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Protein Tyrosine Phosphatase 1B Deficiency in Vascular Smooth Muscle Cells Promotes Perivascular Fibrosis following Arterial Injury

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Abstract

Background Smooth muscle cell (SMC) phenotype switching plays a central role during vascular remodeling. Growth factor receptors are negatively regulated by protein tyrosine phosphatases (PTPs), including its prototype PTP1B. Here, we examine how reduction of PTP1B in SMCs affects the vascular remodeling response to injury.

Methods Mice with inducible PTP1B deletion in SMCs (SMC.PTP1B-KO) were generated by crossing mice expressing Cre.ER^{T2} recombinase under the *Myh11* promoter with PTP1B$^{flox/flox}$ mice and subjected to $FeCl_3$ carotid artery injury.

Results Genetic deletion of PTP1B in SMCs resulted in adventitia enlargement, perivascular SMA$^+$ and PDGFRβ$^+$ myofibroblast expansion, and collagen accumulation following vascular injury. Lineage tracing confirmed the appearance of *Myh11*-Cre reporter cells in the remodeling adventitia, and SCA1$^+$ CD45$^-$ vascular progenitor cells increased. Elevated mRNA expression of transforming growth factor β (TGFβ) signaling components or enzymes involved in extracellular matrix remodeling and TGFβ liberation was seen in injured SMC.PTP1B-KO mouse carotid arteries, and mRNA transcript levels of contractile SMC marker genes were reduced already at baseline. Mechanistically, Cre recombinase (mice) or siRNA (cells)-mediated downregulation of PTP1B or inhibition of ERK1/2 signaling in SMCs resulted in nuclear accumulation of KLF4, a central transcriptional repressor of SMC differentiation, whereas phosphorylation and nuclear translocation of SMAD2 and SMAD3 were reduced. SMAD2 siRNA transfection

Keywords
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- neointima formation
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- smooth muscle cells

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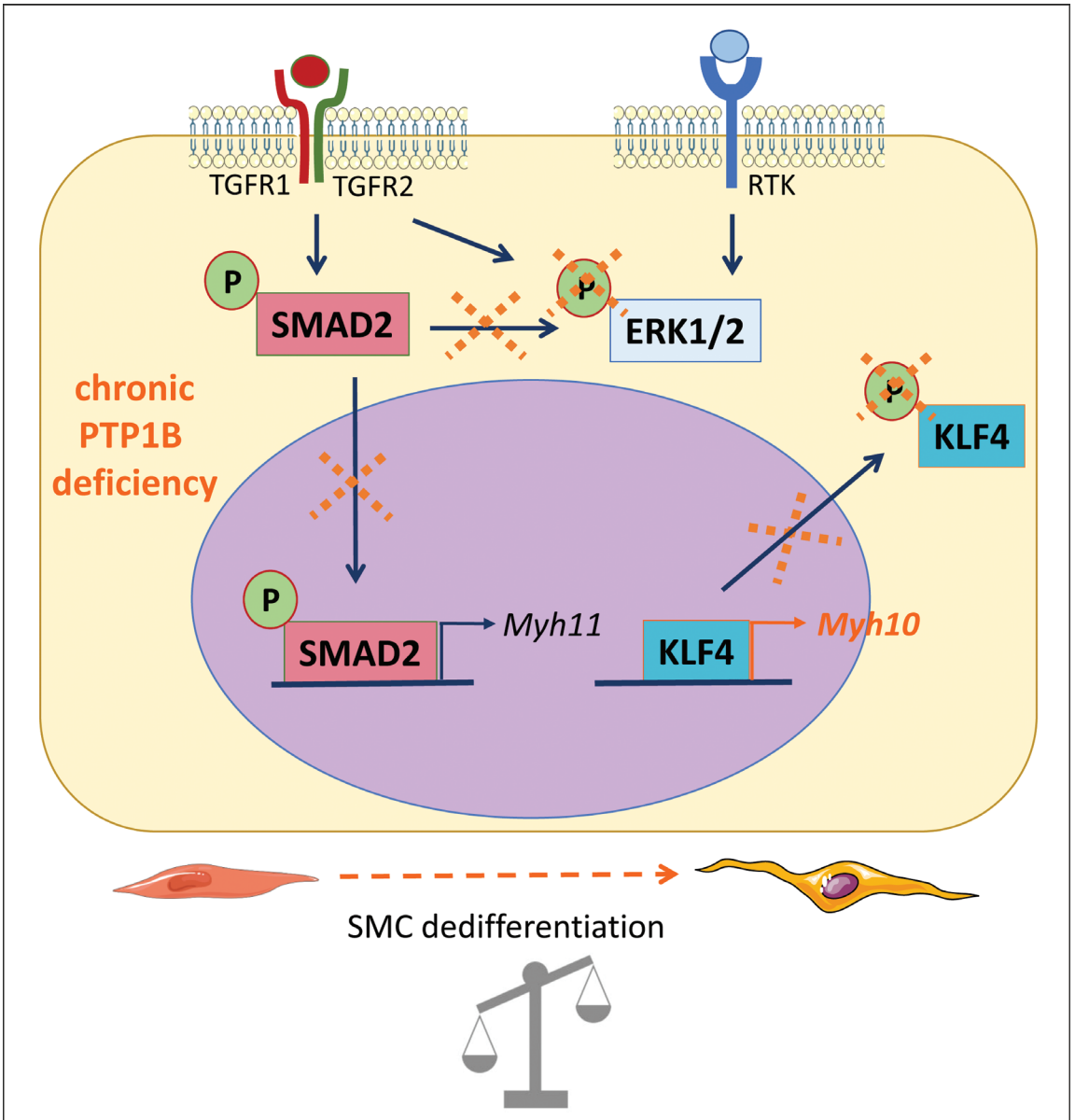

Visual summary. Changes in SMC signaling and differentiation marker expression following genetic deletion of PTP1B and chronic PTP1B deficiency. Reduced phosphorylation of ERK1/2, e.g., downstream of TGFβ type 1 and type 2 receptors (TGFR1 and TGFR2) or receptor tyrosine kinases (RTK) mediating growth factor signaling, impairs the nuclear transport of SMAD2 and its binding to target genes involved in SMC differentiation, such as Myh11, whereas lower ERK1/2-mediated phosphorylation of KLF4 reduces its nuclear export and stabilizes KLF4 bound to the promoter of genes involved in SMC dedifferentiation, such as Myh10. Cellular events that are reduced or inhibited in states of chronic PTP1B deficiency are indicated by orange crosses. Events that are induced are highlighted in bold. SMC, smooth muscle cell.

increased protein levels of PDGFRβ and MYH10 while reducing ERK1/2 phosphorylation, thus phenocopying genetic PTP1B deletion.

Conclusion Chronic reduction of PTP1B in SMCs promotes dedifferentiation, perivascular fibrosis, and adverse remodeling following vascular injury by mechanisms involving an ERK1/2 phosphorylation-driven shift from SMAD2 to KLF4-regulated gene transcription.

Introduction

The excessive proliferation and migration of smooth muscle cells (SMCs) in response to growth factors plays an important role in vascular disease processes, including atherosclerosis and restenosis following vascular injury.[1] Growth factors, such as platelet-derived growth factor (PDGF) or transforming growth factor β (TGFβ), are released from activated platelets, myofibroblasts and inflammatory cells, and bind to their receptors expressed, among others, on SMCs. In response to growth factor stimulation, SMCs may lose the expression of markers of differentiation, such as smooth muscle myosin heavy chain (SMMHC or MYH11), calponin, or smoothelin, and start expressing genes expressed in less differentiated SMCs or other cell lineages, a process commonly referred to as SMC phenotype switching.[2] Moreover, dedifferentiated SMCs exhibited increased expression of extracellular matrix proteins, which promotes vascular lesion growth, but also stability.

