

# The Phosphatase PTP-PEST/PTPN12 Regulates Endothelial Cell Migration and Adhesion, but Not Permeability, and Controls Vascular Development and Embryonic Viability\*

Received for publication, June 1, 2012, and in revised form, October 22, 2012. Published, JBC Papers in Press, October 26, 2012, DOI 10.1074/jbc.M112.387456

Cleiton Martins Souza<sup>‡1</sup>, Dominique Davidson<sup>‡</sup>, Inmoo Rhee<sup>‡§</sup>, Jean-Philippe Gratton<sup>¶||</sup>, Elaine C. Davis<sup>\*\*2</sup>, and André Veillette<sup>‡§||3</sup>

From the Laboratories of <sup>‡</sup>Molecular Oncology and <sup>¶</sup>Endothelial Cell Biology, Clinical Research Institute of Montréal, Montréal, Québec H2W 1R7, Canada, <sup>§</sup>Department of Medicine, McGill University, Montréal, Québec H3G 1Y6, Canada, <sup>||</sup>Department of Medicine, University of Montréal, Montréal, Québec H3T 1J4, Canada, and <sup>\*\*</sup>Department of Anatomy and Cell Biology, McGill University, Montréal, Québec H3A 2B2, Canada

**Background:** PTP-PEST is a phosphatase essential for embryonic viability.

**Results:** PTP-PEST is critical for adhesion and migration of endothelial cells. Its absence in endothelial cells results in mouse embryonic lethality.

**Conclusion:** The embryonic viability seen in constitutive PTP-PEST-deficient mice is due to a defect in endothelial cell functions.

**Significance:** PTP-PEST is a key regulator of endothelial cell functions *in vitro* and *in vivo*.

Protein-tyrosine phosphatase (PTP)-PEST (PTPN12) is ubiquitously expressed. It is essential for normal embryonic development and embryonic viability in mice. Herein we addressed the involvement of PTP-PEST in endothelial cell functions using a combination of genetic and biochemical approaches. By generating primary endothelial cells from an inducible PTP-PEST-deficient mouse, we found that PTP-PEST is not needed for endothelial cell differentiation and proliferation or for the control of endothelial cell permeability. Nevertheless, it is required for integrin-mediated adhesion and migration of endothelial cells. PTP-PEST-deficient endothelial cells displayed increased tyrosine phosphorylation of Cas, paxillin, and Pyk2, which were previously also implicated in integrin functions. By eliminating PTP-PEST in endothelial cells *in vivo*, we obtained evidence that expression of PTP-PEST in endothelial cells is required for normal vascular development and embryonic viability. Therefore, PTP-PEST is a key regulator of integrin-mediated functions in endothelial cells seemingly through its capacity to control Cas, paxillin, and Pyk2. This function explains at least in part the essential role of PTP-PEST in embryonic development and viability.

Protein-tyrosine phosphatase (PTP)<sup>4</sup>-PEST (or PTPN12) is a ubiquitously expressed cytosolic PTP with an amino (N)-termi-

nal PTP domain and a carboxyl (C)-terminal region involved in protein-protein interactions (1, 2). Through its C-terminal domain, it interacts with several cytoskeletal adaptor proteins including Cas, paxillin, and PSTPIP (3–7). Moreover, it associates with the adaptors Grb2 and Shc and through paxillin with the protein-tyrosine kinases (PTKs) FAK and Pyk2 (8–12). These associations are postulated to recruit PTP-PEST to different intracellular locales, especially to the cytoskeleton. Several PTP-PEST-interacting proteins such as Cas, paxillin, Shc, PSTPIP, FAK, and Pyk2 are direct substrates of PTP-PEST.

PTP-PEST is required for normal embryonic development as revealed by the finding that a constitutive PTP-PEST-deficient mouse exhibits embryonic lethality between day 9.5 and 10.5 (E9.5–10.5) (13). This phenotype is accompanied by severe developmental defects affecting blood vessels, neurological tissues, and liver, although the exact cellular basis of this phenotype was not determined. Overexpression studies and analyses of embryo fibroblasts from PTP-PEST-deficient mice showed that PTP-PEST is a regulator of integrin-mediated functions (7, 14–17). Using a tissue-specific PTP-PEST-deficient mouse in which PTP-PEST was specifically eliminated in T cells, it was also found that PTP-PEST is a positive regulator of T cell activation as a result of its capacity to promote homotypic adhesion between T cells (11). Lastly, it was recently uncovered that PTP-PEST is a tumor suppressor in human breast cancer and lung cancer, seemingly as a result of its capacity to control receptor PTK signaling (18).

Endothelial cells form the inner lining of blood vessels (19). They play an essential role during mammalian development, being the first fully functional system in the embryo (19, 20). Endothelial cells are also critical for many processes in postnatal life including exchange of nutrients, blood clotting, and migration of immune cells toward inflamed tissues. Blood vessel development occurs through two consecutive processes, vasculogenesis and angiogenesis. Vasculogenesis involves

\* This work was supported in part by grants from the Canadian Institutes of Health Research and the Canadian Cancer Society Research Institute (to A. V., J.-P. G., and E. C. D.).

<sup>1</sup> Recipient of the Angelo Pizzagalli Fellowship.

<sup>2</sup> Holds a Canada Research Chair in Anatomy and Cell Biology.

<sup>3</sup> Holds the Canada Research Chair in Signaling in the Immune System. To whom correspondence should be addressed: *Clinical Research Inst. of Montréal (IRCM)*, 110 Pine Ave. W., Montréal, Québec H2W 1R7, Canada. Tel.: 514-987-5561; Fax: 514-987-5562; E-mail: veillea@ircm.qc.ca.

<sup>4</sup> The abbreviations used are: PTP, protein-tyrosine phosphatase; PSTPIP, *proline, serine, threonine phosphatase-interacting protein*; PTK, protein-tyrosine kinase; FAK, focal adhesion kinase; E, day of embryonic development; VE, vascular endothelial; UBC, ubiquitin C; EYFP, enhanced yellow fluorescent protein.

blood vessel formation from pluripotent mesenchymal cells, whereas angiogenesis implicates blood vessel formation from existing vessels. Both require extensive endothelial cell proliferation and migration.

Blood vessel integrity is maintained by tight intercellular junctions between endothelial cells as well as by interactions between endothelial cells and surrounding basement membranes, stromal cells, and smooth muscle cells (21–24). Adhesion receptors, especially vascular endothelial (VE)-cadherin and integrins, mediate these various contacts. The VE-cadherin pathway controls vascular permeability (20, 25, 26). It is tightly regulated by PTKs such as Tie2, which dynamically modulates the function of VE-cadherin (25, 26). A PTP, VE-PTP, influences VE-cadherin function through its ability to regulate Tie2 (27, 28). In contrast, the integrin pathway influences adhesion and migration (29–32). The PTK FAK was shown to be a central regulator of the integrin pathway in endothelial cells (29–32). However, the identity of the PTP(s) influencing this pathway in endothelial cells has not been determined.

Here we examined the role of PTP-PEST in endothelial cells. Using primary endothelial cells from an inducible PTP-PEST-deficient mouse, we found that PTP-PEST is required for migration and adhesion of endothelial cells, but not for proliferation, differentiation, and modulation of permeability to macromolecules by these cells. This function correlates with the capacity of PTP-PEST to dephosphorylate Cas, paxillin, and Pyk2. Studies of a mouse in which PTP-PEST was specifically eliminated in endothelial cells provided evidence that the function of PTP-PEST in endothelial cells is critical for normal vascular development and embryonic viability.

## EXPERIMENTAL PROCEDURES

**Mice**—Mice expressing a tamoxifen-inducible Cre (Cre-ERT2) under the control of the ubiquitin C promoter (*UBC-Cre-ERT2*) or the Cre recombinase under the control of the *Tie2* promoter (*Tie2-Cre*), and *ROSA26EYFP* mice were obtained from The Jackson Laboratory (Bar Harbor, ME) (33–35). They were then bred with mice expressing a conditionally deleted allele of the PTP-PEST-encoding gene, *Ptpn12<sup>fl</sup>*, backcrossed for at least 15 generations to the C57BL/6 background (11). A constitutive PTP-PEST-deficient mouse (*Ptpn12<sup>-/-</sup>*) in which PTP-PEST expression was constitutively lacking in all cells was generated by breeding *Ptpn12<sup>fl/+</sup>* mice with mice expressing a ubiquitous Cre transgene (11). Timed matings were set up between male *Ptpn12<sup>fl/+</sup>;Tie2-Cre<sup>+</sup>* and female *Ptpn12<sup>fl/fl</sup>* mice. Day 0.5 of embryonic development (E0.5) was defined as the morning when the vaginal plug was observed. All mice used in this study were bred under specific pathogen-free conditions. Animal experimentation was approved by the Clinical Research Institute of Montreal Animal Care Committee in accordance with the regulations of the Canadian Council for Animal Care.

**Inducible Deletion of PTP-PEST in Adult Mice**—Female *Ptpn12<sup>fl/fl</sup>* mice were bred with male *Ptpn12<sup>fl/+</sup>;UBC-Cre-ERT2<sup>+</sup>* mice. For tamoxifen-induced deletion of PTP-PEST, mice were fed for 5 consecutive days with tamoxifen (200  $\mu$ g/g of weight/day in corn oil; Sigma-Aldrich). After 10 days, mice were sacrificed and used for endothelial cell isolation.

