Cardiovascular, Pulmonary, and Renal Pathology

BDNF/TrkB Signaling Augments Smooth Muscle Cell Proliferation in Pulmonary Hypertension

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Pulmonary hypertension (PH) is a life-threatening disorder that is characterized by pulmonary arterial smooth muscle cell (PASMC) hyperplasia. Until now, little has been known about early changes that underlie the manifestation of PH. To characterize these early changes, we performed whole-genome microarray analysis of lungs from mice exposed to either 24 hours hypoxia or normoxia. TrkB, a member of the tyrosine kinase receptor family, and its ligand, brain-derived neurotrophic factor (BDNF), were strongly up-regulated in hypoxic mouse lungs, as well as in arteries of patients suffering from idiopathic pulmonary arterial hypertension (IPAH). BDNF stimulation of PASMC *in vitro* resulted in increased proliferation, TrkB and ERK1/2 phosphorylation, and nuclear translocation of the transcription factor early growth response factor 1 (Egr-1). In addition, increased Egr-1 expression was observed in idiopathic PAH lungs. The pro-proliferative effect of BDNF was attenuated by TrkB kinase inhibitor (K252a) or ERK1/2 inhibitor (U0126) pretreatment, and by knocking down Egr-1. Consequently, we have identified the BDNF-TrkB-ERK1/2 pathway as a proproliferative signaling pathway for PASMC in PH. Interference with this pathway may thus serve as an attractive reverse remodeling

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Pulmonary hypertension (PH) is a life-threatening disease characterized by a distinct and persistent elevation of pulmonary arterial pressure, which ultimately results in right ventricular failure and death.^{1,2} PH manifests itself in various diseases, including chronic obstructive lung disease and chronic thromboembolism, but also rare idiopathic and familial forms exist.³ PH has a multifactorial pathogenesis, with both vasoconstriction and structural remodeling of the pulmonary vessels contributing to disease progression.³ Structural changes underlying the remodeling processes include abnormal endothelial cell proliferation, as well as increased migration and proliferation of smooth muscle cells (SMC) and fibroblasts.⁴ Enhanced proliferation of pulmonary arterial SMCs (PASMC) can arise from a dysfunctional bone morphogenic protein receptor II; however, these mutations occur in only 10% to 40% of all idiopathic pulmonary arterial hypertension (IPAH) patients.^{5,6} Additionally, changes in growth factor production or receptor levels have been shown to influence the development of PH. These include; platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF),^{7–10} which act as strong mitogens and chemoattractants for pulmonary SMCs.¹¹ Additionally, up-regulation of the tyrosine receptor kinases PDGFR α and β has been observed in lambs with chronic intrauterine pulmonary hypertension¹² and already first clinical evidence for

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successful treatment of PH by PDGFR interference was presented.¹³

Another well-recognized stimulus of pulmonary vasoconstriction and consequent vascular remodeling is chronic hypoxia.^{14–16} In response to low alveolar oxygen, voltage-gated potassium channels are down-regulated, resulting in depolarization of SMCs, subsequent Ca^{2+} influx, and increased vasoconstriction.^{17,18} Other important effectors for hypoxic remodeling are hypoxia-inducible transcription factors (HIFs), which are known to induce a number of genes under hypoxic conditions.^{19–21}

In a previous study, we have demonstrated genes regulated after prolonged hypoxia exposure in mouse model of hypoxia-induced PH.²² However, early genetic changes in response to hypoxia, representing possible trigger mechanisms, leading to vascular remodeling remain poorly understood. Therefore, we have undertaken whole-genome microarray analysis to investigate transcriptional changes occurring in the lung of the hypoxic mouse model during onset of pulmonary hypertension after only 24 hours of hypoxic exposure. We identified the *Ntrk2* gene encoding tyrosine-related kinase B (TrkB), a member of the tyrosine kinase receptor family, as strongly up-regulated in hypoxic mouse lungs. Against the background that i) in our study, TrkB levels were also elevated during sustained hypoxic exposure, ii) TrkB expression has been previously shown to be present on human and rat vascular SMCs and endothelial cells,^{23,24} and iii) other tyrosine kinases have previously been implicated in the development of PH, we have studied in more detail the role of TrkB in vascular remodeling.

Materials and Methods

Exposure to Chronic Hypoxia

Eight-week-old male BALB/c mice were exposed to normobaric normoxia [fraction of inspired oxygen (FiO_2) of 0.21] and to normobaric hypoxia (FiO_2 of 0.10) for 1, 7, and 21 days, respectively. Isolation and preparation of mouse lungs have been described in detail previously.^{22,25} The *Egr-1*^{−/−} animals were a kind gift of Patrick Charnay (Ecole Normale Supérieure, IBENS, Paris Cedex, France). The *BDNF*^{+/−} and *TrkB*^{+/−} mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Microarrays and Data Analysis

