Supplemental Material

Detailed Methods

Mice

MFG-E8 KO mice were generated as described previously.[1] Heterozygous founders were speed backcrossed to C57BL/6NCr mice to generate animals with appropriate and uniform genetic backgrounds using the Speed Congenics Services of the NCI-Frederick Center for Cancer Research Laboratory Animal Sciences Program. This resulted in mice that were ~99% identical to C57BL/6NCr based on the distribution of highly polymorphic microsatellite markers and equivalent to N10 backcrosses to C57BL/6NCr using a standard approach. MFG-E8–deficient mice were generated by interbreeding homozygous animals carrying the targeted *MFG-E8* allele**.** Adult female WT C57BL/6NCr mice (8-12 weeks old) were obtained from the NCI-Frederick Animal Production Program. Mice were bred and housed in a pathogen-free environment and used in experiments in accordance with institutional guidelines.

Cells and Tumor Implantation Studies

B16F10 cells (Tumor Repository of the NCI/Frederick Cancer Research and Development Center) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES-NaOH (pH 7.4), penicillin (100 U/ml), streptomycin (100 μg/ml), and 1 mM sodium pyruvate (RPMI 1640 complete medium). Tumors were initiated by subcutaneous injection of 10^5 washed melanoma cells into the flanks of MFG-E8 WT and KO mice. Tumor dimensions were measured 3x per week using engineer's calipers and tumor sizes reported represent length x width (mm^2). Tumor-bearing mice were euthanized when maximal tumor diameters reached 2 cm or humane endpoints incorporated into the protocol prospectively were reached. Mouse C3H/10T1/2 cells (ATCC) were maintained in BME medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and used before passage 10.

Antibodies

Abs and their sources were as follows: rat anti-mouse CD31 mAb (MEC13.3; BD Bioscience-Pharmingen), rabbit anti-mouse NG2 pAb (Chemicon), rat anti-mouse PDGFRβ mAb (eBioscience), rat anti-mouse CD68 mAb (AbD Serotec), mouse anti-α-smooth muscle actin (SMA) mAb (Sigma). Alexa 488-, Alexa 568-, Alexa 647-conjugated secondary Abs and Zenon Rabbit IgG Labeling Kits were obtained from Molecular Probes (Invitrogen). Hybridomas producing rat anti-mouse CD4 mAb (clone GK1.5) and CD8 mAb (clone 2.43) were from ATCC. Purified mAb were prepared from Hybridoma-SFM Media (Invitrogen) that had been conditioned in CL1000 bioreactors (Argos Technologies) populated with hybridoma cells that were grown to exhaustion using protein G-Sepharose (GE Healthcare Biosciences).

Rabbit anti-mouse MFG-E8 pAb were generated by immunizing and boosting rabbits with a fusion protein comprised of the N-terminal 148 amino acids of MFG-E8 (including the EGF–like domains (E1 and E2) and the linker region (L) in frame with the Fc region of human IgG1 (250 μg per injection in complete (x1) and incomplete Freund's adjuvant (x3) at 3 week intervals). Monospecific pAb were prepared from high titer antisera using serial affinity chromatography that first removed reactivity with human IgG and then selected for binding to MFG-Ig. Ab specificity was verified by ELISA, western blotting and immunofluorescence microscopy with relevant proteins and tissues from control and MFG-E8 KO mice (see Figure 4A). Mouse anti-mouse MFG-E8 were generated by immunizing MFG-E8 KO mice with an MFG-E8-encoding plasmid via gene gun immunization and boosting immunized mice with MFG-E8 protein and/or MFG-E8 producing 293T cells. Splenocytes and lymphocytes were isolated from immunized mice and fused with SP2 myeloma cells. Drug-resistant hybridoma clones producing mAb that reacted with MFG-E8 were identified by ELISA and 4 clones (1H6, B1F10, B10C7, B18A9) were ultimately expanded and utilized. The mAb produced by these clones were purified from culture supernatants via protein G-Sepharose chromatography and their reactivity was characterized by ELISA and immunoblot using recombinant MFG-E8 and MFG-Ig fusion proteins produced in 293-F cells (FreeStyle 293 Expression System) as well as MFG-E8 protein fragments produced in bacteria. mAb

reactivities are as follows: 1H6 - C1 domain; B1F10 - E1, E2 or linker domain; B10C7 - RGD-dependent epitope in E2 domain and B18A9 - C1 domain (data not shown).

Preparative Flow Cytometry

Single cell suspensions were prepared from 100 mm^2 (1 cm diameter) B16 melanoma tumors growing subcutaneously in flank skin. Tumors were minced in 5 mg/ml collagenase D (Roche) in RPMI with 5% FCS and DNase, incubated at 37^o C for 2 hours and erythrocytes were lysed (ACK buffer). Prior to flow sorting, tumor cells were incubated with rat anti-mouse FcγR mAb (2.4G2, reactive with CD16/CD32), washed and stained consecutively at 4°C with Alexa 488-conjugated anti-CD45 mAb (BioLegend), PE-conjugated anti-PDGFRβ (eBioscience) and Alexa 647-conjugated anti-CD31 mAb (BioLegend). Stained cells were sorted via a BD FACSAria™ II Flow Cytometer (BD Biosciences).

Lymphocyte Depletion

To deplete T lymphocytes in tumor-bearing mice, mice were injected i.p. on days -3 and -2 before B16 cell inoculation with 200 μg anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 2.43) alone or in combination. Depletion efficiency was monitored by determining CD4 and CD8 T cell frequencies in peripheral blood, and mAb injections were repeated approximately every 10 days to maintain depletion levels of >95%.

Immunofluorescence Microscopy and Image Analysis

Tumors (100 mm^2) were excised from flank skin and 14 μm frozen tissue sections were prepared and fixed in 4% PFA in PBS or cold acetone. After blocking with 3% dry milk-PBS (Bio-Rad) supplemented with 5% normal donkey serum or 5% normal goat serum (as appropriate) for 1 hour at room temperature, sections were stained with Ab of interest followed by Alexa 488-, Alexa 568-conjugated secondary Ab or they were stained with Alexa 488-, Alexa 568-conjugated Ab and control proteins that were prepared using Zenon Labeling Kits (Invitrogen). Sections were counterstained with 4,6-diamidino-2-phenylindole

(DAPI) to visualize nuclei, mounted in ProLong Gold antifade reagent (Invitrogen) and examined. To visualize proliferating cells in tumors, sections were stained with rabbit anti-Ki-67 pAb (Abcam) and counterstained with DAPI. Percentages of Ki-67 and DAPI double positive nuclei were analyzed using Image J software (NIH). Apoptotic cells were identified by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) using an *in situ* cell death detection kit (Roche) according to the manufacturer's protocol. Percentages of TUNEL and DAPI double positive nuclei were analyzed using Image J software. All immunofluorescence images were collected and visualized with an AxioImager A1 conventional immunofluorescence microscope (Zeiss) or a LSM510 confocal laser-scanning microscope (Zeiss).

Measurement of Vessel Permeability

Tumor vessel permeability was quantified by determining the amount of dye that extravasated after i.v. injection (Miles assay).[2] Mice were injected with Evans blue dye (1% stock; Sigma) at a dose of 30 mg/kg. After 30 minutes, animals were euthanized and perfused with 20 mL of 0.9% NaCl solution using a peristaltic pump. Tumors were removed and weighed, and then incubated in formamide for 18 hours at 37^{o} C. A_{620} of tumor lysates was determined spectrophotometrically. The amount of extravasated dye was calculated and reported after normalization (μg dye per g tumor).

