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Online data supplement

Role of Protein Tyrosine Phosphatase 1B in VEGF Signaling and Cell-Cell Adhesions in Endothelial Cells

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Running title: Role of PTP1B in VEGF signaling

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Materials and Methods

Materials- Antibodies to VEGFR2, phosphotyrosine (pY99), VE-cadherin, and phospho-PLCγ (pY783), actin and GAPDH were from Santa Cruz. Antibodies to phospho-VEGFR2 (pY1175), phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-p38 mitogen-activated protein kinase (MAPK), ERK1/2, and p38 MAPK were from Cell Signaling. Anti-PTP1B monoclonal antibody and rabbit polyclonal antibody were from Calbiochem and Upstate, respectively. Human recombinant VEGF165 was from R&D Systems and BRB Preclinical Repository. Oligofectamine, and Opti-MEMI Reduced-Serum Medium were from Invitrogen Corp. CellTiter 96® solution cell proliferation assay was obtained from Promega. Other materials including anti-vinculin antibody were purchased from Sigma.

Cell Culture- Human umbilical vein ECs (HUVECs) were purchased from VEC Technologies, Inc. (Rensselaer, NY) and were grown in endothelial basal medium2 (EBM2, Clonetics) containing 10% fetal bovine serum (FBS) as described [1]. Experiments were performed using cells between passages 3 and 6.

Immunoprecipitation and Immunoblotting- Growth-arrested HUVECs were stimulated with VEGF (10 ng/ml) and cells were lysed in lysis buffer, pH 7.4 (in mM) 50 HEPES, 5 EDTA, 50 NaCl), 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) and phosphatase inhibitors ((in mmol/L) 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate). Cell lysates were used for immunoprecipitation and immunoblotting as described previously [2].

Transient Transfection of CHO cells- CHO cells were transiently transfected with pcDNA3-myc-VEGFFR2cyto (entire intracellular domain of human KDR, residues 790-1356) and pMT2-GST-PTP1B-WT or PTP1B-D181A or PTP1B-C215S which were kindly provided by Dr. Nicholas Tonks [3] using the Polyfect according to manufacturer's instruction (Qiagene).

Adenovirus Transduction - HUVECs were incubated with 10 multiples of infection (MOI) of either adenovirus expressing wild-type PTP1B (Ad.PTP1B-WT) or PTP1B-C/S (catalytially inactive C215S mutant; Ad.PTP1B-C/S) or Ad.LacZ (control) in 10% FBS

containing culture medium for 24 hr, followed by incubation in 0.5%FBS without virus for 12 hr before experiments, as we described previously [4].

siRNA Transfection- RNA oligonucleotides were obtained from Sigma The sequences of specific siRNA against PTP1B is; 5'-AAATCAACGGAAGAAGGGTCT-3'. The scrambled siRNA control is 5'-GAGATGACACGACUGAGATAA-3'. We performed a Blast search and confirmed that the PTP1B and scrambled siRNA sequences have no overlap with other proteins. HUVECs were grown to 60 % confluence in 100 mm dishes and transfected with 30 nM siRNA using Oligofectamine (Invitrogen), as described previously [5]. Cells were used for experiments at 48 hr after transfection.

PTP1B Activity Assay- HUVEC cells or mice hindlimb muscles were snap-frozen in liquid N_2, and disrupted by scraping into ice-cold, deoxygenated homogenization buffer (150 mM NaCl, 5 mM EDTA, 5 mM EGTA, in 50 mM Hepes, pH 7.5, containing a protease inhibitor mixture (Sigma), 1% (v/v) Triton X-100, and 0.5% (v/v) NP-40, followed by brief sonication. The whole cell lysate was cleared by centrifugation at 15,000 g for 20 min. Specific PTP1B activity was measured by the hydrolysis of p-nitrophenyl phosphate(pNPP; Sigma) in PTP1B immunoprecipitates. Briefly, PTP1B immunoprecipitates from 500 μ g of cell lysates were incubated in a final volume of 100 μ l at 37 °C for 30 min in reaction buffer containing 10 mM pNPP and 2 mM EDTA in 20 mM MES at pH 6.0. The reaction was stopped by the addition of 200 μ l of 5 M NaOH, and the absorption was determined at 410 nm[6].