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) from lung homogenate of 18 BALB/c mice per group exposed for 24 hours to normobaric normoxia (FiO_2 of 0.21) and to normobaric hypoxia (FiO_2 of 0.10). RNA from 6 mice each was used as a pooled sample for labeling and hybridization. Labeled cDNA was generated using Superscript II to reverse transcribe 50 μg of RNA incorporating of Cy3- and Cy5-dCTP (all reagents from Invitrogen, Karlsruhe, Germany). The labeled cDNA was purified using a PCR purification kit (Qiagen). The volume of elute was reduced from 50 μL to ~10 μL using a centrifugal vacuum concentrator. Competitive hybridizations (hypoxia/normoxia) were per-

formed for 18 hours in UltraHyb buffer (Ambion, Austin, TX) at 42°C on 60mer oligonucleotide microarrays (MWG 30K mouse) using the GeneTAC hybridization station (PerkinElmer, Waltham, MA). The data were analyzed using the limma package in R.^{26,27} Intensity values were background subtracted; log ratios were normalized using a loess correction on the MA-plot values.²⁷ Regulated genes were filtered by using moderated t-statistics and adjusting the false discovery rate at 10%.²⁷ Experimental design is provided in Supplemental Figure S1 (available at <http://ajp.amjpathol.org>). Pathways were analyzed using PathwayExpress and OntoExpress of the OntoTools.²⁸

Isolation of Mouse Pulmonary Arteries

Mice exposed to hypoxia ($n = 10$) or normoxia ($n = 10$) for 21 days were euthanized with ketamine (6 mg/100 g) and xylazine (1 mg/100 g) intraperitoneally. Major pulmonary arteries were dissected for isolation of mRNA and expression analysis.

Rat Monocrotaline and Su5416 Models of Pulmonary Arterial Hypertension

Lung homogenate samples from the monocrotaline-induced as well as the Su5416 rat model²⁹ of pulmonary hypertension were obtained as described previously.^{25,30}

Patient Characteristics and Measurements

Samples from human lung tissue were obtained from five donors and five IPAH patients undergoing lung transplantation as described previously.³¹ Patient characteristics are provided in previous publications.^{31,32}

Laser-Assisted Microdissection

Microdissection of pulmonary arteries (40 vessels per animal with a diameter of 100 to 250 μm , four animals each) was performed on mounted cryosections using the Laser Microbeam System (P.A.L.M., Bernried, Germany) as described in detail previously.²²

Immunohistochemistry

All immunohistochemical experiments were performed on paraffin-embedded serial slides. All antibodies were diluted (1:100) in ChemMate Antibody Diluent, (Dako, Glostrup, Denmark): anti-TrkB, anti-BDNF, anti-NT-4/5, anti-Egr-1 (all from Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation in a humid chamber, slides were washed three times in Tris-buffered saline (pH 7.4 to 7.6) and incubated for 45 minutes with corresponding secondary antibodies conjugated with alkaline phosphatase (Rockland, Gilbertsville, PA) at a 1:250 dilution. Negative controls were prepared with the omission of the primary antibody.

Immunofluorescence Staining

For immunofluorescence analysis, untreated or BDNF treated PSMC were grown on glass slides and fixed for 5 minutes in cold methanol. Cells were then blocked for 2 hours with 3% bovine serum albumin in Tris-buffered saline, and incubated overnight at 4°C with anti-TrkB, anti-BDNF, or anti-Egr-1 antibody (all from Santa Cruz Biotechnology). Slides were then incubated with anti-rabbit IgG-Alexa 555 and mounted with fluorescence Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). To investigate proliferation of unstimulated or BDNF-stimulated human PSMC, cells were permeabilized for 5 minutes with 0.25% Triton X-100 in Tris-buffered saline, incubated with a mouse anti-human Ki-67 antibody (Santa Cruz Biotechnology), followed by incubation with fluorescein isothiocyanate-labeled secondary antibody (Dianova, Hamburg, Germany). Cell nuclei were counterstained with DAPI (Sigma-Aldrich, Munich, Germany). For microscopic inspection, a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) was used.

Western Blot Analysis and Kinase Activity

Cell lysates from lung homogenates and PSMC were separated by 12% SDS polyacrylamide gel, followed by electrotransfer to a 0.45- μ m polyvinylidene difluoride membrane (ImmobilonTM-P; Millipore, Bedford, MA). After blocking with 5% nonfat dry milk in Tris-buffered saline + 0.1% Tween20, the membrane was incubated overnight at 4°C with one of the following primary antibodies: anti-BDNF, anti-TrkB, anti-Egr-1 (all from Santa Cruz Biotechnology), or anti- β -actin (Biozol, Eching, Germany). After incubation for 1 hour with horseradish peroxidase-labeled secondary antibodies (Pierce Biotechnology, Rockford, IL; all at 1:3000 in blocking solution) signals were visualized by the ECL detection system (Pierce Biotechnology). For phosphorylation study, a rabbit anti-phospho-TrkB(Tyr516)/A(Tyr490) (Santa Cruz Biotechnology), or a rabbit anti-phospho-p42/44 MAP kinase antibody (Cell Signaling Technology, Boston, MA) was applied. Blots were stripped and reprobed using a rabbit anti-TrkB (Santa Cruz Biotechnology), or an anti-p42/44 MAP kinase antibody, respectively (Cell Signaling Technology).

