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

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Abstract

Vascular endothelial growth factor (VEGF) binding induces phosphorylation of VEGF receptor-2 (VEGFR2) in tyrosine, which is followed by disruption of VE-cadherin-mediated cell-cell contacts of endothelial cells (ECs), thereby stimulating EC proliferation and migration to promote angiogenesis. Tyrosine phosphorylation events are controlled by the balance of activation of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Little is known about the role of endogenous PTPs in VEGF signaling in ECs. In this study, we found that PTP1B expression and activity are markedly increased in mice hindlimb ischemia model of angiogenesis. In ECs overexpression of PTP1B, but not catalytically inactive mutant PTP1B-C/S, inhibits VEGF-induced phosphorylation of VEGFR2 and ERK1/2 as well as EC proliferation, while knockdown of PTP1B by siRNA enhances these responses, suggesting that PTP1B negatively regulates VEGFR2 signaling in ECs. VEGF-induced p38MAP kinase phosphorylation and EC migration are not affected by PTP1B overexpression or knockdown. *In vivo* dephosphorylation and co-transfection assays reveal that PTP1B binds to VEGFR2 cytoplasmic domain *in vivo*, and directly dephosphorylates activated VEGFR2 immunoprecipitates from HUVECs. Overexpression of PTP1B stabilizes VE-cadherin-mediated cell-cell adhesions by reducing VE-cadherin tyrosine phosphorylation, while PTP1B siRNA causes opposite effects with increasing endothelial permeability as measured by transendothelial electrical resistance. In summary, PTP1B negatively regulates VEGFR2 receptor activation via binding to the VEGFR2 as well as stabilizes cell-cell adhesions through reducing tyrosine phosphorylation of VE-cadherin. Induction of PTP1B by hindlimb ischemia may represent important counter-regulatory mechanism that blunts over-activation of VEGFR2 during angiogenesis *in vivo*.

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Keywords

Protein tyrosine phosphatase 1B; vascular endothelial growth factor; endothelial cell; cell-cell adhesions; angiogenesis

Introduction

Vascular endothelial growth factor (VEGF) induces angiogenesis by stimulating endothelial cell (EC) migration and proliferation primarily through the VEGF type2 receptor (VEGFR2, KDR/Flk-1)[1]. VEGF binding initiates autophosphorylation of VEGFR2, which in turn creates docking sites for Src homology domain-2 (SH2) domain containing adaptor molecules. This event is followed by activation of diverse key angiogenic enzymes such as ERK1/2 and p38 MAP kinase which are involved in EC proliferation and migration, respectively [2, 3]. One of the initial responses of quiescent ECs to induce angiogenesis is the loss of established cell-cell contacts, which is followed by EC migration, proliferation and formation of capillary tube networks. The molecule primarily responsible for cell-cell adhesions of ECs is the transmembrane homophilic adhesion molecule, vascular endothelial (VE)-cadherin whose tyrosine phosphorylation is important for disruption of cell-cell contacts [4]. Thus, tyrosine phosphorylation of VEGFR2 and VE-cadherin is important initial signaling events by which VEGF stimulates angiogenesis in ECs. Tyrosine phosphorylation events are controlled by the balance of activation of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). PTP inhibitors accelerate neovascularization in rat ischemia hindlimb models [5, 6]; however, little is known about role of endogenous PTPs in VEGF signaling in ECs.

In preliminary studies to identify PTPs involved in angiogenesis *in vivo*, we identified PTP1B as one of the major PTP whose expression is dramatically induced during angiogenesis in a mouse ischemia hindlimb model. PTP1B is the first PTP isolated in homogenous form, and is ubiquitously expressed enzymes [7]. Studies using PTP1B-deficient mice show that PTP1B is a key negative regulator of insulin and leptin signaling and a therapeutic target for the type 2 diabetes and obesty [8, 9]. PTP1B is also implicated in growth factor- [10] and integrin- [11] mediated signaling. Furthermore, PTP1B binds to E- and N-cadherin and regulates the cell-cell adhesions in neural retina cells and epithelial cells, respectively [12, 13]. However, the specific role of PTP1B in VEGF signaling and VE-cadherin-mediated cell-cell adhesions in ECs is unknown.

We performed the present study to test the hypothesis that PTP1B may play an important role in VEGF signaling linked to angiogenesis. Using overexpression and knockdown strategies, we demonstrate that PTP1B negatively regulates VEGFR2 receptor autophosphorylation via binding to the VEGFR2, which in turn inhibits downstream ERK1/2 phosphorylation and EC proliferation. We also found that PTP1B associates with VE-cadherin, thereby reducing its tyrosine phosphorylation, which may contribute to stabilizing cell-cell adhesions. PTP1B expression and activity are markedly increased in a mouse hindlimb ischemia model, which may represent important counter-regulatory mechanism that blunts over-activation of VEGFR2 during angiogenesis *in vivo*.

Materials and Methods

Materials, cell culture, immunoprecipitation and immunoblotting, transient transfection of CHO cells, Adenovirus transduction, siRNA transfection, PTPase activity assay, In vivo receptor dephosphorylation assays, cell proliferation and migration assays, confocal immunofluorescence microscopy, transendothelial electrical resistance (TER) measurement, mouse ischemic hindlimb model and statistical analyses are described in the Material and Methods section in the online data supplement.

Results

PTP1B overexpression inhibits while PTP1B knockdown enhances VEGF-induced autophosphorylation of VEGFR

To determine the function of PTP1B in VEGF signaling, we examined the effect of PTP1B overexpression on VEGFR2 autophosphorylation in HUVECs. Figure 1 shows that transduction of adenovirus expressing wild-type PTP1B (Ad.PTP1B-WT) significantly increased PTP1B protein expression and activity (Fig. 1A), and inhibited VEGF-induced VEGFR2 tyrosine phosphorylation without affecting VEGFR2 expression (Fig. 1B). In contrast, Ad.PTP1B-C/S (catalytically inactive C215S mutant), which increased PTP1B protein expression at similar extent as PTP1B-WT but had no effect on PTP1B activity (Fig. 1A), significantly enhanced VEGF-induced phosphorylation of VEGFR2 (Fig. 1B). These results suggest that overexpression of PTP1B inhibits VEGFR2 phosphorylation in a catalytic activity-dependent manner, and that PTP1B-C/S protects from dephosphorylation by endogenous PTP1B. In contrast, Figure 2 shows that knockdown of endogenous PTP1B protein and specific activity with siRNA (Fig. 2A) enhanced VEGF-induced VEGFR2 tyrosine phosphorylation (Fig. 2B). These results suggest that PTP1B functions as negative regulator for VEGFR2 autophosphorylation.

