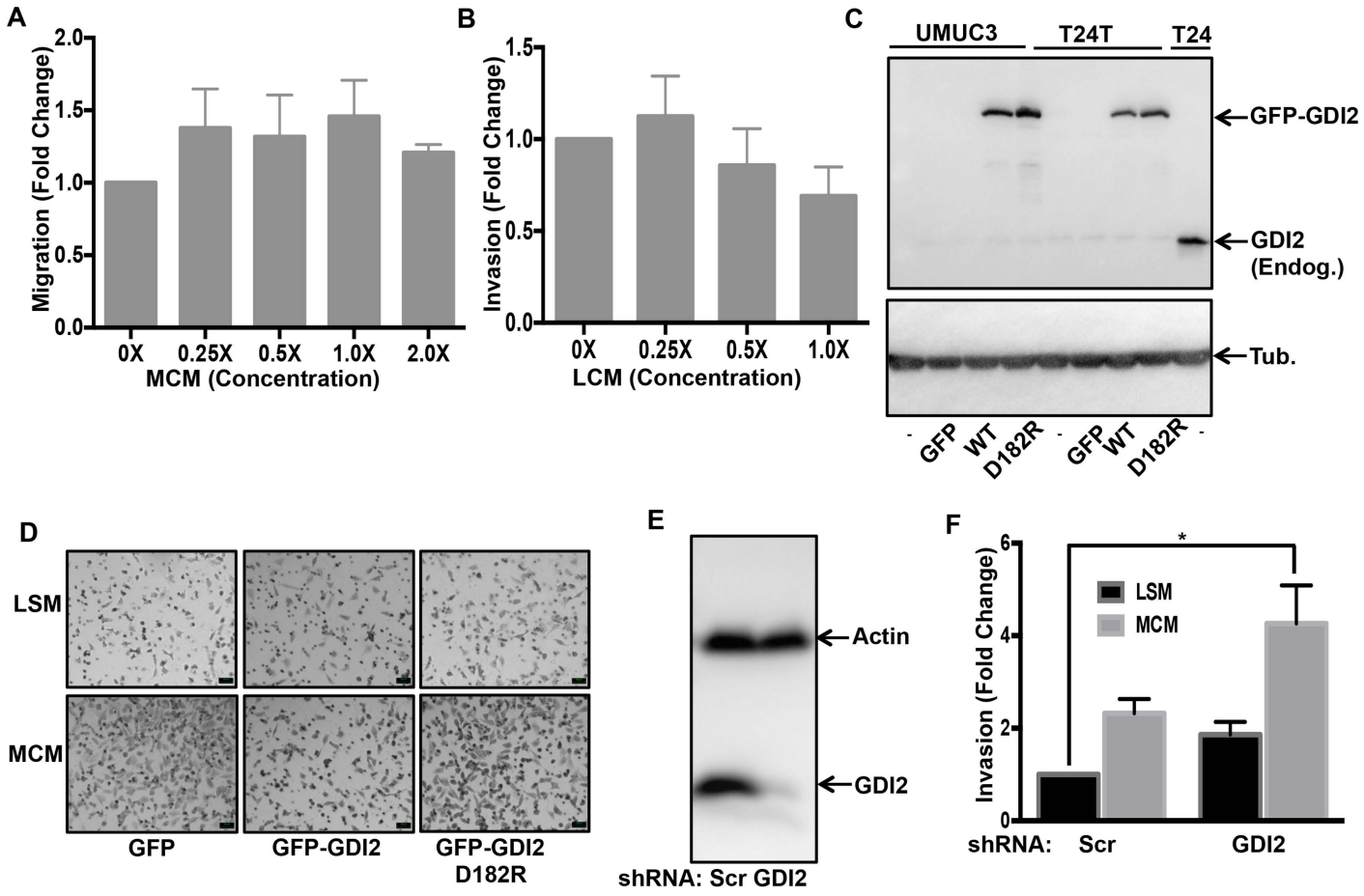

Figure S1, related to Figure 1

RhoGD12 suppresses macrophage-conditioned medium induced invasion.
(**A**) Quantification of transwell migration assay in response to macrophage-condtioned medium. Fold changes relative to control, n=3. (**B**) Quantification of the matrigel invasion assay in response to L cell conditioned medium, n=3.(**C**) Western blot of GFP, RhoGDI2 and D182R mutant stable UMUC3, T24T and T24 cells. Tubulin was used as loading control. (**D**) Representative images from the matrigel invasion assays as described in Figure 1E (scale bar, 100 μM). (**E**) RT4 cells expressing scrambled or RhoGDI2 shRNA. Actin was used as loading control. (**F**) RT4 cells stably expressing scrambled or RhoGDI2 shRNA were analyzed for invasion with or without macrophage-conditioned medium, Values are mean ± SEM, n=4, *$p < 0.05$.