In Vivo Receptor Dephosphorylation Assays- Growth-arrested HUVECs were stimulated with VEGF for 5min, and cell lysates were immunoprecipitated with rabbit anti-VEFGR2 antibody and immobilized on protein A/G agarose for 1.5 hr. Beads were washed and incubated in the presence of recombinant active PTP1B protein (Biomol, 50-200ng) at 30 ^{o}C for 10 min in 50 µl of buffer (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 12 mM MgCl, 1 mM dithiothreitol, 10 µM ATP). The reaction was stopped by adding 2x sample buffer and samples were immunoblotted with anti-phosphotyrosine or VEGFR2 antibodies.

Cell Proliferation Assay- HUVECs (10^5 cells) were seeded in 6-well plates in EBM containing 10% FBS overnight, and incubated in EBM containing 0.5% FBS for 24 hours and then incubated with or without stimulants in EBM containing 0.2% FBS for 48 hours. After culturing, 20 μl of an CellTiter 96® solution was added to each well, and the absorbance was measured at 490 nm according to the manufacturer's instructions after incubation at 37°C for 2-3 h. In some experiments, after trypsinization, the cell number was determined by counting with a hemocytometer [1].

Modified Boyden Chamber Migration Assay- Migration assays using a Modified Boyden Chamber method were conducted in 24-well transwell chambers as described previously [1].

Confocal Immunofluorescence Microscopy- HUVECs growing on 0.1% gelatin-coated glass coverslips were stimulated with VEGF or vehicle alone, and fixed with 4% paraformaldehyde in PBS, and permeabilized in 0.05% Triton X-100/PBS for 5 min. After blocking, cells were incubated with rabbit anti-VE-cadherin antibody for 1 hr, incubated in FITC-conjugated goat anti-rabbit IgG for 1 hr. Images were taken using the confocal laser scanning imaging system Zeiss LSM 510. In some experiments, HUVECs were transiently transfected with plasmid encoding GFP-PTP1B-D/A or GFP only which were kindly provided by Dr. Carlos Arregui [7] using Amaxia Nucleofector System according to manufacturer's instruction, as reported previously [8]. After transfection, cells were stimulated with VEGF, fixed, permeabilized, and were incubated with anti-VE-cadherin antibody, followed by Rhodamine Red X-conjugated goat anti-rabbit IgG..

Transendothelial Electrical Resistance Measurement- The time course of endothelial cell retraction in real time, as a measure of increased endothelial permeability, was recorded as described previously [8].

Mouse Ischemic Hindlimb Model- Study protocols were approved by the Animal Care and Use Committee of University of Illinois at Chicago. Female C57BL/6J mice (8-9 weeks of age) were obtained from The Jackson Laboratory. The right superficial femoral artery was ligated proximally and distally with 5-0 silk ligatures, and excised. To measure hind limb blood flow we used a laser Doppler blood flow (LDBF) analyzer (Lisca AB,

Sweden) as described previously [9]. At 0, 1, 3 7 days after ischemia, thigh adductor muscle in ischemic hindlimbs were used for immunoblotting and immunohistochemistry as described previously [4, 9, 10].

Statistical Analysis- Results are expressed as mean ± S.E. Statistical significance was assessed by Student's paired two-tailed t-test or analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, using the Super ANOVA statistical program (Abacus Concepts, Berkeley, CA). A p value of <0.05 was considered to be statistically significant.

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Figure legends for Supplemental Figures

Supplemental Figure I. PTP1B overexpression inhibits while PTP1B knockdown enhances VEGF-induced phosphorylation of VEGFR2-Tyr1175, PLCγ1 and ERK1/2. HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S (A) or transiently transfected with scrambled (control) or PTP1B siRNAs (B) were stimulated with VEGF (20 ng/ml) for 5 min. Lysates were immunoblotted with anti-phospho-VEGFR2 (pY1175) or phospho-PLCγ1 (pY783) or phospho-ERK1/2 antibodies. Bottom panels show averaged data, expressed as fold change of phosphorylation over basal (means ± S.E., n=3). *P < 0.05 for VEGF-induced changes in cells infected with Ad.PTP or transfected with PTP1B siRNA vs control.

