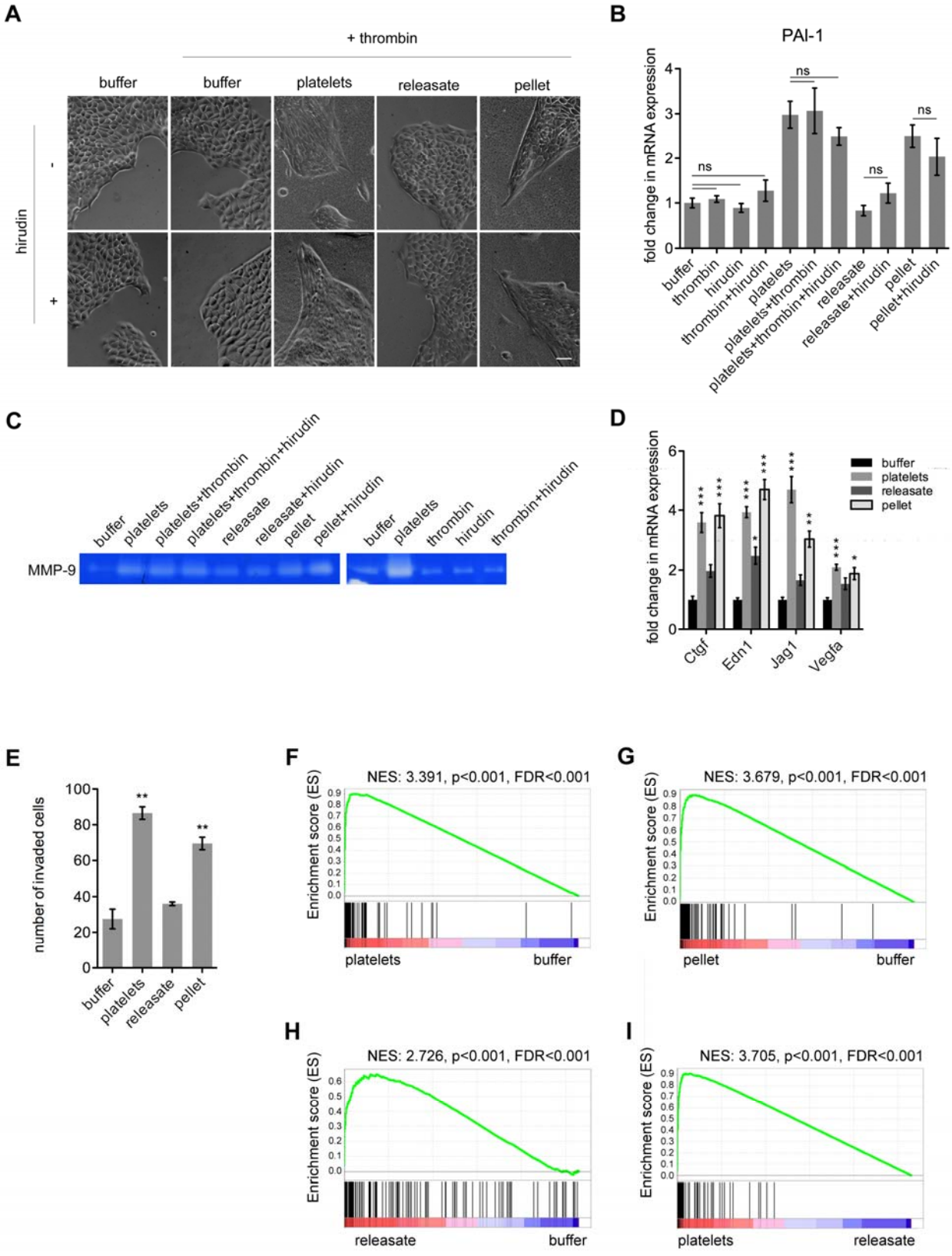
A
+ thrombin
buffer
buffer
platelets
releasate
pellet
hirudin
-
+
B
PAI-1
fold change in mRNA expression
ns
buffer
thrombin
hirudin
thrombin+hirudin
platelets
platelets+thrombin
platelets+thrombin+hirudin
releasate
releasate+hirudin
pellet
pellet+hirudin
C
MMP-9
buffer
platelets
platelets+thrombin
platelets+thrombin+hirudin
releasate
releasate+hirudin
pellet
pellet+hirudin
thrombin
thrombin+hirudin
D
fold change in mRNA expression
buffer
platelets
releasate
pellet
Ctgf
Edn1
Jag1
Vegfa
E
number of invaded cells
F
NES: 3.391, p<0.001, FDR<0.001
Enrichment score (ES)
platelets
buffer
G
NES: 3.679, p<0.001, FDR<0.001
pellet
buffer
H
NES: 2.726, p<0.001, FDR<0.001
releasate
buffer
I
NES: 3.705, p<0.001, FDR<0.001
platelets
releasate