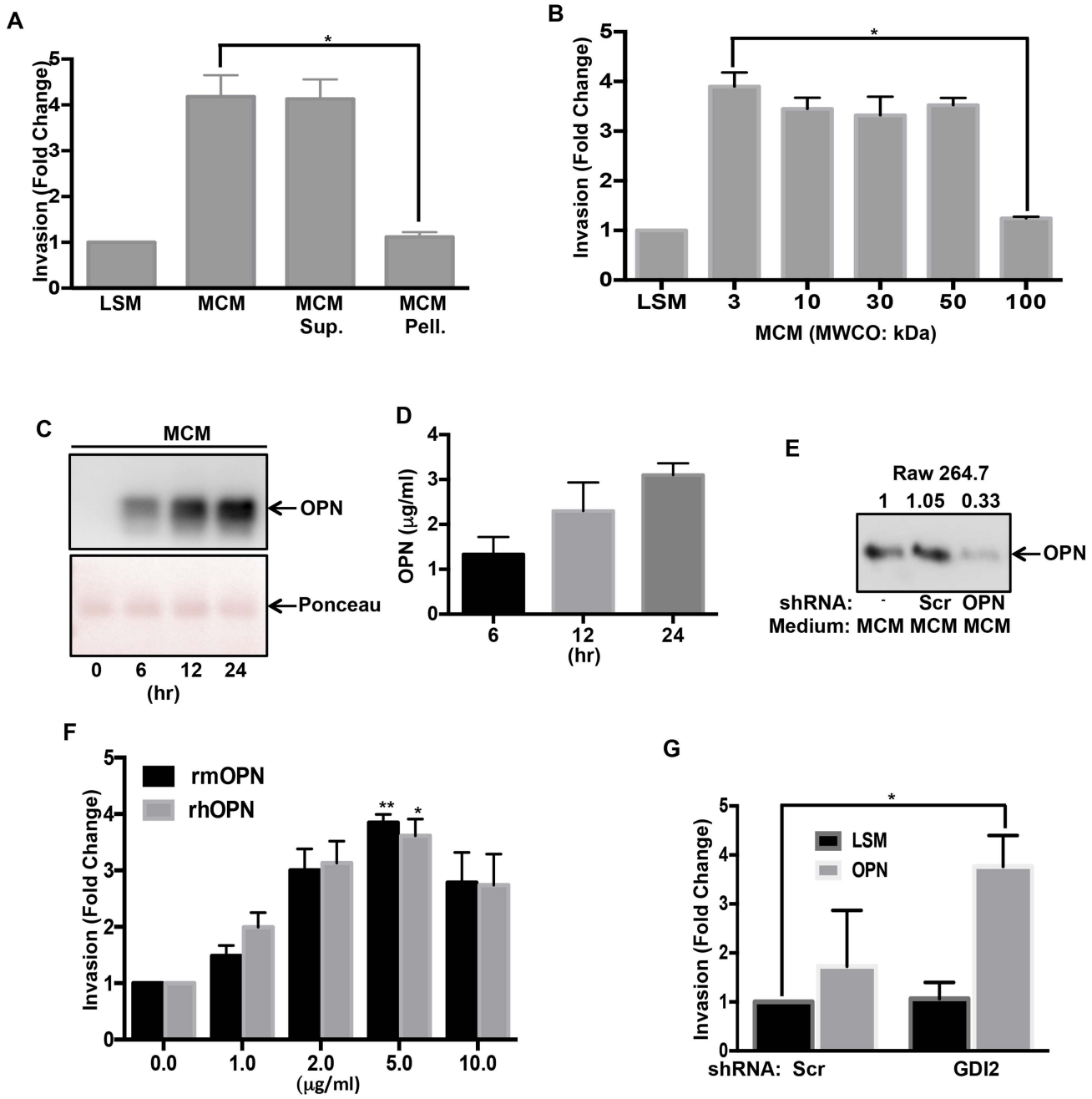

Figure S2, related to Figure 2

Identification of secreted active factor in macrophage-conditioned medium

(**A**) UMUC3 cells were analyzed for matrigel invasion in the presence of whole macrophage-conditioned medium, or macrophage-conditioned medium centrifuged at high speed to separate the supernatant and particulate fractions as described in experimental procedure. After 18 hr, invaded cells were counted and fold change relative to control determined, n=3, *p < 0.05. (**B**) Quantification of matrigel invasion assay using macrophage-conditioned medium retained after filtration through Amicon filters with the indicated molecular weight cut offs. Values are mean ± SEM, n=3, *p < 0.05. (**C**) Western blot for OPN in macrophage-conditioned medium. Ponceau stain of the filter as loading control. (**D**) ELISA for OPN in medium conditioned by macrophages for the indicated times, n=3. (**E**) Western blot for OPN in medium conditioned by Raw 264.7 macrophages stably transduced with scrambled or OPN shRNA. Fold change of OPN expression shown at the top of blot. (**F**) UMUC3 cells were assayed for invasion in the presence of the indicated concentration of recombinant mouse or human OPN for 18 hr. (**G**) RT4 cells stably expressing scrambled or RhoGDI2 shRNA were analyzed for invasion with or without 5 μg/ml rmOPN, n=3, *p < 0.05.