Assessment and Treatment of Oxygen-Induced Retinopathy-Associated Angiogenesis

Angiogenesis associated with oxygen-induced retinopathy (OIR) was studied as previously described.[3,4] Litters of pups born to MFG-E8 heterozygous parents were exposed to 75% O_2 for 5 days (P7-P12) with nursing mothers. Mice were returned to ambient air (20% O_2) on P12, and on day P17 retinal neovascularization was examined in retinal whole mounts or axial sections. Retinal whole mounts were obtained from enucleated eyes, fixed with 4% PFA at 4^{o} C overnight, washed, permeabilized and blocked with 0.5% Triton-X supplemented with 1% BSA/PBS and 5% goat serum for 2 hours at room temperature. Prior to immunofluorescnce microscopy, retinas were stained with FITC-conjugated *Bandeiraea*

simplicifolia isolectin B4 (Sigma-Aldrich), anti-NG2 pAb, anti-MFG-E8 pAb and rat anti-CD68 Ab (clone FA-11; Serotec) as indicated. Thereafter, retinas were stained with Alexa 568-conjugated secondary Abs and visualized with an AxioImager A1 immunofluorescence microscope (Zeiss) equipped with a digital camera.

To quantify neovascularization, 6 μ paraffin-embedded axial sections of retinas were stained with periodic acid Schiff (PAS) and hematoxylin and the number of vessel-associated nuclei that protruded above the inner limiting membrane into the vitreous were counted. The data presented represent the mean of nuclear counts in 10 sections (every third serial section) per eye and were obtained by an observer (S.M.) who was blinded with respect to mouse genotype. In treatment studies, rabbit anti-MFG-E8 pAb, mouse anti-MFG-E8 mAb and appropriate control proteins were injected i.p. on days P12, 14 and 16 (50 μg per injection). On day P17, retinal neovascularisation was quantified.

MFG-E8 Knockdown Experiments and Quantification of MFG-E8 mRNA and Protein

Short interfering RNAs (siRNA) specific for mouse MFG-E8 mRNA was designed using the QIAGEN GeneGlobe Search Center. The targeting sequences were as follows; MFG-E8 siRNA #1 (5'-AAGCGGTGGAGACAAGGAGTT-3'), MFG-E8 siRNA #2 (5'- AAGGCTGAATAATCAGGGCAA-3') and MFG-E8 siRNA #3 (5'-AAAGCAATGGAACCTGCGTGC-3'). siRNAs and AllStars negative control siRNA were purchased from QIAGEN. To inhibit MFG-E8 production, 10T1/2 cells (5×10^5 cells per 60 mm plate) were transfected with 10 nM siRNA using HiPerFect Transfection Reagent (QIAGEN). After 48 hours, MFG-E8 mRNA and protein levels were assessed by quantitative RT-PCR and ELISA. MFG-E8-depleting shRNAs were also designed. Two plasmids containing shRNA oligonucleotides targeting the MFG-E8 sequences [GGCTGGATAATCAGGGCAA (MFG-E8 shRNA#1) and AGACATGGAACCTGCGTGC (MFG-E8 shRNA#2)] were constructed. Corresponding synthetic oligonucleotides were annealed and cloned into the pSUPER.retro.puro vector (Oligoengine) at *Bgl II* and *Xho I* restriction sites. The resulting plasmids were characterized by restriction enzyme digestion and DNA sequencing. MFG-E8 shRNA or the control empty vector plasmids were transfected into Phoenix-

Ampho cells (from Dr. Gary Nolan, Stanford University), and virus-containing supernatants were collected and used to infect 10T1/2 cells. Stable MFG-E8 shRNA transductants were established by selection with 2 μg/ml puromycin for 1 week.

MFG-E8 mRNA levels were determined using quantitative RT-PCR. Total RNA was isolated with RNeasy Kits (Qiagen) and reverse transcribed with SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR was performed using the following primers: MFG-E8 (forward, 5'-ATCTACTGCCTCTGCCCTGA-3'; reverse, 5'-ACACAGACGAGGCGGAAATC-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward. 5'-ACCCAGAAGACTGTGGATGG-3'; reverse, 5'-CACATTGGGGGTAGGAACAC-3'). PCR products were generated and quantified with SYBR Green PCR Master Mix (Applied Biosystems) using CFX96 Real-Time PCR Detection System (Bio-Rad) equipped with CFX Manager™ Software version 1.5.

MFG-E8 protein was quantified by ELISA. Mouse anti-mouse MFG-E8 mAb 1H6 (reactive with the C1 domain) was adsorbed (100 ng/well) onto Maxisorp ELISA plates (Nunc) via overnight incubation at 4°C. After blocking with 2%BSA/PBS, conditioned medium was added and incubated for 90 minutes at 37°C. After washing, plates were incubated with rabbit anti-mouse MFG-E8 pAb (reactive with the N-terminus) for 60 minutes at 37°C. Washed plates were then incubated with HRP-conjugated goat anti-rabbit IgG (Jackson) for 60 minutes, developed with TMB substrate solution (Pierce) and enzymatic reactions were halted with 1 N H_2SO_4 before determining the A_{450}. Absolute concentrations were determined using appropriate standard curves.

10T1/2 Cell Migration Studies

10T1/2 cells were incubated in low serum (0.5% FCS)-containing media overnight and cell migration was subsequently assayed using FluoroBlok™ 24-Multiwell Insert System transwell culture plates (3 μ pore size; BD Biosciences). Cells were stained with Calcein AM (BD Biosciences), resuspended in 0.5% FCS-containing BME medium, and 10^5 cells were seeded into upper compartments of the transwell

apparatus. Medium containing 0.5% FCS and 50 ng/ml PDGF-BB (PeproTech) was added to the lower compartments. After 4 hours incubation at 37°C, images of cells that were attached to the undersurfaces of transwell filters were acquired with an inverted phase fluorescent microscope equipped with a digital camera. Areas covered by migrated cells in each well were quantified using Image J.

To assess the involvement of MFG-E8 in 10T1/2 cell migration in the transwell assay, MFG-E8 production by 10T1/2 cells was inhibited with siRNAs prior to assessment of migratory ability. To accomplish this, 10T1/2 cells were transfected with appropriate siRNA oligonucleotides, and transfected cells were used for transwell migration assays 42 hours later. As indicated, siRNA transfected 10T1/2 cells and control cells were preincubated with or without 100 ng/ml recombinant MFG-E8 (R&D systems) for 15 minutes prior to addition to the upper chamber to reconstitute the effect of MFG-E8 depletion. The involvement of MFG-E8 in migration in transwell assays was also assessed using anti-MFG-E8 Ab. In these experiments, 10T1/2 cells were incubated for 20 minutes with anti-MFG-E8 mAb (1H6, B1F10, B10C7, B18A9), rabbit anti-MFG-E8 pAb, anti-αV integrin mAb (RMV-7; Chemicon) or with corresponding control Abs (all at 20 μg/ml unless otherwise specified) before migration assessment. After treatment with Abs, 10T1/2 cells were incubated with or without 100 ng/ml recombinant MFG-E8 for 15 minutes prior to addition to the upper chamber. Ab and control proteins were also added into the media that was placed in the lower chambers of transwell plates to avoid Ab dilution.

The involvement of MFG-E8 in 10T1/2 cell migration was also assessed in an *in vitro* wound healing ("scratch") assay. In these experiments, MFG-E8 knockdown and control 10T1/2 cells were incubated in 0.5% FCS-containing medium overnight, the confluent cell monolayer was scratched with a pipette tip and displaced cells were removed with washing. "Wounded" monolayers were incubated for 16 hours, images of wounded areas were acquired at identical locations at the beginning and the end of the assay and the areas of residual "wounds" were determined using Image J. Both MFG-E8 shRNA and siRNA transfected 10T1/2 cells were used for scratch assays. In studies involving siRNA transfected cells, the scratch assay was performed 32 hours after transfection. As indicated, the ability of MFG-E8 to reverse of effects of MFG-E8 knockdown with shRNA was tested by adding recombinant MFG-E8 (100

ng/ml; R&D Systems) into the medium at the beginning of the 16 hour recovery period.