Endogenous control mechanisms involved in the termination of activated growth factor signaling and thus the prevention of uncontrolled SMC growth include the dephosphorylation of tyrosine residues on receptors and downstream signaling intermediates by protein tyrosine phosphatases. Although beneficial to terminate signaling events, absence or inactivation of this "molecular brake" and subsequent chronic growth factor overstimulation may result in premature senescence[3] and promote vascular disease.[4] A major protein tyrosine phosphatase expressed in SMCs is protein tyrosine phosphatase 1B (PTP1B).[5] Earlier studies in rats revealed upregulated PTP1B mRNA expression levels in proliferating and migrating intimal and medial SMCs following balloon catheter-induced vascular injury.[6,7] However, the question of whether PTP1B upregulation is an adaptive mechanism or causally involved in restenosis development still remains unanswered. In cultivated cells, adenoviral overexpression of PTP1B was shown to inhibit SMC proliferation and migration in response to PDGF and fibroblast growth factor.[8] In vivo, overexpression of dominant negative PTP1B[8] increased neointima formation after balloon injury in rats, whereas periadventitial application of a general protein tyrosine kinase inhibitor inhibited intimal hyperplasia.[9] PTP1B is expressed in several cell types involved in the vascular remodeling response, including endothelial and inflammatory cells, and those previous studies therefore do not allow making definite conclusions about its role in SMCs.

Genetic fate mapping studies using *Myh11*-CreER^{T2} reporter mice have documented the plasticity of SMC lineages, and that vascular injury is associated with dedifferentiation events and cellular phenotype changes.[10,11] First experimental evidence underlines the importance of the negative regulation of transdifferentiation events by phosphatases, as shown for phosphatase and tensin homolog phosphatase and tensin homolog (PTEN) during atherosclerosis.[12–14] The aim of the present study was to determine how inducible deletion of PTP1B in SMCs affects their differentiation and phenotype during neointima formation following vascular injury. Our findings suggest a role for PTP1B in MYH11-Cre-expressing cells in controlling SMC differentiation and the development of perivascular fibrosis which involves changes in the extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated phosphorylation and the nuclear transport of the transcription factors Krüppel-like factor 4 (KLF4) and mothers against decapentaplegic homolog 2 (SMAD2).

Methods

A detailed description of the material and methods is provided in the **Supplementary Material** (►**Supplementary Methods** [available in the online version]).

Studies Involving Mice

To generate mice lacking PTP1B in SMCs (SMC.PTP1B knockout, KO), mice with loxP-flanked (floxed, fl/fl) *Ptp1b* alleles (courtesy of Benjamin G. Neel)[15] were crossed with mice expressing a Cre recombinase-estrogen receptor fusion protein (ER^{T2}-Cre) under control of the SMMHC (*Myh11*) promoter,[16] regarded as one of the most specific Cre driver lines for SMCs to date.[17] Cre recombinase activity was induced by feeding male mice with tamoxifen-containing rodent chow (Harlan Laboratories; TD.55125) for 6 weeks, as reported previously.[18] To visualize Cre recombinase expression, SMMHC.ER^{T2}-Cre mice were mated with IRG mice,[19] in which Cre-mediated recombination results in green fluorescent protein (GFP) expression. At the age of 12 to 14 weeks, male littermate mice were anesthetized by intraperitoneal injection of xylazine (CP-Pharma; 16 mg/kg body weight) and ketamine hydrochloride (Pharmanovo; 80 mg/kg body weight) and subjected to carotid artery injury using 10% ferric chloride ($FeCl_3$), as described.[20] At different time points after vascular injury, deeply anesthetized mice were killed by perfusion with sterile 0.9% sodium chloride solution via the left ventricle and the injured as well as the contralateral, uninjured carotid arteries harvested and prepared for subsequent molecular and histological analyses. All animal care and experimental procedures had been approved by the institutional Animal Research Committee (G-16–1-081) and complied with national guidelines for the care and use of laboratory animals.

Studies Involving Cells

Primary SMCs were isolated from aortas of uninjured C57BL/6J wild-type mice or from uninjured SMC.PTP1B-WT (wild-type) and SMC.PTP1B-KO littermates, as described in the **Supplementary Material** (►**Supplementary Methods** [available in the online version]), and analyzed using quantitative *real-time* polymerase chain reaction (PCR), western blot, and immunocytochemistry. In some experiments, cells were incubated with the MAP kinases MEK1 and MEK2 inhibitor PD98059 (Calbiochem; 10 μM in dimethyl sulfoxide [DMSO]). Human primary aortic SMCs (HAoSMCs; PromoCell) were treated with a cell-permeable, selective, reversible, and noncompetitive allosteric inhibitor of PTP1B (Calbiochem; 539741) or transfected with small-interfering RNA (siRNA) targeting PTP1B (Santa Cruz Biotechnology; sc-36328) or SMAD2 (ThermoFisher Scientific; Cat #AM16708) or with

fluorescein-labeled control siRNA (Santa Cruz Biotechnology; sc-36869) using lipofectamine (ThermoFisher Scientific) and analyzed 48 to 72 hours later.

Statistical Analysis

Quantitative data are reported as mean ± standard error of the mean or median with interquartile range, depending on the presence of normal distribution or not, as determined using the D'Agostino–Pearson omnibus normality test. Two groups were compared using Student's *t*-test, if normal distribution was present, or nonparametric Mann–Whitney test, if not. If more than two groups were compared, One-way analysis of variance test followed by Sidak's multiple comparison test was performed. Statistical differences were assumed if p reached a value less than 0.05. All analyses were performed using GraphPad PRISM data analysis software (version 9.3.0; GraphPad Software Inc.).

Results

Increased Vascular PTP1B Expression following Carotid Artery Injury

In preliminary analyses, we observed that vascular injury, induced by application of 10% $FeCl_3$ onto the adventitia of the common carotid artery of C57BL/6J wild-type mice ($n = 3$ mice per time point), was associated with an increased PTP1B protein expression, particularly at day 7 (►**Supplementary Fig. S1A**, **B** [available in the online version]). PTP1B immunosignals localized to cells within the media and developing neointima as well as the outer layers of the vessel wall (►**Supplementary Fig. S1C** [available in the online version]). Similar observations were reported by others[6,7]; however, whether PTP1B plays a causal role for vascular remodeling processes has never been directly examined to date.

Generation of Mice with Inducible Deletion of PTP1B in Smooth Muscle Cells

To determine the role of PTP1B for neointima formation, we generated mice with tamoxifen-inducible, *Myh11*-CreER^{T2} recombinase-driven deletion of PTP1B in SMCs (SMC.PTP1B-KO mice; ►**Supplementary Fig. S2A** [available in the online version]). Significantly reduced PTP1B mRNA and protein levels in primary SMCs isolated from the aorta of SMC.PTP1B-KO mice compared with SMC.PTP1B-WT controls were confirmed using quantitative *real-time* PCR (►**Supplementary Fig. S2B** [available in the online version]) and Western blot analysis (►**Supplementary Fig. S2C** [online only]) and also confirmed using confocal immunofluorescence microscopy (►**Supplementary Fig. S2D** [available in the online version]). Low PTP1B immunosignals were also seen in vivo in the aortic media of SMC.PTP1B-KO mice (►**Supplementary Fig. S2E** [available in the online version]).