Figure S3, related to Figure 4. Platelet-Derived TGFβ1 and Platelet-Bound Factors Cooperate to Promote Metastasis

(A) Phase-contrast micrographs of Ep5 cells treated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) +/- thrombin and hirudin for 24h. The releasate and pellet fractions were generated by treating platelets with thrombin (0.5U/ml) and separated by centrifugation. For some conditions, thrombin was blocked with hirudin (5U/ml) prior dilution in culture medium and co-incubation with the tumor cells. Scale bar=50μm.

(B) Relative fold change in *PAI-1* mRNA expression in Ep5 cells treated as in (A) for 40h (n=3). Values are normalized to *Gapdh* expression. ns ($p>0.05$) was determined by one-way ANOVA followed by Tuckey's post test.

(C) Zymography for MMP-9 in the conditioned medium of Ep5 cells treated as in (B).

(D) Relative fold change in mRNA expression in Ep5 cells treated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) (n=3). Values are normalized to *Gapdh* expression. Bars represent the mean ± SEM, and *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs buffer were determined by one-way ANOVA followed by Tuckey's post test.

(E) Ep5 cells were added at the top of transwells coated with Matrigel and treated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet). The total numbers of cells that invaded to the bottom of the transwell were counted after 48h. Each bar represents the mean ± SEM of n=2. **$p<0.01$ vs buffer were determined by one-way ANOVA followed by Tuckey's post test.

(F-I) Enrichment plots for the platelet-induced gene signature (genes upregulated by more than 2 fold; Table S1) in an independent set of microarray data generated with Ep5 cells treated with

buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) (n=3). Enrichment in platelet-, platelet pellet- or releasate-treated cells versus untreated cells (buffer) are shown in F, G and H. Enrichment in the platelet-treated cells in comparison to the releasate-treated cells is presented in I. Each vertical black line represents a platelet-induced gene. The left-to-right position of each line indicates the relative position of the gene within the rank ordering of the 13,243 genes represented in the dataset from the gene most upregulated upon platelet treatment (position 1 on the left) to the most down-regulated (position 13,243 on the right). The genes near the middle are unaffected by the platelet treatment. The platelet-induced gene signature is clearly enriched in the platelet-treated Ep5 cells (E; $p<0.001$, $FDR<0.001$), as evidenced by the cluster of vertical black lines at the very left of the distribution and the positive enrichment score marked by the green line, validating the platelet-induced gene signature in this data set. Similarly, the gene signature is also highly enriched in the pellet-treated cells (F; $p<0.001$, $FDR<0.001$). Interestingly, while the platelet-induced gene signature is overall also enriched in releasate-treated cells (G; $p<0.001$, $FDR<0.001$), there is a subset of genes which are less affected by this treatment and are redistributed towards the right of the plot, suggesting that treatment with the releasate only induces partial gene expression changes in comparison to treatment with platelets in Ep5 cells. The overall lower magnitude of gene expression changes observed in the releasate-treated cells in comparison with platelet-treated cells is further illustrated by the enrichment of the platelet-induced gene signature in platelet-treated cells directly compared to releasate-treated cells (H; $p<0.001$, $FDR<0.001$). The NES (normalized enrichment score), p-value and FDR (false discovery rate) are indicated at the top of each plot.