**Isolation of Lung-derived Endothelial Cells**—Primary lung-derived endothelial cells were extracted from lung tissue of 8–10-week-old mice as described below for embryonic endothelial cells. Cells were then cultured in low glucose DMEM/F-12 medium (1:1 mixture; Invitrogen) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT), endothelial growth supplement (5  $\mu$ g/ml; BD Biosciences), and heparin (100  $\mu$ g/ml; Sigma-Aldrich) using gelatin-coated tissue culture dishes. Two successive rounds of endothelial cell purification were then performed using rat anti-mouse ICAM-2 antibody (mAb 3C4, BD Biosciences) coupled to sheep anti-rat IgG-coated magnetic beads (Dynabeads, Invitrogen). The purity of endothelial cells was confirmed by flow cytometry analysis using antibodies directed against the endothelial cell markers CD31 (rat anti-mouse CD31 mAb 390, eBioscience, San Diego, CA) and ICAM-2 (rat anti-mouse mAb 3C4, Biolegend, San Diego, CA). After 16 days in culture, cells were used for experimentation. At that time, >95% of cells expressed the endothelial cell-specific markers.

**Microscopy Analyses of Mouse Embryos**—Embryos and yolk sacs were harvested using a dissecting microscope (Model MZ12, Leica) and photographed using QImaging MicroPublisher with a real time viewing camera (QImaging, Surrey, British Columbia, Canada) and Northern Eclipse 7.0 software (Empix Imaging Inc., Mississauga, Ontario, Canada). Yolk sacs were used to genotype the embryos. For whole-mount staining, embryos were fixed at 4 °C overnight in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (Canemco, Québec, Canada). After blocking for 2 h at room temperature in a PBS solution containing 2% skim milk and 2.5% Triton X-100, embryos were incubated overnight with purified rat anti-mouse CD31 monoclonal antibody (mAb MEC13.3, BD Biosciences). After several washes, embryos were incubated overnight at 4 °C in the presence of a 1:500 dilution of Alexa Fluor 488-coupled goat anti-rat IgG (Invitrogen). After several washes, images were obtained by confocal microscopy using an LSM710 microscope (Zeiss). For cross-section analyses, fixed embryos were embedded in Epon, sectioned (0.5- $\mu$ m thickness), and stained with toluidine blue. They were then visualized by microscopy (Axiophot, Zeiss) as described above.

**Isolation of Embryonic Endothelial Cells**—Male *Ptpn12<sup>fl/+</sup>;Rosa26EYFP<sup>+</sup>;Tie2-Cre<sup>+</sup>* mice were bred with female *Ptpn12<sup>fl/fl</sup>;Rosa26EYFP<sup>+</sup>* mice. E10.5 embryos were harvested from timed pregnancies, washed, minced, and digested at 37 °C for 1 h with collagenase A (1 mg/ml; Roche Applied Science). To remove undigested material, the cell suspension was washed through a cell strainer (BD Biosciences). Isolated cells were then incubated on ice for 1 h with the antibodies described below. Cells were washed, and enhanced yellow fluorescent protein-positive (EYFP<sup>+</sup>) cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences).

**Flow Cytometry**—Embryonic cells or primary lung-derived endothelial cells were stained and analyzed by flow cytometry using the following antibodies (from Biolegend, BD Biosciences, or eBioscience): Alexa Fluor 647-coupled rat anti-mouse CD31 (mAb 390), Alexa Fluor 647-coupled rat anti-mouse ICAM-1 (mAb YN1/1.7.4), Alexa Fluor 647-coupled rat anti-mouse ICAM-2 (mAb 3C4), Alexa Fluor 647-coupled rat anti-

## PTP-PEST in Endothelial Cells

mouse VE-cadherin (mAb VECD1), phycoerythrin-coupled hamster anti-mouse  $\beta$ 1 integrin (CD29; mAb HMb1-1), fluorescein isothiocyanate (FITC)-coupled rat anti-mouse  $\beta$ 2 integrin (CD18; mAb M18/2), and rat anti-mouse isotype control (mAb RTK2758).

**Proliferation Assays**—Primary lung-derived endothelial cells ( $4 \times 10^4$  cells) were seeded in 24-well, gelatin-coated tissue culture dishes. At the desired time point, cells were harvested by trypsin (Invitrogen) and counted.

**Adhesion Assays**—96-well flat-bottom Immuno plates (Nunc, ThermoFisher, Rochester, NY) were coated overnight with fibronectin (10  $\mu$ g/ml; BD Biosciences), type I collagen (10  $\mu$ g/ml; BD Biosciences), or bovine serum albumin (BSA; 2 mg/ml; Sigma-Aldrich). After washing, plates were blocked with BSA, and  $10^4$  endothelial cells were allowed to adhere for 30 min at 37 °C. After washing away non-adherent cells, adherent cells were fixed and stained with crystal violet (Fisher Scientific). Cell adhesion was estimated by measuring the absorbance at 562 nm in a microplate scanning spectrometer (Power Wave X, Bio-Tek Instruments, Inc., Winooski, VT). Photographs were also obtained using an inverted microscope (Model Axiovert S100TV, Zeiss) with a QImaging camera and Volocity image analysis software (PerkinElmer Life Sciences).

**Spreading Assays**—Coverslips were coated at 4 °C overnight with fibronectin or collagen as above. Then coverslips were washed with PBS and placed in 6-well dishes. Endothelial cells were harvested and seeded ( $1 \times 10^5$  cells) on the coverslips. After 90 min at 37 °C, unbound cells were washed, and the remaining cells were fixed with 2% paraformaldehyde and mounted on a glass slide for examination by contrast microscopy. Data from 10 independent fields were acquired.

**Migration Assays**—For Transwell migration assays, endothelial cells were deprived of growth supplement and FBS overnight. Then  $10^5$  cells were loaded in the upper chamber of a Transwell insert (8- $\mu$ m pores; Costar, Corning, Lowell, MA) previously coated with fibronectin (5  $\mu$ g/ml). The medium in the lower chamber was then replaced by medium containing 20% FBS, endothelial growth supplement (5  $\mu$ g/ml; BD Biosciences), and heparin (100  $\mu$ g/ml; Sigma-Aldrich). Cells were allowed to migrate for 24 h. Cells having migrated in the lower chamber were counted using Volocity image analysis software. For wound healing assays, endothelial cells were cultured in gelatin-coated 6-well tissue culture plates (Nunc). Once the monolayer was confluent, a scratch was performed using a 200- $\mu$ l pipette tip. Wound closure was monitored with a time lapse microscope (DM IRE2, Leica), capturing images every 10 min. The speed of migration and persistence index, which is calculated as the ratio of the distance between the first and the last points of the tracked cells over the total distance traveled, were assessed after labeling cells with Hoechst using Volocity image analysis software. Image analyses and measurements were performed using Matlab software (MathWorks Inc., Torrance, CA).

**Permeability Assays**—Permeability was measured using type I collagen-coated Transwell units (0.4- $\mu$ m pores; Costar). The indicated numbers of endothelial cells were seeded in the upper chamber and allowed to form a monolayer. Once a monolayer

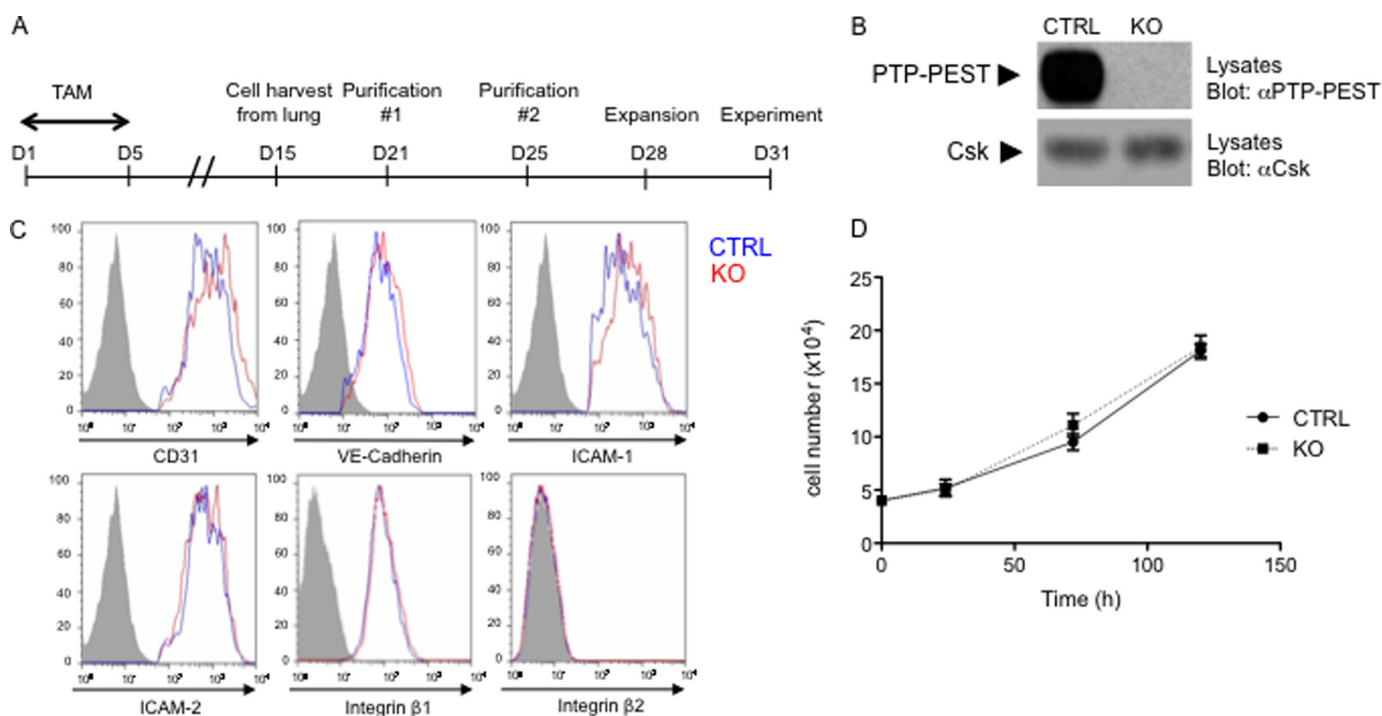
was formed, cells were deprived of serum and endothelial growth supplement for 1 or in some cases 4 h. After deprivation, FITC-dextran (molecular weight, 40,000; 1 mg/ml; Invitrogen) was added to the upper chamber and allowed to diffuse into the lower chamber for 30 or 60 min at 37 °C in the absence or presence of vascular endothelial growth factor (VEGF; 75 ng/ml) (36). Endothelial permeability was determined by measuring the fluorescence in the lower chamber at 520 nm with an excitation wavelength of 492 nm. Triplicates measurements were taken.