PTP1B dephosphorylates and associates with VEGFR2

To determine if VEGFR2 can serve as a substrate for PTP1B, we performed *in vivo* dephosphorylation assay. Figure 3A shows that recombinant active PTP1B protein dephosphorylated VEGFR2 obtained from VEGFR2 immunoprecipitates of VEGF-stimulated HUVEC lysates, in a dose-dependent manner. To assess the mechanism by which PTP1B negatively regulates VEGFR2 phosphorylation, we examined whether PTP1B physically interacts to VEGFR2. Figure 3B shows that PTP1B was co-immunoprecipitated with VEGFR2 in HUVECs infected with Ad.PTP1B-WT, but not with Ad.LacZ, in basal state and after VEGF stimulation. These results suggest that PTP1B associates with VEGFR2 in a phosphotyrosine-independent manner, and that endogenous PTP1B interaction with VEGFR2 is unstable to be detected by co-precipitation. To confirm further their interaction *in vivo*, GST-tagged PTP1B-WT or substrate-trapping mutants PTP1B-D/A (D181A) or PTP1B-C/S, and Myc-tagged entire intracellular domain of human VEGFR2 (VEGFR2cyto, residues 790-1356) [14] were co-transfected in CHO cells. Figure 3C shows that either PTP1B-WT or PTP1B-D/A or PTP1B-C/S, but not empty vector alone, co-precipitated with Myc-VEGFR2cyto. These results suggest that PTP1B associates with VEGFR2 kinase domain, independent of phosphorylation status of the receptor and phosphatase activity.

PTP1B overexpression inhibits while PTP1B knockdown enhances VEGF-induced phosphorylation of ERK1/2, but not p38MAPK

To characterize further the effect of PTP1B on VEGF signaling, we examined the effect of overexpression of PTP1B on phosphorylation of downstream signaling such as ERK1/2 and p38MAPK, which are involved in endothelial proliferation and migration, respectively [2, 3]. Figure 4A shows that Ad.PTP1B-WT significantly inhibited, while Ad.PTP1B-C/S slightly enhanced, VEGF-induced ERK1/2 phosphorylation without affecting p38MAPK phosphorylation. Conversely, PTP1B siRNA significantly enhanced VEGF-induced phosphorylation of ERK1/2 without affecting p38MAPK phosphorylation (Fig. 4B). We also found that PTP1B siRNA has no effect on Akt phosphorylation (data not shown). These results suggest that PTP1B is endogenous negative regulator for VEGFR2-ERK1/2 pathway. This notion is further supported by the observation that overexpression of PTP1B-WT inhibited, but PTP1B siRNA enhanced, VEGF-stimulated phosphorylation of VEGFR2 at Tyr1175 and PLCγ, which are upstream of ERK1/2 [2] (Supplemental Figure I).

PTP1B negatively regulates VEGF-stimulated EC proliferation

Since VEGF-induced ERK1/2 is involved in EC proliferation [2], we examined the effects of overexpression of PTP1B and PTP1B siRNA on this response using MTT assay. Figure 5A shows that Ad.PTP1B-WT, but not either LacZ or PTP1B-C/S, significantly inhibited VEGF-stimulated EC proliferation. Either treatment did not affect on basal proliferation. Conversely, PTP1B siRNA significantly enhanced VEGF-stimulated EC proliferation without affecting basal response (Fig. 5B). Similar responses are obtained by cell number measurement (data not shown). In contrast, both PTP1B overexpression and knockdown had no effects on VEGF-mediated migration (Supplemental Figure II) and cell survival (data not shown), which are dependent on p38MAPK [3] and Akt, respectively.

PTP1B stabilizes VE-cadherin-mediated cell-cell adhesion

Disruption of cell-cell contacts by reducing VE-cadherin from the cell-cell adhesion sites or tyrosine phosphorylation of VE-cadherin is important for initiating angiogenesis [4]. We thus examined the role of PTP1B in localization of VE-cadherin at adherence junctions in confluent monolayer of ECs. Figure 6A shows that Ad.PTP1B-WT increased VE-cadherin staining at sites of cell-cell contact, while Ad.PTP1B-C/S markedly reduced it, resulting in small gaps between adjacent cells in basal state. VEGF stimulation reduced VE-cadherin staining at sites of cell-cell contact in LacZ-infected cells, which was prevented by PTP1B-WT while further decreased by PTP1B-C/S. Furthermore, basal and VEGF-induced tyrosine phosphorylation of VE-cadherin was significantly reduced by overexpression of PTP1B-WT, while enhanced by PTP1B-C/S (Fig. 6B and Supplemental Figure IIIA). Conversely, PTP1B siRNA markedly reduced VE-cadherin staining at cell-cell contacts and significantly enhanced basal and VEGF-induced tyrosine phosphorylation of VE-cadherin (Fig. 7AB and Supplemental Figure IIIB). We also determined transendothelial electric resistance (TER), a measure of intercellular adhesion between ECs and found that PTP1B-WT significantly increased, while PTP1B-C/S and PTP1B siRNA significantly decreased TER (Fig. 6C and 7C). Either Ad.PTP1B-WT or Ad.PTP1B-C/S or PTP1B siRNA did not affect protein expression of VE-cadherin (data not shown). These results suggest that PTP1B activity stabilizes VE-cadherin-mediated cell-cell adhesion in ECs.

We also found that PTP1B was co-immunoprecipitated with VE-cadherin in unstimulated and VEGF-stimulated confluent HUVECs infected with Ad.LacZ, Ad.PTP1B-WT or Ad.PTP1B-C/S (Supplemental Figure IIIC). Moreover, confocal analysis with HUVEC transiently transfected with GFP-PTP1B-D181A (D/A) revealed that PTP1B colocalized with VE-cadherin at cell-cell contacts but majority of PTP1B was found at perinucleus as reported [15, 16] (data not shown). Colocalization of GFP-PTP1B-WT with VE-cadherin was weaker than the D/A mutant, indicating that stabilization of enzyme-substrate complex is necessary for visualization of PTP1B-VE-cadherin complex. These results suggest that PTP1B associates with VE-cadherin at cell-cell junctions and dephosphorylates VE-cadherin in confluent ECs, thereby stabilizing cell-cell adhesions.

Increase of PTP1B expression and activity in mouse ischemic hindlimb model of angiogenesis

To assess the role of PTP1B in angiogenesis *in vivo*, we confirmed the expression of PTP1B and examined its activity in a mouse hindlimb ischemia model. Figure 8A shows that hindlimb blood flow recovery after femoral artery ligation was markedly decreased immediately after surgery (day 0) and recovered to the level of that of the nonischemic limb by day 7. Western analysis shows that PTP1B protein was robustly increased in the ischemic hindlimb at 7 days after operation compared with nonischemic legs (Fig. 8C), which was associated with the increase in PTP1B activity (Fig. 8B). Of note, their increase was time-dependent with a peak at day 7, and there was no significant difference between day 0 and 1 (data not shown).

Furthermore, immunofluorescence analysis demonstrated that PTP1B expression was increased at lectin-positive capillary ECs and skeletal myocyte at 3 and 7 days after ischemic injury, in a time-dependent manner (Supplemental Figure IV). In contrast, expression of SHP-2 was not changed after hindlimb ischemia (Fig. 8C). We also found that SHP-2 siRNA had no effect on VEGFR2 autophosphorylation and ERK1/2 phosphorylation in HUVECs (data not shown). Given the functional role of PTP1B in VEGF signaling and cell-cell adhesion in ECs, these data suggest that PTP1B may be involved in the process by which new blood vessels are formed *in vivo*.