Deletion of PTP1B in *Myh11*.ER^{T2}-Cre-Expressing Cells Does Not Alter Inward Vascular Remodeling but Leads to Adventitia Enlargement

To test the importance of PTP1B for the response of SMCs to growth factors, as released from activated platelets, immune and other cell types, mice were exposed to $FeCl_3$-induced carotid artery injury and thrombosis. Morphometric analysis of vascular lesion formation 3 weeks after injury revealed that the neointima (►**Supplementary Fig. S3A** [available in the online version]) or media (►**Supplementary Fig. S3B** [online only]) area and the degree of lumen stenosis (►**Supplementary Fig. S3C** [available in the online version]) did not differ between SMC.PTP1B-WT ($n = 14$) and SMC-PTP1B-KO ($n = 14$) mice. These findings were unexpected given the previously reported negative regulatory role of PTP1B in growth factor signaling in SMCs in vitro.[8,9,21] On the other hand, morphometric analysis of the outer layers of the vessel wall revealed that the mean area (►**Fig. 1A**) and

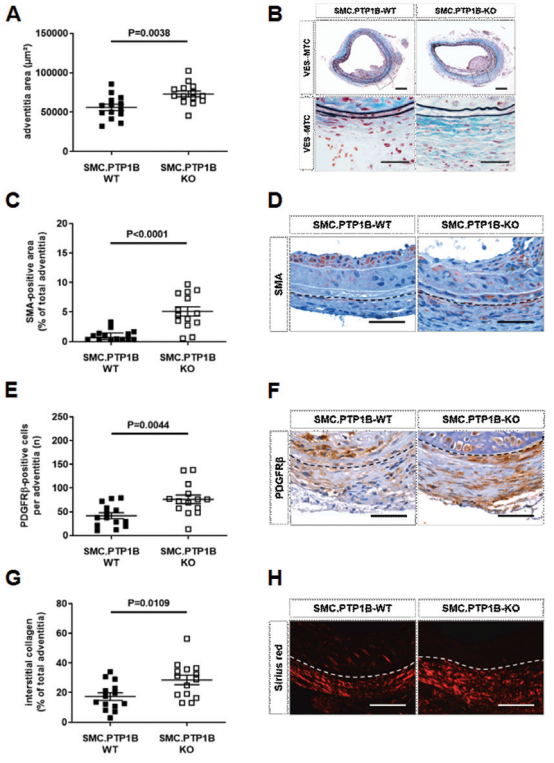

Fig. 1 Histochemical analysis of vascular lesion size and composition in mice lacking PTP1B in vascular smooth muscle cells (SMCs). Serial cross-sections through the left carotid artery of male SMC.PTP1B-WT ($n = 14$) and SMC.PTP1B-KO ($n = 14$) mice 3 weeks after induction of $FeCl_3$ vascular injury were stained with a combined VES – MTC staining protocol, photographed, and morphometrically analyzed (using ImagePro Plus software). **(A)** Summarized results after analysis of the adventitia area. **(B)** Representative images of VES – MTC-stained cross-sections through injured carotid arteries of SMC.PTP1B-WT and SMC.PTP1B-KO mice. Scale bars in (B) represent 150 µm (*top row*) or 50 µm (*bottom row*). Immunohistochemistry was performed to detect (activated) myofibroblasts: the results of the quantitative analysis and representative images of the SMA-immunopositive area (**C, D**; *red signal*) and the number of PDGFRβ-immunopositive cells (**E, F**; *brown signal*) in the adventitia of male SMC.PTP1B-WT and SMC.PTP1B-KO mice are shown. Interstitial collagen was visualized by Sirius Red staining followed by microscopy under polarized light (**G, H**; *red signal*). Graphs in (A), (C), (E), and (G) show individual values as well as the mean ± SEM (A, E, G) or median with interquartile range (C) per group. Statistical analysis was performed using unpaired Student's *t*-test (A, E, G) or Mann–Whitney test (C). Scale bars in (D), (F), and (H) represent 50 µm. A *dotted line* was added to indicate the media–adventitia interface. SEM, standard error of the mean.

thickness (►**Supplementary Fig. S3D** [available in the online version]) of the adventitia were significantly increased in SMC.PTP1B-KO mice compared with SMC.PTP1B-WT controls. The increase in the adventitia area continued to be significant if normalized to the total area of the vessel ($p < 0.0001$; data not shown). Representative Verhoeffs Elastica – Masson TriChrome stained cross-sections of vascular lesions are shown in ►**Fig. 1B**. Analysis of tamoxifen-fed *Myh11*.ER^{T2}-Cre transgenic mice not carrying the floxed PTP1B transgene demonstrated that differences in adventitia remodeling were not the result of tamoxifen administration (►**Supplementary Fig. S4** [available in the online version]).

Deletion of PTP1B in *Myh11*.ER^{T2}-Cre-Expressing Cells Promotes Adventitial Myofibroblast Expansion and Perivascular Fibrosis

Because differences in the vascular remodeling response of mice with PTP1B deletion in *Myh11*.ER^{T2}-Cre-expressing cells were primarily observed in the adventitia, we focused our subsequent analyses on the outer layers of the vessel wall. Significantly increased numbers of cells immunopositive for the myofibroblast markers smooth muscle α-actin (SMA; ►**Fig. 1C, D**) or PDGF receptor β (PDGFRβ; ►**Fig. 1E, F**) and higher amounts of interstitial collagen (►**Fig. 1G, H**) were observed in the adventitia of SMC.PTP1B-KO mice compared with SMC.PTP1B-WT controls 3 weeks after injury. Higher total cell numbers ($p = 0.0019$; not shown) and higher relative (expressed per total number of cells) numbers of PCNA-positive cells (►**Fig. 2A, B**) in the adventitia of SMC.PTP1B-KO mice compared with SMC.PTP1B-WT controls suggested increased perivascular cell proliferation. On the other hand, relative numbers of neural/glial antigen 2 (NG2)-positive pericytes (►**Fig. 2C, D**) or adventitial F4/80-positive macrophages (►**Fig. 2E, F**) did not differ between both groups.