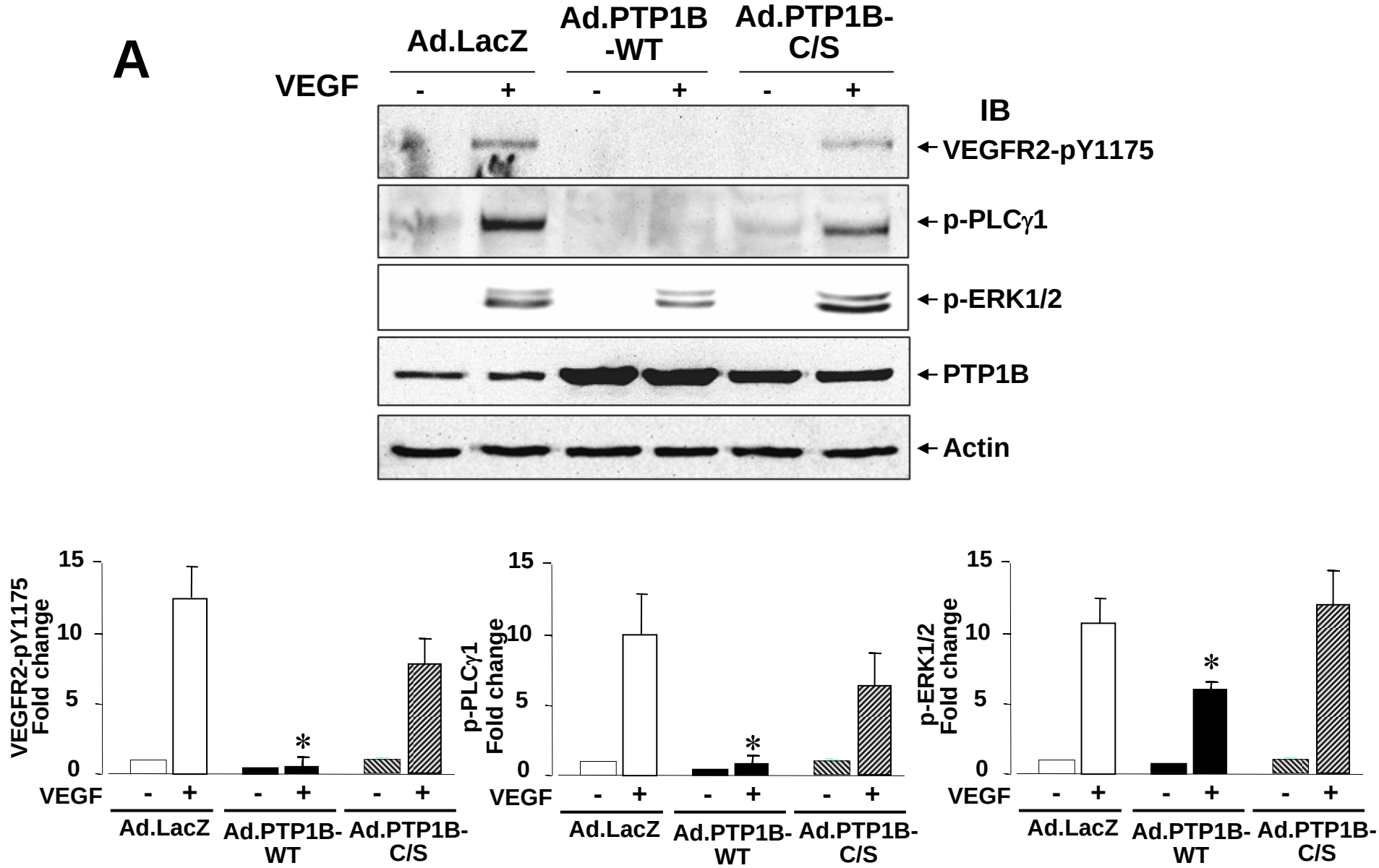
Supplemental Figure II. PTP1B overexpression or knockdown has no effects on VEGF-induced cell migration. HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S (A) or transiently transfected with scrambled or PTP1B siRNAs (B) were used for measurement of cell migration with the modified Boyden chamber method. Cells were stimulated with 50 ng/ml VEGF for 6 hours. Bar graph represents averaged data, expressed as cell number counted per 10 fields (X200) and fold change in cell number over that in unstimulated LacZ-infected (A) or scrambled siRNA-transfected (B) cells (control).