Discussion

Many studies have focused on mechanism of VEGFR2 signal activation as opposed to signal *termination*, which is much less studied. The present study provides evidence that: 1) Overexpression of PTP1B inhibits VEGFR2 autophosphorylation, ERK1/2 phosphorylation and EC proliferation, while knockdown of PTP1B using siRNA significantly enhances these responses; 2) PTP1B binds to VEGFR2 in HUVEC overexpressing PTP1B as well as in CHO cells co-transfected with PTP1B and VEGFR2 cytoplasmic domain; 3) Recombinant active PTP1B protein dephosphorylates VEGFR2 immunoprecipitates of VEGF-stimulated HUVECs; 4) Overexpression of PTP1B stabilizes endothelial cell-cell junction by reducing VE-cadherin tyrosine phosphorylation and increasing TER, while PTP1B siRNA causes opposite effects; 5) PTP1B physically associates with VE-cadherin in HUVECs; 6) PTP1B expression and activity are dramatically increased in ischemic hindlimb model.

Previous studies demonstrated that several PTPs including SHP-1, SHP-2 and HCPTPA bind to VEGFR2 directly or indirectly [1]. However, their endogenous role in VEGFR2 autophosphorylation under physiological condition remains unclear. SHP-1 is involved in TNFα-mediated prevention of VEGFR2 phosphorylation [17], while SHP-2 inhibits tyrosine phosphorylation of VEGFR2 in ECs when they are cultured only on type I collagen [18]. HCPTPA overexpression attenuates VEGFR2 autophosphorylation, but this has not been studied with a loss of function approach [19]. Using overexpression and knockdown approaches as well as co-precipitation and co-transfection assays, the present study demonstrates that PTP1B functions as a negative regulator for VEGFR2 autophosphorylation in ECs via binding to the VEGFR2 cytoplasmic kinase domain. Of note, VEGFR2 is co-precipitated with PTP1B in HUVEC overexpressing PTP1B-WT as well as in CHO cells transfected with PTP1B-WT or substrate-trapping mutants of PTP1B (PTP-D/A and PTP1B-C/S), suggesting that PTP1B binds to VEGFR2 in a phosphorylation-independent manner, as demonstrated for its binding to p130Cas [20]. We could not detect PTP1B association with VEGFR2 in LacZ-infected cells, which may due to the unstable complex formation of endogenous PTP1B with its substrate [10]. In addition, VEGFR2 dephosphorylation assay demonstrates that ligand-induced, activated VEGFR2 is dephosphorylated by PTP1B. Consistent with our findings, PTP1B has been shown to bind to and dephosphorylate PDGF receptor [21], EGF receptor (EGFR) [10] and insulin receptor [7, 11], indicating that PTP1B is a biologically relevant receptor tyrosine kinase phosphatase. Our study cannot rule out the possibility that PTP1B-VEGFR2 interaction is indirect, perhaps through intermediate proteins, which is required for PTP1B-induced dephosphorylation of VEGFR2.

VEGFR2 autophosphorylation is required for activation of diverse signaling pathways such as ERK1/2 and p38MAPK which are involved in EC proliferation and migration, respectively [2, 3]. The downstream signaling events that derive from the autophosphorylated tyrosines within VEGFR2 as well as dephosphorylation of RTKs by PTPs are highly site-specific. Previous reports show that phosphorylation of Y1175 within VEGFR2 is essential for activation of PLCγ, and subsequent ERK phosphorylation and cell proliferation [2], while pY1214 is involved in VEGF-stimulated p38MAPK activation and cell migration [3]. The present study demonstrates

that PTP1B siRNA enhances, while overexpression of PTP1B inhibits, phosphorylation of Y1175 of VEGFR2, PLCγ, ERK and EC proliferation without affecting phosphorylation of p38MAPK or Akt as well as EC migration. Consistent with our result, Milarski et al. [22] reported that PTP1B specifically dephosphorylates EGFR pY992 that is specific binding site for PLCγ. On the other hand, using porcine aortic ECs transfected with chimeric murine VEGFR2, Holmqvist et al. [23] and Dayanir et al. [24] showed that VEGFR2-pY1175/1173 is required for PI3 kinase activation which is involved in EC migration and proliferation, respectively. Moreover, PTP1B inhibitors enhance phosphorylation of Akt and eNOS, and improve peripheral endothelial dysfunction in chronic heart failure model of mice [25]. This conflicting evidence may be attributable to differences in experimental conditions (cell culture conditions or transfection system with chimeric murine VEGFR2 or cell types or species). It is possible that PTP1B may preferentially bind to the VEGFR2-PLCγ complex, but not to the VEGFR2-PI3 kinase complex, which may form appropriate conformation or localize at PTP1B-associated subcellular compartments. Furthermore, PTP1B may affect other VEGFR2 phosphorylation sites in addition to pY1175 of the receptor. Nevertheless, our data clearly demonstrate that PTP1B negatively regulates EC proliferation at least in part by selectively inhibiting VEGFR2-pY1175-PLCγ-ERK pathway in intact human ECs. Detailed analysis using mutant Y1175F-VEGFR2, characterization of the interaction of PTP1B and VEGFR2 and their localization, and identification of the dephosphorylation site(s) of VEGFR2 by PTP1B are objective of future studies.

VE-cadherin-mediated cell-cell adhesion is important for preserving endothelial integrity of ECs. Tyrosine phosphorylation of VE-cadherin is associated with a loss of cell-cell contacts in confluent monolayer of ECs and an increase in endothelial permeability, which is initial events to promote angiogenesis [4]. PTPs activity also regulates the integrity of cell-cell junctions [26]. We found that PTP1B binds to and colocalizes with VE-cadherin at cell-cell contacts in basal state. Overexpression of PTP1B stabilizes endothelial cell-cell junction by reducing VE-cadherin tyrosine phosphorylation and increasing TER, while PTP1B siRNA causes opposite effects. Thus, PTP1B functions to maintain endothelial barrier integrity. Consistent with our results, PTP1B is shown to localize at cell-cell junctions in confluent rat corneal ECs [27], and binds directly to E-cadherin [12] or N-cadherin [13] in other systems, which are involved in stabilizing intercellular junctions. Given that a fraction of VEGFR2 is localized at VE-cadherin-containing cell-cell contacts [28-31], it is conceivable that PTP1B localized at adherens junction may form multiple complex with VEGFR2 and VE-cadherin to downregulate VEGF signaling, thereby limiting angiogenic program. Density-enhanced phosphatase-1 (DEP1)/CD148 indirectly associates with VE-cadherin/VEGFR2 complex, which in turn promotes contact-dependent inhibition of VEGFR2 phosphorylation, ERK activation and EC proliferation [32]. Thus, PTP1B-mediated inhibition of VEGFR2-ERK-EC proliferation may be at least in part through promoting cell-cell contacts, thereby contributing to DEP-1-mediated contact-dependent inhibition of VEGFR2 signaling in ECs. Additionally, vascular endothelial PTP (VE-PTP) [33] and SHP-2 [34] bind to VE-cadherin and β-catenin, respectively, which are involved in VE-cadherin-mediated barrier function in confluent ECs. These suggest that PTP1B and other PTPs which localize at cell-cell contacts may collaborate to maintain low levels of tyrosine phosphorylation, and thus stabilizing junctional integrity, which may contribute to contact-dependent inhibition of VEGF signaling and EC proliferation.