Proliferation assay

Cell proliferation was measured using the MTS assay. 10T1/2 cells were transfected with siRNA oligos (MFG-E8 siRNA or control siRNA). Sixteen hours after transfection, cells were treated with trypsin-EDTA and plated at a density of 5,000 cells per well with 0.5% FCS containing medium in 96 well plates. Twenty four hours after transfection, cells were stimulated with PDGF-BB (5, 10, 50, 100 ng/ml) or 10% FCS medium as a positive control. After 48 hours incubation at 37°C, 20 μl of CellTiter 96 AQueous One Solution Reagent (Promega) was added. After an additional incubation at 37°C for 2 hours, the absorbance at 490 nm was measured using an ELISA plate reader.

10T1/2 differentiation assays

To assess the possible role of MFG-E8 in the differentiation of 10T1/2 cells, expression of α smooth muscle actin (SMA) by TGF-β1-treated 10T1/2 cells was analyzed via Western blot assays and immunofluorescence microscopy. To induce differentiation of 10T1/2 cells into pericytes/vascular SMC, 10T1/2 cells were cultured with TGF-β1 (1 ng/ml; R&D Systems) for 24 hours. To assess the involvement of MFG-E8 in 10T1/2 cell differentiation, MFG-E8 production by 10T1/2 cells was inhibited with siRNA prior to induction of differentiation by TGF-β1. 10T1/2 cells were transfected with appropriate siRNA oligonucleotides, and transfected cells were cultured with TGF-β1 for an additional 24 hours. The involvement of MFG-E8 in differentiation was also assessed using neutralizing anti-MFG-E8 pAb. In these experiments, 10T1/2 cells were incubated for 1 hour with rabbit anti-MFG-E8 pAb or with corresponding control Ab (20 μg/ml) before TGF-β1 treatment. After treatment with Ab, 10T1/2 cells were incubated with or without 1 ng/ml TGF-β1 for 24 hours. After washing with ice-cold PBS, cells were disrupted in lysis buffer (20 mM Tris-HCl (pH 7.6) 140 mM NaCl, 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche) (complete lysis buffer) on ice. Cell

lysates were centrifuged at 10,000xg for 15 minutes at 4°C, and the resulting supernatants were subjected to SDS-PAGE. Ten μg of total protein was loaded per lane. Immunoblot analysis utilized mouse anti-αSMA mAb and anti-mouse HRP-conjugated secondary antibodies (Jackson) in conjunction with ECL (Pierce). Densitometric analysis of exposed films was accomplished using Image J software.

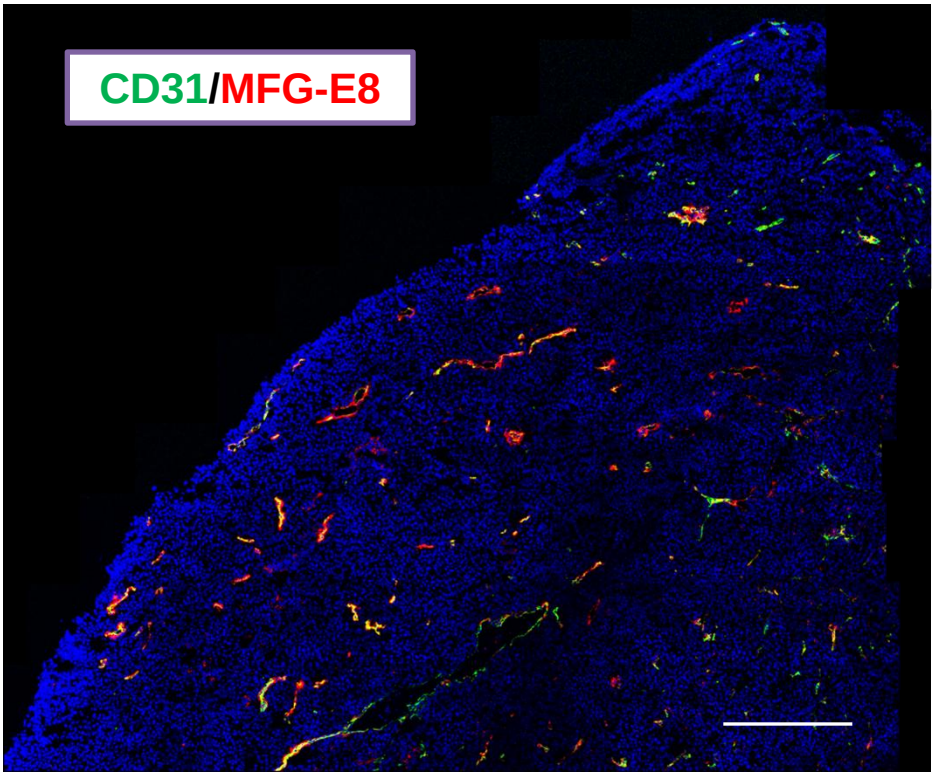
Statistics

P values were calculated using the Student's *t*-test (two-sided) or by analysis of one-way ANOVA followed by Bonferroni's post test as appropriate. Survival differences were subjected to statistical analysis using a log-rank test. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

Supplemental References

1. Neutzner M, Lopez T, Feng X, Bergmann-Leitner ES, Leitner WW, Udey MC. MFG-E8/Lactadherin Promotes Tumor Growth in an Angiogenesis-Dependent Transgenic Mouse Model of Multistage Carcinogenesis. *Cancer Res.* 2007;67(14):6777-6785.
2. Lin MI, Yu J, Murata T, Sessa WC. Caveolin-1-deficient mice have increased tumor microvascular permeability, angiogenesis, and growth. *Cancer Res.* 2007;67(6):2849-2856.
3. Connor KM, Krah NM, Dennison RJ, Aderman CM, Chen J, Guerin KI, Sapieha P, Stahl A, Willett KL, Smith LE. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc.* 2009;4(11):1565-1573.
4. Economopoulou M, Langer HF, Celeste A, Orlova VV, Choi EY, Ma M, Vassilopoulos A, Callen E, Deng C, Bassing CH, Boehm M, Nussenzweig A, Chavakis T. Histone H2AX is integral to hypoxia-driven neovascularization. *Nat Med*. 2009;15(5):553-558.

A



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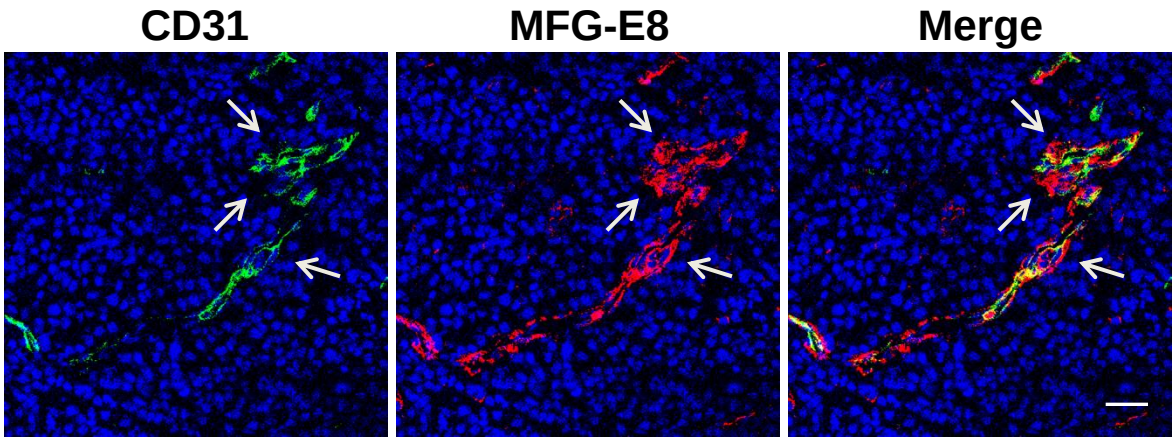

