SUPPLEMENTARY MATERIALS AND METHODS

Immunoblotting

Total protein from cultured cells was extracted using a RIPA buffer with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Twenty micrograms of total protein (assessed using BCA protein assay) were subjected to SDS-PAGE using Any kD polyacrylamide gels (Bio-Rad Laboratories). Proteins were transferred to Immun-Blot PVDF Membrane (Bio-Rad Laboratories) and stained using a goat anti-human CXCL1 antibody (sc-1374, dilution 1/250; Santa Cruz Biotechnology), mouse anti-human CXCR2 antibody (ab24963, dilution 1:500; Abcam), rabbit anti-human IL6 antibody (sc-1265, dilution1:200, Santa Cruz Biotechnology), goat anti-human TIMP4 antibody (sc-9375, dilution 1:200, Santa Cruz Biotechnology) and mouse anti-human β-actin antibody (AC-15, dilution 1:10,000, Sigma-Aldrich). Stained proteins were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Equal loading was confirmed by β-actin staining.

Stable transfection of cell lines

Plasmids with sequence verified human CXCL1 cDNA cloned within pCMV6-Entry vector and plasmid with vector alone (Origene Technologies) were transfected into DU145 cells using Fugene HD transfection reagent (Roche Diagnostics) to create DU145-CXCL1 and DU145-Empty. Similarly, CXCL1 short hairpin RNA (shRNA) cloned within pRS vector was transfected into T24 and PC3 cells as well as CXCL1 plasmid scramble (Scr) non-effective shRNA construct within pRS vector (Origene) using Fugene HD. Stable transfectants were selected with 1,200 μg/ml of G418 (Life Technologies, Inc., Carlsbad, CA) for DU145 clones and 0.25 μg/ml of puromycin (Life Technologies) for T24 and PC3 clones for 14 days and subcloned by limiting dilution in 96-well plates. Integration of the transfected gene into the genome was confirmed by RT-PCR. Stable cell lines were

maintained in media containing 500 µg/ml of G418 for DU145 clones and in media containing 0.25 µg/ml of puromycin for T24 and PC3 clones.

Quantitative reverse transcriptase-PCR

RNA was extracted from cells using RNeasy mini kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Conversion to cDNA was achieved through High Capacity cDNA Reverse Transcription kit (Life Technologies). Quantitative reverse transcriptase (RT)-PCR was carried out using ABI 7300 Real-Time PCR System (Life Technologies) in a 20 µl reaction volume containing 1 µl of the first-strand cDNA, 1 µl of gene-specific TaqMan primer and probe mix. Gene-specific TaqMan primer and probe sets used in this study were Hs00236937_m1 for CXCL1 and Hs01060665_g1 for β -actin. Relative fold changes in mRNA levels were calculated after normalization to β-actin using the comparative Ct method [1].

Capillary tube formation assays

Matrigel (BD Biosciences) was added to 96-well plates (50 µl per well) and allowed to solidify for 30 min at 37^0C. HUVEC cells were incubated in serum- and growth factor-free EBM2 basal media containing 0.1% delipidated BSA for 5 hrs. HUVECs were seeded on top of Matrigel in triplicates at a density of 10^4 cells per well in conditioned media and incubated for 6 hrs. Similarly, HUVEC cells were resuspended in EBM-2 basal media with or without 20 µg/ml or 100 µg/ml of CXCL1 monoclonal antibody (HL2401) prior to seeding on top of Matrigel and incubated for 6 hrs. Images were acquired with a Nikon ECLIPS E400 microscope (Nikon, Melville, NY). The total length of tube-like structures in at least 4 viewed fields per well was measured using ImageJ. At least three independent experiments consisting of each condition tested in triplicate wells was used to calculate mean ± SD values.

Cell proliferation and soft agar colony formation assay

Briefly, 10^3 cells (T24, DU145 and PC3) per well were plated in 96-microwell plates and incubated for 6, 24, 48 and 72 hrs with the indicated concentration of HL2401 for 72 hrs. Each condition was tested in triplicate wells. Cell proliferation was determined by incorporation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described [28, 29]. At least three independent experiments were performed in triplicate.