**Immunoprecipitation and Immunoblots**—Cells were lysed directly on the plate with TNE buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 2 mM EDTA) supplemented with protease and phosphatase inhibitors as described (37). Immunoprecipitation was accomplished by incubating 400  $\mu$ g of total cell lysates with a protein-specific antibody for 45 min at 4 °C. To collect immune complexes, Pansorbin (Calbiochem, EMD Millipore) was added for an additional 45 min. Unbound proteins were washed away, and the precipitated protein was loaded for 8% SDS-PAGE. Immunoblots were performed as outlined elsewhere (37). The following antibodies were used for immunoprecipitation: Cas (sc-860, Santa Cruz Biotechnology, Santa Cruz, CA), paxillin (610272, BD Biosciences), and FAK (sc-558, Santa Cruz Biotechnology). Phosphospecific antibodies against FAK (Tyr(P)-397 (3483) and Tyr(P)-576/577 (3281)) and antibodies recognizing phospho-extracellular signal-regulated kinase (ERK) (mAb E10) were from Cell Signaling Technology (Danvers, MA). The following antibodies were used for reprob-ing the membrane: Cas (sc-860 (Santa Cruz Technology, Santa Cruz, CA)), paxillin (610272, BD Biosciences), FAK (610088, BD Biosciences), and ERK (06-182, Millipore, Burlington, Ontario, Canada). Rabbit antibodies directed against Pyk2, PTP-PEST, and Csk were described elsewhere (12, 38). To evaluate VEGF-induced signals, endothelial cells were deprived of serum and endothelial growth supplement for 2–4 h. They were then stimulated with VEGF (50 ng/ml) for the indicated periods of time, and total cell lysates were probed by immunoblotting with antibodies recognizing phospho-ERK.

**Statistical Analyses**—*t* test analyses were performed using GraphPad version 5.00 for Mac (GraphPad Software, San Diego, CA).

## RESULTS

**PTP-PEST Is Not Required for Endothelial Cell Proliferation and Differentiation in Vitro**—The midgestation embryonic lethality previously observed in a constitutive PTP-PEST-deficient mouse prevented establishing primary cultures of PTP-PEST-deficient endothelial cells to address the possible role of PTP-PEST in endothelial cells (13). To resolve this issue, we bred mice carrying a conditional allele of the PTP-PEST-encoding gene (*Ptpn12<sup>fl/fl</sup>*) with mice expressing an estrogen-responsive Cre (Cre-ERT2), under the control of the ubiquitin C (UBC) promoter (Fig. 1A) (34). In the absence of tamoxifen, Cre-ERT2 is cytosolic and not functional. However, when mice are fed tamoxifen, Cre-ERT2 translocates to the nucleus where it can mediate deletion of the conditional allele. This enables deletion of the *Ptpn12* gene in adult mouse tissues.



**FIGURE 1. Generation and characterization of PTP-PEST-deficient primary endothelial cells.** *A*, protocol used to generate PTP-PEST-deficient endothelial cells. Adult mice were fed for 5 consecutive days (D1–5) with tamoxifen (TAM). On day 15, lung cells were harvested and seeded in culture. Two purifications of endothelial cells were done on days 21 and 25 using anti-CD102 antibody-coupled magnetic beads. After an expansion on day 28, cells were used for experimentation on day 31. *B*, expression of PTP-PEST and Csk (as control) in primary endothelial cells from tamoxifen-fed *Ptpn12*<sup>+/+</sup>; *UBC-Cre-ERT2*<sup>+</sup> (control (CTRL)) or tamoxifen-fed *Ptpn12*<sup>fl/fl</sup>; *UBC-Cre-ERT2*<sup>+</sup> (knock-out (KO)) mice was analyzed by immunoblotting. Data are representative of >10 experiments. *C*, flow cytometry analyses of surface markers on primary endothelial cells from control (blue) and KO (red) mice. Staining with an isotype control antibody is depicted by the filled gray curves. Data are representative of at least two experiments. *D*, primary endothelial cells from control and KO mice were seeded in growth medium containing endothelial growth supplement. They were then harvested at different times (in hours) and counted. Error bars representing standard deviations of values from duplicate wells are shown. Data are representative of two experiments.

Primary cultures of endothelial cells were established from the lungs of tamoxifen-fed *Ptpn12*<sup>fl/fl</sup>; *UBC-Cre-ERT2*<sup>+</sup> mice or, as control, tamoxifen-fed *Ptpn12*<sup>+/+</sup>; *UBC-Cre-ERT2*<sup>+</sup> mice (Fig. 1A). Immunoblot analyses of total cell lysates with anti-PTP-PEST antibodies showed that cells from tamoxifen-fed *Ptpn12*<sup>fl/fl</sup>; *UBC-Cre-ERT2*<sup>+</sup> mice completely lacked expression of PTP-PEST (Fig. 1B). However, they exhibited normal expression of the endothelial cell markers CD31, VE-cadherin, ICAM-1, ICAM-2, and  $\beta 1$  integrin, suggesting that their differentiation was normal (Fig. 1C). In all experiments reported herein, >95% of cells expressed these markers. In the presence of endothelial cell growth supplement, these cells also proliferated at a normal rate in culture (Fig. 1D). Hence, PTP-PEST was not needed for endothelial cell proliferation and differentiation *in vitro*.

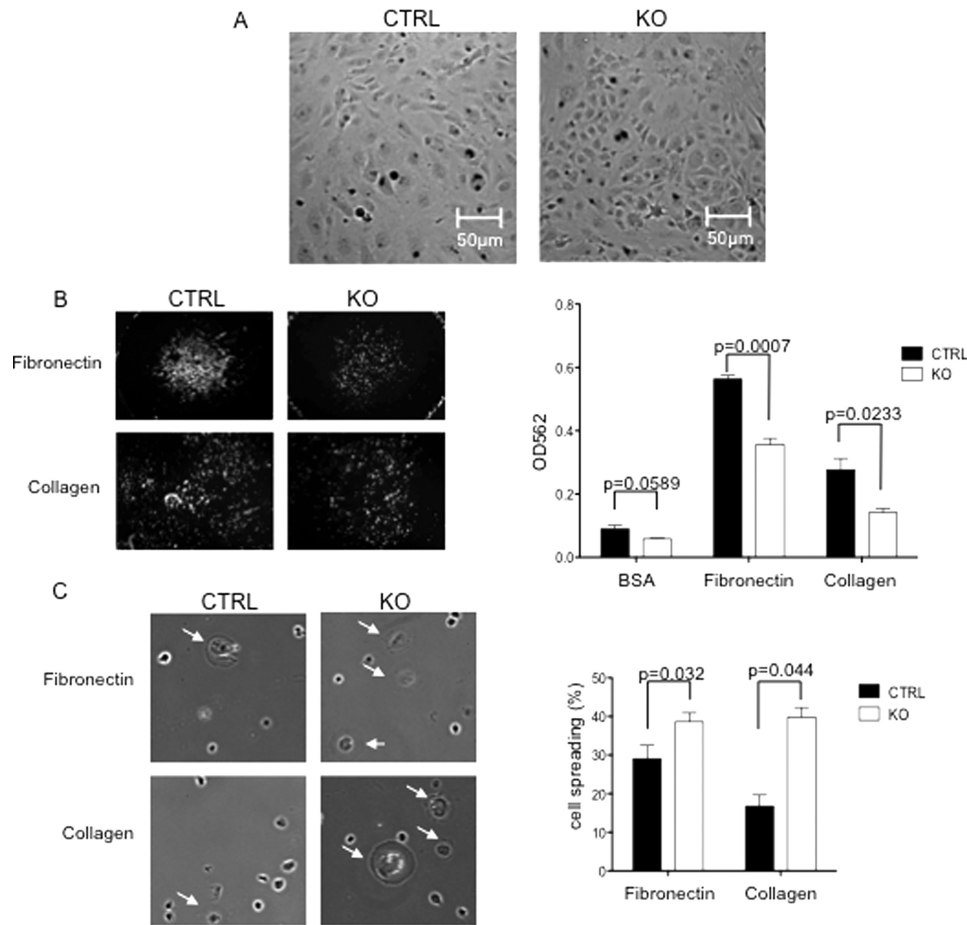
**PTP-PEST Regulates Integrin-mediated Adhesion and Spreading in Endothelial Cells**—Integrins are adhesion molecules involved in cell-cell and cell-substratum interactions (23, 29, 32). Compared with control cells, endothelial cells lacking PTP-PEST had more irregular sizes and shapes (Fig. 2A). To address the possibility that this alteration was the result of abnormal integrin function, the ability of endothelial cells to adhere in response to the integrin ligands fibronectin and collagen was tested (Fig. 2B). Fibronectin binds  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  integrins, whereas collagen recognizes  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins on endothelial cells. After 30 min of adhesion to integrin ligand-coated surfaces, cells were washed, and adherent cells

were detected by staining with crystal violet. In comparison with cells expressing PTP-PEST, cells lacking PTP-PEST had a decrease in adhesion to fibronectin and collagen (Fig. 2B).