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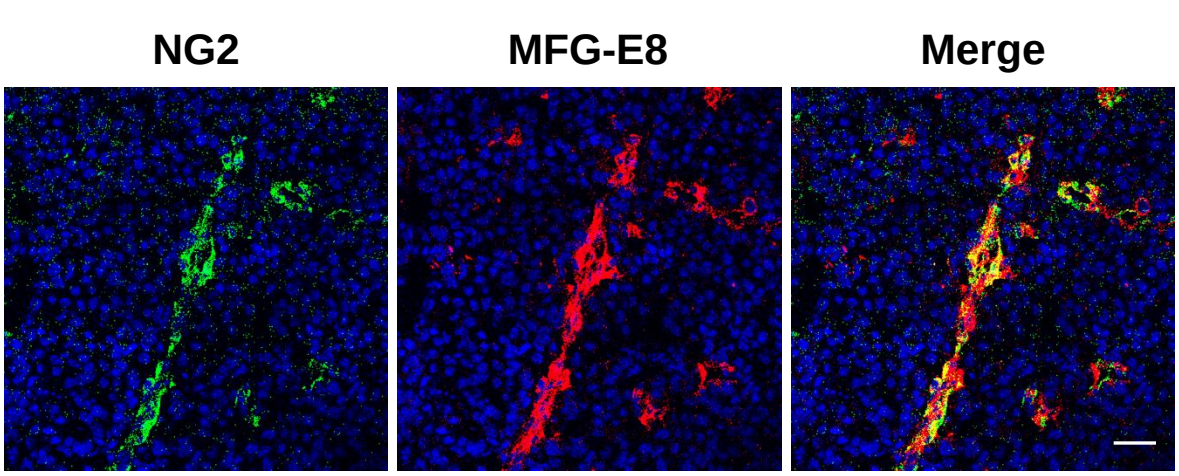