RT^2 Profiler PCR Arrays for Angiogenesis and Metastasis

Cellular RNA from DU145-Empty, DU145-CXCL1-OE8, T24-shSCR, T24-CXCL1-KD4, PC3-shSCR and PC3-CXCL1-KD7 recovered and converted to cDNA as described above. RT^2 Profiler 'human angiogenesis' PCR arrays, (Catalog # PAHS-024ZA; SABiosciences Corporation, Frederick, MD, USA) and RT^2 Profiler 'human metastasis' PCR arrays, (Catalog # PAHS-028ZA; SABiosciences) were analyzed in duplicate according to the manufacturer's instructions (www.sabiosciences.com/pcrarraydataanalysis.php) by quantitative reverse transcriptase (RT)-PCR carried out using ABI 7300 Real-Time PCR system (Life Technologies, Carlsbad, CA). The specificity of the SYBR Green assay was confirmed by melting curve analysis. Relative fold changes in mRNA levels were calculated after normalization to housekeeping control gene targets using the comparative Ct method.

Cell migration and invasion assays

Migration assays were performed in 6 well two-tier invasion chambers (Collaborative Biomedical Products, Bedford, MA, USA) [2]. Polycarbonate membranes were coated with 4 mg/mL growth factor reduced Matrigel (BD Biosciences, San Jose, CA) as described for

invasion assays, control inserts (migration only) contained no coating. Two separate experimental designs were tested. First, DU145-CXCL1-OE3&8, DU145-Empty, T24-CXCL1-KD4&8, T24-shSCR, PC3-CXCL1-KD7 and PC3-shSCR cells were added to each insert at a density of 10^5 cells/ml/well in RPMI media. The lower chamber contained RPMI media with 10% FBS as a chemoattractant. The cells were maintained in a humidified incubator in 5% CO_2 at 37°C for 24 hours. After the designated time, the cells on the top of the polycarbonate membrane were removed. The cells attached to the bottom of the membrane were stained for 1 hour with cell viability indicator Calcein AM Fluorescent Dye (BD Biosciences, Franklin Lakes, NJ) and quantified using the FLUOstar OPTIMA at 495mm excitation and 515nm emission (BMG LABTECH Inc., Cary, NC).

In addition, T24, DU145 and PC3 cells (10^5 cells/mL/well) were exposed to 0-200 µg/ml of CXCL1 monoclonal antibody (HL2401) in RPMI media. The lower chamber contained RPMI media with 10% FBS as chemoattractant. After 24 hours, the T24, DU145 and PC3 cells on the top of the polycarbonate membrane were removed, while T24, DU145 and PC3 cells attached to the bottom of the membrane were stained for 1 hour with cell viability indicator Calcein AM Fluorscent Dye and quantified using the FLUOstar OPTIMA. For the migration and invasion assays, at least three independent experiments consisting of each condition tested in triplicate wells was used to calculate mean ± SD values.

To check reliability of the proliferation assay and invasion assay, preliminary experiment using different numbers of cells was performed to generate the standard curves (*Supplemental Fig. 7A and B*).

Antibody protein array

DU145-CXCL1-OE3&8, DU145-Empty, T24-CXCL1-KD4&8, T24-shSCR, PC3-CXCL1-KD7 and PC3-shSCR were harvested from three individual experiments and lyzed with 2X Cell Lysis Buffer (RayBiotech, Norcross, GA). Protein levels were quantified using a bicinchoninic acid protein assay kit (Applygen Technologies Inc., Beijing, China). Lysates were then analyzed for angiogenesis-related cytokines (60 cytokines total) and Matrix metalloproteinase-related proteins (10 proteins total) using a RayBio® C-Series Human Angiogenesis Antibody Array 1000 Kit (RayBiotech) and RayBio® C-Series Human Matrix Metalloproteinase Antibody Array 1 Kit (RayBiotech), respectively. The assay was performed according to the manufacturer's instruction. Briefly, 100 μl blocking buffer was added into each well and incubated at room temperature for 60 min to block slides. After removing the blocking buffer, 30 μg of samples or serial diluted standards were added to each well containing 70 μl sample diluent and incubated overnight at 4°C. The samples were decanted and wash three times with wash buffer I at room temperature with gentle shaking. Next, the array was washed twice with wash buffer II, then 70 μl diluted detection antibody was added to each well and incubate at room temperature for 2 h, followed by three washes with wash buffer I at room temperature. Subsequently, 80 μl diluted Cy3 equivalent dye-conjugated streptavidin was added to each well. The array was incubated in dark room at room temperature for 1 h and washed as above.