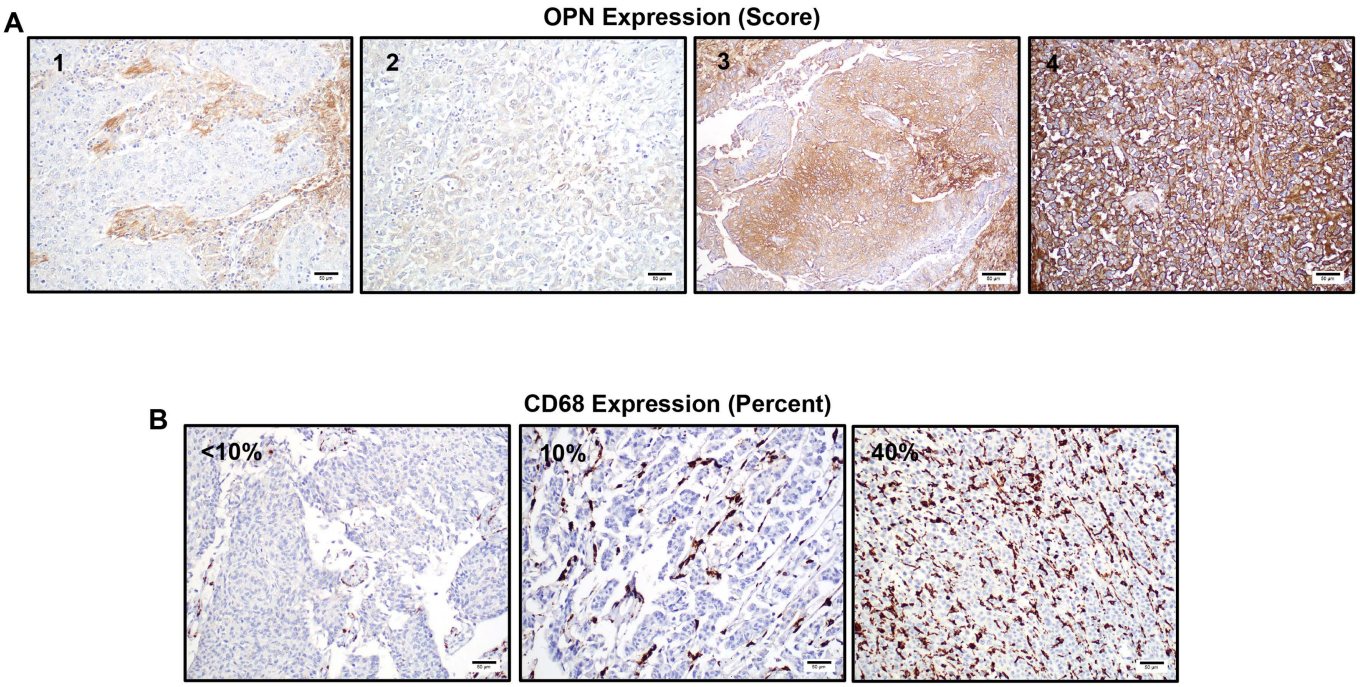

Figure S3, related to Figure 3

OPN and CD68 expression in human bladder cancer specimens

(**A&B**) Representative images of OPN and CD68 expression from the IHC staining of human bladder cancer specimens as described in experimental procedures. Osteopontin immunostaining was scored, 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (intense staining). CD68 positive cells were scored as a percentage, in steps of 10% (scale bar, 50 μM).

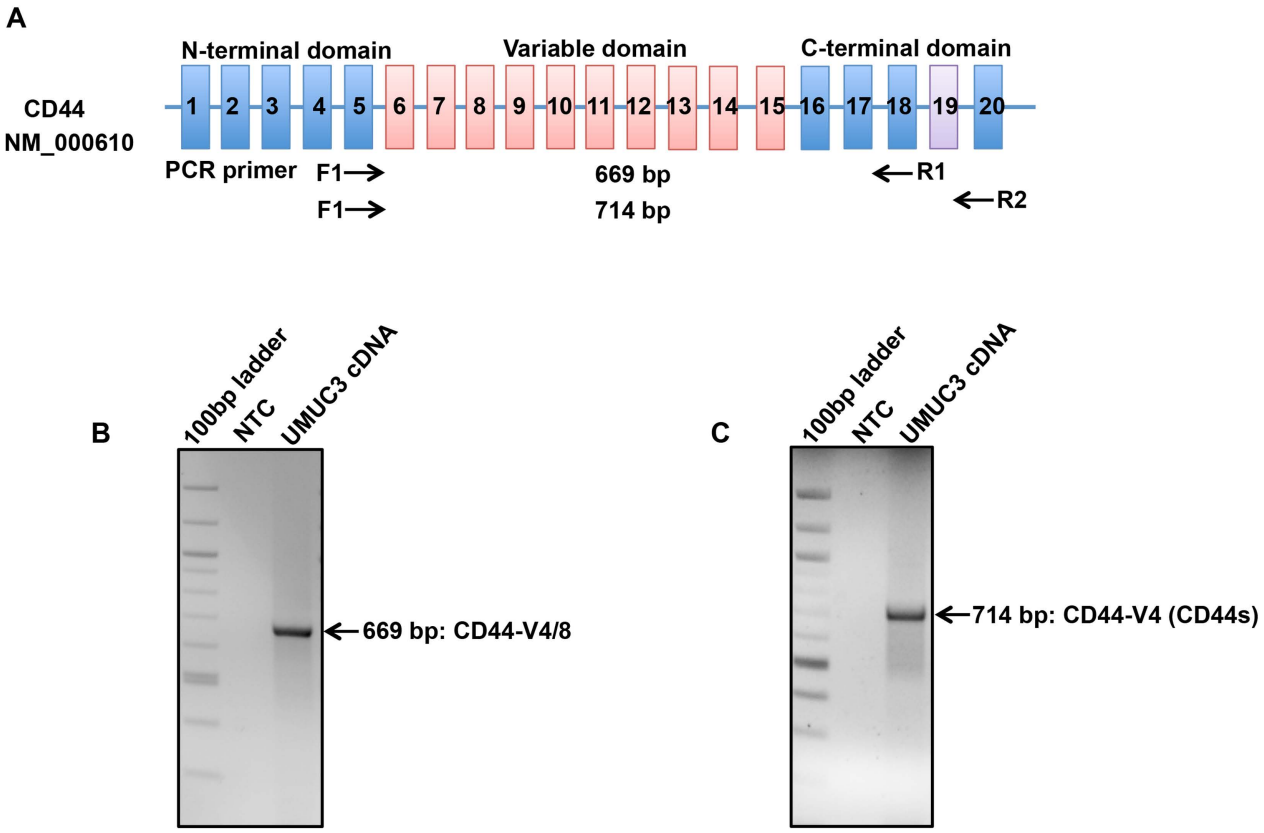

Figure S4, related to Figure 4

Standard isoform of CD44 (CD44s) is expressed in UMUC3 cells

(**A**) Schematic diagram of CD44 transcript and PCR design. (**B**) DNA gel of CD44 PCR using no template control (NTC) or UMUC3 cDNA; 669 bp product corresponds to CD44-V4 or V8. (**C**) DNA gel of PCR of UMUC3 cDNA using primers for the CD44s isoform. 714 bp corresponds to CD44s (isoform V4).