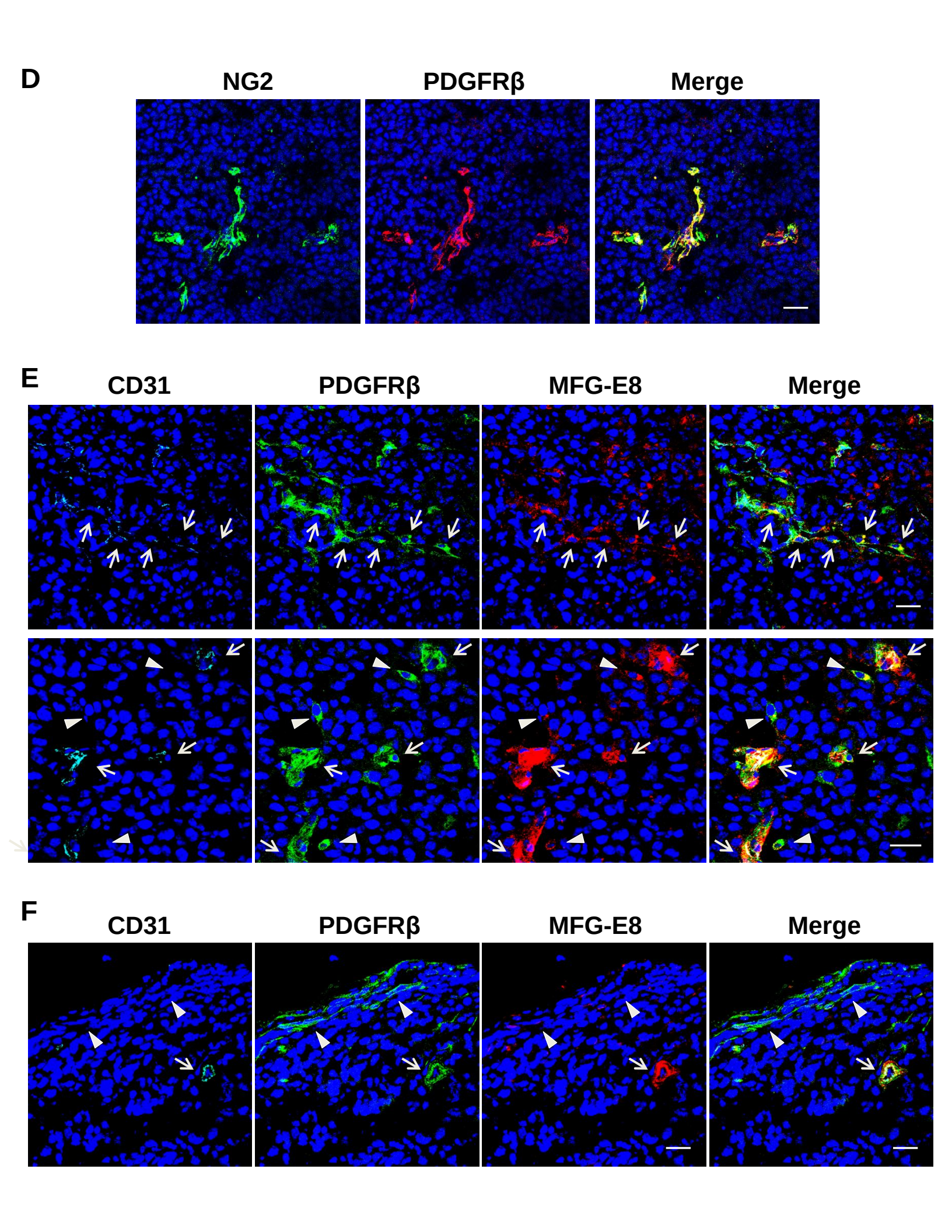
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NG2
PDGFRβ
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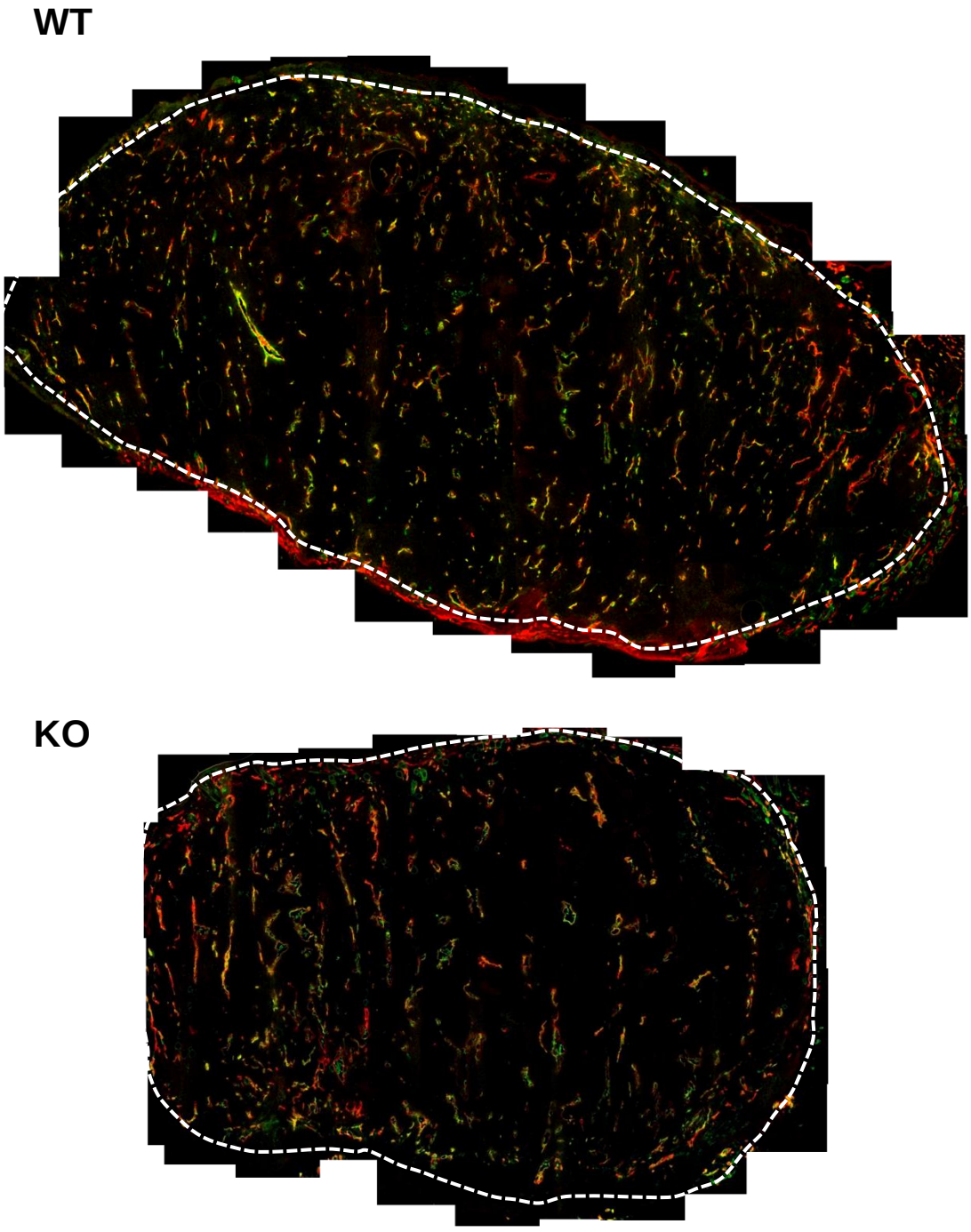
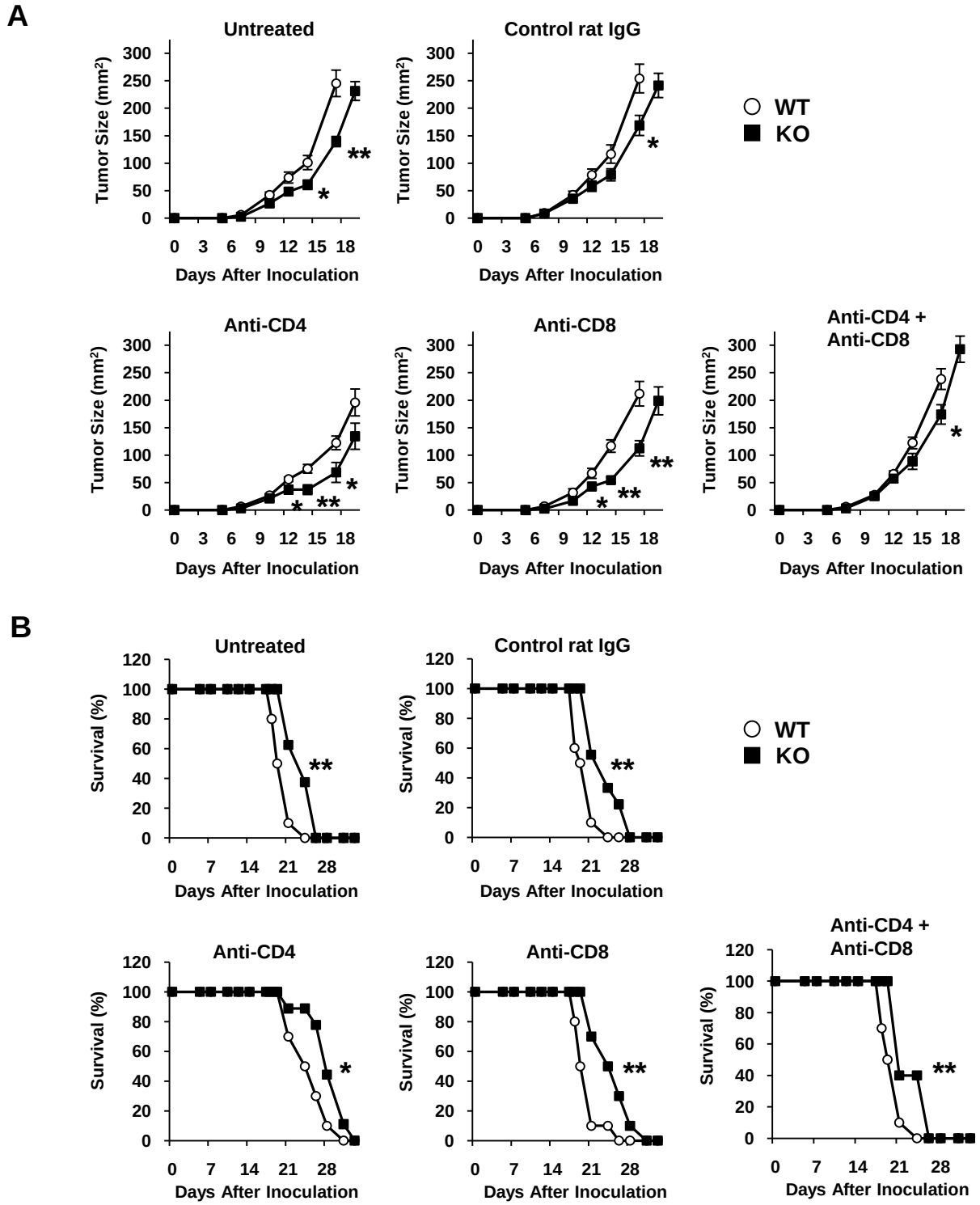
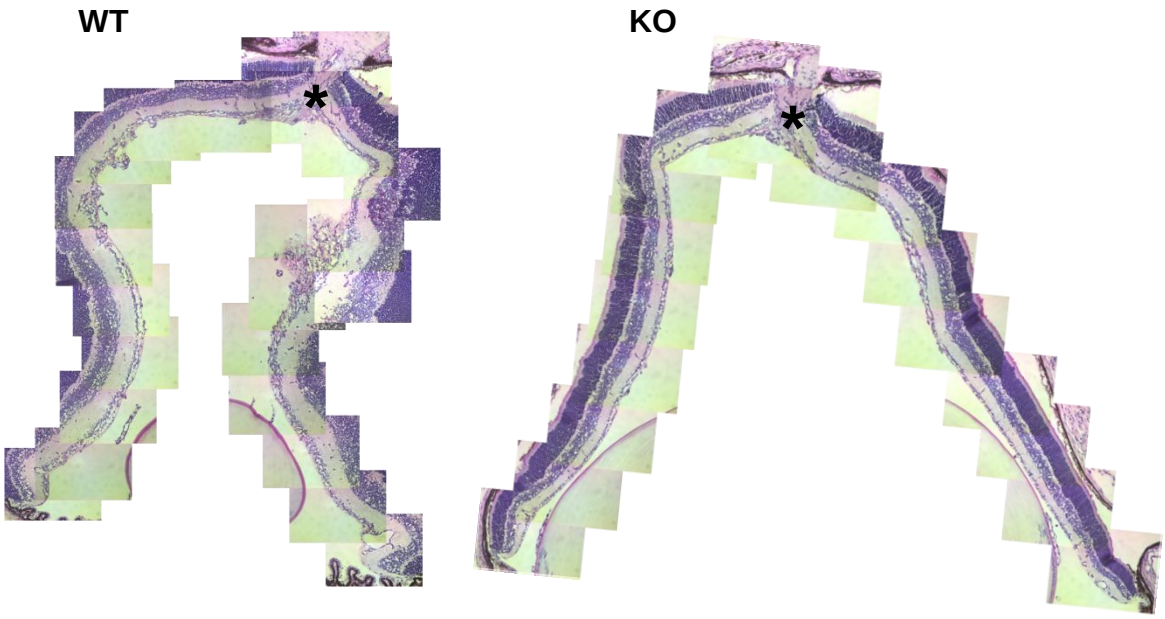
Supplemental Figure I. MFG-E8 localizes in close proximity to pericytes in tumors.
(A) Localization of MFG-E8 in relationship to EC (CD31+) in low magnification view of B16 melanoma (Bar=500 μ).
(B) Localization of MFG-E8 in relationship to EC (CD31+) in B16 melanoma (Arrow: MFG-E8+ CD31- cells, Bar=50 μ).
(C) Localization of MFG-E8 in relationship to PC (NG2+) in B16 melanoma (Bar=50 μ).
(D) Localization of PDGFRβ+ and NG2+ PC in B16 melanoma (Bar=50 μ).
(E) Localization of MFG-E8 in relationship to PC (PDGFRβ+) and EC (CD31+) in B16 melanoma (Arrow: PDGFRβ+ MFG-E8+ PC, Arrowhead: PDGFRβ+ MFG-E8+ stromal cells, Bar=50 μ).
(F) Localization of MFG-E8 in relationship to PDGFRβ+ stromal cells or PDGFRβ+ PC in the periphery of B16 melanoma (Arrow: PDGFRβ+ MFG-E8+ PC, Arrowhead: PDGFRβ+ MFG-E8- spindle cells, Bar=50 μ).