Once cells adhere to an integrin ligand-coated surface, they undergo integrin-dependent signaling events that lead to cell spreading. In light of this, the ability of endothelial cells to spread in response to integrin ligands was evaluated (Fig. 2C). Cells were added to fibronectin- or collagen-coated glass coverslips. After 90 min, non-adherent cells were washed away, and spreading of the remaining adherent cells was assessed. Whereas PTP-PEST-deficient cells exhibited less frequent adhesion than control cells, they underwent more frequent spreading (Fig. 2C).

Therefore, PTP-PEST was critical for integrin-mediated adhesion of endothelial cells. It also regulated endothelial cell spreading.

**PTP-PEST Is Not Required for Integrity of Endothelial Cell-Cell Junctions**—Endothelial wall integrity in the embryo and postnatal life is maintained by close contacts between endothelial cells (20–22). This is mediated in large part by VE-cadherin, a self-binding endothelial cell-specific member of the cadherin family of adhesion molecules. Given the impact of PTP-PEST on integrin-mediated adhesion noted above, we also examined the role of PTP-PEST in endothelial cell permeability. To this end, we first stained primary endothelial cells expressing or not expressing PTP-PEST with antibodies against VE-cadherin, which identifies cell-cell junctions (Fig. 3A). Staining was then



**FIGURE 2. Altered morphology, adhesion, and spreading of PTP-PEST-deficient endothelial cells.** *A*, light microscopy analysis of primary endothelial cells from tamoxifen-fed *Ptpn12*<sup>+/+</sup>;*UBC-Cre-ERT2*<sup>+</sup> (control (CTRL)) or tamoxifen-fed *Ptpn12*<sup>fl/fl</sup>;*UBC-Cre-ERT2*<sup>+</sup> (KO) mice. Data are representative of at least 10 experiments. *B*, the ability of control and KO endothelial cells to adhere to plastic coated with BSA, fibronectin, or collagen was analyzed. After the reaction was stopped, cells were stained with crystal violet. Low magnification photographs of representative wells are shown on the left. On the right, a microplate scanning spectrometer was used to quantitate the intensity of staining with crystal violet by measuring the optical density (OD) at 562 nm. Error bars representing standard deviations of triplicates with mean values and *p* values are shown. Data are representative of six (fibronectin) or three (collagen) experiments. *C*, spreading of endothelial cells was analyzed using fibronectin- or collagen-coated glass coverslips. Left, representative cells are shown. White arrows represent cells that have undergone spreading. Right, the percentages of cells (from 10 independent fields) showing full spreading are indicated. Error bars representing standard deviations with mean values and *p* values are shown. Data are representative of four experiments.

detected by confocal microscopy. As was the case for control cells, PTP-PEST-deficient cells exhibited staining with anti-VE-cadherin antibodies at the cell surface. Although cell sizes and shapes were distinct between the two cell populations, the anti-VE-cadherin antibody-decorated intercellular junctions did not seem to be appreciably affected by PTP-PEST deficiency.

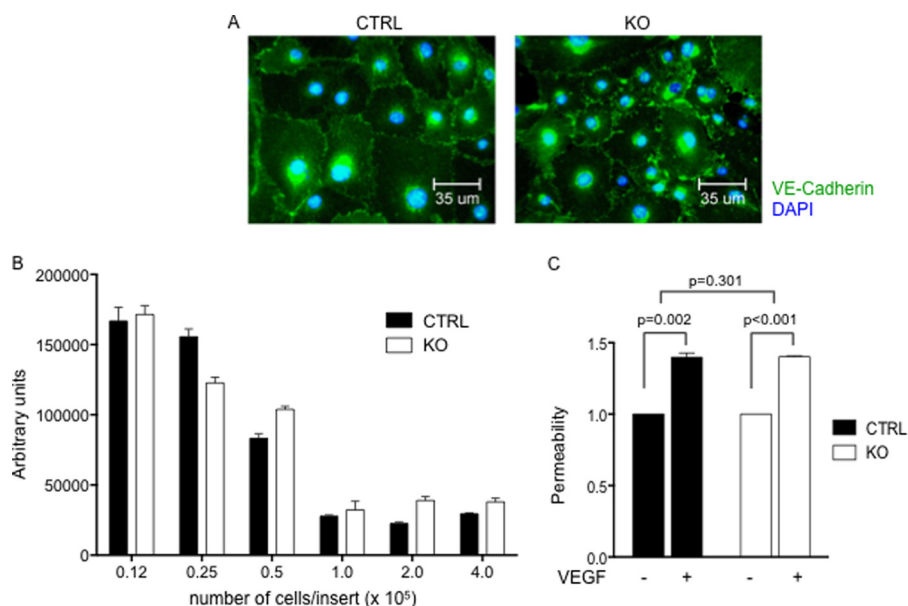
Next, endothelial cell integrity was examined using a vascular permeability assay (Fig. 3, *B* and *C*). In this assay, we monitored the ability of a small molecule, FITC-dextran, to diffuse across endothelial cells plated on a membrane with small pores (0.4- $\mu$ m pores). First, we assessed basal permeability (Fig. 3*B*). By plating progressively higher numbers of endothelial cells, we found that lack of PTP-PEST had no appreciable effect on the ability of endothelial cells to block transfer of FITC-dextran. Second, we examined the ability of the growth factor VEGF to induce an increase in vascular permeability (Fig. 3*C*). In three independent experiments, the absence of PTP-PEST had no impact on the capacity to augment transfer of FITC-dextran.

These findings indicated that PTP-PEST did not influence basal or VEGF-induced permeability of endothelial cells. They

also implied that PTP-PEST had no effect on VE-cadherin function.

*PTP-PEST Is Required for Endothelial Cell Migration*—Next, we ascertained the impact of PTP-PEST on the ability of endothelial cells to migrate, which is required for vascular development (Fig. 4). Migration was first analyzed using a Transwell migration assay (Fig. 4*A*). Endothelial cells were put in the upper chamber of a Transwell apparatus (4- $\mu$ m pores), and their migration toward the bottom chamber, which contained endothelial growth supplement and serum, was analyzed. Lack of PTP-PEST significantly reduced (by ~60%) the ability of endothelial cells to migrate in this assay.

Migration was also examined in a wound healing assay (Fig. 4, *B–E*). Primary endothelial cells were plated and, when confluent, inflicted with a scratch wound. Cell migration was then monitored over 24 h by time lapse microscopy and still photography (Fig. 4*B*). Endothelial cells from control mice migrated toward the wound and enabled its closure after 18 h. Although PTP-PEST-deficient endothelial cells also migrated toward the wound, they failed to close the wound after 24 h. Quantitation of the speed of wound closure (Fig. 4*C*) and the area repaired



**FIGURE 3. Cell-cell junctions and permeability of PTP-PEST-deficient endothelial cells.** *A*, immunostaining of primary endothelial cells from tamoxifen-fed *Ptpn12*<sup>+/+</sup>; *Ubc-Cre-ERT2*<sup>+</sup> (control (CTRL)) or tamoxifen-fed *Ptpn12*<sup>fl/fl</sup>; *Ubc-Cre-ERT2*<sup>+</sup> (KO) mice with anti-VE-cadherin antibodies (shown in green). Nuclei were stained with DAPI (shown in blue). The sizes and shapes of PTP-PEST-deficient endothelial cells were more irregular than those of control cells. Data are representative of three experiments. *B*, the permeability to macromolecules of primary endothelial cell monolayers from control and KO mice was analyzed using progressively higher numbers of endothelial cells seeded in the Transwell apparatus and FITC-dextran. The extent of diffusion of FITC-dextran (in arbitrary units) into the lower chamber was determined using a fluorescence spectrometer. Error bars representing standard deviations of triplicates with mean values are shown. Data are representative of at least three experiments. *C*, VEGF-induced increase in permeability was examined as detailed for *B* except that 3.0–3.5 × 10<sup>5</sup> cells/insert were plated. Cells were then stimulated or not with VEGF (75 ng/ml) for 60 min. Data are standardized so that base-line permeability (in the absence of VEGF) is given a value of 1.0. The results shown are derived from three independent experiments. Error bars representing standard deviations and *p* values are shown.

(Fig. 4D) showed that the speed of closure by cells lacking PTP-PEST cells was ~50% of that of control cells. Nonetheless, tracking of individual cells after staining nuclei with 4',6-diamidino-2-phenylindole (DAPI) showed that PTP-PEST-deficient cells moved directionally toward the wound as was the case for control cells (Fig. 4E). The “persistence index,” a measurement of directed migration, was not affected by PTP-PEST deficiency (Fig. 4F). Hence, the inability of PTP-PEST-deficient cells to close the wound was not due to compromised directional movement but rather was caused by reduced speed of migration.