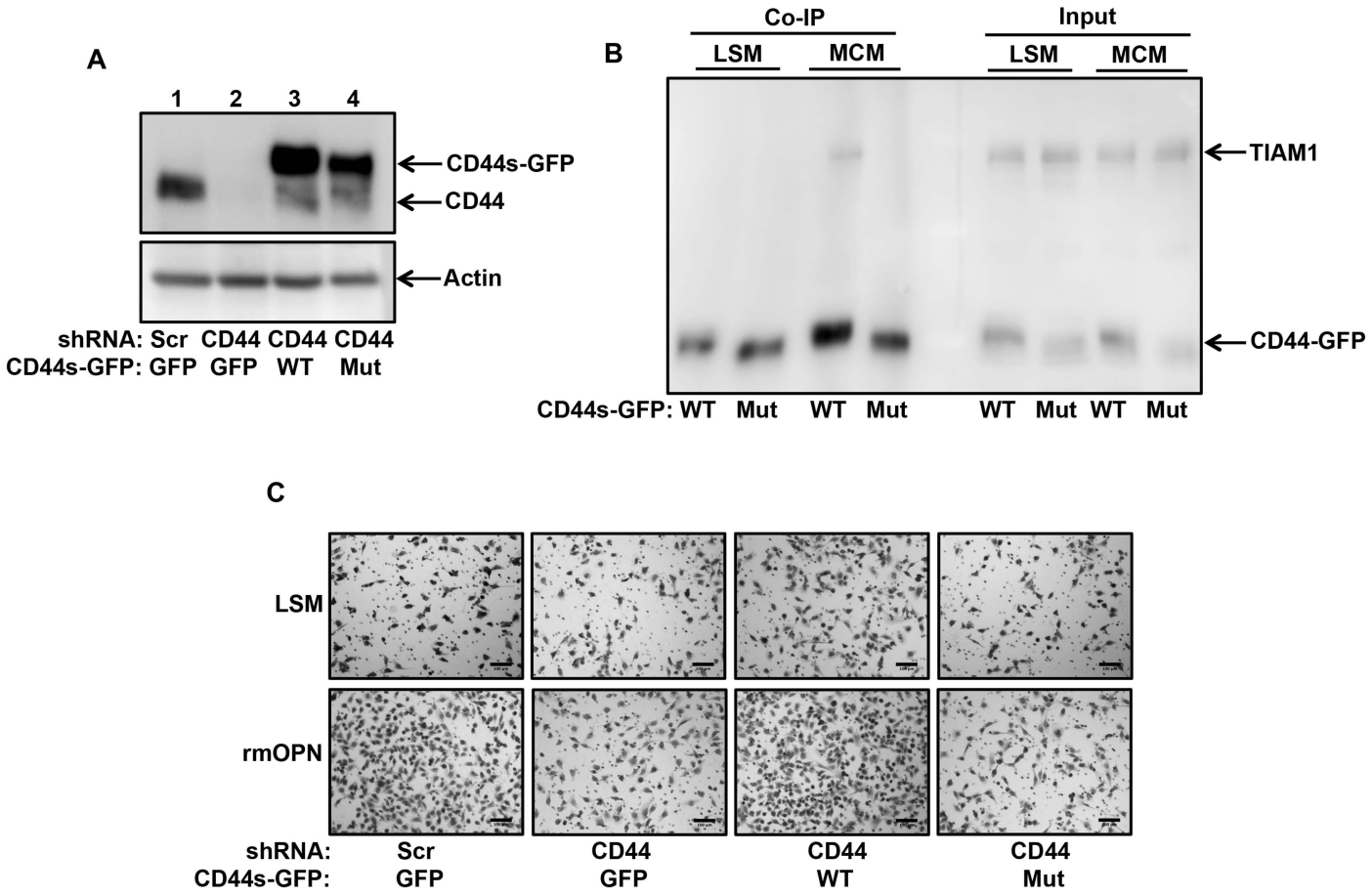

Figure S5, related to Figure 5

CD44-TIAM1 interaction regulates macrophage-conditioned medium / OPN induced invasion of UMUC3 cells

(**A**) Western blot for CD44 in lysates from UMUC3 cells stably expressing the indicated constructs, with actin as loading control. (**B**) UMUC3 cells expressing the indicated constructs were treated with macrophage-condtioned medium for 5 min, GFP immunoprecipitated from cell lysates and immunoblotted for CD44 and TIAM1 (**C**) Representative images from the experiments in Figure 5E (scale bar, 100 μM).