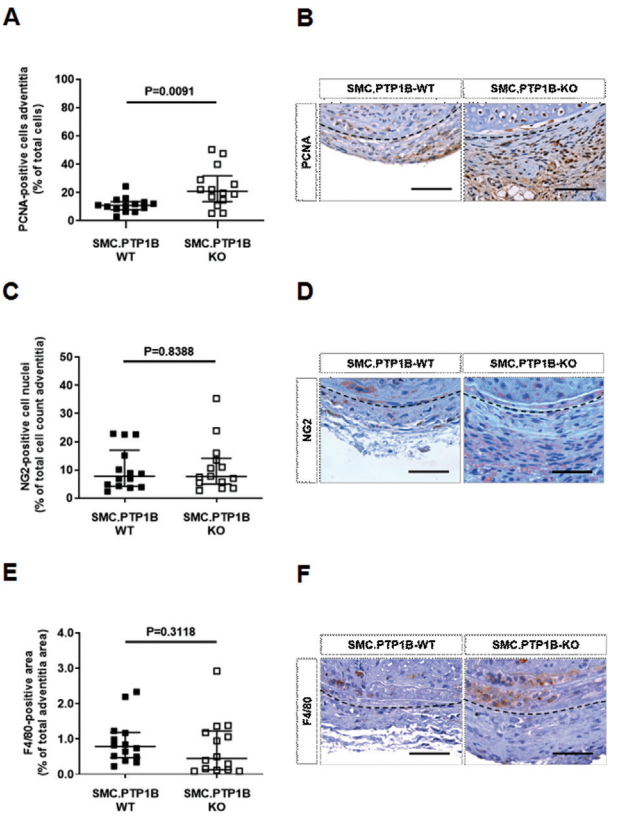

Fig. 2 Cellular composition of injured carotid arteries of male SMC.PTP1B-WT and -KO mice. Immunohistochemistry was performed on cross-sections through vascular lesions of SMC.PTP1B-WT and SMC.PTP1B-KO mice 3 weeks after $FeCl_3$-induced carotid artery injury. Quantitative analysis and representative pictures of the relative number (expressed as percent of total number of cells on the same section) of PCNA-immunopositive cells **(A, B)** or NG2-immunopositive pericytes **(C, D)** and the area immunopositive for the macrophage marker F4/80 **(E, F)** within the adventitia are shown. Graphs show individual values as well as the median and interquartile range per group. Statistical analysis was performed using the Mann–Whitney test. Scale bars in (B), (D), and (F) represent 50 µm.

Genetic PTP1B Deletion Results in Smooth Muscle Cell Dedifferentiation

RT2 qPCR array profiler analysis of carotid artery homogenates showed upregulation of several extracellular matrix constituents and remodeling enzymes in carotid arteries of SMC.PTP1B-KO mice ($n = 3$) compared with SMC-PTP1B-WT controls ($n = 3$) 21 days after vascular injury (►**Fig. 3A**). Although morphometric parameters did not differ between SMC.PTP1B-WT and SMC.PTP1B-KO mice (►**Supplementary Fig. S5A–D** [available in the online version]), quantitative real-time PCR analysis revealed reduced transcript levels of markers of contractile, differentiated SMCs, such as smooth muscle calponin-1 (*Cnn1*; ►**Fig. 3B**), smoothelin (*Smtn*; ►**Fig. 3C**), or SMMHC (*Myh11*; ►**Fig. 3D**) in uninjured carotid arteries, whereas mRNA levels of collagen type I α1 chain (*Col1a1*) were significantly increased (►**Fig. 3E**). Significantly lower mRNA transcript levels of *Cnn1* (►**Fig. 3F**) and *Myh11* (►**Fig. 3G**) and higher mRNA levels of *Col1A1* (►**Fig. 3H**) and the collagenase matrix metalloproteinase 2 (*Mmp2)* (►**Fig. 3I**) were also observed in primary SMCs isolated from the aorta of SMC.PTP1B-KO mice compared with those from SMC.PTP1B-WT controls, suggesting cell-intrinsic alterations. Western blot analysis demonstrated elevated PDGFRβ protein levels in primary SMCs isolated from SMC.PTP1B-KO mice (►**Fig. 3J, K**), in line with the in vivo findings.

PTP1B Gene Deletion Is Associated with Increased Adventitial Vascular Progenitor Cell Numbers following Injury

To further study the cell types involved in the observed perivascular fibrosis in SMC.PTP1B-KO mice, vascular injury was induced in mice expressing a green fluorescent reporter gene under control of the *Myh11* promoter. Immunofluorescence microscopy analysis of uninjured carotid arteries confirmed the expression of *Myh11*.Cre in more than 90% of cells within the media as well as few scattered cells in the adventitia (►**Fig. 4A**, ►**Supplementary Fig. S6A** [available in the online version]), whereas lectin-positive endothelial cells (►**Supplementary Fig. S6B** [available in the online version]) or NG2-positive pericytes (►**Supplementary Fig. S6C** [available in the online version]) did not express