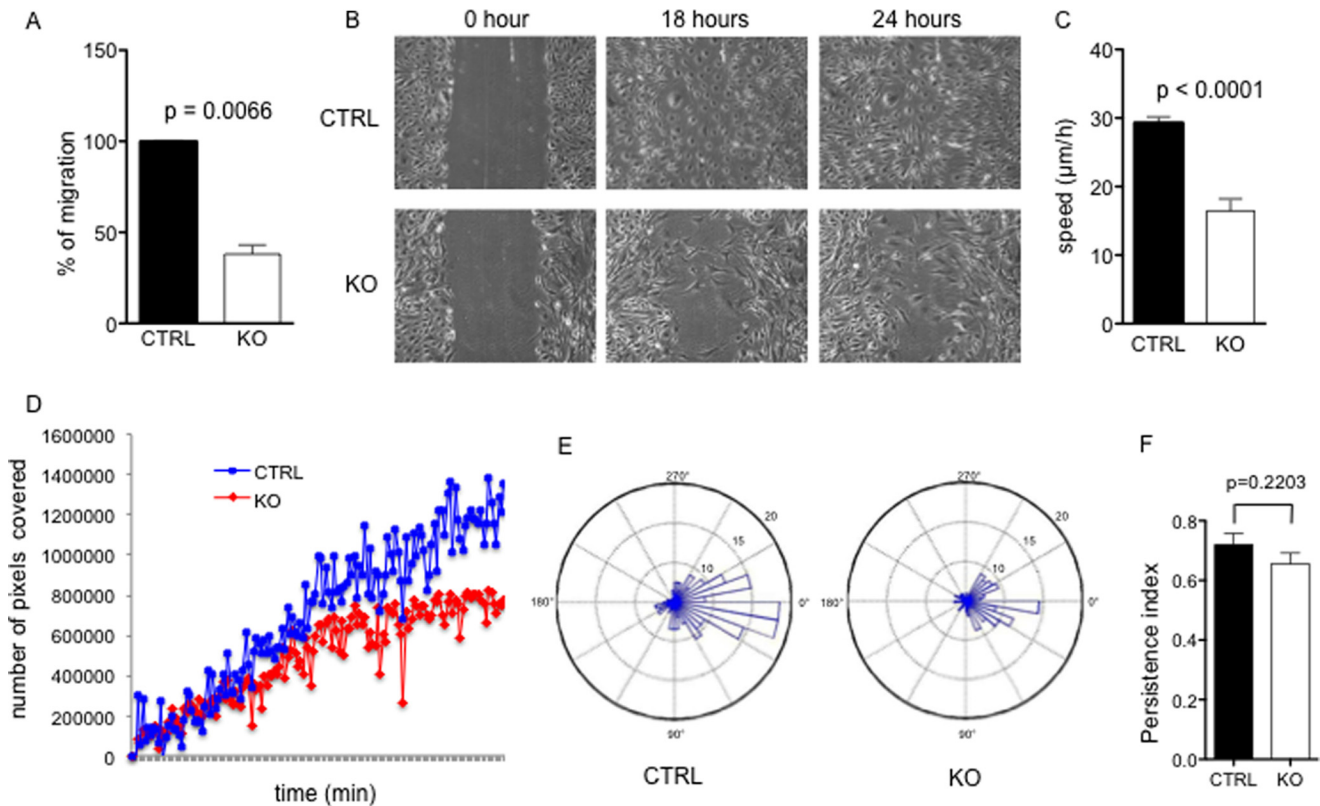
**PTP-PEST Regulates Cas, Paxillin, and Pyk2 in Endothelial Cells**—To ascertain the biochemical mechanism by which PTP-PEST influences endothelial cell functions, protein tyrosine phosphorylation was examined by probing total cell lysates with anti-phosphotyrosine immunoblotting (Fig. 5A). The lack of PTP-PEST selectively enhanced the tyrosine phosphorylation of polypeptides of ~115–130 kilodaltons (kDa) and less prominently 70 and 90 kDa. To identify these products, tyrosine phosphorylation of canonical PTP-PEST substrates was evaluated by immunoprecipitation (Fig. 5B) (1, 2). PTP-PEST deficiency resulted in a marked enhancement (3–6-fold) of tyrosine phosphorylation of Cas (~130 kDa) and paxillin (~70 kDa). A smaller increase (~2-fold) in the phosphotyrosine content of Pyk2 (~115 kDa) was seen. No effect was observed on FAK (~120 kDa). Similar results were obtained when phospho-specific antibodies directed against known phosphorylation sites of FAK, tyrosine 397 and tyrosines 576/577, were used (Fig. 5C).

We also analyzed the impact of PTP-PEST deficiency on VEGF-induced signals (Fig. 5D). Cells were stimulated or not for various periods of time with VEGF, and activation of ERK was monitored by immunoblotting total cell lysates with antibodies recognizing activated phospho-ERK. The lack of PTP-PEST had little or no effect on VEGF-induced ERK activation in keeping with the lack of effect on vascular permeability noted earlier.

Thus, the lack of PTP-PEST in endothelial cells resulted in augmented tyrosine phosphorylation of Cas, paxillin, and, to a lesser extent, Pyk2. All three substrates are known regulators or effectors of the integrin pathway. However, it had no obvious effect of VEGF-induced signals.

**PTP-PEST Expression in Endothelial Cells Is Necessary for Vascular Development and Embryonic Viability**—Previous analyses of a constitutive PTP-PEST-deficient mouse in which PTP-PEST was missing from all cells showed that PTP-PEST is critical for embryonic development and viability (13). To examine whether this was due to a role of PTP-PEST in endothelial cells, *Ptpn12*<sup>fl/fl</sup> mice were crossed with mice expressing Cre under the control of the *Tie2* (or *Tek*) promoter (Tables 1 and 2 and Fig. 6) (29, 32, 33). *Tie2-Cre* is active in all endothelial cells and a subset of hematopoietic cells and is widely used to delete conditional alleles in endothelial cells. Heterozygous *Ptpn12*<sup>fl/+</sup>; *Tie2-Cre*<sup>+</sup> mice were then bred with *Ptpn12*<sup>fl/fl</sup> mice. Strikingly, genotype analyses of a total of 339 mice born from this cross failed to identify any *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> pups (Table 1). Normally, ~25% of live pups would be expected to be *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup>. This finding suggested that, as reported for consti-

## PTP-PEST in Endothelial Cells



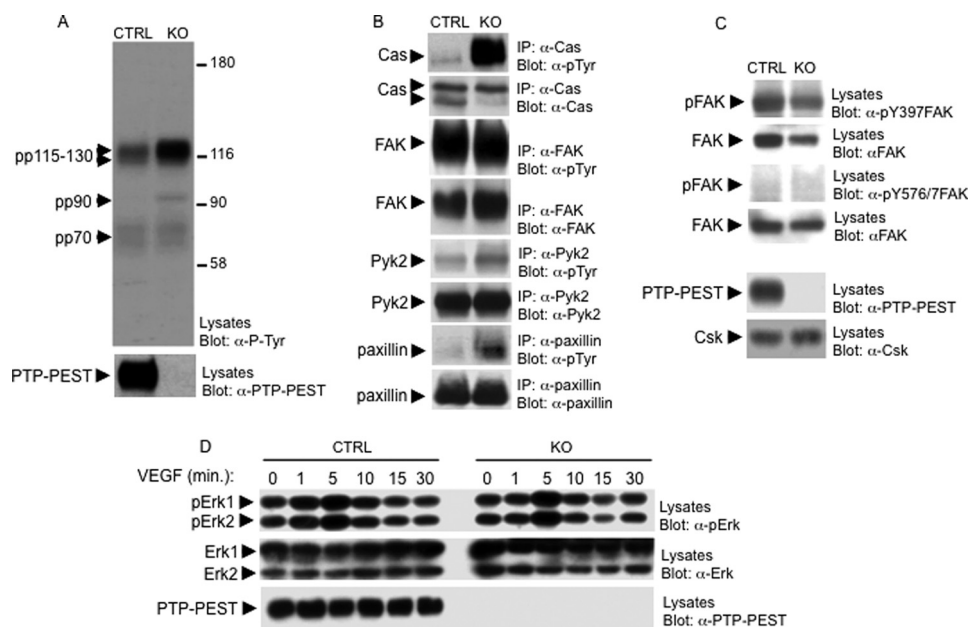
**FIGURE 4. Reduced migration by PTP-PEST-deficient endothelial cells.** *A*, the migration of primary endothelial cells from tamoxifen-fed *Ptpn12*<sup>+/+</sup>; *Ubc-Cre-ERT2*<sup>+</sup> (control (CTRL)) or tamoxifen-fed *Ptpn12*<sup>fl/fl</sup>; *Ubc-Cre-ERT2*<sup>+</sup> (KO) mice was examined in a Transwell migration assay. Data from three independent experiments with average values and error bars representing standard deviations are graphically presented. To facilitate the analysis, the migration of control cells in each experiment was considered as 100%. *B–F*, wound healing assay. A scratch wound was inflicted in monolayers of endothelial cells. The closure of the wound was monitored by time lapse microscopy. Still images taken at various times are shown in *B*, and the speed of migration is presented in *C*. Error bars representing standard deviations of triplicates with *p* values and mean values are shown. The surface of the wound covered by cells (depicted as pixels covered) was also estimated over time (*D*). Lastly, individual cells (stained with Hoechst) were tracked over a period of 6 h, and the direction and extent of their migration were analyzed (*E*). In the latter analysis, plotting the angle with respect to the initial position of the cell in a rose plot diagram depicts directionality of cell migration. The area of each bin represents the number of cells moving in the same direction of migration. For simplification, all cells in *E* are shown to migrate toward a hypothetical wound located on the right side of the field. For control mice, 176 cells were analyzed, whereas for KO mice, 106 cells were analyzed. The persistence index was assessed in *F*. It is the ratio between the shortest distance of the cells at time 3 h and their original position at time 0 over the actual distance traveled by the cells. 128 cells were analyzed for each mouse. Error bars representing standard deviations are shown. Image analyses and measurements were performed using Volocity software (*A* and *C*) or Matlab software (*D–F*). Data are representative of 15 (*B* and *D*), seven (*C*), or three (*E* and *F*) experiments.

tutive PTP-PEST-deficient mice, the embryos lacking PTP-PEST in cells targeted by *Tie2-Cre* were dying *in utero*. To evaluate this possibility, embryos from timed pregnancies were studied (Table 2). Live *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> embryos were observed at E10.5 but not at E11.5. This implied that *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> embryos died between E10.5 and E11.5.