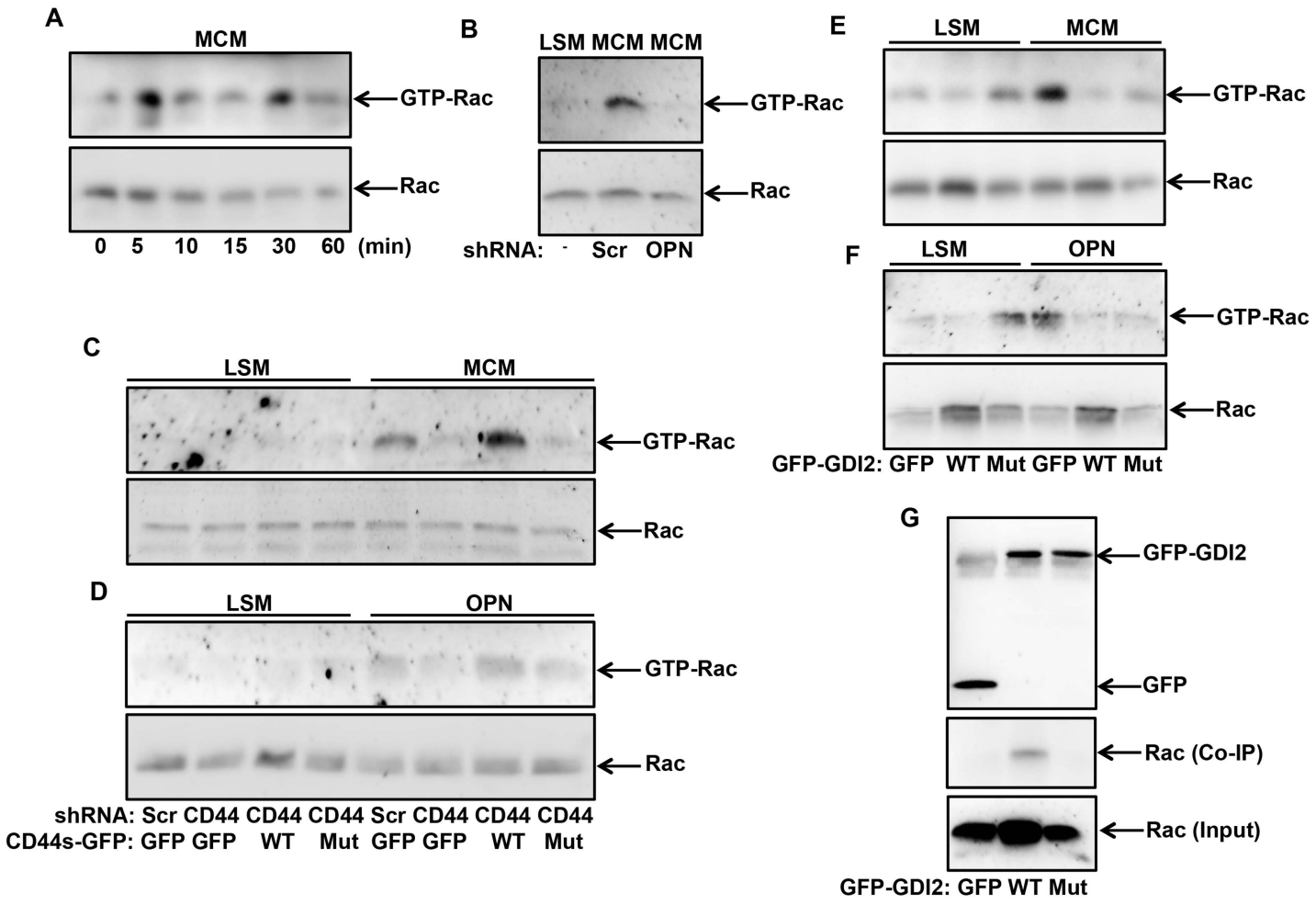

Figure S6, related to Figure 5

CD44 / TIAM1 regulates macrophage-conditioned medium / OPN induced Rac1 activation
(**A**) UMUC3 cells were treated for the indicated time with macrophage-conditioned medium and lysates analyzed for Rac1 activation as described in experimental procedures (upper panel). Immunoblotted for total Rac1 using 2% of the total cell lysates (lower panel). (**B**) UMUC3 cells were treated for 5 min with macrophage-conditioned medium from control or OPN knock down Raw 264.7 cells and analyzed for Rac1 activation as in A. (**C, D, E & F**) UMUC3 cells expressing the indicated constructs were treated with either macrophage-conditioned medium or 5 µg/ml rmOPN and analyzed for Rac1 activation as described in A. (**G**) Lysates from UMUC3 cells expressing indicated constructs were immunoprecipitated for GFP and immunoblotted for Rac1 and GFP.

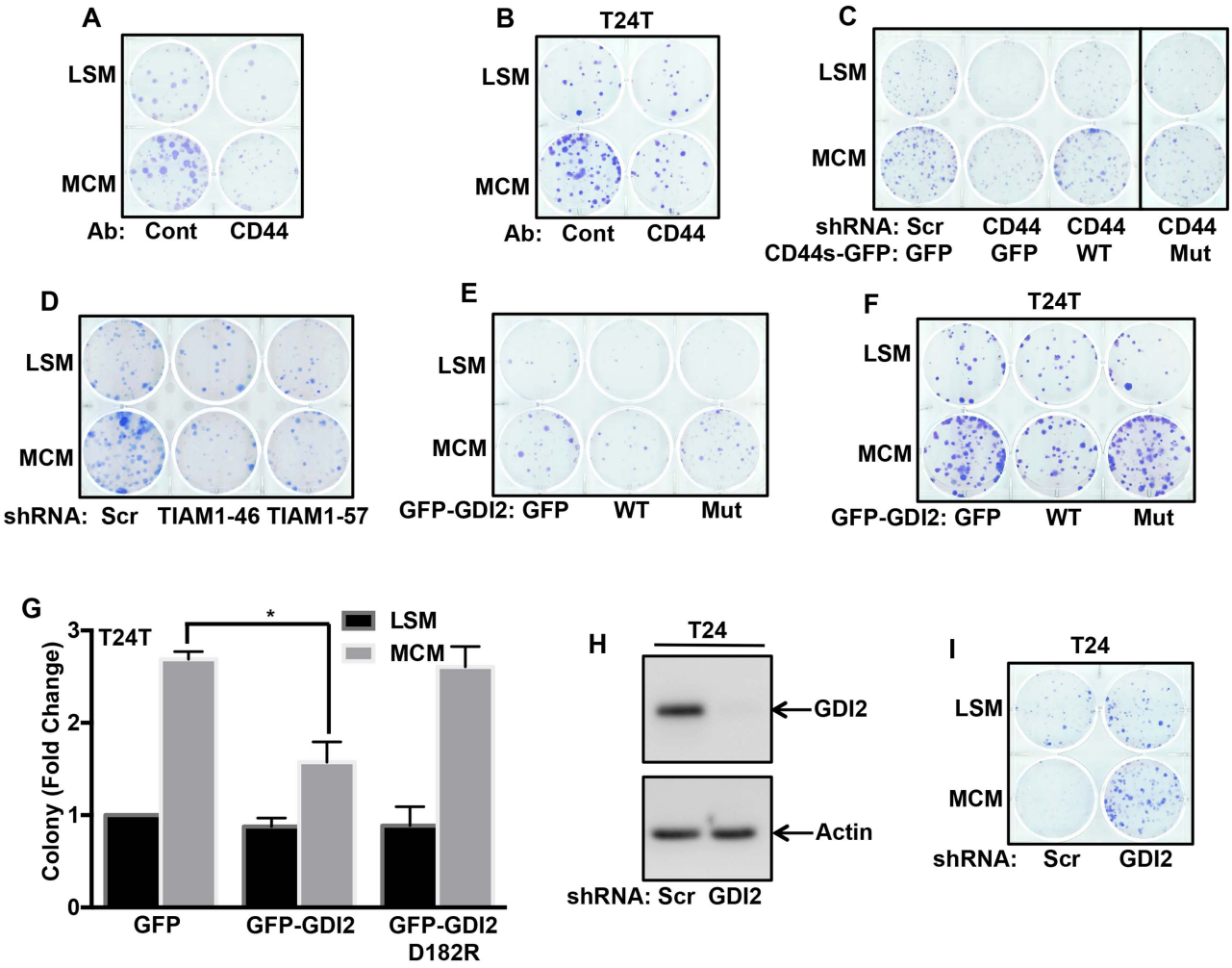

Figure S7, related to figure 7

RhoGD12 / CD44 regulates OPN-induced clonal growth

Representative image of the clonal growth assay quantified (**A**) in Figure 7A, (**B**) in Figure 7B, (**C**) in Figure 7C, (**D**) Figure 7D, (**E**) in Figure 7E. (**F**) Clonal growth assay for T24T cells stably expressing GFP, GFP-GDI2 or GFP-GDI2D182R (**G**) Quantification of F, values are mean ± SEM, n=4, *$p < 0.05$. (**H**) Western blot for RhoGDI2 in scrambled and GDI2 shRNA stable lines, with actin as loading control. (**I**) Representative Image of clonal growth assay quantified in Figure 7F.