PTPs have a reactive Cys residue in the enzyme active site (e.g., Cys-215 in human PTP1B) which is deprotonated at neutral pH and is susceptible to the reversible oxidative inactivation by agonist-induced ROS [35]. We and others demonstrated that ROS are involved in VEGFR2 autophosphorylation [36-38] as well as loss of cell-cell adhesions through increasing tyrosine phosphorylation of VE-cadherin and/or other junctional proteins [39-41]. Whether VEGF-induced ROS are involved in oxidative inactivation of PTP1B in ECs is currently under investigation.

General PTP inhibitors promote VEGFR2 activation, blood flow recovery or angiogenesis after hindlimb ischemia [5, 6], suggesting PTPs as potential therapeutic targets to promote neovascularization. Using mouse hindlimb ischemia model, we demonstrate that PTP1B expression is increased at lectin positive ECs and skeletal myocyte in a time-dependent manner with a peak at 7days after hindlimb ischemia, which is associated with an increase in PTP1B activity. This staining pattern is consistent with the previous report for the induction of VEGFR2 in ischemic skeletal muscle [42]. Together with our *in vitro* data, ischemia-induced upregulation of PTP1B may represent an important compensatory mechanism that blunts over-activation of angiogenic signaling *in vivo* at least in part by inhibiting tyrosine phosphorylation of VEGFR2 and VE-cadherin. Our results are consistent with previous reports that PTP1B inhibits PDGF- or FGF-induced proliferation of cultured vascular smooth muscle, while PTP1B expression and neointima formation are increased in vascular injury model [43, 44]. This was interpreted as counter-regulatory function of PTP1B in neointima formation in response to vascular injury. In contrast, SHP-2 expression was not changed after ischemia and SHP-2 siRNA had no effects on VEGFR2 autophosphorylation in cultured ECs, suggesting specific involvement of PTP1B in angiogenic responses *in vitro* and *in vivo*. Sugano et al. [17] reported that SHP-1 protein is increased in a rat hindlimb ischemia model, which prevents TNFα-induced negative inhibitory effects on VEGF signaling. We also found that SHP-1 protein is increased after ischemia, but the extent of its increase is much less than that of PTP1B. Previous reports show that PTP1B inhibitor enhances VEGF-mediated angiogenesis using mouse Matrigel model [45]; which cannot eliminate the non-specific effects. Since our ischemia hindlimb data implicates but does not conclude that PTP1B may play a role in VEGF-induced angiogenesis *in vivo*, further investigation will be required using $PTP1B^{-/-}$ mice in future study. Given that $PTP1B^{-/-}$ mice prevent type 2 diabetes and obesty [8, 9] in which ischemia-induced angiogenesis is impaired, inhibiting PTP1B may be important therapeutic strategy to promote new vessel formation in cardiovascular diseases.

In summary, we demonstrate that PTP1B functions as negative regulator of VEGF signaling by dephosphorylating VEGFR2 via binding to the receptor as well as by stabilizing adherens junctions via inhibiting tyrosine phosphorylation of VE-cadherin that mediates contact-dependent inhibition of VEGFR2 signaling. These mechanisms may contribute to specific inhibition of ERK-EC proliferation pathway. Induction of PTP1B by hindlimb ischemia may represent essential counter-regulatory mechanism that blunts widespread, uncontrolled activities of VEGFR2 and other RTKs during angiogenesis *in vivo*. These findings should provide novel insight into PTP1B as a potential therapeutic target to promote neovascularization in ischemic cardiovascular diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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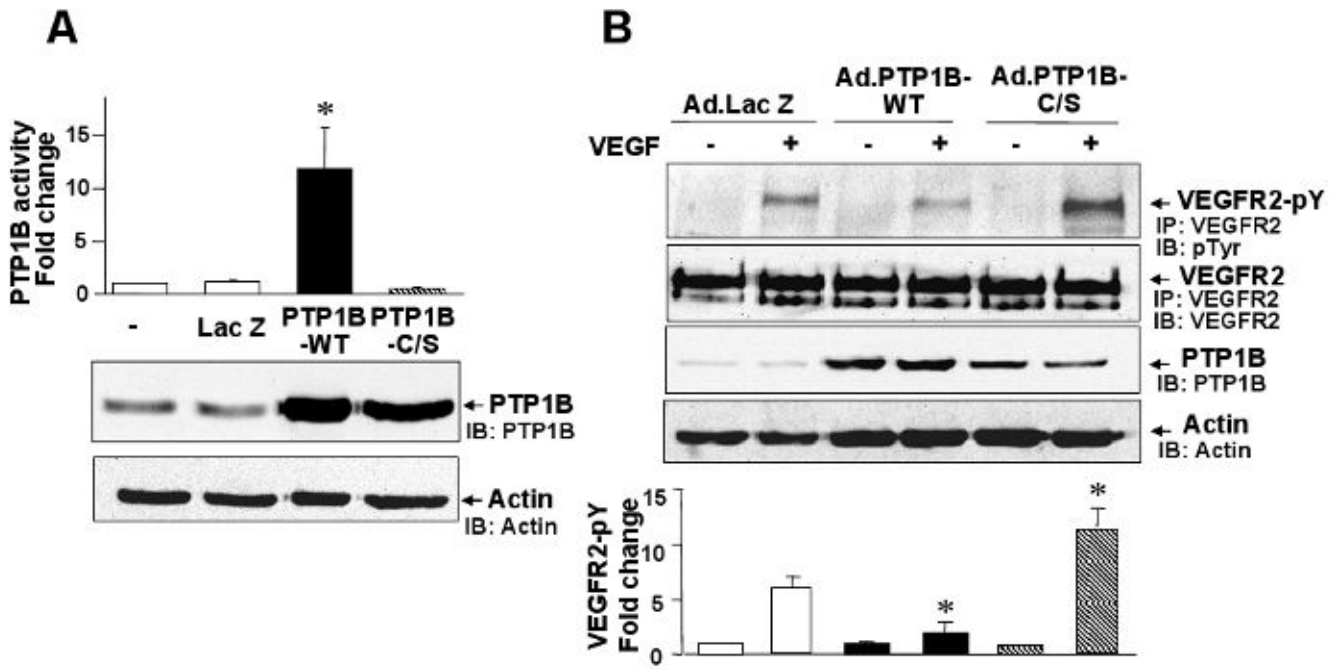


Figure 1. PTP1B overexpression inhibits VEGF-induced autophosphorylation of VEGFR2

HUVECs were infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S. A, PTP1B activity (upper graph) and PTP1B protein expression (lower blot) measured by western analysis with anti-PTP1B or actin antibody (loading control). PTP1B activity was expressed as fold change over basal (the ratio in untreated cells was set to 1). * $P<0.05$ for the increase induced by PTP1B overexpression. B, Cells were stimulated with VEGF (20 ng/ml) for 5 min, and lysates were immunoprecipitated with anti-VEGFR2 antibody, followed by immunoblotting with anti-phosphotyrosine (pTyr) antibody. Bottom panel shows averaged data, expressed as fold change over basal (means ± S.E., n=3). *$P<0.05$ vs. Ad.LacZ.