Macroscopic examination of these embryos showed that E9.5 and E10.5 *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> embryos were smaller than control embryos (control embryos were either *Ptpn12*<sup>fl/fl</sup> or *Ptpn12*<sup>fl/+</sup>; *Tie2-Cre*<sup>+</sup>) (Fig. 6A). This was especially true for E10.5 embryos. Nonetheless, both were still alive as reflected by the presence of active heart contractions. Whole-mount staining of embryos with an antibody against CD31, a marker of endothelial cells, revealed that *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> embryos exhibited formation of some large blood vessels but had dramatically altered sprouting of smaller blood vessels (Fig. 6B). Many of the smaller blood vessels appeared dilated. Furthermore, toluidine blue-stained, semithin sections of E10.5 embryos showed that the dorsal aorta of PTP-PEST-deficient embryos had multiple hypersegmented lumens instead of a single lumen as found in control embryos (Fig. 6C).

We also performed a side-by-side comparison of anti-CD31-stained *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> embryos with constitutive *Ptpn12*<sup>-/-</sup> embryos (Fig. 6B). As reported elsewhere (13), constitutive PTP-PEST-deficient embryos died at ~E9.5–10.5, that is ~1 day earlier than *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> embryos. Moreover, at E9.5 and E10.5, they were smaller and more distorted and showed more extensive loss of vascular sprouting in comparison with *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> embryos. Possible explanations for the distinctions between *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> and *Ptpn12*<sup>-/-</sup> embryos will be provided under Discussion.

To assess whether lack of PTP-PEST affected endothelial cell differentiation *in vivo*, *Ptpn12*<sup>fl/fl</sup>; *Tie2-Cre*<sup>+</sup> mice were bred with *ROSA26-EYFP* mice in which a stop sequence flanked by *loxP* sites is inserted upstream of the *EYFP* gene to prevent expression of EYFP (Fig. 6D). In the presence of Cre, however, the stop sequence is removed, thereby enabling expression of EYFP and detection of *Ptpn12*-deleted cells (35). Flow cytometry analyses of EYFP<sup>+</sup> cells from disaggregated embryos revealed that large proportions of cells from PTP-PEST-deficient mice expressed CD31, ICAM-1, and ICAM-2. However, the proportions of cells expressing higher levels of these mark-



**FIGURE 5. Altered protein tyrosine phosphorylation in PTP-PEST-deficient endothelial cells.** *A*, overall tyrosine phosphorylation in primary endothelial cells from tamoxifen-fed *Ptpn12*<sup>fl/+</sup>; *UBC-Cre-ERT2*<sup>+</sup> (control (CTRL)) or tamoxifen-fed *Ptpn12*<sup>fl/fl</sup>; *UBC-Cre-ERT2*<sup>+</sup> (KO) mice was examined by anti-phosphotyrosine (*P-Tyr*) immunoblotting of total cell lysates. The positions of the substrates showing enhanced tyrosine phosphorylation are shown on the *left*, whereas those of prestained molecular weight markers are indicated on the *right*. Data are representative of at least five experiments. *B*, the extent of tyrosine phosphorylation of the indicated substrates was analyzed by immunoprecipitation (IP) with substrate-specific antibodies followed by immunoblotting with anti-phosphotyrosine (*pTyr*) antibodies. The abundance of the proteins in the immunoprecipitates was verified by reprobing the immunoblot membranes with substrate-specific antibodies. Data are representative of at least five experiments. *C*, tyrosine phosphorylation at specific sites on FAK (tyrosine 397 (*pY397*) and tyrosines 576 and 577 (*pY576/7*)) was assessed by immunoblotting of cell lysates with phosphospecific antibodies against these sites. Data are representative of three experiments. *D*, VEGF-induced activation of ERK was examined by stimulating cells or not with VEGF for the indicated periods of time. After lysis, activation of ERK was monitored by immunoblotting of cell lysates with a phosphospecific antibody against ERK (*pErk*). Data are representative of three experiments.

**TABLE 1**

**Distribution of genotypes in pups from cross between *Ptpn12*<sup>fl/+</sup>; *Tie2-Cre*<sup>+</sup> and *Ptpn12*<sup>fl/fl</sup>**

The absolute and relative numbers of live pups was examined at birth. Normally, each of the genotypes listed should encompass 25% of the total numbers of pups. 52 pregnant females were used.

Genotype	Birth
<i>Ptpn12</i> <sup>fl/fl</sup> <i>Tie2-Cre</i> <sup>+</sup>	0 (0%)
<i>Ptpn12</i> <sup>fl/fl</sup> <i>Tie2-Cre</i> <sup>-</sup>	113 (33.4%)
<i>Ptpn12</i> <sup>fl/+</sup> <i>Tie2-Cre</i> <sup>+</sup>	110 (32.4%)
<i>Ptpn12</i> <sup>fl/+</sup> <i>Tie2-Cre</i> <sup>-</sup>	116 (34.2%)
Total	339 (100%)

ers were lower in PTP-PEST-deficient mice compared with control mice. This alteration might be secondary to the poor general condition of the embryos or be an effect of PTP-PEST deficiency on the expansion, survival, or both of differentiated endothelial cells. Thus, PTP-PEST expression in endothelial cells was critical for embryonic viability and vascular development, although it did not seem to be required for emergence of the endothelial cell lineage.

## DISCUSSION

To generate PTP-PEST-deficient endothelial cells, *Ptpn12*<sup>fl/fl</sup> mice were bred with *UBC-Cre-ERT2* transgenic mice. Adult *Ptpn12*<sup>fl/fl</sup>; *UBC-Cre-ERT2*<sup>+</sup> mice were then fed tamoxifen, and primary cultures of lung-derived endothelial cells were established. These cells exhibited a total absence of PTP-PEST expression. Despite this, they proliferated normally *in vitro* and displayed normal expression of endothelial cell markers. The lack of effect of PTP-PEST deficiency on proliferation or differentiation of endo-

thelial cells was consistent with similar findings made in other cell types, namely fibroblasts and T cells in which lack of PTP-PEST had no appreciable impact on these functions (7, 11).

Nonetheless, PTP-PEST deficiency resulted in alterations of the morphology of endothelial cells. Moreover, although integrin expression was not altered, PTP-PEST-deficient endothelial cells had markedly reduced adhesion to fibronectin- or collagen-coated surfaces. Once adhered to these surfaces, they also exhibited enhanced spreading. In addition, PTP-PEST-deficient endothelial cells exhibited a pronounced defect in migration. This effect was noted in Transwell migration and wound healing assays. In the latter assay, lack of PTP-PEST reduced the speed of migration but had no effect on directional movement. From this observation, it was inferred that PTP-PEST is not required for sensing environmental cues that trigger migration but rather is needed for the migration process itself.

Although PTP-PEST is necessary for homotypic adhesion between activated T cells (11), it was not needed for homotypic interactions between endothelial cells (this report). In support of the latter, staining with anti-VE-cadherin antibodies showed that the intercellular junctions between endothelial cells were not detectably affected by lack of PTP-PEST. Additionally, in a vascular permeability assay using the small molecule FITC-dextran, endothelial cell integrity was not compromised in the absence or presence of VEGF. Although this distinction between T cells and endothelial cells might seem surprising, it likely reflects differences in the molecular basis of the homotypic interactions between these cells. Interactions between T cells are principally mediated by integrins, whereas interactions