Table S4, related to Figure 4. Gene Set Enrichment Analysis (GSEA) for Ep5 Cells Treated with Platelets, Platelet Pellet or Platelet Releasate

Gene sets	Platelets vs Buffer			Pellet vs Buffer			Releasate vs Buffer		
	NES	Nominal p-value	FDR	NES	Nominal p-value	FDR	NES	Nominal p-value	FDR
EMT Signatures									
BLICK_EMT-SIG_UP	1.550	0.011	0.044	1.426	0.033	0.063	-1.501	0.018	0.032
TAUBE_EMT_UP	1.430	0.041	0.034	1.220	0.120	0.122	1.024	0.414	0.411
TAUBE_EMT_DN	-1.937	<0.001	0.002	-1.929	<0.001	<0.001	-1.968	<0.001	<0.001
ONDER_CDH1_TARGETS_2_UP	1.470	0.004	0.001	1.350	0.010	0.010	1.093	0.240	0.275
ONDER_CDH1_TARGETS_2_DN	-1.897	<0.001	0.003	-1.863	<0.001	<0.001	-2.058	<0.001	0.002
TGFβ Signatures									
GIAMPIERI_TGFB_UP	2.194	<0.001	<0.001	1.856	<0.001	<0.001	-1.103	0.229	0.221
GIAMPIERI_TGFB_DN	-2.583	<0.001	<0.001	-2.466	<0.001	<0.001	-1.789	<0.001	<0.001
VALCOURT_TGFB_UP	1.976	<0.001	0.002	1.390	0.046	0.039	-1.498	0.013	0.059
VALCOURT_TGFB_DN	-2.130	<0.001	<0.001	-2.162	<0.001	<0.001	-1.725	0.005	0.037
Cancer Stem Cell Signatures									
CREIGHTON_CSC_UP	1.570	0.004	0.001	1.380	0.028	0.021	1.060	0.336	0.313
CREIGHTON_CSC_DN	-1.480	0.004	0.006	-1.540	<0.001	0.002	-1.240	0.063	0.084
Tumor progression and Metastasis Signatures									
VANTVEER_BREAST_CANCER_POOR_PROGNOSIS	1.762	0.004	0.012	1.721	0.005	0.005	1.247	0.155	0.361
JAEGER_METASTASIS_UP	1.790	0.006	0.010	1.663	0.010	0.006	1.273	0.140	0.086
NF-κB Signatures									
HINATA_NFKB_TARGETS_KERATINOCYTE_UP	1.552	0.012	0.046	1.377	0.034	0.074	-1.499	0.026	0.034
SANA_TNF_SIGNALING_UP	1.672	0.007	0.023	1.840	<0.001	<0.001	-1.290	0.083	0.150

Enrichment of gene sets from the literature. Positive normalized enrichment score (NES) indicates enrichment in either platelet-, pellet- or releasate-treated Ep5 cells; negative NES indicates enrichment in untreated Ep5 cells (buffer). FDR (false discovery rate).
NF-κB signatures are enriched in platelet- or pellet-treated Ep5 cells, but not in releasate-treated Ep5 cells (note the negative NES for releasate-treated cells), suggesting a dependence on platelet-bound factors for activation of this pathway. Similarly, while genes upregulated during EMT, upon TGFβ treatment, in cancer stem cells or during tumor progression and metastasis are significantly enriched in platelet- or pellet-treated cells ($p<0.05$ and/or $FDR<0.25$), this enrichment is not observed in releasate-treated cells ($p>0.05$ and/or $FDR>0.25$). However, genes downregulated upon TGFβ treatment and during EMT are significantly depleted upon all three treatments, suggesting that partial TGFβ and EMT responses are maintained in releasate-treated cells.