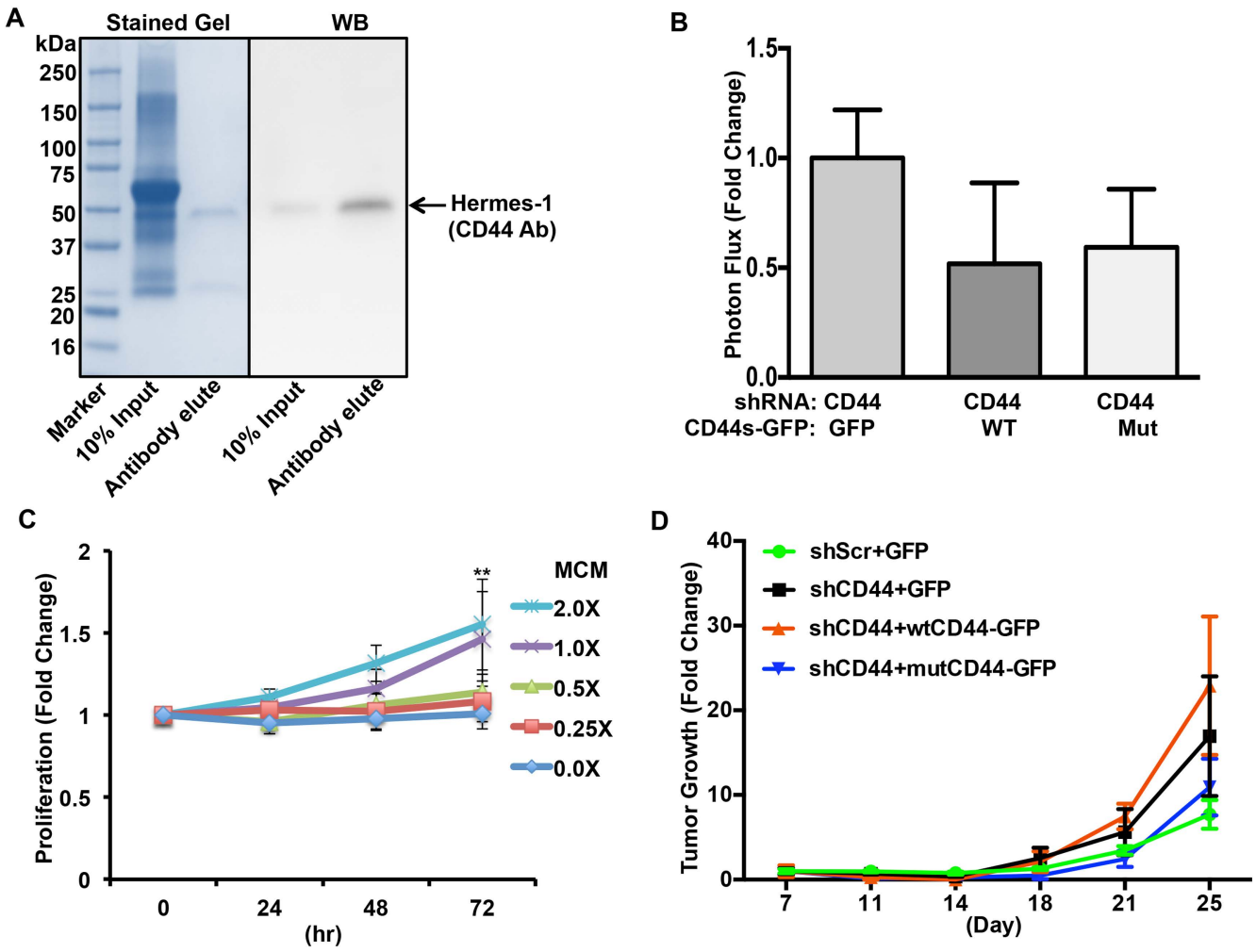

Figure S8, related to Figure 8

Weak effect of macrophage-conditioned medium on UMUC3 proliferation in denser culture
(**A**) Supernatant from hermes-1 (CD44) hybridoma and the antibody elute as described in experimental procedures, resolved with SDS-PAGE, stained with gel code blue stain (Thermo Scientific) or western blotted for IgG. (**B**) Total normalized photon flux at the end of orthotopic experiment as in Figure 8C. Fold change relative to control. CD44 shRNA + GFP (n=7); CD44 shRNA + WT CD44s-GFP (n= 8); CD44 shRNA + Mut CD44s-GFP (n=8) (**C**) $2x10^3$ UMUC3 cells were seeded per well in 96 well plates. After 24 hr of seeding, cells were grown with the indicated amounts of macrophage-conditioned medium for 72 hr. Proliferation was measured as described in experimental procedures, n=3, **p < 0.01. (**D**) The indicated UMUC3 cell lines were injected subcutaneously in nude mice and tumor growth was measured over 25 days as described in experimental procedures. Values (change relative to control at t=7) are mean ± SEM; scrambled shRNA + GFP, n=5; CD44 shRNA + GFP, n=4; CD44 shRNA + WT CD44s-GFP, n=4; CD44 shRNA + Mut CD44s-GFP, n=4.