Transient transfection of small interfering RNA (siRNA)

IL6 targeting siRNA, TIMP4 targeting siRNA and Scr-siRNA were purchased from Life Technologies. For siRNA transfection, cells were seeded at a density of 10,000 cells/well in 6-well plates and transfected with 10 nM siRNA using Lipofectamine™ RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer's instruction. The transfection was performed by the reverse transfection method, in which cells were transfected and plated simultaneously. In order to avoid cytotoxicity of the transfection

reagent, the medium was routinely replaced with fresh medium at 24 h after siRNA transfection. After incubation of 48 hours in the fresh medium, the conditioned media was collected for in vitro tube formation assay using HUVEC or invasion assay using Fluoroblok insert system (BD Biosciences, San Jose, CA).

Monoclonal Anti-CXCL1 Antibody Production

A mouse monoclonal antibody against CXCL1 was commercially produced using a standard protocol of the Hybridoma and Protein Core Laboratories, University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) [3]. Briefly, two female Balb/cByJ mice were immunized with approximately 100 µg of native CXCL1 protein diluted in sterile physiologic phosphate buffered saline (PBS) and emulsified in Ribi MPL +TDM adjuvant. The immunogen was administered on day 1, 21, 44, and 192. The test bleeds were collected 11 to 14 days after the second and third immunizations. The presence of anti-CXCL1 antibodies in the post-immunized serum was determined by western blots and ELISA. Six days after the fourth immunization, mouse #1 was euthanized and the splenic lymphocytes were collected and fused with mouse myeloma cells to form hybridoma cells [4]. The cultured media of the growing hybridoma mass cultures (n = 30) were collected and screened for anti-CXCL1 antibody production by ELISA. The mass cultures that tested positive by ELISA were subsequently tested for biologic effect in a proliferation assay utilizing HUVEC cells. The cultures (n = 3) that showed reactivity to CXCL1 in ELISA and exhibited anti-proliferative effects were grown out, and further cloned by limiting dilution. The cultured media collected from each clone were tested again by ELISA. The monoclonal antibodies were isotyped by ELISA and IsoStrip tested following manufacturer's protocol. The cultured medium of the final selected hybridoma clone (HL2401) was harvested, and purified through a protein G column (GE Healthcare Protein G Sepharose 4 Fast Flow). The concentration of the

purified monoclonal anti-CXCL1 antibody (HL2401) was determined by Bradford Protein Assay and stored at 4°C for future validation. A gel clot LAL assay from Lonza (Basel, Switzerland) ensured the antibody was free of endotoxins.

In vivo administration of CXCL1 antibody HL2401

The importance of CXCL1 expression for tumorigenicity and angiogenesis was assessed *in vivo* using bladder cancer and prostate cancer mouse xenograft models. Animal care was in compliance with the recommendations of *The Guide for Care and Use of Laboratory Animals* (National Research Council) and approved by University of Hawaii local IACUC. The subcutaneous tumorigenicity assay was performed in athymic BALB/c nu/nu male mice (6 to 8 weeks old) purchased from Envigo by inoculating 2×10^6 parental T24 cells and 2×10^6 parental PC3 cells as described previously [5, 6]. After one week, mice bearing subcutaneously xenograft tumors were divided randomly into three groups (Control or 8 mg/kg) of HL2401 and treatment was initiated. Each group was comprised of at least 10 mice. No toxicity or weight loss was noted in any of the treatment groups (*Supplemental Figure 6*). HL2401 (100 µl diluted in sterile PBS) was administered *via* intraperitoneal injection twice weekly for four weeks. Control mice received IgG alone on the same schedule. Tumor volumes were measured weekly with digital calipers and calculated by $V\ (mm^3) = \text{length} \times (\text{width})^2 \times 0.5236$. After five weeks of cell inoculation, the mice were sacrificed, tumors and key organs were resected and analyzed histologically and by immunohistochemical staining.