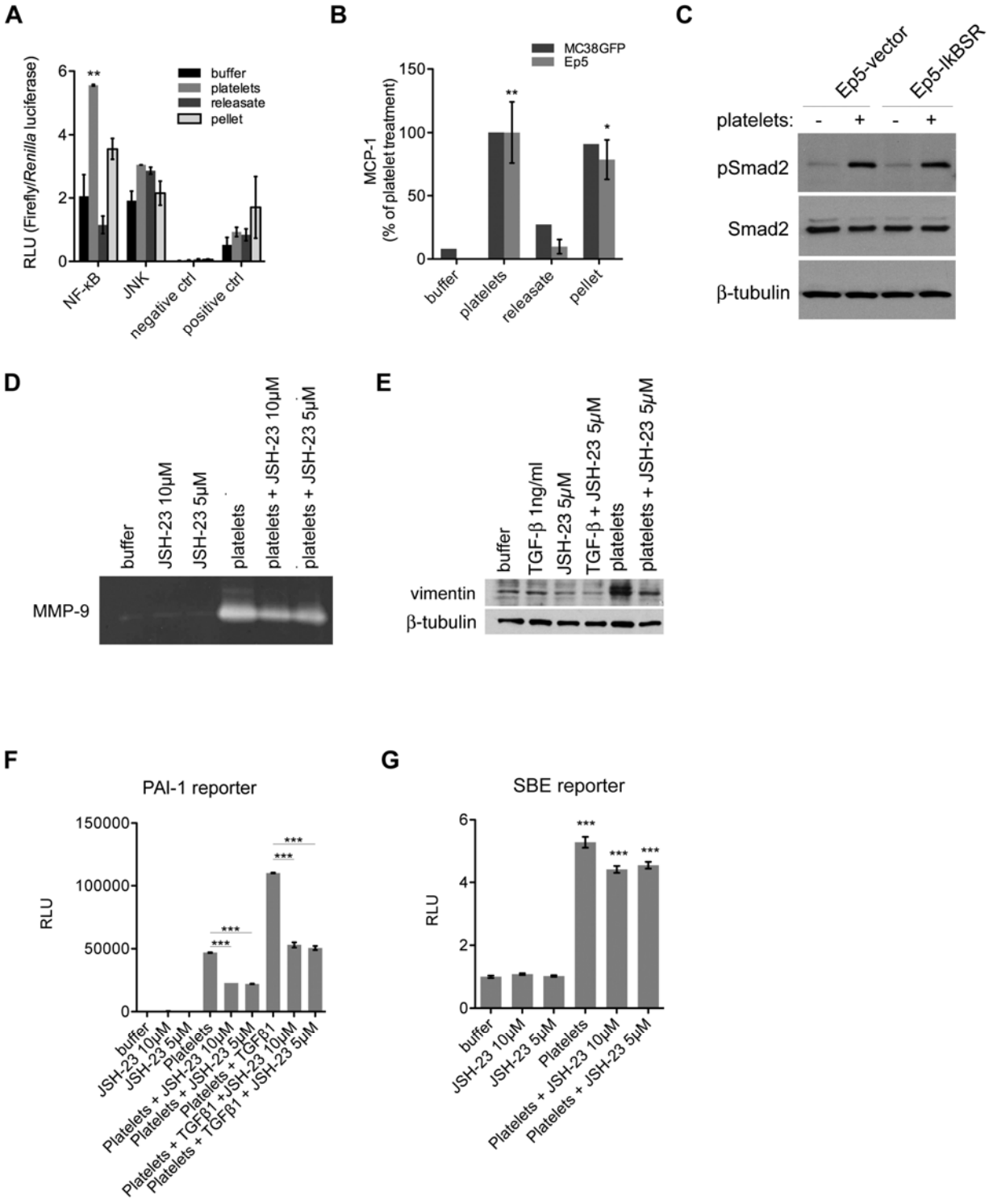
A
RLU (Firefly/Renilla luciferase)
buffer
platelets
releasate
pellet
NF-κB
JNK
negative ctrl
positive ctrl
B
MC38GFP
Ep5
MCP-1
(% of platelet treatment)
buffer
platelets
releasate
pellet
C
Ep5-vector
Ep5-IkBSR
platelets:
pSmad2
Smad2
β-tubulin
D
buffer
JSH-23 10μM
JSH-23 5μM
platelets
platelets + JSH-23 10μM
platelets + JSH-23 5μM
MMP-9
E
buffer
TGF-β 1ng/ml
JSH-23 5μM
TGF-β + JSH-23 5μM
platelets
platelets + JSH-23 5μM
vimentin
β-tubulin
F
PAI-1 reporter
RLU
buffer
JSH-23 10μM
JSH-23 5μM
Platelets
Platelets + JSH-23 10μM
Platelets + JSH-23 5μM
Platelets + TGFβ1
Platelets + TGFβ1 +JSH-23 10μM
Platelets + TGFβ1 + JSH-23 5μM
G
SBE reporter
RLU
buffer
JSH-23 10μM
JSH-23 5μM
Platelets
Platelets + JSH-23 10μM
Platelets + JSH-23 5μM