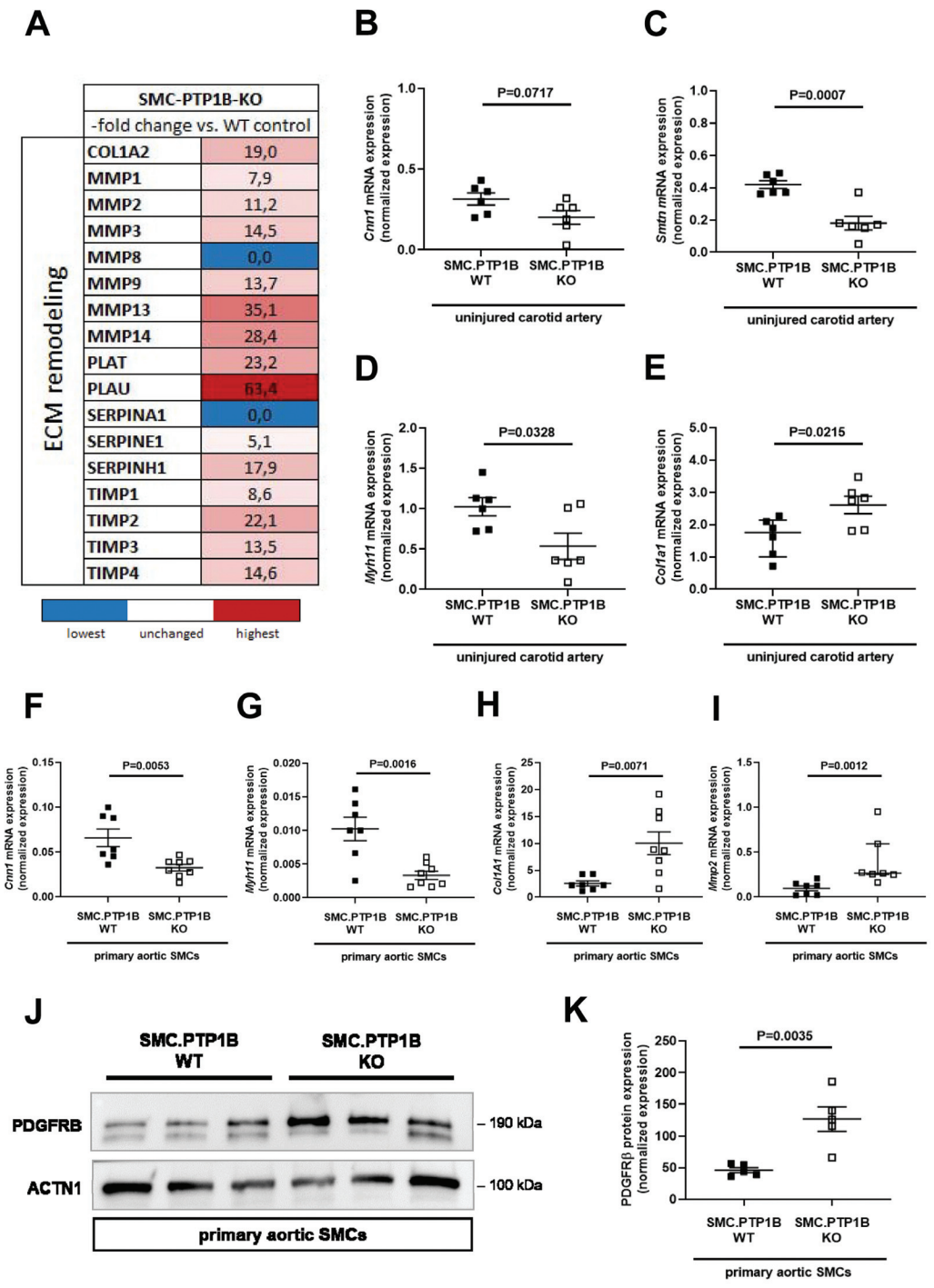

Fig. 3 Differences in vascular smooth muscle differentiation marker and extracellular remodeling enzyme expression following genetic PTP1B deletion in SMCs. **(A)** Total RNA was isolated from carotid artery homogenates 21 days after $FeCl_3$-induced vascular injury and changes in the mRNA expression of genes involved in extracellular matrix (ECM) remodeling analyzed using the RT2 PCR Profiler array "fibrosis." A heatmap showing -fold changes in mRNA transcript levels in SMC.PTP1B-KO mice (mean of $n = 3$ mice) versus SMC.PTP1B-WT controls (mean of $n = 3$ mice) is shown. Upregulated factors are *red*, downregulated factors are *blue*. **(B–E)** Total RNA was isolated from uninjured carotid arteries of SMC.PTP1B-KO mice ($n = 6$) versus SMC.PTP1B-WT controls ($n = 6$) and examined for differences in mRNA expression levels of calponin (*Cnn1*; [B]), smoothelin (*Smtn*; [C]), smooth muscle myosin heavy chain (*Myh11*; [D]) and collagen1A1 (*Col1A1*; [E]), using quantitative *real-time* PCR. **(F–I)** Messenger RNA levels of *Cnn1* (F), *Myh11* (G), *Col1A1* (H), and *Mmp2* (I) were examined in primary smooth muscle cells (SMCs) isolated from the aorta of SMC.PTP1B-WT and SMC.PTP1B-KO mice and expanded ex vivo (7–8 biological replicates per group) using quantitative *real-time PCR. Protein was isolated from SMCs and subjected to Western blot analysis of PDGFRβ expression.* ***(J)*** *Membrane showing PDGFRβ protein levels in SMCs from SMC-PTP1B-WT and -KO mice ($n = 3$ per group). Alpha-actinin (ACTN1) protein expression levels were used for normalization.* ***(K)*** *Results of the quantitative analysis ($n = 5$ biological replicates, $n = 2$ independent experiments). Graphs show individual values as well as the mean ± SEM (C–H and K) or median and interquartile range (B and I). Statistical analyses were performed using the Mann–Whitney test (B and I) and unpaired Student's t-test (C–H and K). PCR, polymerase chain reaction; SMC, smooth muscle cell.*

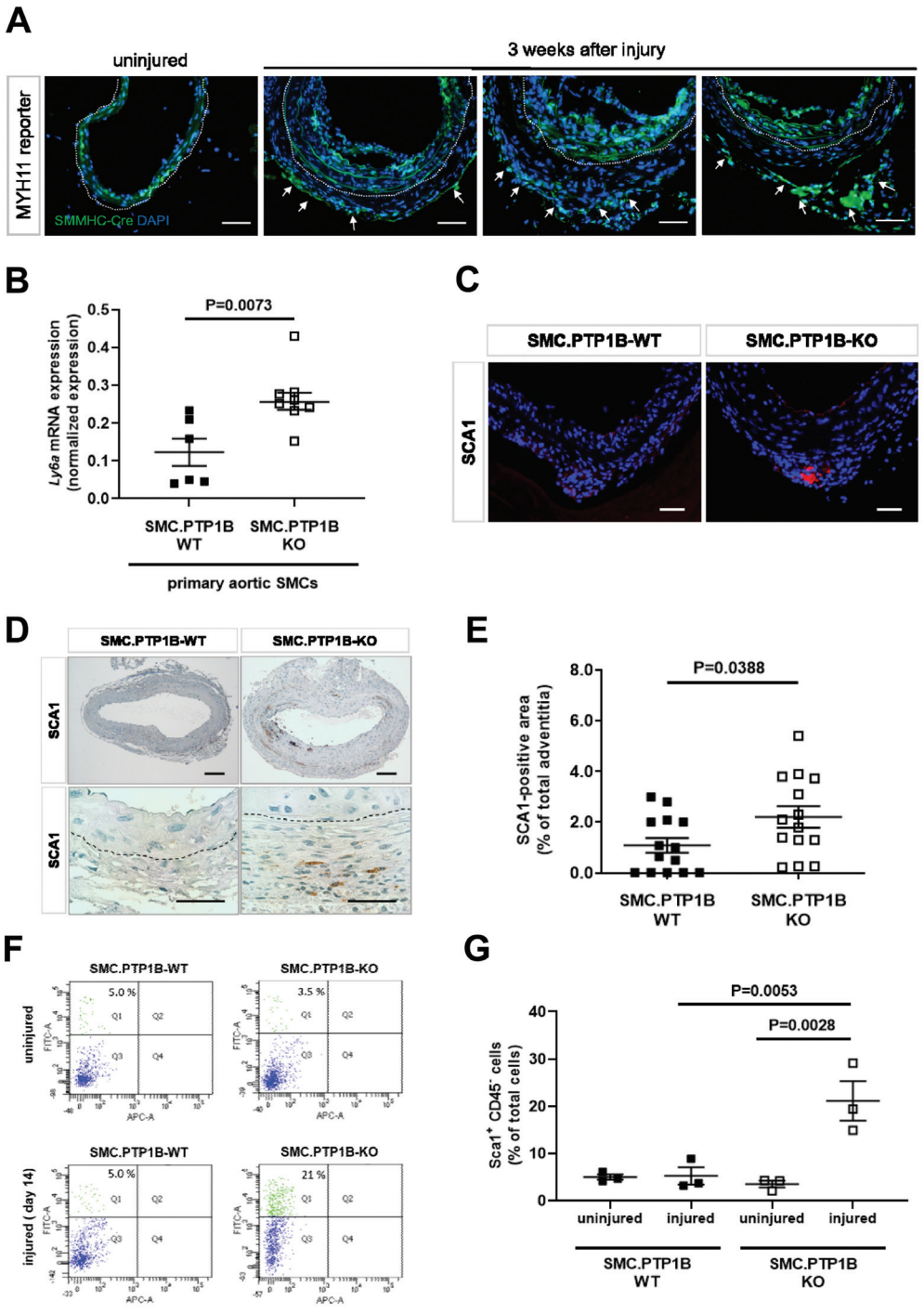

Fig. 4 Lineage tracing of MYH11-expressing cells and changes in adventitial SCA1 vascular progenitor cell numbers in mice and primary smooth muscle cells lacking PTP1B. **(A)** SMMHC/MYH11-Cre reporter gene expression (GFP; *green signal*) in the vessel wall was examined 21 days after vascular injury in the injured carotid artery (three representative examples are shown) as well as the contralateral uninjured carotid artery (one representative example is shown). *Arrows* point to GFP reporter-expressing cells in the adventitia. A *dotted line* was added to indicate the media–adventitia interface. Scale bars represent 25 µm. **(B)** qPCR analysis of *Ly6a* mRNA expression in primary aortic SMCs isolated from SMC.PTP1B-WT and SMC.PTP1B-KO mice. Graph shows individual values as well as the mean ± SEM. Statistical analysis was performed using Student's *t*-test. **(C–E)** Immunohistochemical detection of SCA1-positive cells in the adventitia of male SMC.PTP1B-WT and SMC.PTP1B-KO mice at day 21 after carotid artery injury. Representative images using fluorescence (C; *red signal*) or bright field (D; *brown signal*) microscopy and the results of the quantitative analysis (E) are shown. Graphs show individual values as well as the mean ± SEM. Statistical analysis was performed using Student's *t*-test for unpaired means. Scale bars in (C) and (D) represent 150 µm. **(F)** Flow cytometry analysis of CD45-negative (CD45 − ; APC-labeled) and SCA1-positive (SCA1 + ; FITC-labeled) cells in injured and contralateral uninjured carotid arteries of male SMC.PTP1B-WT and SMC.PTP1B-KO mice at day 14 after injury. Representative dot blots (F) and the results of the quantitative analysis (**G**; $n = 3$ biological replicates) are shown. Graphs show individual values as well as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. Nonsignificant differences are not shown. ANOVA, analysis of variance; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean; SMC, smooth muscle cell.