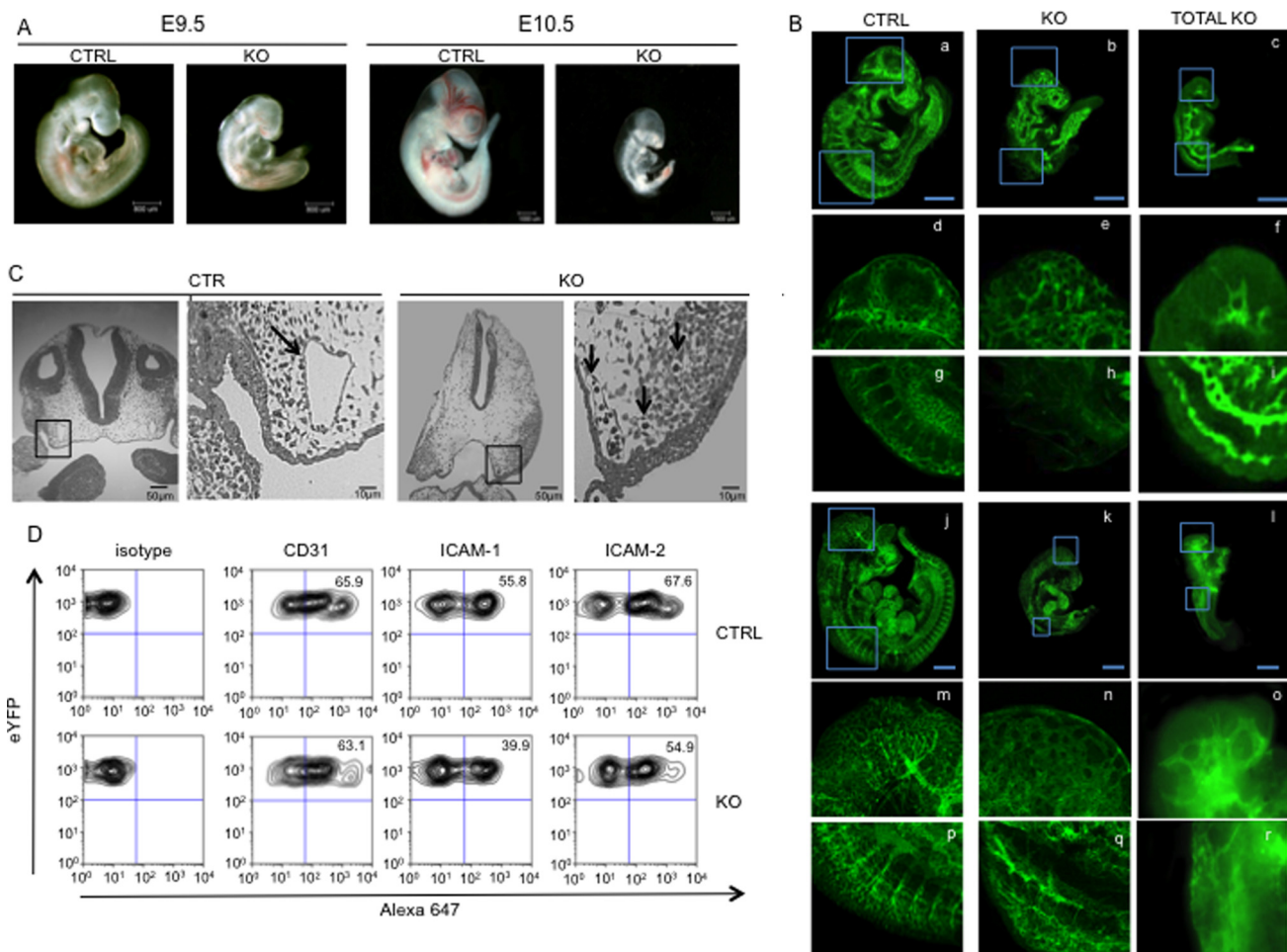


**TABLE 2**

**Distribution of genotypes in embryos from cross between  $Ptprn12^{fl/fl};Tie2-Cre^+$  and  $Ptprn12^{fl/fl}$**

The absolute and relative numbers of live embryos was examined at the indicated ages. Normally, each of the genotypes listed should encompass 25% of the total numbers of embryos. No live embryos were detected after E10.5. Eight or 13 pregnant females were used for E9.5 and E10.5, respectively.

Genotype	E9.5	E10.5	E11.5	E12.5	E13.5
$Ptprn12^{fl/fl};Tie2-Cre^+$	28 (23.0%)	39 (25.8%)	0 (0%)	0 (0%)	0 (0%)
$Ptprn12^{fl/fl};Tie2-Cre^-$	29 (23.8%)	39 (25.8%)	38 (33.9%)	35 (33.0%)	38 (33.0%)
$Ptprn12^{fl/+};Tie2-Cre^+$	32 (26.2%)	36 (23.8%)	36 (32.2%)	35 (33.0%)	39 (33.9%)
$Ptprn12^{fl/+};Tie2-Cre^-$	33 (27.0%)	37 (24.6%)	38 (33.9%)	36 (34.0%)	38 (33.1%)
Total	112 (100%)	151 (100%)	112 (100%)	106 (100%)	115 (100%)



**FIGURE 6. Analyses of  $Ptprn12^{fl/fl};Tie2-Cre^+$  embryos.** **A**, E9.5 and E10.5 embryos from  $Ptprn12^{fl/fl};Tie2-Cre^+$  (KO) and  $Ptprn12^{fl/+};Tie2-Cre^+$  (control (CTRL)) mice were photographed. Data are representative of >9 experiments. **B**, whole-mount staining with anti-CD31 antibodies of E9.5 (panels a–i) and E10.5 (panels j–r) embryos from KO and control mice. Embryos from a constitutive PTP-PEST-deficient mouse (TOTAL KO) are also shown for comparison. Regions defined in the blue-bordered insets in panels a–c and j–l were analyzed at greater magnification in panels d–i and m–r. Scale bars for panels A–C represent 800  $\mu$ m, whereas those for panels J–L represent 1000  $\mu$ m. Data are representative of two (E9.5) or three (E10.5) experiments. **C**, toluidine blue-stained cross-sections of E10.5 embryos from control and KO mice. For each mouse, the region defined in the inset in the left-hand panel was analyzed at greater magnification in the right-hand panel. Arrows show the location of the dorsal aorta. **D**, flow cytometry analyses of cells from disaggregated E10.5 embryos from control and KO mice. Analyses were gated on EYFP-positive cells. The percentages of cells positive for the various markers are shown in the upper right quadrant. Data are representative of three experiments.

between endothelial cells are primarily due to VE-cadherin. Seemingly, PTP-PEST influences the function of integrins but not that of VE-cadherin.

It is probable that dysregulated tyrosine phosphorylation of Cas, paxillin, and Pyk2 explains the adhesion and migration defects seen in PTP-PEST-deficient endothelial cells. All three molecules are known regulators of the integrin pathway (23, 24). More importantly, previous data showed that Cas plays a critical role in integrin-mediated endothelial cell migration and vascular development (39, 40). Likewise, Pyk2 was shown to be

involved in the control of angiogenesis (41). At this time, however, we cannot rule out that additional, as yet unidentified, substrates also contributed to the adhesion and migration defects seen in endothelial cells lacking PTP-PEST.

To examine the role of PTP-PEST expression in endothelial cells *in vivo*, we crossed  $Ptprn12^{fl/fl}$  mice with  $Tie2-Cre$  mice. Genotype analyses of embryos and born pups showed that all  $Ptprn12^{fl/fl};Tie2-Cre^+$  embryos died *in utero* between E10.5 and E11.5. Furthermore, prior to death, these embryos showed markedly retarded growth. By staining embryos for CD31, it

was found that *Ptpn12* deletion resulted in compromised vascular development, in particular angiogenesis. This was likely due to a defect in endothelial cell adhesion and migration as both processes are critical for normal vascular development (20, 24). The time at which embryonic lethality occurred was also consistent with a vascular defect. Indeed, several other gene mutations affecting the vasculature result in embryonic lethality at ~E9.5–10.5 (20, 25–27, 29–32, 39).

Of note, however, the embryonic lethality observed in *Ptpn12<sup>fl/fl</sup>;Tie2-Cre<sup>+</sup>* mice arose approximately 1 day later than that previously noted in constitutive PTP-PEST-deficient mice (13). Moreover, a side-by-side comparison showed that *Ptpn12<sup>-/-</sup>* embryos showed more extensive loss of vascular sprouting in comparison with *Ptpn12<sup>fl/fl</sup>;Tie2-Cre<sup>+</sup>* embryos. These disparities might be explained by the inducible nature of conditional Cre-mediated deletion. Although *Tie2-Cre* is expressed as early as E6.5, deletion of both copies of the conditional allele and degradation of all PTP-PEST RNA and protein (the half-life of the PTP-PEST protein has been estimated to be at least 4 h (42)) are needed for complete PTP-PEST deficiency. This might require a few days and likely enabled partial expression of PTP-PEST between E6.5 and E10.5. Alternatively, it might reflect a role of PTP-PEST in additional cell types that are not targeted by *Tie2-Cre*.

Adhesion receptors play critical roles in vascular functions during embryogenesis and adult life. The VE-cadherin pathway is a key regulator of vascular permeability. Previous data showed that Tie2, a PTK, and VE-PTP, a PTP, regulate the activity of VE-cadherin and that both are essential for vascular development and embryogenesis (20, 25–27). By opposition, the integrin pathway is important for the control of endothelial cell adhesion and migration. Earlier results, in addition to the findings herein, revealed that another PTK, FAK, and another PTP, PTP-PEST, control the activity of integrins. Both also have pivotal roles in vascular and embryonic development (29–32). In this manner, unique pairs of PTKs and PTPs regulate the two major adhesion receptor families controlling vascular functions.

Together, the findings reported herein show that PTP-PEST is a key regulator of integrin-mediated adhesion and migration of endothelial cells *in vivo* and *in vitro*. This function is likely mediated by the ability of PTP-PEST to dephosphorylate the cytoskeleton regulators Cas, paxillin, and Pyk2. Moreover, such an activity likely explains the critical role of PTP-PEST in vascular development and embryonic viability. Given the continuing importance of angiogenesis after birth, it is probable that PTP-PEST also plays a key function in postnatal processes involving endothelial cells. These processes may include wound healing and tissue repair and pathological situations such as tumor formation, inflammation, and diabetic retinopathy. The possible involvement of PTP-PEST in pathological angiogenesis raises the possibility that pharmacological inhibitors of PTP-PEST or of PTP-PEST-regulated pathways may have therapeutic benefit in these conditions.

*Acknowledgments*—We thank the members of our laboratory for discussions. We also thank Dominic Filion for help with microscopy and image analyses.