Supplemental Figure III. PTP1B negatively regulates tyrosine phosphorylation of VE-cadherin through binding to VE-cadherin. HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S (A) or transiently transfected with scrambled or IQGAP1 siRNAs (B) were stimulated with VEGF (20 ng/ml). Lysates were immunoprecipitated with anti-pTyr antibody, and then immunoblotted with VE-cadherin antibody, and averaged data for tyrosine phosphorylation of VE-cadherin, as expressed by mean±SE for 3 independent experiments are shown. C, HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S were stimulated with VEGF (20 ng/ml) for 5 min, and lysates were immunoprecipitated with anti-PTP1B antibody, and then immunoblotted with VE-cadherin antibody. The blots are representative of 3 separate

experiments.

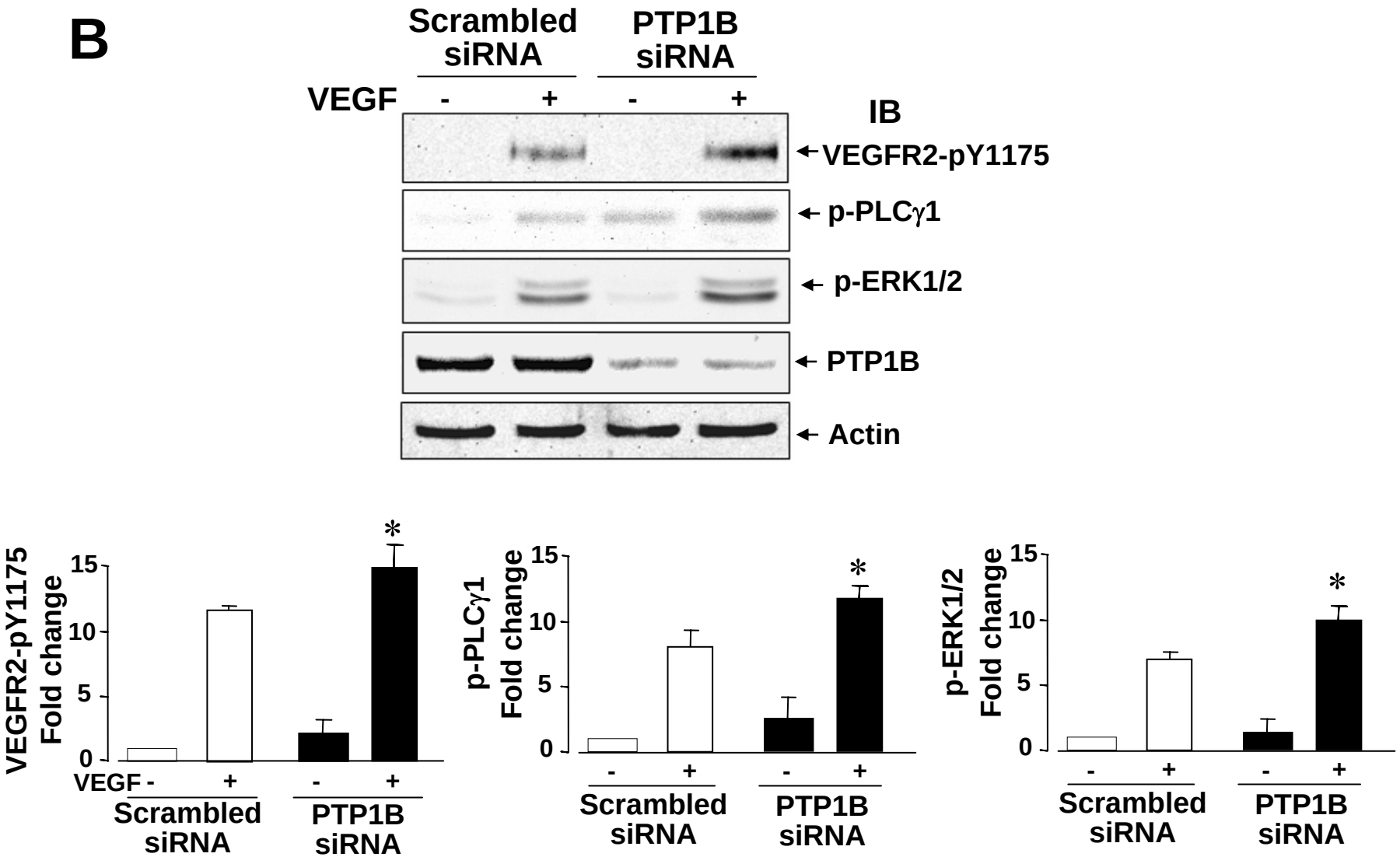
Supplemental Figure IV. Increase of PTP1B expression at lectin-positive ECs and skeletal myocyte in mouse ischemic hindlimb model of angiogenesis. Hindlimb ischemia was induced by the right femoral artery ligation as described in Materials and Methods. Immunostaining of ischemic adductor skeletal muscle with anti-PTP1B antibody (green) or lectin which stains ECs of capillaries (red) at 0, 3 and 7 days after ischemia was visualized with a confocal fluorescence microscope. NC indicates staining without primary antibody (negative control).