GFP. Three weeks after vascular injury, reporter gene expression was also detectable in numerous cells within the adventitia (representative microscopy images are shown in ►**Fig. 4A**). Earlier studies have shown that *Myh11*-expressing cells are not terminally differentiated, but capable of phenotypic transition and numerical expansion after vascular injury.[22,23] Extending previous findings, mRNA transcript levels of stem cell antigen-1 (SCA1 or *Ly6a*) (►**Fig. 4B**) and CD34 ($p = 0.0476$; not shown), which are markers of adventitial vascular progenitors with the potential to differentiate into SMCs,[22,24,25] were significantly increased in primary SMCs isolated from the aorta of SMC.PTP1B-KO mice. Immunofluorescence microscopy (►**Fig. 4C**), immunohistochemistry (►**Fig. 4D, E**), and flow cytometry (►**Fig. 4F, G**) confirmed increased SCA1-positive cell numbers in the remodeling adventitia of SMC.PTP1B-KO mice compared with their wild-type counterparts.

Deletion of PTP1B in Smooth Muscle Cells Stabilizes the Nuclear Localization of KLF4 via Mechanisms Involved in Reduced ERK1/2 Phosphorylation

A key transcriptional regulator of SMC phenotype switching and SCA1-positive progenitor cell reprogramming is KLF4.[22,26] Immunofluorescence microscopy revealed significantly increased numbers of KLF4-positive cells in the injured adventitia of SMC.PTP1B-KO mice compared with SMC.PTP1B-WT controls (►**Fig. 5A, B**). Increased KLF4 protein levels were confirmed in primary aortic SMCs isolated from SMC.PTP1B-KO mice (►**Fig. 5C**), whereas KLF4 mRNA transcript levels did not differ (►**Fig. 5D**).[27] High-resolution immunofluorescence microscopy revealed significantly higher relative numbers of SMCs with KLF4-immunopositive cell nuclei in those isolated from SMC.PTP1B-KO mice (►**Fig. 5E, F**). The export of KLF4 from the cell nucleus is regulated by activation of ERK1/2 and phosphorylation of KLF4 at Ser132 to initiate interaction with the nuclear export factor.[28] Whereas short-term (60 minutes) incubation of primary SMCs with a PTP1B inhibitor was associated with significantly increased phosphorylation of ERK1/2 (►**Supplementary Fig. S7A**, **B** [available in the online version]) as well as of KLF4 (►**Supplementary Fig. S7C**, **D,** available in the online version]), ERK1/2 phosphorylation was markedly reduced in primary SMCs following genetic, chronic deletion (►**Fig. 5G, H**). Supporting a role for downregulated ERK1/2 in the observed nuclear accumulation of KLF4, inhibition of ERK1/2 signaling using PD98059 (10 µM for 18 hours) significantly increased nuclear KLF4 immunosignals also in SMCs isolated from SMC.PTP1B-WT mice (►**Fig. 5E, F**).

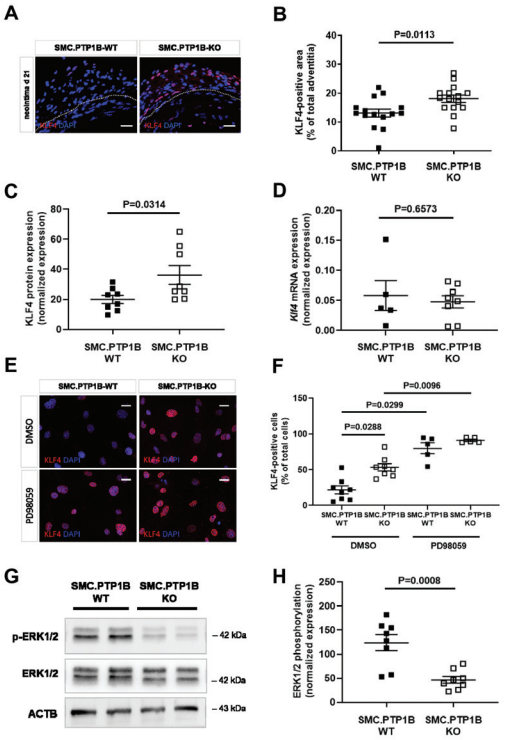

Fig. 5 Changes in the vascular expression and subcellular localization of KLF4 in mice and primary smooth muscle cells lacking PTP1B. Immunofluorescence microscopic detection of KLF4 on cross-sections through vascular lesions of SMC.PTP1B-WT and SMC.PTP1B-KO mice 3 weeks after $FeCl_3$-induced carotid artery injury. Representative pictures (**A**; size bars represent 50 µm) and the results of the quantitative analysis of the relative number of KLF4-immunopositive cells in the adventitia are shown **(B)**. Individual values as well as the mean ± SEM are shown. Statistical analyses were performed using Student's *t*-test. Analysis of KLF4 protein (**C**; $n = 8$ biological replicates) and *Klf4* mRNA (**D**; $n = 5–8$ biological replicates) expression in primary SMCs isolated from the aorta of SMC.PTP1B-WT and SMC.PTP1B-KO mice. Graphs show individual values as well as the mean ± SEM. Statistical analysis was performed using Student's *t*-test. Primary aortic SMCs were grown on coverslips and the subcellular presence of KLF4 (*red signal*), at baseline and following incubation with the ERK1/2 inhibitor PD98059, was examined using fluorescence microscopy. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue signal*). Representative findings are shown in panel **(E)** (size bars represent 25 µm), and the results of the quantitative analysis in **(F)**. Individual values as well as the mean ± SEM are shown. Statistical analysis was performed using one-way ANOVA. Representative Western blot membrane **(G)** and the results of the quantitative analysis **(H)** showing changes in ERK1/2 phosphorylation in SMCs isolated from the aorta of SMC.PTP1B-WT and SMC.PTP1B-KO mice. Graph shows individual values as well as the mean ± SEM. Findings in SMC.PTP1B-WT mice were normalized to 1. Statistical analysis was performed using Student's *t*-test. ANOVA, analysis of variance; SEM, standard error of the mean; SMC, smooth muscle cell.