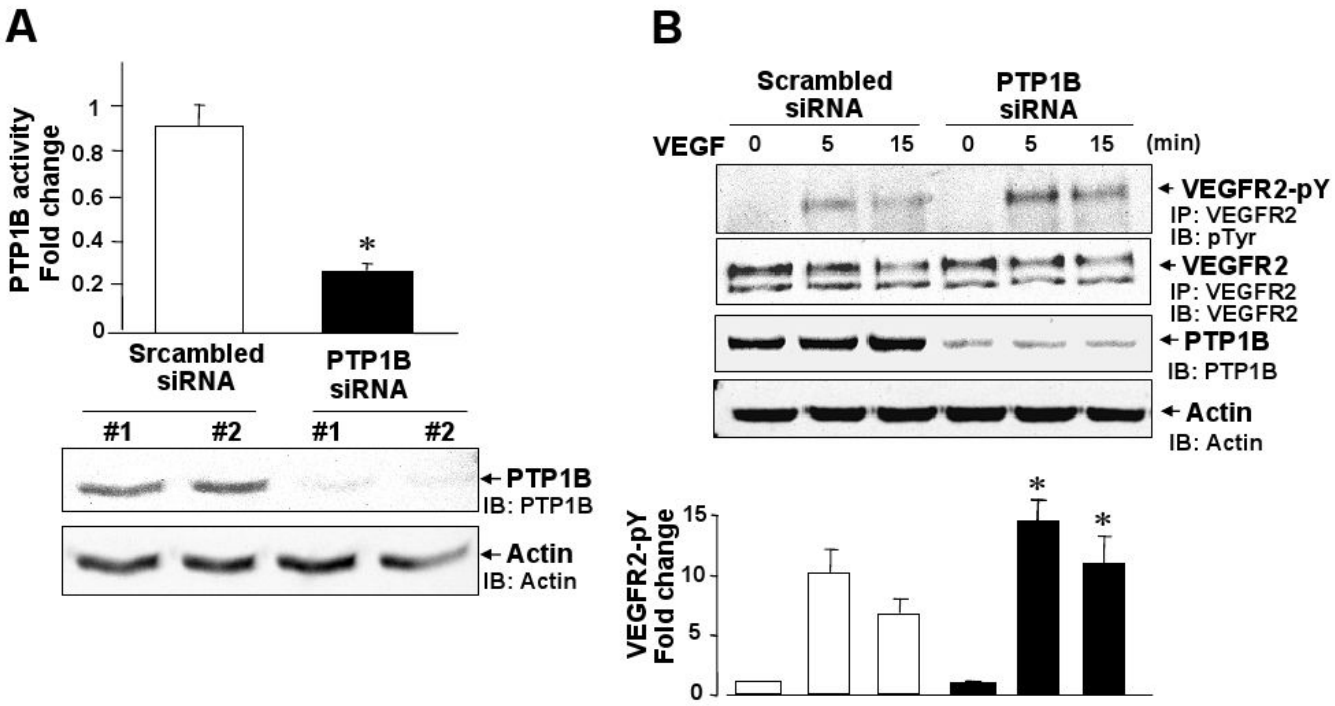


Figure 2. Knockdown of endogenous PTP1B by siRNA enhances VEGFR2 autophosphorylation

HUVECs were transfected with scrambled or PTP1B siRNA. A, PTP1B activity (upper graph) and PTP1B protein expression (lower blot). PTP1B activity was expressed as fold change from control (the ratio in untransfected cells was set to 1). * P<0.05 vs. Scrambled siRNA. B, Cells were stimulated with VEGF (20 ng/ml) and lysates were immunoprecipitated with anti-VEGFR2 antibody, followed by immunoblotting with anti-pTyr or VEGFR2 antibody. Bottom panel shows averaged data, expressed as fold change of phosphorylation over basal (means ± S.E., n=3). *P<0.05 vs. Scrambled siRNA at each time point.