Supplemental Experimental Procedures

Cell culture and conditioned medium preparation

Human bladder cancer cells UMUC3 and mouse fibroblast L cells (MEM, 1 mmol/L sodium pyruvate), T24T, T24, RT4 (DMEM/F12), and mouse macrophage line, Raw 264.7 cells (RPM1640) were grown in medium with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen) in a humidified atmosphere with 5% CO_2 at 37 ^{o}C. For macrophage conditioned medium (MCM) preparation, 5×10^6 Raw 264.7 cells were seeded in 10 cm dish in complete growth medium. After 24 hr, medium was changed to 0.2% FBS. 24 hr later, conditioned medium was collected and filtered through 0.45-μm pore filters. In some cases, conditioned media was concentrated 20X with 3 kDa cut off Amicon Ultra-15 centrifugal filter units (Millipore), stored at -80 ^{0}C in small aliquots and used for 2-3 months. For MWCO experiment, macrophage conditioned medium was concentrated with 3, 10, 30, 50 and 100 kDa Amicon filters. For some experiments, macrophage conditioned medium was cleared at 2×10^3xg for 10 min and then ultra-centrifuged at $100\times10^3\times$g for 1 hr, the supernatants was removed and the pellet (exosome) resuspended in medium for testing on cells. For fibroblast conditioned medium preparation, 5×10^6 L cells were seeded in 10 cm dish in complete growth medium. After 24 hr, medium was changed to 0.2% FBS. 24 hr later, conditioned medium was collected and filtered through 0.45-μm pore filters, and used for the experiment (Luga et al., 2012).

Plasmid construction

WT *RhoGDI2* and mutant *D182R* were sub cloned from pEGFP-C1 (Moissoglu et al., 2009) into pBOB-GFP lentiviral vector. For knockdowns, shRNA sequence corresponding to, human *CD44* (5' AATATAACCTGCCGCTTTGCA 3') in the constant region, mouse *OPN* (5' GTTTCACAGCCACAAGGACAA 3'), *RhoGDI2* (5' TGATGAGAGTCTAATTAAG 3') and *scramble* (5' GATTGACGCAACCGAGAACA 3') were cloned into the plko.1-TRC vector. *TIAM1* shRNA (5' TTCGAAGGCTGTACGTGAATA 3': TRCN0000256946; 5' GCTTGAGAAGGTTGATCAATT 3': TRCN0000267657) was purchased from Sigma. The human *CD44* standard isoform (*CD44s*) was amplified from UMUC3 cDNA and cloned into pBOB-GFP lentiviral vector. To make *CD44s* rescue vector, it was further mutated to make it resistant to *CD44* shRNA by inserting three silent mutations in *CD44* shRNA target sequence (AA***C***AT***T***AC***T***TGCCGCTTTGCA). The TIAM1 binding mutant CD44s-E335K/E338K-GFP was generated by site directed mutagenesis from pBOB-CD44s-GFP rescue vector. For labeling CD44 cell lines, luciferase gene (Luc2) was sub cloned from pGL4.51 into Sal1 and BstB1 site in pLenti6 lentiviral vector.

Lentivirus particle generation and stable expression.

For lentivirus production, 70% percent confluent HEK 293T cells were transfected with lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Briefly, lentiviral expression vectors were cotransfected with the 3rd generation packaging plasmid (Addgene). Conditioned medium was collected after 48 hr and 72 hr, filtered through 0.45-μm filters and used for infection of cells. To achieve stable expression of lentiviral expression vectors, semi confluent UMUC3 / T24T / T24 / RT4 / Raw 264.7 cells were infected with virus for 8 hr in 5 μg/ml polybrene. Two days after infection cells were shifted to growth medium with 2 μg/ml puromycin for 3 days or 20 μg/ml blasticidin for 1 week or cells were FACS sorted for GFP at Yale FACS facility to obtain the desired expression level and used for the experiments.

Immunoprecipitation and Western blotting

Equal numbers of cells were seeded for the experiment and at the termination of experiment cells were washed with chilled PBS and lysed in buffer (TIAM1 Co-IP: 10 mM Tris base (pH-7.5), 50 mM KCl, 1% Nonidet P-40, 8% glycerol, 20 μM Calpain Inhibitor (Calbiochem); Rac1 Co-IP: 10 mM Tris base (pH-7.5), 150 mM NaCl, 1% Nonidet P-40, 8% glycerol; other experiments: 50 mM Tris base (pH- 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxy-cholate, 5 mM dithiothreitol) containing 1x protease and phosphatase inhibitor. The samples were cleared with centrifugation at 21×10^3xg for 15 min and supernatants were either treated with SDS sample buffer or incubated with 1 μg antibody and 2 μl of protein A/G beads (Santa Cruz) over night, washed 3X and then treated with SDS sample buffer. Samples were resolved by SDS-PAGE and electro-transferred to nitrocellulose (Bio-Rad Laboratories). Membranes were blocked with 5% milk for 1 hr and probed with primary antibodies: rabbit anti-D4-GDI (0.2 μg/ml;

Spring Bioscience-E2432), goat anti-Actin (0.2 μg/ml; Santa Cruz-SC-1615), mouse anti-CD44 (0.2 μg/ml; R&D-MAB7045), goat anti-OPN (0.2 μg/ml; R&D-AF808), mouse anti-Tubulin (0.4 μg/ml; Sigma-clone DM1A), mouse anti-Rac1 (0.4 μg/ml; Millipore-clone 23A8) and sheep anti-TIAM1 (0.2 μg/ml; R&D-AF5038) and respective secondary antibodies at similar concentration, for 3 hr at room temperature / overnight. Membranes were washed, and detected by super signal west femto (Thermo scientific). For OPN depletion, equal volumes of low serum medium or macrophage-conditioned medium were incubated with control IgG or anti-OPN IgG conjugated with protein A/G beads for 5 hr at 4^{o}C. 10% of the supernatants and beads were analyzed for OPN by western blotting.