Immunohistochemical (IHC) analysis of xenograft tumors

Paraffin embedded tumor sectioned were deparaffinized in xylene, rehydrated using graded percentages of ethanol. Slides were treated with 1% hydrogen peroxide in methanol to block endogenous peroxidase activity. Staining was conducted using goat

anti-human CXCL1 antibody (sc-1374, dilution 1:200, Santa Cruz Biotechnology), mouse anti-human CXCR2 antibody (ab24963, dilution 1:200; Abccam), human IL6 antibody (sc-1265, dilution1:200, Santa Cruz Biotechnology) human TIMP4 antibody (sc-9375, dilution 1:200, Santa Cruz Biotechnology), cleaved caspase-3 antibody (5A1E, dilution 1:1,500, Cell Signaling Technology), mouse anti-human PECAM-1 (sc-1506-R, dilution 1/1,000, Santa Cruz Biotechnology) and mouse anti-human Ki-67 (MIB-1, dilution, 1:100, Dako). Biotin-labeled horse anti-mouse IgG, rabbit IgG or goat IgG (2 μg/ml in blocking buffer) was used as secondary antibody. Immunoreactive signals were amplified by formation of avidin-biotin peroxidase complexes and visualized using 3, 3'- diaminobenzidine (DAB). Nuclear counterstaining was conducted with hematoxylin. Apoptotic Index (AI), Microvessel density (MVD) and Proliferative Index (PI) analyses were determined as previously described [6-8].