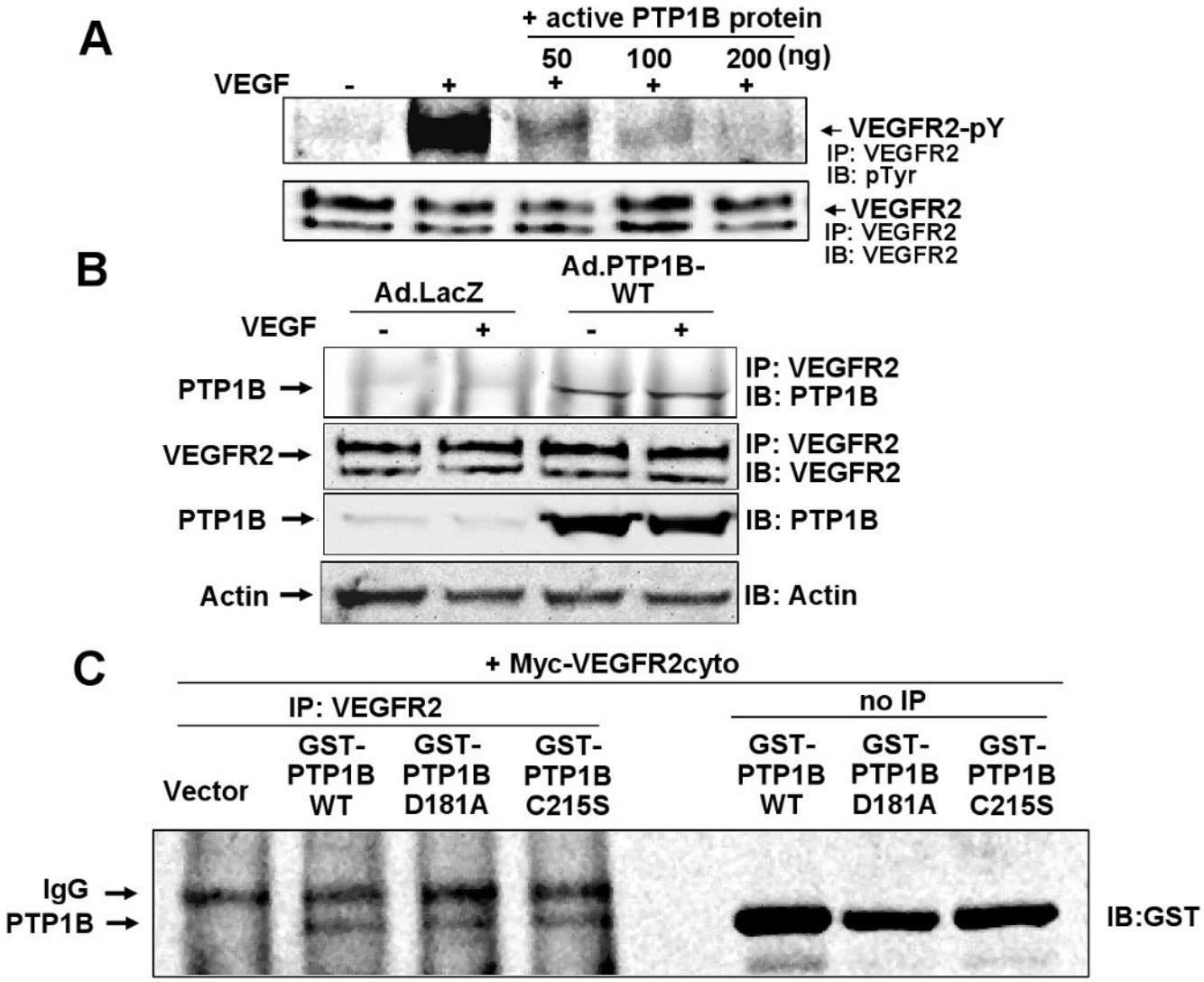
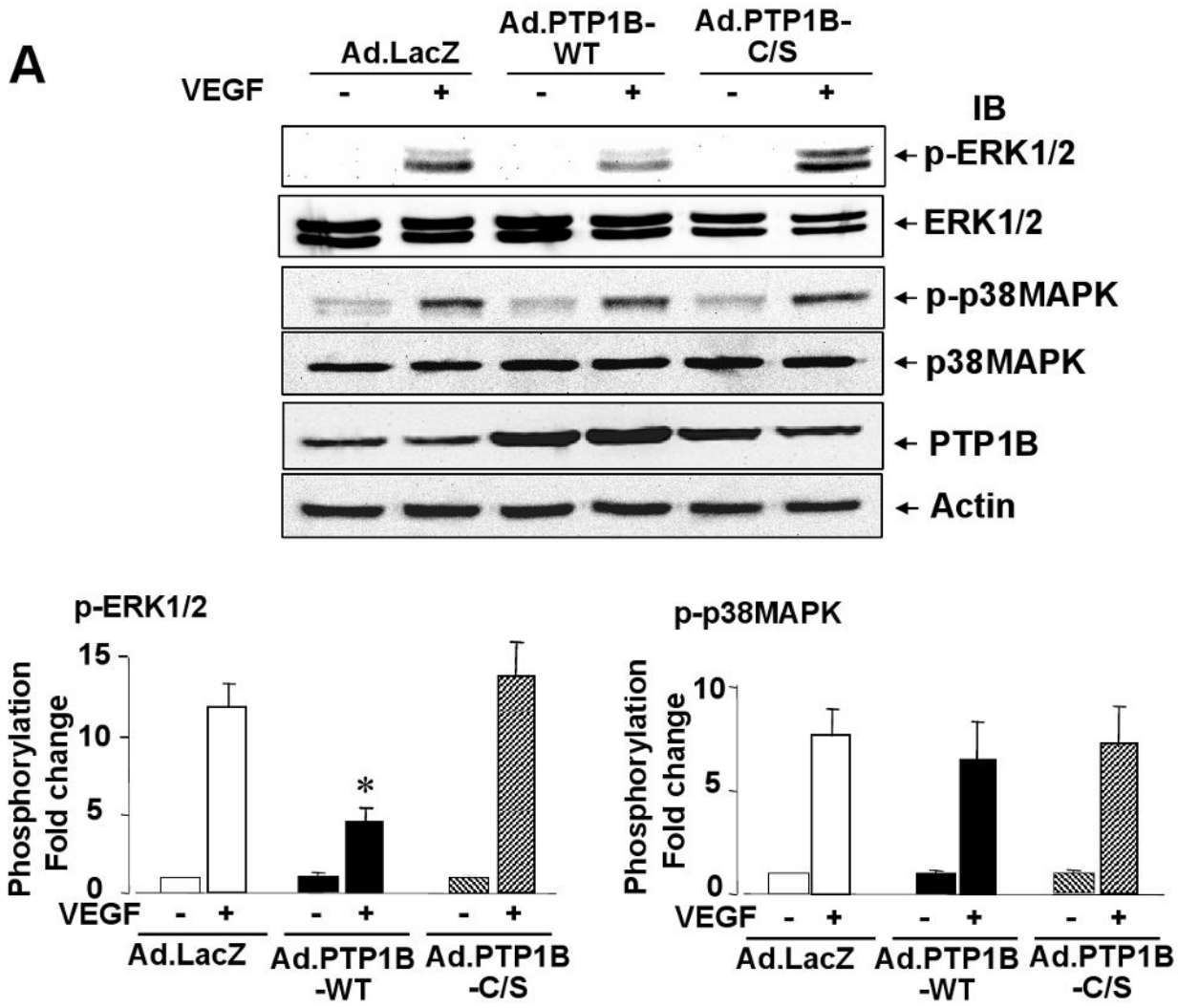


Figure 3. PTP1B dephosphorylates and associates with VEGER2 *in vivo*

A, *In vivo* receptor dephosphorylation assay. VEGFR2 immunoprecipitates obtained from HUVECs stimulated with VEGF for 5 min were incubated with active recombinant PTP1B protein at 30 °C for 10 min. Samples were immunoblotted with anti-pTyr or VEGFR2 antibody. B, HUVECs infected with Ad.LacZ (control) or Ad.PTP1B-WT were stimulated with VEGF (20 ng/mL) for 5 min. Lysates were immunoprecipitated with anti-VEGFR2 antibody, followed by immunoblotting with anti-PTP1B or VEGFR2 antibody. Bottom blot shows western analysis with anti-PTP1B antibody. C, CHO cells were co-transfected with Myc-tagged VEGFR2 cytoplasmic domain (myc-VEGFR2cyto) and GST-tagged various mutants of PTP1B (GST-PTP1B-WT, D181A, C215A) or empty vector. Lysates immunoprecipitated with VEGFR2 antibody (IP: VEGFR2) or without immunoprecipitation (no IP) were immunoblotted with anti-GST antibody.