Pull down Assays

Equal numbers of cells were seeded in complete growth medium. After 24 hr cell were serum starved for further 24 hr in 0.2% FBS and then treated with 1X macrophage conditioned medium or 5 μg/ml OPN for the indicated time. At the termination of the experiment cells were washed with chilled PBS and lysed in buffer containing 50 mM Tris base (pH-7.5), 200 mM NaCl, 10 mM MgCl2, 1% Nonidet P-40, 5% glycerol and 1x protease and phosphatase inhibitor. The samples were cleared with centrifugation at $21\times10^{3}\times$g for 5 min and supernatants were either treated with SDS buffer or incubated with pre-conjugated GST-PBD beads for 30 min, washed 3X and then treated with SDS sample buffer, and analyzed with immunoblotting for Rac1 activation.

RNA isolation and RT PCR

To identify isoforms of CD44 in UMUC3 cells, cells were scraped into TRIzol reagent, RNA was extracted with phenol / chloroform and digested with DNase for 15 min, and cleaned up using the PureLink RNA mini kit (ambion) as per the manufacturer's protocol. cDNA was synthesized using iScript cDNA synthesis kits (Bio-Rad). To identify *CD44* variants, PCR primers were designed to differentiate between different variants based on size of the PCR products. Primers used were: forward (5' GAGCATCGGATTTGAGACCTGC 3'; exon 5) and reverse (5' CTTCGACTGTTGACTGCAATGC 3'; exon 18). These primers give a product of 669 bp, which represents transcript variant four and eight. To specifically identify transcript variant four, the second reverse primer (5' CCATTGCCACTGTTGATCACTAG 3'; exon 20) was used in combination with forward primer. PCR gives a product of 714 bp, specific to transcript variant four, which represents CD44s.

Co-culture assays

$1x10^{5}$ stable GFP or GFP-RhoGDI2 expressing UMUC3 cells were seeded in the bottom chamber of trans-well (8.0 μm pores) in 0.2% FBS containing medium (MEM). $3x10^{5}$ Raw 264.7 cells were seeded in the top chambers in 0.2% FBS containing medium (RPMI 1640). After 18 hr, these cells were harvested and $1x10^{5}$ pre-conditioned UMUC3-GFP or UMUC3-GFP-RhoGDI2 cells were seeded in co-culture conditioned medium (CCM) from the co-culture wells into the upper chamber of matrigel invasion wells. Complete growth medium (CGM) was added to the lower chambers. After 7 hr, cells were fixed and cells on the lower surface of the membranes stained with DIFF Quik Stain (IMEB INC). Images were taken from five randomly selected fields and invaded cells were counted using Image J.

Invasion assays

For invasion assays, $4x10^{4}$ UMUC3, $3x10^{4}$ T24T and $2x10^{5}$ RT4 cells were seeded in matrigel trans well invasion chambers (354480, BD Bioscience) with or without 1X macrophage conditioned media / 5 μg/ml recombinant OPN / fibroblast (L cells) conditioned medium for 18 hr, complete growth medium was added in the bottom chambers. In some cases, cells were incubated with 2.5 μg control, CD44 (hermes-1) or a_vb_3 (LM609) or b1 or b4 IgG for 30 min in 50 μl medium. Cells were then seeded onto invasion chamber with or without 500 μl conditioned medium or 5 μg/ml recombinant human (Sigma) or mouse (R&D) OPN for 18 hr. To deplete OPN, macrophage conditioned mediim or LSM (low serum medium; 0.2% FBS) was incubated with protein A/G beads conjugated to control IgG or anti-OPN IgG for 5 hr at 4 ^{0}C. Samples were spun at $3x10^{3}$xg for 5 min, and supernatants used for invasion assays.

Migration assays

$2.5x10^{4}$ cells were seeded into the upper chambers of 8.0 μm pore trans well (353097, Corning) in 0.2% FBS containing medium, and complete growth medium was added in the bottom chambers as a chemo-

attractant. Conditioned media was added to both upper and lower chambers. After 18 hr, cells on the lower surface were fixed, stained and counted as in invasion assays.

Colony assays

Cells were seeded in 6 well plates (300 cells / well) in low serum (UMUC3-2%; T24 and T24T-1%) containing medium with or without macrophage-conditioned medium. For antibody blocking, $4x10^4$ UMUC3 or $3x10^4$ T24T cells were incubated with 2.5 μg control or CD44 antibody for 30 min in 50 μl medium and 300 cells per well were seeded and analyzed in the same way. After 10 days, colonies were fixed and stained with crystal violet solution (0.05% crystal violet, 1% formaldehyde and 1% methanol in 1X PBS). Wells were washed with water to remove excess dye; dried plates were scanned and numbers of colonies were counted.

Proliferation assays

UMUC3 cells were seeded in 96 well plates at 2×10^3 cells / well in complete growth medium. After 24 hr, cells were treated with 20X macrophage conditioned medium to final concentrations of (0.0X-2.0X) in 0.2% FBS containing medium for the indicated times. Cell number was determined by adding MTT (Sigma) at 0.5 mg/ml in medium for 3 hr, then medium was aspirated, wells extracted with DMSO, and absorbance at 570 and 630 nm measured using plate reader (BioTEK).

Osteopontin ELISA

OPN in conditioned medium was determined by using the mouse / rat OPN ELISA kit (R&D) as per the manufacturer's protocol.

Antibody purification

Hybridoma cells for the CD44 function blocking antibody, hermes-1 (developmental studies hybridoma bank, University of Iowa, IA) were grown at 37 ^oC in serum free growth medium (HB101 + T151; Irvine scientific). After 14 days, culture supernatant was collected, filtered through 0.45 μm filters and concentrated with 100 kDa MWCO Amicon Ultra-15 centrifugal filter units (Millipore). For further purification, concentrated supernatant was incubated with protein A/G beads (Santa Cruz). After O/N incubation, beads were centrifuge at $3\times10^3\times g$ for 2 min and washed 3X with TBST (0.1% Tween). Antibody was eluted from beads with 0.2M pH 2.5 glycine, neutralized with 1M pH-7.5 Tris base, and concentrated using 100 kDa MWCO Amicon filters.

Hematoxylin and eosin staining

Lung were harvested from mice and fixed in 4% formaldehyde for 48 hr. Fixed lungs were embedded in paraffin, sectioned, and H&E stained at the Yale histology laboratory.

Supplemental References

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