Figure S4, related to 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

(A) Ep5 cells were transfected with firefly luciferase reporters of NF-κB or JNK activity and constitutively active control *Renilla* luciferase reporters. 24h after transfection, the cells were treated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) for 20h, and the relative luciferase activity (RLU) was measured (n=3).

(B) MCP-1 concentration in the conditioned medium from MC38GFP or Ep5 cells incubated with buffer, platelets, releasate from activated platelets (releasate), or the pellet fraction from activated platelets (pellet) for 40h (n=3).

(C) Detection of phospho-Smad2 and total Smad2 protein levels by immunoblotting in Ep5 cells stably expressing an IκB super-repressor (Ep5-IkBSR) or a control vector (Ep5-vector) and treated with buffer or platelets for 40h. β-tubulin is used as loading control.

(D) Zymography for MMP-9 in the conditioned medium from Ep5 cells treated with buffer, platelets, JSH-23, or platelets + JSH-23 for 40h.

(E) Detection of vimentin protein levels by immunoblotting in Ep5 cells treated with buffer, platelets, JSH-23 5 μM or platelets + JSH-23 5 μM. β-tubulin is used as loading control.

(F) Relative luciferase activity in MLEC cells stably expressing a luciferase reporter under the control of the *PAI-1* promoter construct and treated with buffer, platelets, JSH-23, platelets + JSH-23, or platelets + TGFβ1 (1 ng/ml) + JSH-23 for 20h (n=2).

(G) Relative luciferase activity in Ep5 cells stably expressing a luciferase reporter under the control of the SBE promoter and treated with buffer, platelets, JSH-23 or platelets + JSH-23 for 20h (n=2).

For panels A, B, F and G, bars represent the mean ± SEM, and *p<0.05, **p<0.01, ***p<0.001 vs buffer were determined by one-way ANOVA followed by Tuckey's post test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Tissue Culture

Ep5 (EpRas) cells (obtained from R. Weinberg), MC38GFP cells (obtained from A. Varki), and MLEC cells stably expressing a *PAI-1* promoter-luciferase reporter construct (Abe et al., 1994) were cultivated in DMEM 10% FCS, 1% Penicillin/Streptomycin and 2 mM L-Glutamine. HMLER cells (H-Ras transformed human mammary epithelial cell line; obtained from R. Weinberg) were cultivated as described previously (Elenbaas et al., 2001). MCF10A cells (Soule et al., 1990) were maintained in DMEM/F12 (50:50) supplemented with 5% horse serum, 10mM HEPES, 10µg/ml insulin, 20ng/ml epidermal growth factor, 0.5µg/ml hydrocortisone, 100ng/ml cholera toxin, 1% Penicillin/Streptomycin.

Generation of Cell Lines Stably Expressing ZsGreen and IkBSR Reporters

Retroviral vectors coding for ZsGreen or IκBα super-repressor and GFP (IkBSR) were transduced in Ep5 cells. Packaging of the vectors was obtained by cotransfection of 293FT cells (ATCC) with 1ng transfer vector, 1µg MLV gag-pol, and 1µg VSVg expression vectors using Fugene6 (Roche) as described previously (Stern et al., 2008). 48h after transfection, 293FT cell-conditioned medium was collected, filtered through a 0.45µm filter, and applied to Ep5 cells with 4µg/ml polybrene (Sigma). Ep5 cells were then selected on the basis of GFP or ZsGreen expression by FACS sorting.