Figure 4



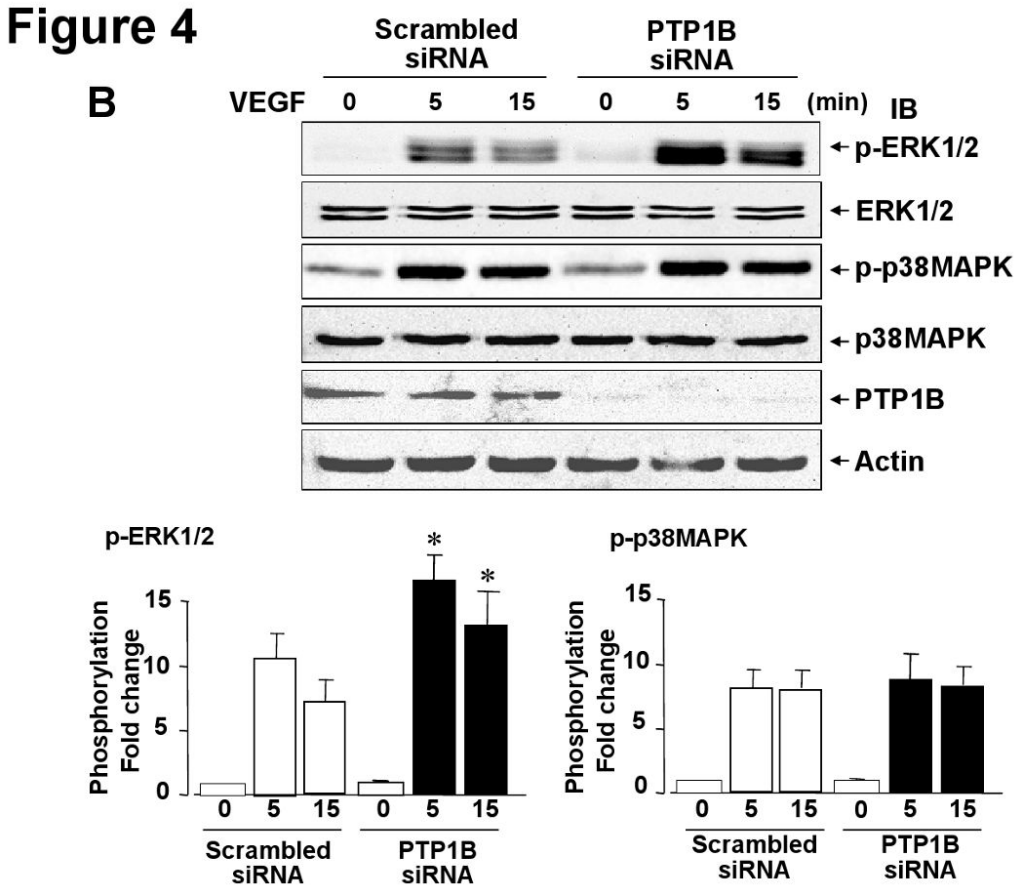


Figure 4. PTP1B overexpression inhibits while PTP1B knockdown enhances VEGF-induced phosphorylation of ERK1/2, but not p38MAPK

HUVECs infected with Ad.LacZ (control) or Ad.PTP1B-WT (A) or transfected with scrambled or PTP1B siRNAs (B) were stimulated with VEGF (20 ng/mL) for 5 min. Lysates were immunoblotted with phospho-ERK1/2, ERK1/2, phospho-p38MAPK, p38MAPK, PTP1B or actin antibody. Representative blots for ERK1/2 and actin are reprobe from p-ERK1/2 membrane. The representative p-p38MAPK blot is derived from different lysates used for p-ERK1/2 blot, and is reprobed for p38MAPK blot. Bottom panels show averaged data obtained from more than 3 independent experiments, expressed as fold change of phosphorylation over basal (means ± S.E., n=3). *$P < 0.05$ vs. Ad.LacZ (A) or Scrambled siRNA (B) at each time point.

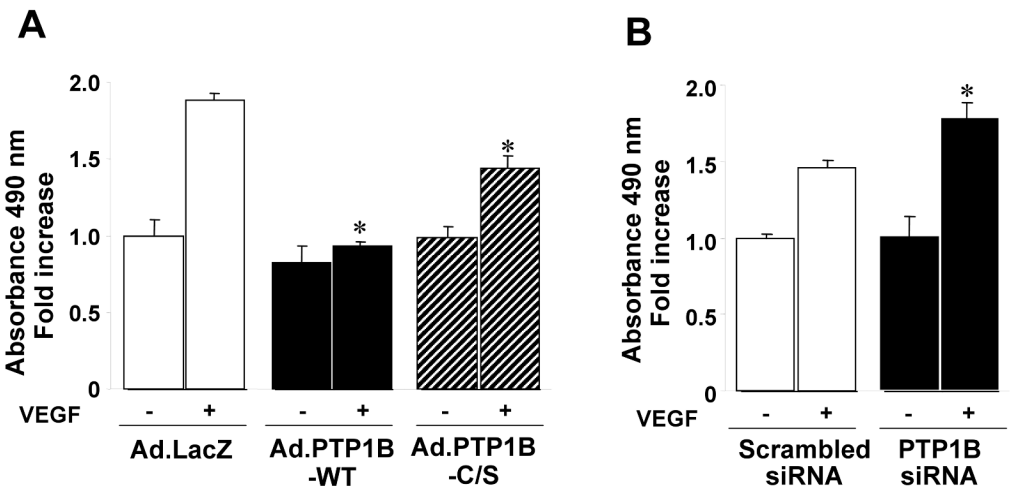


Figure 5. PTP1B negatively regulates VEGF-induced cell proliferation

HUVECs infected with Ad.LacZ (control) or Ad.PTP1B-WT or Ad.PTP1B-C/S (A) or transfected with scrambled or PTP1B siRNA (B) were used for measurement of cell proliferation. Values are the mean ± SE for 3 independent duplicate experiments. *P < 0.05 vs. Ad.LacZ (A) or scrambled siRNA (B).

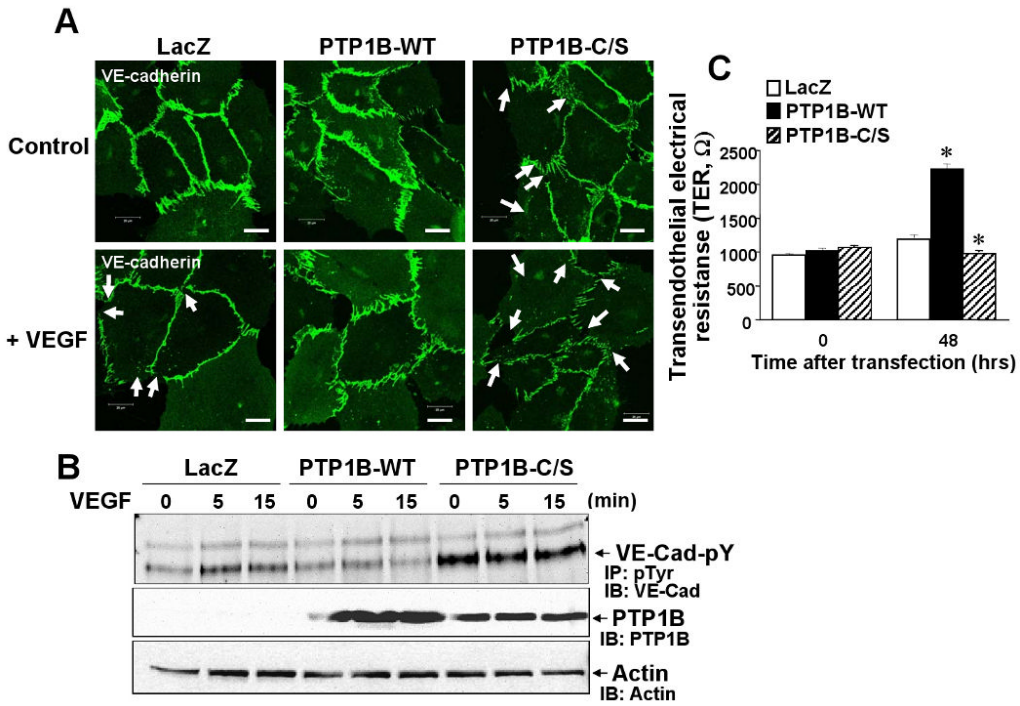


Figure 6. Overexpression of PTP1B stabilizes VE-cadherin-mediated cell-cell adhesion and reduces VE-cadherin tyrosine phosphorylation

HUVECs infected with Ad.LacZ, PTP1B-WT or PTP1B-C/S were stimulated with VEGF (50 ng/mL). A, Cells were permeabilized, fixed and stained with anti-VE-cadherin antibody, followed by FITC-conjugated goat anti-rabbit antibody. White arrows show VEGF-induced decrease in VE-cadherin staining at cell-cell contact with formation of small gaps. Images were taken at 5 different fields/well, and representative of more than 3 independent experiments. B, Lysates were immunoprecipitated with anti-pTyr antibody, followed by immunoblotting with VE-cadherin antibody (Upper). Lysates without immunoprecipitation were immunoblotted with anti-PTP1B antibody (bottom). Results are representative of 3 independent experiments. C, Real-time measurement of transendothelial electrical resistance (TER). Values are the mean ± SE for 3 independent duplicate experiments. *$P < 0.05$ vs. Ad.LacZ.