PTP1B Downregulation in Smooth Muscle Cells Reduces SMAD2 Phosphorylation and Nuclear Localization, and SMAD2 siRNA Transfection Phenocopies PTP1B Deficiency in Smooth Muscle Cells

KLF family members, including KLF4, have been implicated in TGFβ signaling, and KLF4 phosphorylation was found to initiate the interaction of KLF4 with SMAD2 and their cooperative binding to the TGFßRI promoter.[29] In line with reduced ERK1/2-mediated KLF4 phosphorylation, lower protein levels of SMAD2 phosphorylated (at Ser465/467) were observed in HAoSMCs transfected with PTP1B siRNA compared with control siRNA-treated cells studied in parallel (►**Fig. 6A, B**). The efficiency of the siRNA-mediated HAoSMC transfection and PTP1B protein reduction is demonstrated in ►**Supplementary Fig. S8(A–D)** (available in the online version). Nuclear immunosignals of phosphorylated SMAD2 increased in SMCs isolated from SMC.PTP1B-WT mice stimulated with TGFβ1 (10 ng/mL for 60 minutes), but not in those isolated from SMC-PTP1B-KO mice (►**Fig. 6C**), and

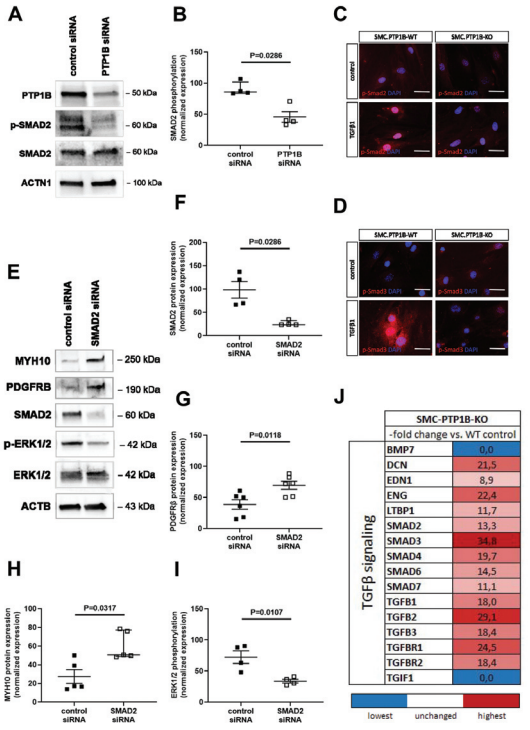

Fig. 6 Changes in TGFβ signaling and SMAD2/3 nuclear localization in mice with reduced PTP1B expression in vascular smooth muscle cells. Western blot analysis of SMAD2 phosphorylation in human aortic SMCs following siRNA-mediated downregulation of PTP1B. Representative Western blot membranes are shown in panel **(A)**, and the results of the quantitative analysis of phosphorylated (p)-SMAD2 levels in $n = 4$ experimental replicates in shown in panel **(B)**. Primary murine aortic SMCs were grown on coverslips and the subcellular presence of p-SMAD2 (**C**; *red signal*) and p-SMAD3 (**D**; *red signal*), at baseline and following stimulation with recombinant TGFβ1 (10 ng/mL for 60 minutes), was examined using fluorescence microscopy. Cell nuclei were stained with DAPI (*blue signal*). Size bars represent 25 µm. Western blot analysis of total MYH10, PDGFRβ, SMAD2, and ERK1/2 protein levels and ERK1/2 phosphorylation in human aortic SMCs following siRNA-mediated downregulation of SMAD2. A representative Western blot membrane is shown in panel **(E)**, and the results of the quantitative analysis in $n = 4–7$ experimental replicates in panels **(F)–(I)**. Graphs show individual values as well as the mean ± SEM. Statistical analyses were performed using Student's *t*-test. **(J)** Total RNA was isolated from carotid arteries 21 days after $FeCl_3$-induced injury and changes in the mRNA expression of TGFβ pathway signaling components analyzed using RT2 PCR Profiler array. A heatmap showing -fold changes in mRNA transcript levels in SMC. PTP1B-KO mice (mean of $n = 3$ mice) versus SMC.PTP1B-WT controls (mean of $n = 3$) is shown. Upregulated factors are *red*, and downregulated factors are *blue*. PCR, polymerase chain reaction; SEM, standard error of the mean; SMC, smooth muscle cell.

similar findings were observed for phosphorylated SMAD3 (►**Fig. 6D**). Importantly, siRNA-mediated knockdown of SMAD2 in HAoSMCs (►**Fig. 6E, F**) resulted in increased protein levels of PDGFRβ (►**Fig. 6E, G**) and MYH10 (►**Fig. 6E, H**), whereas protein levels of phosphorylated ERK1/2 decreased (►**Fig. 6E, I**), thus phenocopying the effects of PTP1B deletion in primary SMCs and the murine adventitia following injury. Interestingly, and in addition to proteases involved in extracellular matrix remodeling and TGFβ liberation, as shown in ►**Fig. 3A**, RT2 profiler qPCR array analysis revealed increased mRNA levels of TGFβ and TGFβ signaling components in carotid arteries of SMC. PTP1B-KO mice 21 days after injury compared with SMC-PTP1B-WT controls (►**Fig. 6J**), including mRNA transcript levels of the TGFβ target gene endothelin-1 (ET-1 or *Edn1*), a potent vasoconstrictor also involved in fibrosis and fibroblast recruitment.

Discussion

The development of a restenosis following intracoronary balloon inflation and/or stent implantation continues to be an important complication of interventional revascularization therapies to limit luminal obstruction by atherosclerotic plaques.[30] While the selective promotion of re-endothelialization and inhibition of SMC growth would be desirable to control lesion progression, antimitogenic medication released from drug-eluting stents nonspecifically inhibits proliferation of both SMCs and endothelial cells. To improve the results of current revascularization therapies and to develop alternative strategies, a better understanding of the cell-specific control mechanisms of growth factor signaling events during neointima formation is a prerequisite.

The proliferation and migration of vascular SMCs in response to growth factors, such as PDGF and TGFβ released from activated platelets, myofibroblasts, and other cell types, involves the phosphorylation and activation of tyrosine or serine/threonine kinase receptors and their downstream signaling intermediates. Our findings suggest that the function of PTP1B is not restricted to dephosphorylation as a short-term protective mechanism to limit excessive growth factor signaling; rather, chronic inactivation or knockdown of PTP1B was associated with an attenuated phosphorylation of signaling intermediates and transcription factors involved in the control of SMC differentiation and growth. The exact molecular basis underlying this observation has yet to be determined, but may involve the induction of counter-regulatory mechanisms.

PTP1B and other protein tyrosine phosphatases are expressed, among others, in vascular SMCs.[5] Previous work demonstrated the association of PTP1B with PDGF receptors[31] and the role of PTP1B in the control of PDGF and other growth factor signaling by receptor tyrosine dephosphorylation.[7,8] PDGF is known to suppress SMC marker expression and to enhance SMC proliferation and migration.[32,33] Our findings show that deletion or inhibition of PTP1B in primary SMCs reduced the expression of markers of SMC differentiation, whereas mRNA levels of immature SMC or myofibroblast markers were increased. Thus, they are consistent with activated PDGF receptor signaling in the absence of PTP1B, which was present already at baseline in aortic SMCs expanded ex vivo, and increased PDGFRβ immunosignals were observed after vascular injury in SMC.PTP1B-KO mice. Although we did not observe significant differences in the relative levels of PDGFRβ phosphorylation in SMCs isolated from SMC.PTP1B-WT and -KO mice

(data not shown), increased total PDGFRβ protein levels in SMCs lacking PTP1B may have obscured the detection of such differences.