Genotyping

DNA from mouse tail biopsies was amplified by PCR using the primers listed in the table below.

Genotyping Primers

For detection of the *Tgfb1* flox and WT alleles	forw rev	5'CCCAGGCTAGCCTTGAACTTCT3' 5'AGGGGTGGAGATGTAGTTTGG3'	(Li et al., 2007)
For simultaneous detection of the *Tgfb1* null (*egfp* knockin) and WT alleles	forw rev1 rev2	5'CGCATCCCACCTTTGCCGAG3' 5'GGCGTCAGCACTAGAAGCCA3' 5'GCCGTAGGTCAG GGTGGTCA3'	(Li et al., 2007)
For detection of the *Pf4-cre* transgene	forw rev	5'CCCATACAGCACACCTTTTG3' 5'TGCACAGTCAGCAGGTT3'	(Tiedt et al., 2007)

***In Vivo* Metastasis Assays**

For lung metastasis assays, cells treated with platelets for 40h were washed in PBS, and either trypsinized (Ep5) or lifted with 2mM EDTA in PBS (MC38GFP). Cells were then rinsed 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). 100µl of cell suspension were then injected via the tail vein of syngeneic mice. After 14 days, animals were sacrificed and the numbers of metastatic foci at the surface of lungs were counted under a fluorescence stereomicroscope (Nikon SMZ1500). For 3h and 48h time points, pictures were taken (magnification 3x) and the number of cells/picture was automatically counted with Cell Profiler (Lamprecht et al., 2007). For metastasis experiments with the *Pf4-cre*$^{+}$*; TGFβ1*$^{fl/fl}$ mice, untreated MC38GFP cells cultivated in DMEM 10%FCS were lifted and washed as described above and 100µl of a 1x10E7/ml cell suspension were injected via the tail vein.

RT-qPCR Analysis

RNA was isolated from total cell lysates using RNeasy Mini kit (Qiagen), and reverse-transcribed with TaqMan Reverse Transcription Reagents and random hexameric primers (Applied Biosystems). Human specific PCR primers were designed using Primer3 and BLAST. RT-qPCR was performed with the iQ SYBR Green Supermix (Biorad). Primers used are listed in

the tables below. Data were normalized to *GAPDH* expression. Relative mRNA levels were calculated using the comparative C_T method.

RT-qPCR Primers used with mouse cells

Gapdh forw	5'CAGTATGACTCCACTCACGGC3'
Gapdh rev	5'GAGGGGCCATCCACAGTCTTC3'
Snail forw	5'GGAAGCCCAACTATAGCGAGC3'
Snail rev	5'CAGTTGAAGATCTTCCGCGAC3'
Fibronectin forw	5'CGTAAATTGCCCCATTGAGTG3'
Fibronectin rev	5'GAGGGTCTGCTAACATCACTG3'
Serpine1 (PAI-1) forw	5'CCCGCCTCCTCATCCTGCCT3'
Serpine1 (PAI-1) rev	5'GCCACTGTGCCGCTCTCGTT3'
Claudin-1 forw	5'GCGTTTCGCAAAGCACCGGG3'
Claudin-1 rev	5'GGCTCGGGTTGCCTGCAAAGT3'
Vimentin forw	5'AATGCTTCTCTGGCACGTCT3'
Vimentin rev	5'GCTCCTGGATCTCTTCATCG3'
Slug forw	5'CATCCTTGGGGCGTGTAAGTC3'
Slug Rev	5'GCCCAGAGAACGTAGAATAGGTC3'
Twist forw	5'GGACAAGCTGAGCAAGATTCA3'
Twist rev	5'CGGAGAAGGCGTAGCTGAG3'
Zeb1 forw	5'CGCCATGAGAAGAACGAGGAC3'
Zeb1 rev	5'TGTATGCAAAGGTGTAACTGCAC3'
Zeb2 forw	5'CAGGCTCGGAGACAGATGAAG3'
Zeb2 rev	5'CTTGCAGAATCTCGCCACTG3'
Ctgf forw	5'CTCCACCCGAGTTACCAATG3'
Ctgf rev	5'TGGCGATTTTAGGTGTCC3'
Edn1 forw	5'TTTCCCGTGATCTTCTCTCTGC3'
Edn1 rev	5'CTGAGTTCGGCTCCCAAGAC3'
Jag1 forw	5'TTCAGTTTCGCCTGGCCGAG3'
Jag1 rev	5'TCAGTGTCTGCCATTGCCGG3'
Vegfa forw	5'CTTGTTCAGAGCGGAGAAAGC3'
Vegfa rev	5'ACATCTGCAAGTACGTTCGTT3'