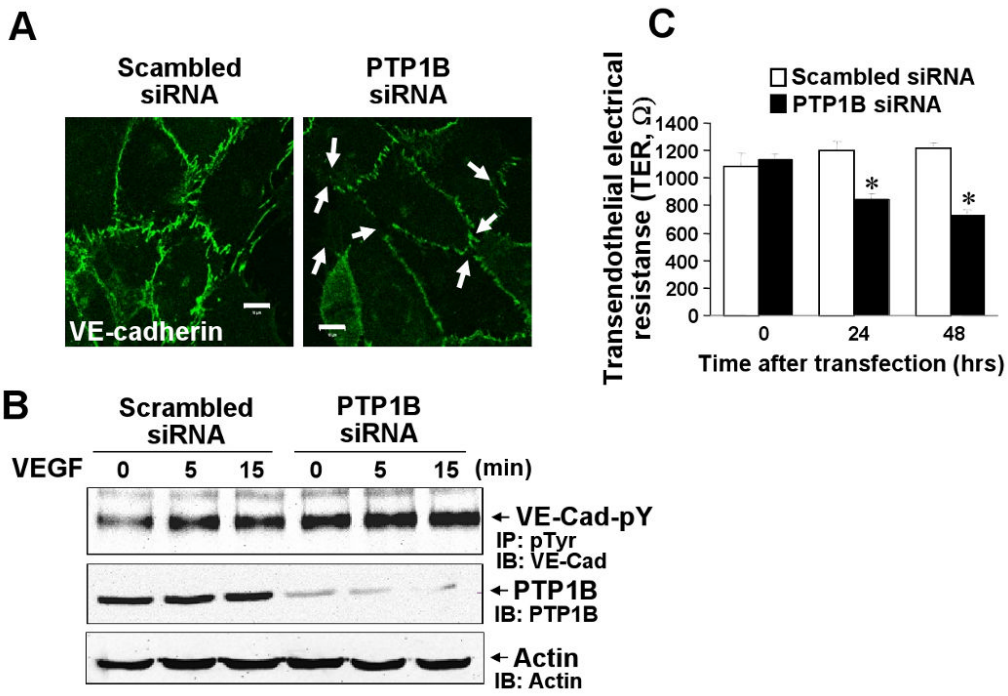


Figure 7. PTP1B knockdown reduces VE-cadherin-mediated cell-cell adhesion and increases VE-cadherin tyrosine phosphorylation

HUVECs transfected with scrambled or PTP1B siRNA were used for VE-cadherin staining (A), VE-cadherin tyrosine phosphorylation analysis (B) and real-time measurement of transendothelial electrical resistance (TER)(C). A, White arrows show decrease in VE-cadherin staining at cell-cell contacts with formation of small gaps. B, Cells stimulated with VEGF (20 ng/ml) were used for measurement of VE-cadherin tyrosine phosphorylation and PTP1B protein expression as described in Figure 6 legend. Results are representative of 3 independent experiments. C, Values are the mean ± SE for 8 independent duplicate experiments. *P < 0.05 vs. scrambled siRNA.

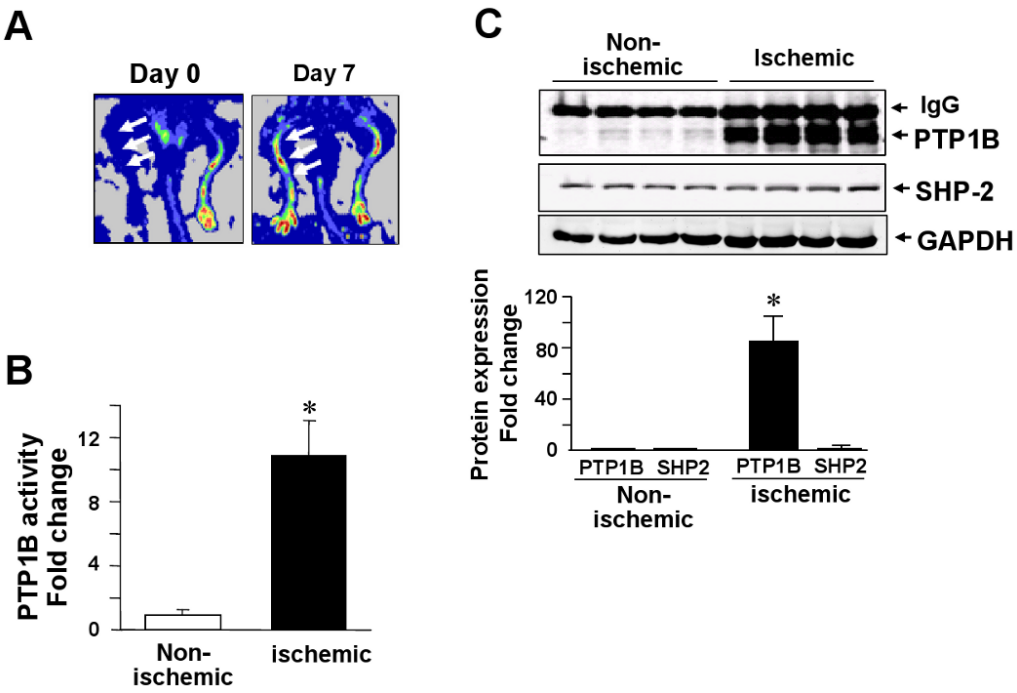


Figure 8. Increase of PTP1B expression and activity in mouse ischemic hindlimb model of angiogenesis

Hindlimb ischemia was induced by the right femoral artery ligation. A, Laser Doppler blood flow analysis: Arrows indicate a low perfusion signal (dark blue) at immediately after operation (day 0) and a high perfusion signal (yellow to red) detected on day 7 in the ischemic hindlimbs. B, PTP1B specific activity in nonischemic and ischemic tissue at 7 day after ischemia. C, Upper blots show PTP1B and SHP2 protein expression in nonischemic and ischemic tissues at 7 days after ischemia. Each lane indicates different mouse. For B and C, bar graph shows averaged data, expressed as fold change over basal (the value in nonischemic tissue was set to 1). Values are the mean ± SE (n = 4). *P < 0.05 vs. hindlimb ischemia.