## REFERENCES

1. Veillette, A., Rhee, I., Souza, C. M., and Davidson, D. (2009) PEST family phosphatases in immunity, autoimmunity, and autoinflammatory disorders. *Immunol. Rev.* **228**, 312–324
2. Hallé, M., Tremblay, M. L., and Meng, T. C. (2007) Protein tyrosine phosphatases: emerging regulators of apoptosis. *Cell Cycle* **6**, 2773–2781
3. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) Identification of p130(cas) as a substrate for the cytosolic protein tyrosine phosphatase PTP-PEST. *Mol. Cell. Biol.* **16**, 6408–6418
4. Garton, A. J., Burnham, M. R., Bouton, A. H., and Tonks, N. K. (1997) Association of PTP-PEST with the SH3 domain of p130cas; a novel mechanism of protein tyrosine phosphatase substrate recognition. *Oncogene* **15**, 877–885
5. Shen, Y., Schneider, G., Cloutier, J. F., Veillette, A., and Schaller, M. D. (1998) Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin. *J. Biol. Chem.* **273**, 6474–6481
6. Côté, J. F., Chung, P. L., Thériberge, J. F., Hallé, M., Spencer, S., Lasky, L. A., and Tremblay, M. L. (2002) PSTPIP is a substrate of PTP-PEST and serves as a scaffold guiding PTP-PEST toward a specific dephosphorylation of WASP. *J. Biol. Chem.* **277**, 2973–2986
7. Angers-Loustau, A., Côté, J. F., Charest, A., Dowbenko, D., Spencer, S., Lasky, L. A., and Tremblay, M. L. (1999) Protein tyrosine phosphatase-PEST regulates focal adhesion disassembly, migration, and cytokinesis in fibroblasts. *J. Cell Biol.* **144**, 1019–1031
8. Charest, A., Wagner, J., Kwan, M., and Tremblay, M. L. (1997) Coupling of the murine protein tyrosine phosphatase PEST to the epidermal growth factor (EGF) receptor through a Src homology 3 (SH3) domain-mediated association with Grb2. *Oncogene* **14**, 1643–1651
9. Charest, A., Wagner, J., Jacob, S., McGlade, C. J., and Tremblay, M. L. (1996) Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase-PEST. Evidence for extended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity. *J. Biol. Chem.* **271**, 8424–8429
10. Lyons, P. D., Dunty, J. M., Schaefer, E. M., and Schaller, M. D. (2001) Inhibition of the catalytic activity of cell adhesion kinase  $\beta$  by protein-tyrosine phosphatase-PEST-mediated dephosphorylation. *J. Biol. Chem.* **276**, 24422–24431
11. Davidson, D., Shi, X., Zhong, M. C., Rhee, I., and Veillette, A. (2010) The phosphatase PTP-PEST promotes secondary T cell responses by dephosphorylating the protein tyrosine kinase Pyk2. *Immunity* **33**, 167–180
12. Davidson, D., and Veillette, A. (2001) PTP-PEST, a scaffold protein tyrosine phosphatase, negatively regulates lymphocyte activation by targeting a unique set of substrates. *EMBO J.* **20**, 3414–3426
13. Sirois, J., Côté, J. F., Charest, A., Uetani, N., Bourdeau, A., Duncan, S. A., Daniels, E., and Tremblay, M. L. (2006) Essential function of PTP-PEST during mouse embryonic vascularization, mesenchyme formation, neurogenesis and early liver development. *Mech. Dev.* **123**, 869–880
14. Angers-Loustau, A., Côté, J. F., and Tremblay, M. L. (1999) Roles of protein tyrosine phosphatases in cell migration and adhesion. *Biochem. Cell Biol.* **77**, 493–505
15. Garton, A. J., and Tonks, N. K. (1999) Regulation of fibroblast motility by the protein tyrosine phosphatase PTP-PEST. *J. Biol. Chem.* **274**, 3811–3818
16. Jamieson, J. S., Tumbarello, D. A., Hallé, M., Brown, M. C., Tremblay, M. L., and Turner, C. E. (2005) Paxillin is essential for PTP-PEST-dependent regulation of cell spreading and motility: a role for paxillin kinase linker. *J. Cell Sci.* **118**, 5835–5847
17. Sastry, S. K., Rajfur, Z., Liu, B. P., Cote, J. F., Tremblay, M. L., and Burrridge, K. (2006) PTP-PEST couples membrane protrusion and tail retraction via VAV2 and p190RhoGAP. *J. Biol. Chem.* **281**, 11627–11636
18. Sun, T., Aceto, N., Meerbrey, K. L., Kessler, J. D., Zhou, C., Migliaccio, I., Nguyen, D. X., Pavlova, N. N., Botero, M., Huang, J., Bernardi, R. J., Schmitt, E., Hu, G., Li, M. Z., Dephoure, N., Gygi, S. P., Rao, M., Creighton, C. J., Hilsenbeck, S. G., Shaw, C. A., Muzny, D., Gibbs, R. A., Wheeler, D. A., Osborne, C. K., Schiff, R., Bentires-Alj, M., Elledge, S. J., and Westbrook, T. F. (2011) Activation of multiple proto-oncogenic tyrosine kinases in breast cancer via loss of the PTPN12 phosphatase. *Cell* **144**,

19. Pober, J. S., and Sessa, W. C. (2007) Evolving functions of endothelial cells in inflammation. *Nat. Rev. Immunol.* **7**, 803–815
20. Gory-Fauré, S., Prandini, M. H., Pointu, H., Roullot, V., Pignot-Paintrand, L., Vernet, M., and Huber, P. (1999) Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* **126**, 2093–2102
21. Dejana, E., Tournier-Lasserre, E., and Weinstein, B. M. (2009) The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev. Cell* **16**, 209–221
22. Vestweber, D., Winderlich, M., Cagna, G., and Nottebaum, A. F. (2009) Cell adhesion dynamics at endothelial junctions: VE-cadherin as a major player. *Trends Cell Biol.* **19**, 8–15
23. Lamalice, L., Le Boeuf, F., and Huot, J. (2007) Endothelial cell migration during angiogenesis. *Circ. Res.* **100**, 782–794
24. Silva, R., D'Amico, G., Hodivala-Dilke, K. M., and Reynolds, L. E. (2008) Integrins: the keys to unlocking angiogenesis. *Arterioscler. Thromb. Vasc. Biol.* **28**, 1703–1713
25. Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A., and Breitman, M. L. (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8**, 1897–1909
26. Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70–74
27. Dominguez, M. G., Hughes, V. C., Pan, L., Simmons, M., Daly, C., Anderson, K., Noguera-Troise, I., Murphy, A. J., Valenzuela, D. M., Davis, S., Thurston, G., Yancopoulos, G. D., and Gale, N. W. (2007) Vascular endothelial tyrosine phosphatase (VE-PTP)-null mice undergo vasculogenesis but die embryonically because of defects in angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3243–3248
28. Winderlich, M., Keller, L., Cagna, G., Broermann, A., Kamenyeva, O., Kiefer, F., Deutsch, U., Nottebaum, A. F., and Vestweber, D. (2009) VE-PTP controls blood vessel development by balancing Tie-2 activity. *J. Cell Biol.* **185**, 657–671
29. Tanjore, H., Zeisberg, E. M., Gerami-Naini, B., and Kalluri, R. (2008)  $\beta$ 1 integrin expression on endothelial cells is required for angiogenesis but not for vasculogenesis. *Dev. Dyn.* **237**, 75–82
30. Shen, T. L., Park, A. Y., Alcaraz, A., Peng, X., Jang, I., Koni, P., Flavell, R. A., Gu, H., and Guan, J. L. (2005) Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. *J. Cell Biol.* **169**, 941–952
31. Braren, R., Hu, H., Kim, Y. H., Beggs, H. E., Reichardt, L. F., and Wang, R. (2006) Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. *J. Cell Biol.* **172**, 151–162
32. Carlson, T. R., Hu, H., Braren, R., Kim, Y. H., and Wang, R. A. (2008) Cell-autonomous requirement for  $\beta$ 1 integrin in endothelial cell adhesion, migration and survival during angiogenesis in mice. *Development* **135**, 2193–2202
33. Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A., and Yanagisawa, M. (2001) Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis *in vivo*. *Dev. Biol.* **230**, 230–242
34. Ruzankina, Y., Pinzon-Guzman, C., Asare, A., Ong, T., Pontano, L., Cot-sarelis, G., Zediak, V. P., Velez, M., Bhandoola, A., and Brown, E. J. (2007) Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell* **1**, 113–126
35. Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M., and Costantini, F. (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* **1**, 4
36. Thibeault, S., Rautureau, Y., Oubaha, M., Faubert, D., Wilkes, B. C., Delisle, C., and Gratton, J. P. (2010) S-Nitrosylation of  $\beta$ -catenin by eNOS-derived NO promotes VEGF-induced endothelial cell permeability. *Mol. Cell* **39**, 468–476
37. Veillette, A., Bookman, M. A., Horak, E. M., and Bolen, J. B. (1988) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* **55**, 301–308
38. Cloutier, J. F., and Veillette, A. (1996) Association of inhibitory tyrosine protein kinase p50csk with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells. *EMBO J.* **15**, 4909–4918
39. Honda, H., Oda, H., Nakamoto, T., Honda, Z., Sakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., Katsuki, M., Yazaki, Y., and Hirai, H. (1998) Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. *Nat. Genet.* **19**, 361–365
40. Ohmori, T., Yatomi, Y., Okamoto, H., Miura, Y., Rile, G., Satoh, K., and Ozaki, Y. (2001) G<sub>i</sub>-mediated Cas tyrosine phosphorylation in vascular endothelial cells stimulated with sphingosine 1-phosphate: possible involvement in cell motility enhancement in cooperation with Rho-mediated pathways. *J. Biol. Chem.* **276**, 5274–5280
41. Weis, S. M., Lim, S. T., Lutu-Fuga, K. M., Barnes, L. A., Chen, X. L., Göthert, J. R., Shen, T. L., Guan, J. L., Schlaepfer, D. D., and Cheresch, D. A. (2008) Compensatory role for Pyk2 during angiogenesis in adult mice lacking endothelial cell FAK. *J. Cell Biol.* **181**, 43–50
42. Charest, A., Wagner, J., Shen, S. H., and Tremblay, M. L. (1995) Murine protein tyrosine phosphatase-PEST, a stable cytosolic protein tyrosine phosphatase. *Biochem. J.* **308**, 425–432