RT-qPCR Primers used with human cells

GAPDH forw	5'GGTCTCCTCTGACTTCAACA3'
GAPDH rev	5'GTGAGGGTCTCTCTCTTCCT3'
Snail forw	5'TCGGAAGCCTAACTACAGCGA3'
Snail rev	5'AGATGAGCATTGGCAGCGAG3'
Fibronectin forw	5'CCATCGCAAACCGCTGCCAT3'
Fibronectin rev	5'AACACTTCTCAGCTATGGGCTT3'
Serpine1 forw	5'ACCGCAACGTGGTTTTCTCA3'

Serpine1 rev	5’TTGAATCCCATAGCTGCTTGAAT3’
N-cadherin forw	5’ATCCTACTGGACGGTTCG3’
N-cadherin rev	5’TTGGCTAATGGCACTTGA3’
Vimentin forw	5’GAACGCCAGATGCGTGAAATG3’
Vimentin rev	5’CCAGAGGGAGTGAATCCAGATTA3’
Slug forw	5’AAGCATTTCAACGCCTCCAAA3’
Slug Rev	5’GGATCTCTGGTTGTGGTATGACA3’
Twist forw	5’CCGGAGACCTAGATGTCATTG3’
Twist rev	5’CCACGCCCTGTTTCTTTG3’
Zeb1 forw	5’GATGATGAATGCGAGTCAGATGC3’
Zeb1 rev	5’ACAGCAGTGTCTTGTTGTTGT3’
Zeb2 forw	5’GGAGACGAGTCCAGCTAGTGT3’
Zeb2 rev	5’CCACTCCACCCTCCCTTATTTC3’

Zymography

Conditioned media from cells treated for 48h were collected and centrifuged to remove cellular debris and platelets. Volumes of conditioned media normalized to the number of cells were then mixed with 2X Laemmli sample buffer (BioRad) and loaded onto a 7.5% acrylamide/bisacrylamide separating gel containing 0.2% (w/v) gelatin. After electrophoresis, the gel was incubated in 2.5% Triton X-100, rinsed in distilled water and incubated at 37°C in buffer containing 50mM Tris pH 7.6, 20mM NaCl, 5mM $CaCl_2$. Finally, the gel was stained in 0.1% Coomassie blue R-250, 30% methanol, 10% acetic acid, and destained in the same solution without the Coomassie blue dye.

Immunoblotting

Immunoblot analysis was performed as described previously (Labelle et al., 2008). The primary antibodies were anti-β-tubulin (Sigma), anti E-cadherin (BD Biosciences), anti-Smad2/3, anti-phosphoSmad2 (Cell Signaling Technology) and anti-vimentin (Sigma). The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson

Immunoresearch).

Invasion Assay

Invasion assays were performed in 24-well BD Biocoat™ Matrigel™ Invasion Chambers (8 μm pore size; BD Biosciences). 50,000 cells were plated in transwell inserts and either left untreated, treated with SB431542 (10 μM), anti-TGFβ1 blocking antibody (6μg/ml) or the different platelet fractions. Both upper and lower chambers contained DMEM. After 48h, cells remaining in the upper part of the transwell were removed with a cotton swab. Migrated cells were then stained with Crystal Violet 0.5% and the total number of cells was counted with a Zeiss Axiovert 200 microscope.