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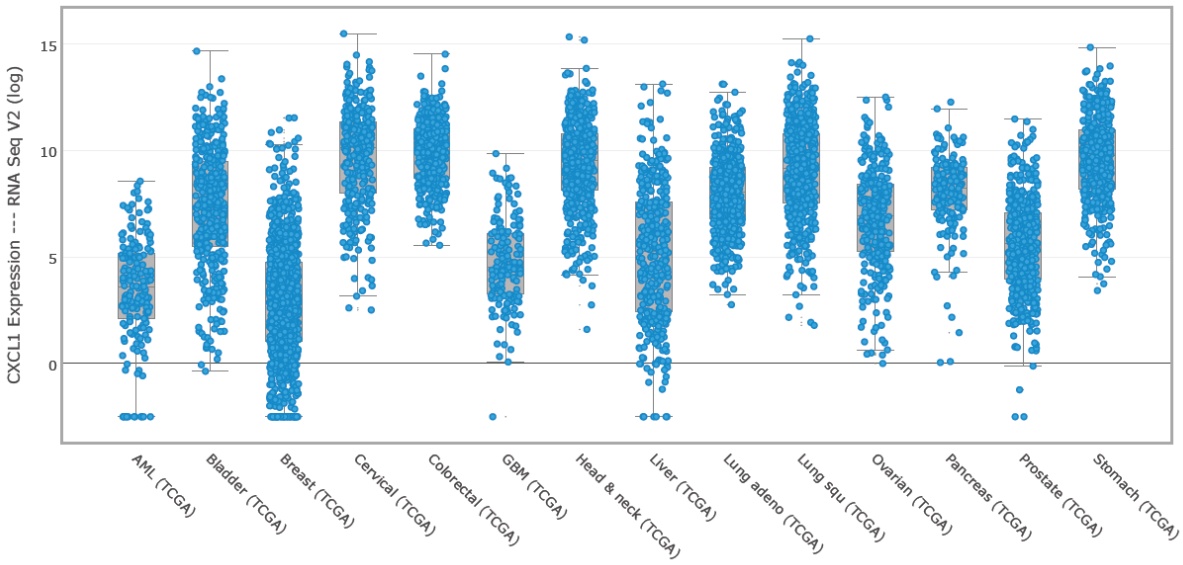
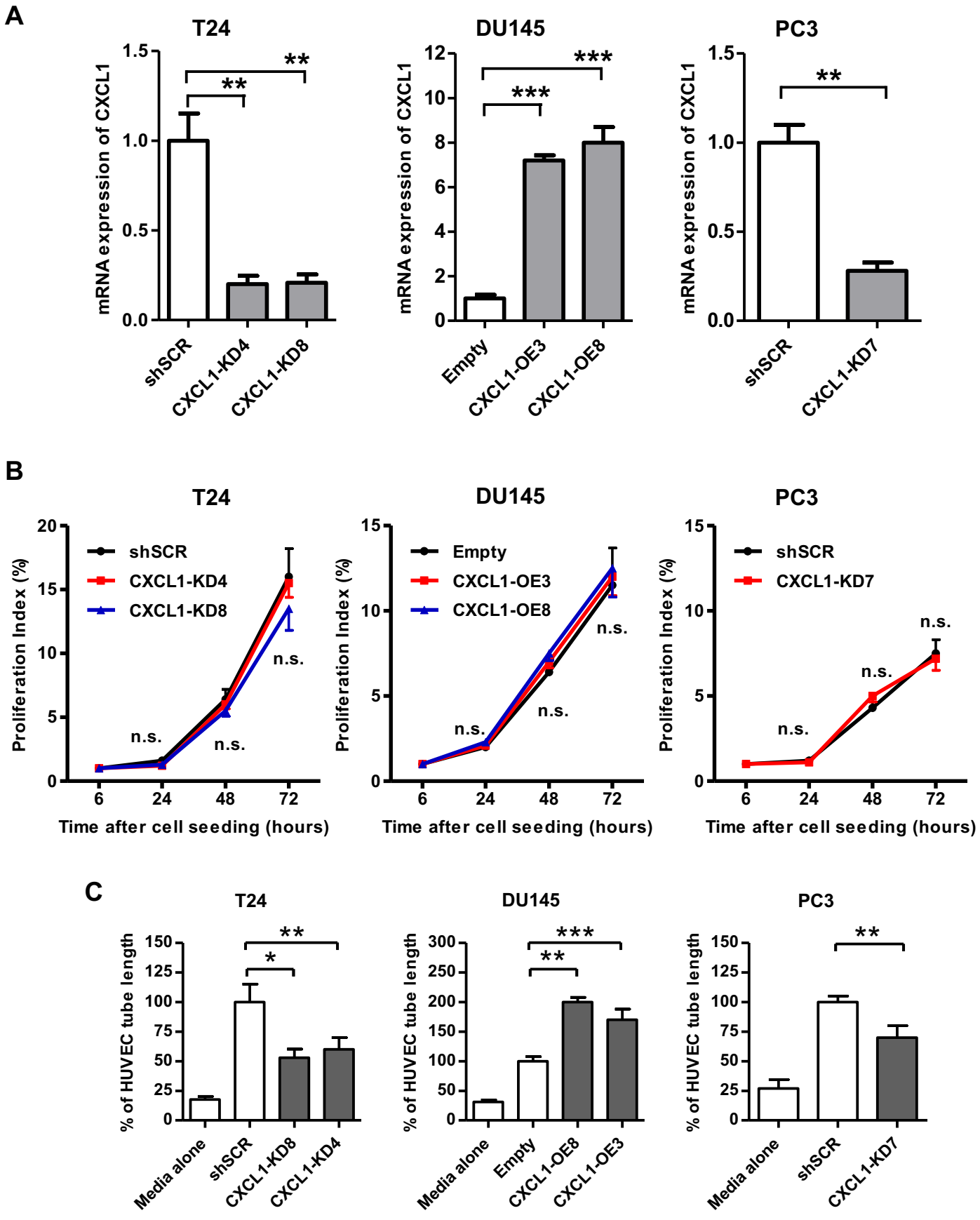