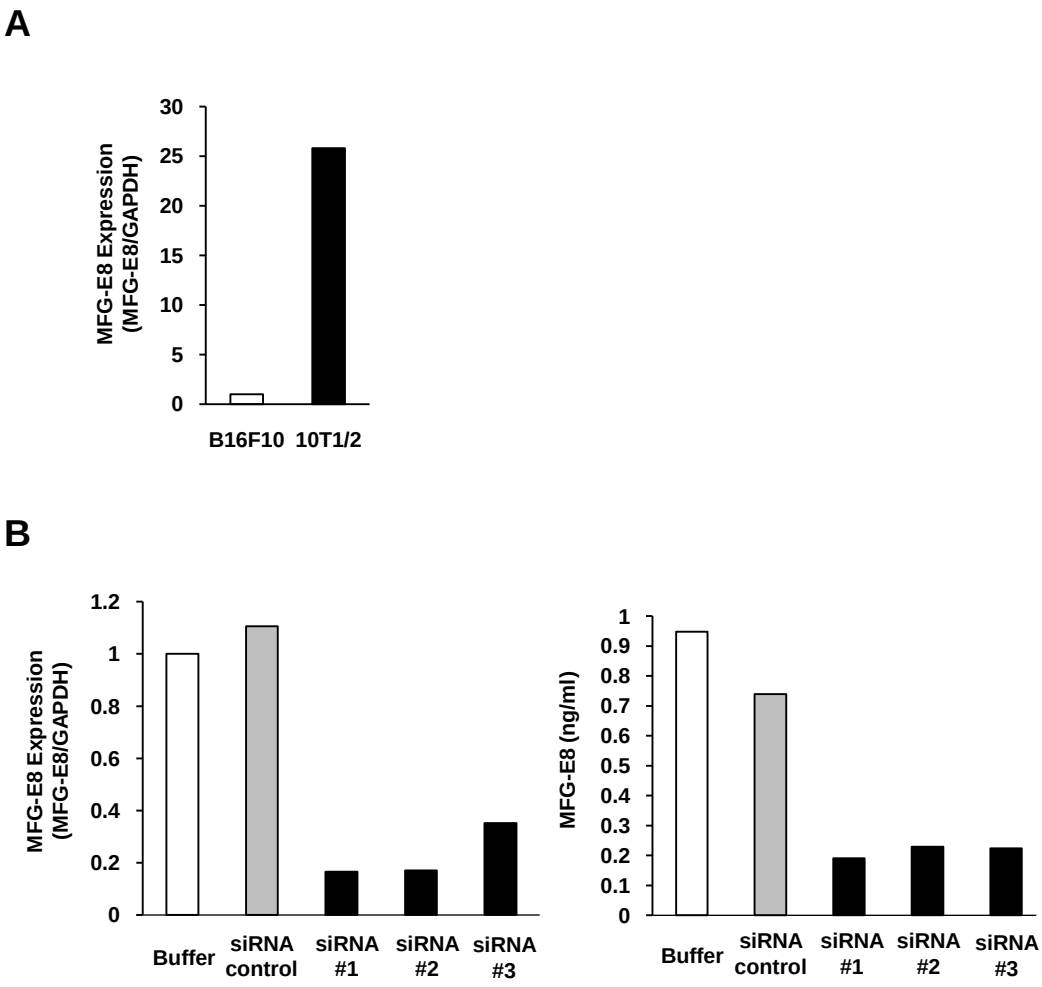
Supplemental Figure II. Tumor-associated blood vessels in wild type and MFG-E8 knockout mice.
Composite views of tumor-associated vessels in 1 cm B16 melanomas growing in WT and MFG-E8 KO C57BL/6 mice (CD31 immunofluorescence staining in red and NG2 staining in green).



Supplemental Figure III. Melanoma growth delay and extended survival in lymphocyte-depleted wild type and MFG-E8 knockout mice. WT and KO mice were treated with lymphocyte-depleting anti-CD4 mAb and/or anti-CD8 mAb such that depletion of circulating lymphocytes was maintained at >95%. Tumor sizes were determined as indicated (A) and mice were euthanized when humane endpoints were reached (B) (*$p< 0.05$; **$p<0.01$; data are representative of n=2 experiments (n=8-10 mice in each group)).

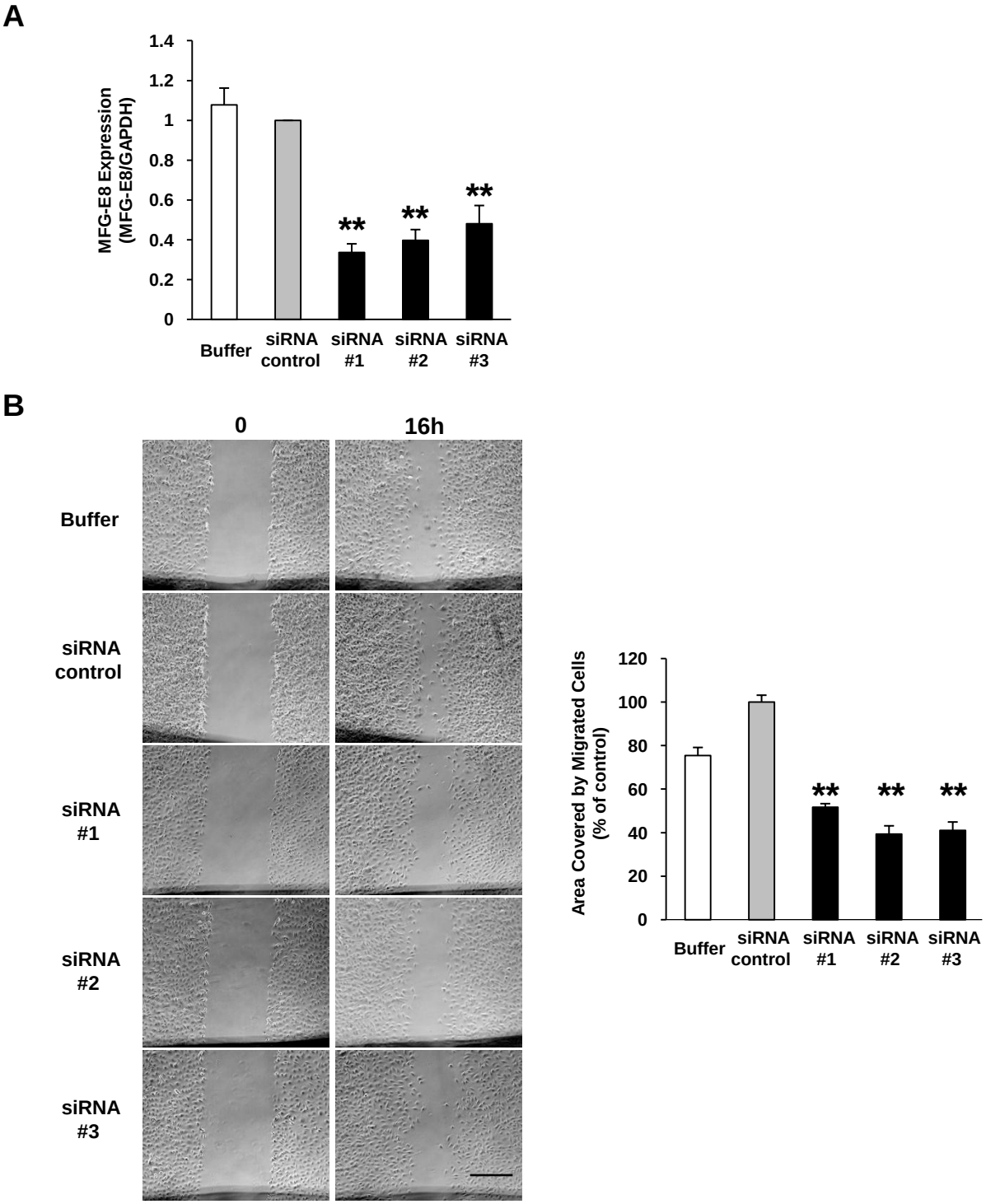


Supplemental Figure IV. Angiogenesis associated with oxygen-induced retinopathy in wild type and MFG-E8 knockout mice Composite views of retinal axial sections obtained from littermate control and MFG-E8 KO mice on day P17 after initiation of the oxygen induced retinopathy protocol described in Methods (* identifies optic nerve).