Immunofluorescence Staining

For visualization of blood vessels and quantification of extravasation, lungs were fixed by tracheal perfusion with PBS, 4% formaldehyde, 0.3% Triton X-100 for 15 min and removed en bloc. Lung lobes were then cut in 4 pieces, washed in PBS, 0.3% triton X-100 and blocked in PBS 10% normal goat serum followed by the primary antibody (anti-PECAM-1, BD Biosciences). After washing, samples were incubated with Alexa 594-conjugated goat anti-rat IgG (Molecular probes) and DAPI. Images of lobe pieces from 3 or more mice per group were taken at 60X with Z-sections every 1μm on an Olympus FV10i inverted confocal microscope. 3D rendering and XYZ views were generated with Volocity (Perkin Elmer). XYZ views were then examined and cells clearly within blood vessels (directly surrounded by PECAM-1 staining; red) were scored as intravascular, while cells outside blood vessels were scored as extravascular.

For platelet immunostaining in lungs, 20 μm-thick sections were fixed in acetone and stained

with anti-GP1bβ (Emfret). The secondary antibody was Alexa 594-conjugated goat anti-rat IgG (Molecular Probes). Images were taken at 60X with Z-sections every 1μm on an Olympus FV10i inverted confocal microscope.

For platelet immunostaining in cell suspensions, cells were prepared as for *in vivo* metastasis assays. The suspensions were then stained with anti-CD41 (BD Biosciences), washed once with PBS and incubated with Alexa 594-conjugated goat anti-rat IgG (Molecular probes). After a final wash in PBS, cells were resuspended in PBS and 5μl of cell suspensions were loaded on a microscope slide.

For immunostaining of tumor cells in tissue culture, cells were rinsed with PBS, fixed with 4% formaldehyde and stained with anti-E-cadherin or anti-N-cadherin (BD Biosciences). The secondary antibody was Alexa 594-conjugated goat anti-rat IgG (Molecular probes). Images were taken with a Zeiss LSM510 microscope.

Tail Bleeding Assay

For tail bleeding assays, mice were anesthetized with 2.5% isoflurane in oxygen. The tail was cut at 5mm and bled onto a Whatman filter paper. The filter paper was dabbed to the wound every 30 seconds without disrupting the forming clot. The experiment was continued until bleeding stopped completely.

Microarray Analysis

For data presented in Fig. S3 and Table S4, total RNA was isolated from Ep5 cells treated with buffer, platelets, platelet pellet or platelet releastate (n=3). Samples where then processed with

the Nugen Applause® WT-Amp Plus ST System and hybridized on Affymetrix Mouse Gene 1.0 ST arrays, according to manufacturer's instructions (Affymetrix).

Data are deposited in Gene Expression Omnibus (GEO) under accession number GSE27456.

Gene Set Enrichment Analysis (GSEA)

GSEA was performed using GSEA v2.07 (www.broadinstitute.org/gsea; Mootha et al., 2003; Subramanian et al., 2005). The signal-to-noise metric and permutation of gene sets were used to rank the genes and calculate nominal p-values and FDR. Probe sets were collapsed to unique gene symbols and used to interrogate the gene sets from the literature listed in the table below, some of which were provided by the Molecular Signatures Database (MSigDB; www.broadinstitute.org/gsea/msigdb).

Gene set	Source
BLICK_EMT-SIG	(Blick et al.)
TAUBE_EMT	(Taube et al.)
ONDER_CDH1_TARGETS_2	MSigDB, (Onder et al., 2008)
GIAMPIERI_TGFB	(Giampieri et al., 2009)
VALCOURT_TGFB	(Valcourt et al., 2005)
HINATA_NFKB_TARGETS_KERATINOCYTE	MSigDB, (Hinata et al., 2003)
SANA_TNF_SIGNALING	MSigDB, (Sana et al., 2005)
CREIGHTON_CSC	(Creighton et al., 2009)
VANTVEER_BREAST_CANCER_POOR_PROGNOSIS	MSigDB, (van 't Veer et al., 2002)
JAEGER_METASTASIS	MSigDB, (Jaeger et al., 2007)

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