Supplemental Figure 1



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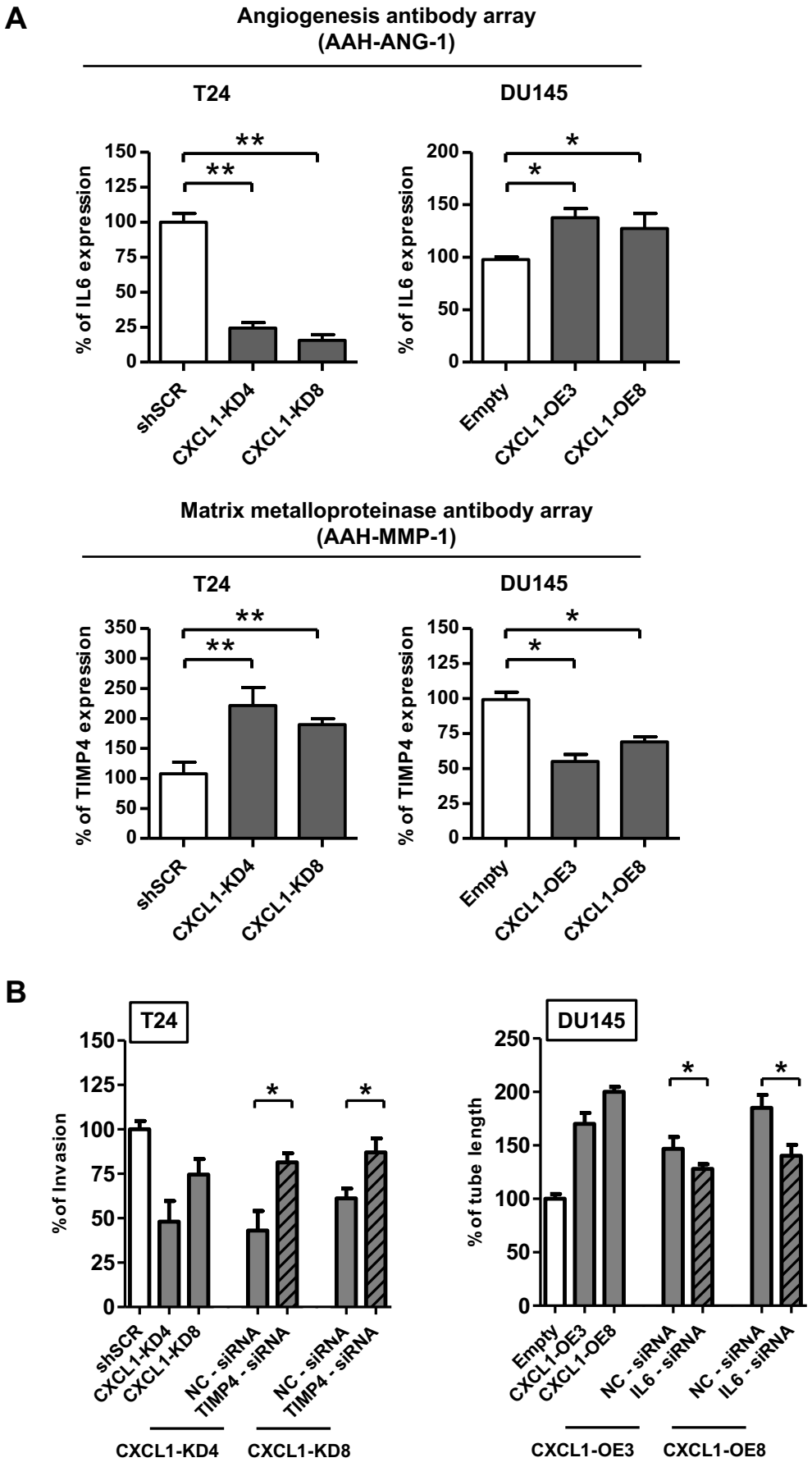