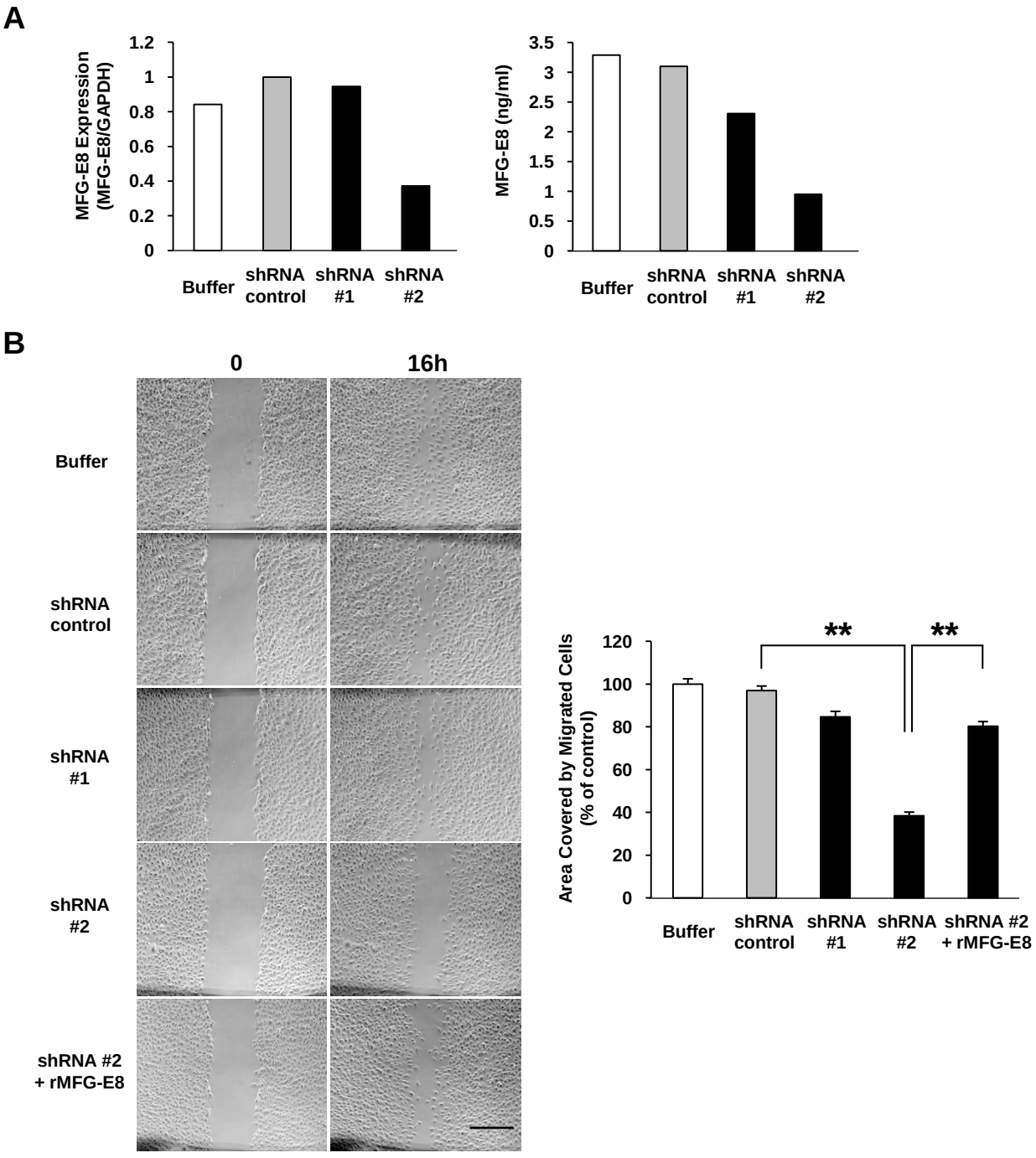


Supplemental Figure V. Expression of MFG-E8 mRNA and protein by 10T1/2 cells and modulation by siRNAs.

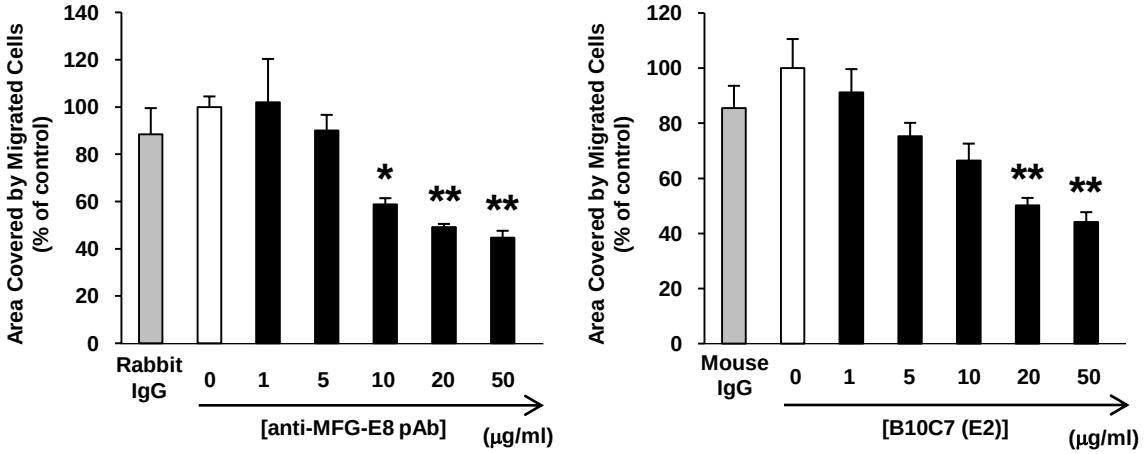
(A) Comparison of MFG-E8 mRNA levels in 10T1/2 and B16 melanoma cells using quantitative RT-PCR (normalized to GAPDH mRNA levels in each cell type). (B) Effects of MFG-E8 and control siRNAs on MFG-E8 mRNA (measured using quantitative RT-PCR), and protein secreted into the media during a 24 hour incubation (assessed via ELISA) in a single experiment 48 hours after transfection of 10T1/2 cells.



Supplemental Figure VI. Inhibition of 10T1/2 cell migration by MFG-E8 siRNAs.
(A) Documentation of inhibition of MFG-E8 mRNA expression 48 hours after transfection of 10T1/2 cells using quantitative RT-PCR (**p< 0.01 relative to siRNA control; Values determined in 3 independent "scratch assay" experiments). (B) Inhibition of 10T1/2 cell migration in a "scratch" (*in vitro* wound healing) assay as documented in phase contrast photomicrographs (bar= 0.5 mm) and quantified via analysis of digital images using Image J software (**p<0.01, representative of n=3 experiments).