Supplemental Figure I

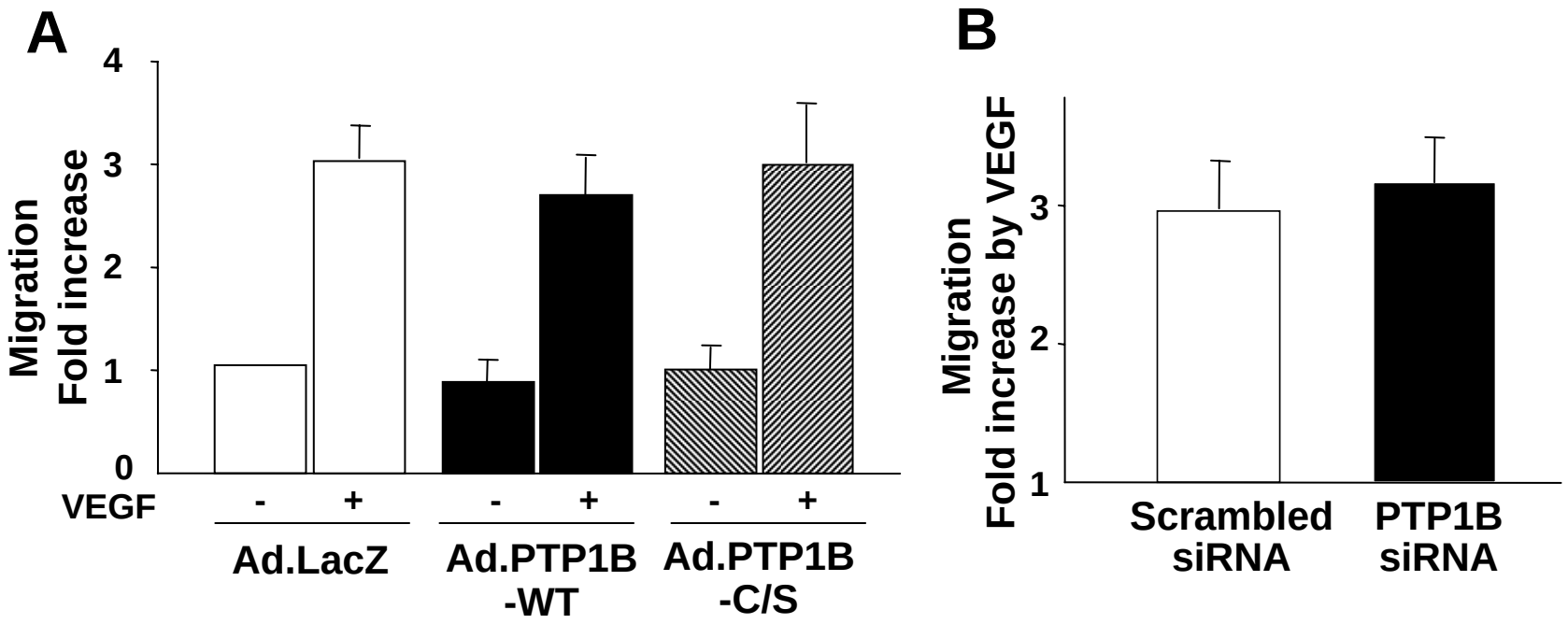


Supplemental Figure I