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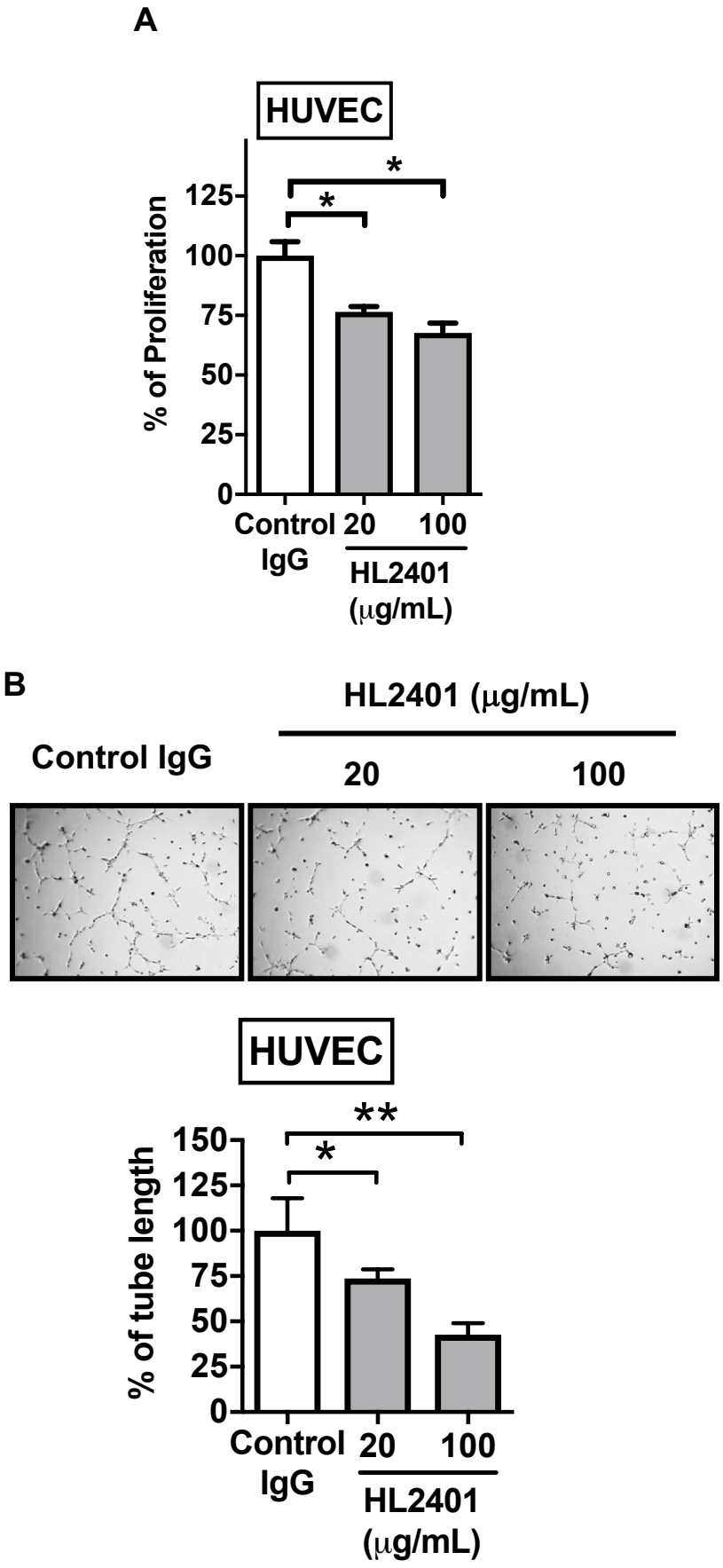

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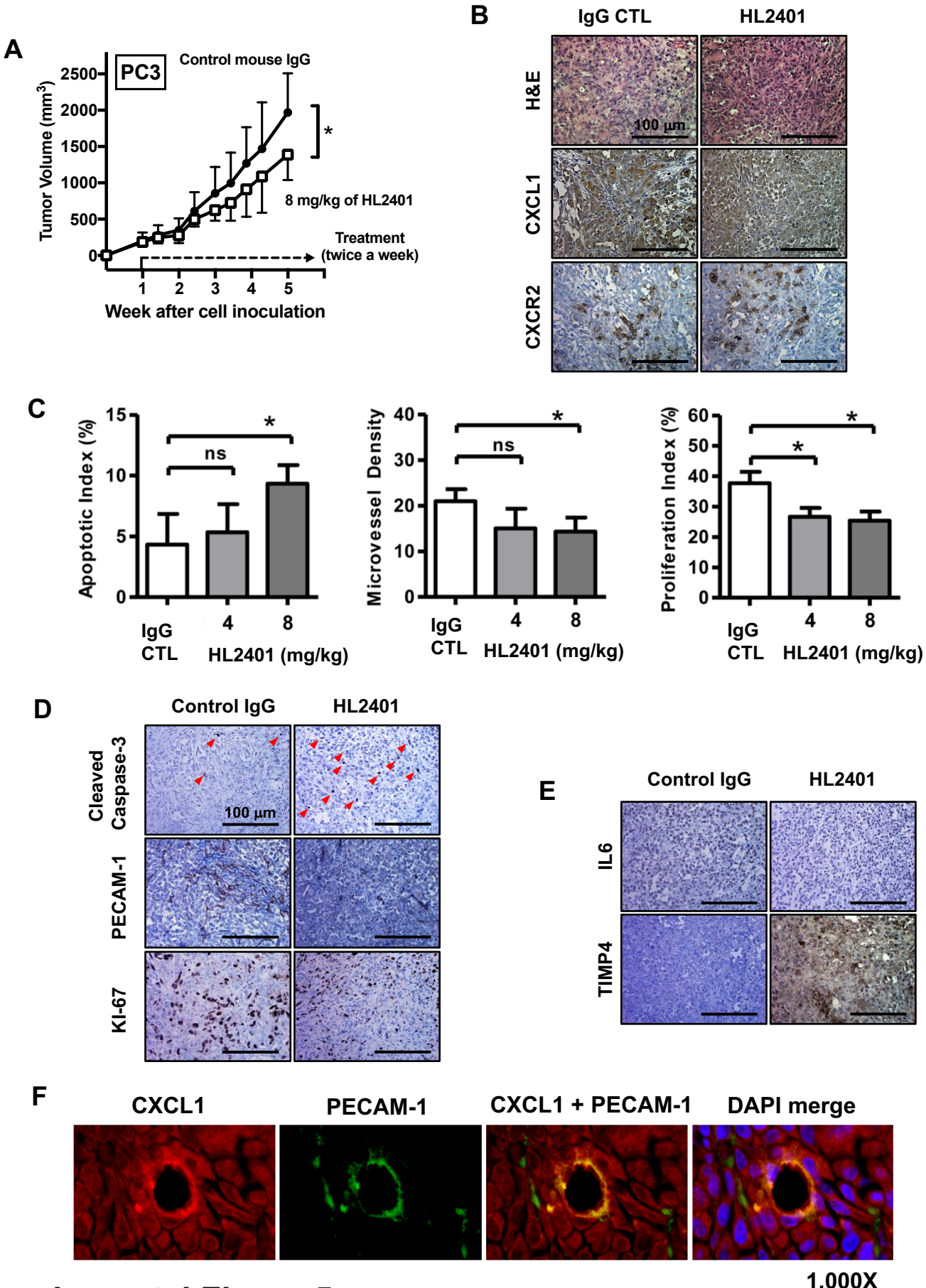