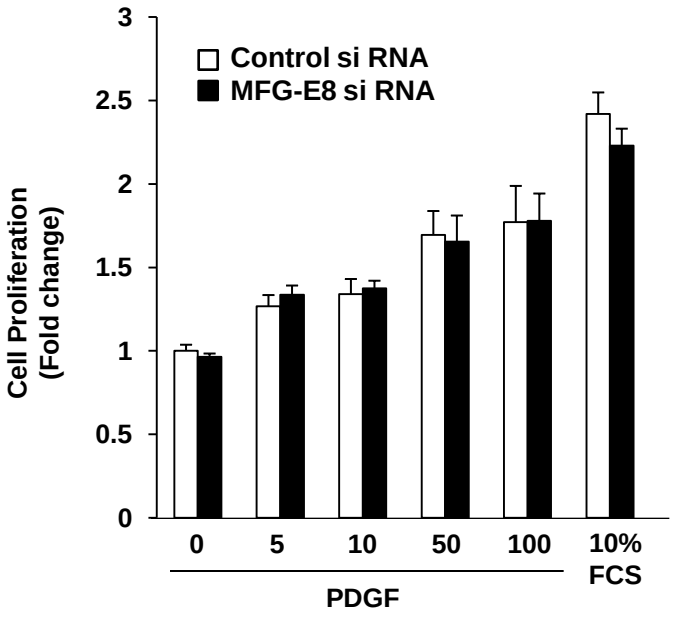


Supplemental Figure VII. Inhibition of 10T1/2 cell migration by MFG-E8 shRNAs and reversal with recombinant MFG-E8. (A) Documentation of inhibition of MFG-E8 expression in a single experimet after selection of long-term 10T1/2 transductants expressing MFG-E8 shRNAs using quantitative RT-PCR and ELISA. (B) Inhibition of 10T1/2 cell migration in "scratch" assays as documented in photomicrographs (bar= 0.5 mm), quantification via analysis of digital images using Image J software and reversal with recombinant MFG-E8 protein (100 ng/ml) (**p<0.01, representative of n=2 experiments).

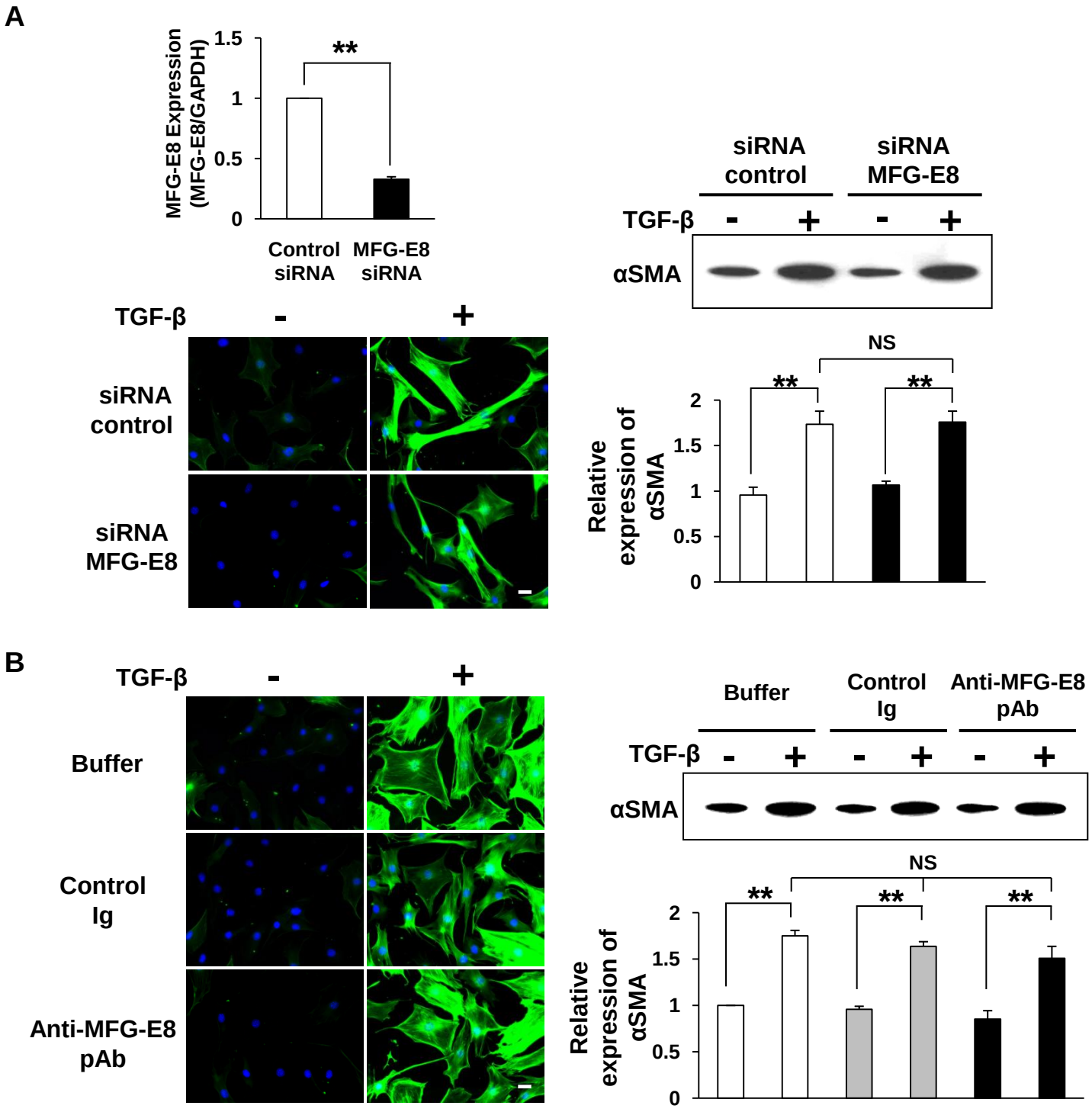


Supplemental Figure VIII. Dose-dependent of inhibition of 10T1/2 cell migration by anti-MFG-E8 Ab.

PDGF-B-induced 10T1/2 cell migration was assessed in a FluoroBlok™ migration assay in the presence and the absence of anti-MFG-E8 mAb and pAb or appropriate control IgG (20 μg/ml) as indicated. Ab were added into lower and upper chambers 20 minutes prior to addition of PDGF-B (50 ng/ml) and migration was quantified 4 hours later via image analysis (*p< 0.05; **p< 0.01).



Supplemental Figure IX. Lack of effect of MFG-E8 on 10T1/2 cell proliferation. PDGF-induced proliferation of 10T1/2 cells transfected with siRNA targeting MFG-E8 or control siRNA. Control siRNA transfected cells cultured in medium without PDGF for 48 hours were assigned a value of 1. Data are means±SEM from n=3 experiments performed in triplicate.



Supplemental Figure X. Lack of effect of MFG-E8 on 10T1/2 cell differentiation. (A) Documentation of inhibition of MFG-E8 mRNA expression 48 hours after transfection of 10T1/2 cells with siRNA using quantitative RT-PCR (**p< 0.01 relative to siRNA control; Values determined in 3 independent "differentiation" experiments). Immunofluorescence photomicrographs depicting TGF-β-induced αSMA expression in 10T1/2 cells transfected with siRNA targeting MFG-E8 or control siRNA (Bar=40 μ). TGF-β-induced expression of αSMA protein in 10T1/2 cells transfected with siRNA targeting MFG-E8 or control siRNA (Western Blot). Quantification of αSMA expression in digitized images using Image J (Histogram). The amount of αSMA in control siRNA transfected cells cultured in medium without TGF-β was assigned a value of 1 (**p<0.01; values determined in 3 independent experiments, NS = not significant). (B) Immunofluorescence photomicrographs depicting TGF-β-induced αSMA expression in 10T1/2 cells treated with control Ig or anti-MFG-E8 pAb (Bar=40 μ). TGF-β-induced expression of αSMA protein in 10T1/2 cells treated with control Ig or anti-MFG-E8 pAb (Western Blot). Quantification of αSMA expression in digitized images using Image J (Histogram). The amount of αSMA in cells cultured in medium without Ab and without TGF-β was assigned a value of 1 (**p<0.01; values determined in 3 independent experiments, NS = not significant).