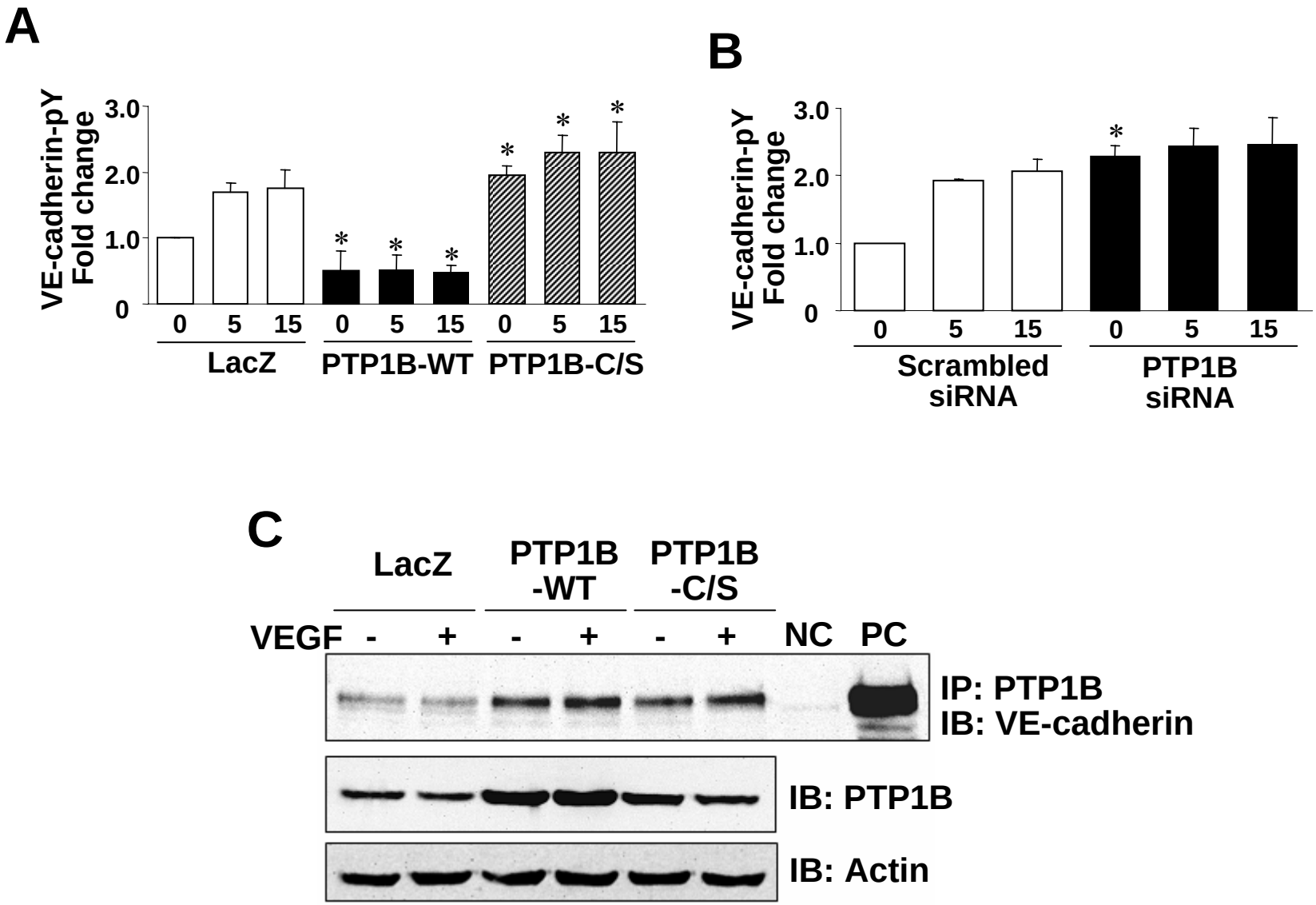
B



Supplemental Figure II



Supplemental Figure III



Supplemental Figure IV