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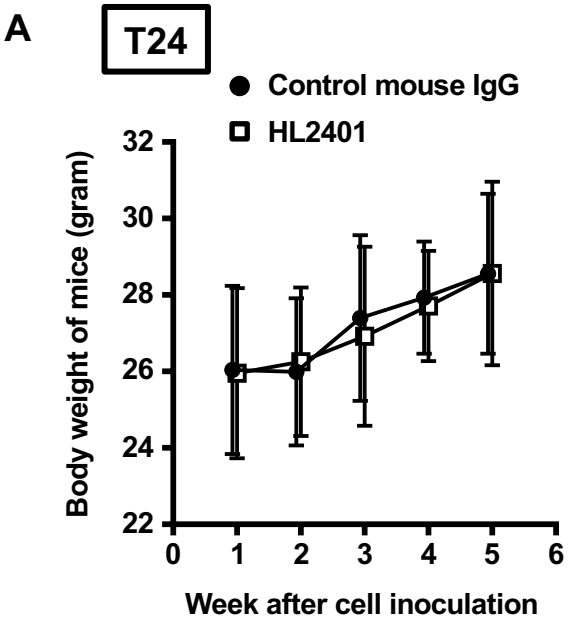

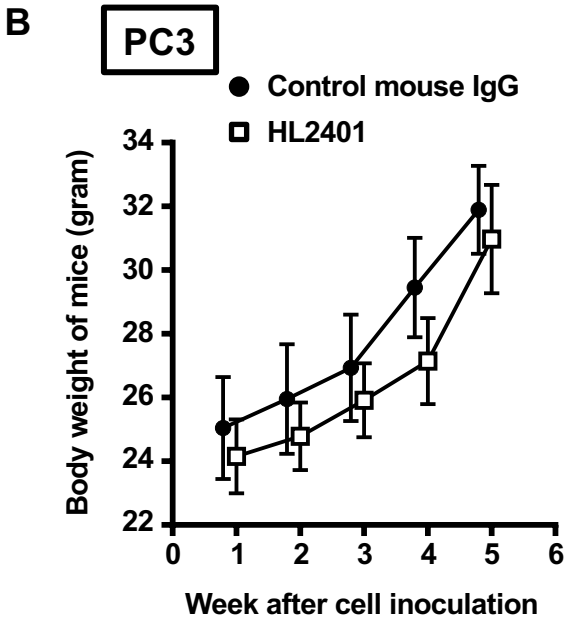

Supplemental Figure 6