Whereas several studies examined the effects of PTP1B in cultivated SMCs, much less is known about its function during vascular remodeling processes in vivo. Local adenoviral overexpression of dominant-negative PTP1B was shown to increase neointimal and medial cell proliferation and restenotic lesion size.[8] Of note, mutant PTP1B protein overexpression was observed primarily in the media, and to a much lesser extent in the neointima or adventitia.[8] We have shown that deletion of PTP1B in endothelial cells promotes neointima formation via increased oxidative stress resulting in endothelial senescence and failure to induce SMC quiescence.[4] Given those previous reports in cells and mice, it was surprising to find that genetic PTP1B deletion in *Myh11*/SMMHC-expressing cells did not alter neointima and media size following vascular injury. Instead, we observed that lesion growth occurred primarily in the outer layers of the vessel wall and was associated with a significant enlargement of the adventitia and perivascular collagen accumulation. Deleterious effects of MYH11-positive mural cell activation resulting in myofibroblast differentiation, proliferation, and fibrosis were reported in other vascular injury models.[34] Lineage tracing studies localized MYH11 reporter-positive cells in the adventitia following carotid or femoral artery ligation injury,[22] and our observations are in line with those previous findings. The *Myh11*-CreER^{T2} transgene is linked to the Y chromosome thus limiting our analyses to male mice. Of note, an X-linked *Myh11*-CreER^{T2} mouse line also has been reported,[35] which allows the study of female mice.

SMCs exhibit a remarkable phenotypic plasticity, and tyrosine kinase signaling has been implicated in the maturation of SCA1+ progenitors to differentiated SMCs.[36] SMMHC (or *MYH11*) expression is believed to be restricted to mature, differentiated SMCs[37] and Myh11.Cre, the most specific driver for gene deletion in SMCs.[17] Using this line, genetic fate-mapping studies employing *Myh11* as Cre driver revealed that differentiated SMCs retain the ability to dedifferentiate and contribute to the vascular progenitor cell pool residing in a niche at the adventitia–media border.[22,34] Our analyses using MYH11 reporter mice confirm and extend these previous findings by showing that genetic ablation of PTP1B in *Myh11*/SMMHC-positive cells increased the adventitial SCA1-positive cell numbers following arterial injury. The observed nuclear accumulation of KLF4, shown to be essential for the induction and maintenance of a smooth muscle progenitor phenotype,[22] may have played a role in this finding. Previous studies demonstrated that SCA1-positive progenitors within the adventitia increase in number and participate in vascular lesion formation.[24,38] Of note, and similar to a previous study,[22] arterial injury was induced *after* tamoxifen administration, and the effects of PTP1B gene deletion in *Myh11*/SMMHC-expressing cells and their progeny were examined, suggesting a negative regulatory role of PTP1B during smooth muscle dedifferentiation and progenitor cell expansion. However, and because the MYH11 reporter cells did not lack PTP1B, definite conclusions as to whether this observation was a direct consequence of the PTP1B deletion or rather due to secondary or paracrine effects cannot be made.

Phosphorylation plays a major role in modulating the transcription of genes involved in SMC differentiation by recruiting transcription factors and coordinating their binding to G/C-rich repressor or activator sites within the promoter region of target genes.[39,40] The transcription factor KLF4 is critically involved in the regulation of SMC differentiation and phenotypic transition,[41,42] and was shown to prevent the transcription of SMC contractile marker genes following stimulation with PDGF[43] or TGFβ.[29] Previous work established that the interaction and cooperative binding of phosphorylated KLF4 with SMAD2 regulates SMC differentiation and growth by binding to control elements in the TGFßRI promoter.[29] In line with this, differential changes in the expression of KLF4 (e.g., *Myh10*) and SMAD2 target genes (e.g., *Myh11*) were observed following genetic deletion of PTP1B. The absence of tyrosine phosphorylation sites within KLF4 suggests that PTP1B exerted its effects via indirect mechanisms. KLF4 phosphorylation (at Ser132) has been shown to be mediated by ERK1/2, resulting in nuclear export and proteasomal degradation of the transcription factor.[28,44] Supporting the role of changes in ERK1/2 signaling in our study, reduced ERK1/2 phosphorylation was observed in SMCs isolated from SMC.PTP1B-KO mice, and ERK1/2 inhibition in SMC.PTP1B-WT SMCs reproduced the phenotype of nuclear KLF4 accumulation seen in SMC.PTP1B-KO SMCs. Others found that the PDGF-induced transcriptional repression of SMC differentiation markers could be prevented by the MEK1/2 inhibitor U0126.[45] KLF4 may also be phosphorylated by activated AKT,[46] and previous studies demonstrated the importance of phosphatase and tensin homolog (PTEN), a major inhibitor of the PI3-K/AKT signaling pathway, in the control of KLF4 activity and SMC differentiation.[14,47]

Our findings that siRNA-mediated downregulation of SMAD2 reproduces several characteristic findings observed in SMCs lacking PTP1B strongly suggest that downregulation of SMAD2 and reduced nuclear translocation of SMAD2 are crucially involved in mediating the effects of PTP1B deficiency or inhibition. In fact, phosphorylation of KLF4 was shown to enable complex formation with SMAD2 in response to TGFβ1 stimulation and its recruitment to SMAD-responsive promoter regions.[29] Others found that genetic deletion of SMAD2 in tubular epithelial cells promotes renal fibrosis and collagen accumulation by enhancing TGFβ/SMAD3 signaling.[48] In addition to the canonical signaling pathway via SMAD proteins, TGFβ may exert its effects also via activation of other, less specific signaling intermediates,[49] and growth factors other than TGFβ also may have played a role in the observed perivascular fibrosis seen in mice lacking PTP1B in SMCs following vascular injury.

Regarding the potential translational relevance of our findings, reduced PTP1B expression has been shown to occur in

aortic SMCs following chronic stimulation with angiotensin II or insulin,[21,50] and our findings may have implications in particular for patients with arterial hypertension or hyperinsulinemia, i.e., patient populations at increased risk for developing restenosis following revascularization.[51] Also, reactive oxygen species (ROS) are known to inactivate PTP1B,[52] suggesting that downregulation or inhibition of PTP1B may occur in the natural course of vascular disease processes involving ROS, such as atherosclerosis. In this regard, we could show that PTP1B expression is reduced in the ascending aortic of patients with advanced coronary atherosclerosis compared with the internal mammary artery, a vessel that is relatively protected from atherosclerosis.[53] Of note, our analyses were performed in an animal model without additional known risk factors of atherosclerosis in humans, such as hypercholesterolemia or diabetes, to minimize the effects of potential confounders on our findings.

Taken together, our findings following genetic deletion of PTP1B using transgenic mice and primary SMCs suggest that chronically reduced levels of PTP1B promote the expansion of perivascular vascular progenitor cells, myofibroblast activation, and fibrosis by altering the ERK1/2-mediated phosphorylation and nuclear translocation of KLF4 and SMAD2 (please also see the **Visual Summary**). The reactive overactivation of the TGFβ signaling pathway may have contributed to the development of perivascular fibrosis following carotid artery injury in mice lacking PTP1B in SMCs.

What is known about this topic?

- Protein tyrosine phosphatases, including its prototype PTP1B, are negative regulators of growth factor signaling.
- Overexpression of PTP1B in smooth muscle cells is observed following experimental vascular injury, whereas reduced PTP1B levels are seen in patients at risk for developing vascular disease.
- The significance of PTP1B in smooth muscle cells for the vascular remodeling response to injury is unknown.

What does this paper add?

- Inducible deletion of PTP1B in *Myh11*-Cre-expressing cells promoted adventitia enlargement, perivascular myofibroblast activation, and fibrosis following vascular injury in mice.
- Lower ERK1/2 phosphorylation in the presence of chronically reduced PTP1B and a shift from SMAD2 to KLF4 mediated gene expression downstream of activated TGFβ signaling were identified as potential mechanisms.
- Our findings suggest that chronically reduced expression of PTP1B is an important cellular event and a potential causal factor in vascular disease processes involving alterations in smooth muscle cell differentiation.

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Conflict of Interest
None declared.

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