Quantitative Real-Time PCR

RNA from laser-microdissected material was isolated by RNeasy Micro Kit (Qiagen), whereas RNA from lung tissues and cell culture experiments was isolated by RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Quality and quantity of RNA was determined by 1.5% (m/v) agarose gel and UV absorbance (Nanodrop spectrophotometer; Thermo Scientific, Wilmington, DE). RNA was reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (Applied Biosystems, Foster City, CA). Real-time PCR was performed in an ABI 7900HT Sequence Detection System (Applied Biosystems) using SYBR-Green I (Qiagen) as

fluorogenic probe. Primer pairs were as follows, mouse: *BDNF*: forward, 5'-TTCGAGAGGTCTGACGACGAC-3', reverse, 5'-ACCCGGGAAGTGTAAGTCC-3'; *PBGD*: forward, 5'-GGTACAAGGCTTTCAGCATCGC-3', reverse, 5'-ATGTCCGGTAACGGCGGC-3'; *TrkB*: forward, 5'-GCAAACCAGAAAAGGCTAGAAATC-3', reverse, 5'-TGTAAGCCACAACTTTAAGCCG-3'; human: *BDNF*: forward, 5'-CAAGGCAGGTTCAGAGGCTT-3', reverse, 5'-CTGGACGTGTACAAGTCTGCG-3'; *CCND1*: forward, 5'-CCGTCCATGCGGAAGATC-3', reverse, 5'-TAGTTCATGGC-CAGCGGG-3'; *Egr-1*: forward, 5'-GTTTGCCAGGAGCGAT-GAAC-3', reverse, 5'-CCGAAGAGGCCACAACACTT-3'; *FN1*: forward, 5'-CCGACCAGAAGTTTGGGTTCT-3', reverse, 5'-CAATGCGGTACATGACCCCT-3'; *PBGD*: forward, 5'-CCCACGCGAATCACTCTCAT-3', reverse, 5'-TGTCTGGTA-ACGGCAATGCG-3'; *TrkB*: forward, 5'-ACATTTCCGTCAC-CTTGACTTGT-3', reverse, 5'-GGATGGATTAGCCTCTTG-GAG-3'. Each gene was measured in duplicate in at least three independent experiments. The ΔC_T values ($\Delta C_T = C_{\text{Reference}} - C_{\text{Target}}$) for each target gene were calculated using *PBGD* as the reference gene.

Cell Culture

Primary human PSMC were purchased from Lonza (Basel, Switzerland) or PromoCell (Heidelberg, Germany) or isolated from human pulmonary arteries from nontransplantable donor lungs, as well as from lungs explanted from IPAH patients. The purity of PSMC cultures was confirmed using indirect immunofluorescence antibody staining for smooth muscle-specific isoforms of α -actin and myosin (minimum 95% of cells stained positive) and lack of staining for von Willebrand factor. Recombinant human BDNF (R&D Systems, Wiesbaden, Germany) was used at a final concentration of 5 or 10 ng/mL, ERK1/2 kinase inhibitor U0126 (final concentration 20 mmol/L) were obtained from Alamone Labs (Jerusalem, Israel) and Calbiochem (Darmstadt, Germany), respectively. The BDNF blocking antibody was purchased from Chemicon International (Temecula, CA; final concentration 2.5 μ g/mL).

Electrophoresis Mobility Shift Assay

The electrophoretic mobility shift assay was performed with nuclear extracts (Pierce Biotechnology) from human PSMC using the oligonucleotide probe (5'-GGATC-CAGCGGGGGCGAGCGGGGGCGA-3') containing the *Egr* consensus sequence (Santa Cruz Biotechnology) as described previously.²⁵

Proliferation Assay

Cell proliferation of PSMC was assessed by [³H]-thymidine incorporation as described previously.³¹ For siRNA studies, proliferation was measured by bromodeoxyuridine incorporation according to the manufacturer's protocol (Roche, Mannheim, Germany).

Assessment of Apoptosis

Identification of apoptotic cells after 1 and 24 hours of BDNF (10 ng/mL) treatment was performed using allophycocyanin-conjugated annexin V (Invitrogen), following the recommendations of the manufacturer. Necrotic cells were excluded by counterstaining with 2 μ g/mL propidium iodide. Data were collected using a FACSCanto flow cytometer and analyzed using a FACSDiva software package (both from BD Biosciences, Heidelberg, Germany). A minimum of 10,000 cells were analyzed per sample. Gates based on forward and side scatter were set to eliminate cellular debris and cell clusters.

Transfection with siRNA

PASMC were transfected with small interference RNA (siRNA) at (100 nmol/L) using X-treme Gene siRNA Transfection Reagent (Roche) or Lipofectamine2000 (Invitrogen). Predesigned, commercially available siRNA against human *Egr-1* (siGenomeSMART pool) was purchased from Dharmacon (Chicago, IL), *TrkB* (siGenomeSMART pool) from Dharmacon and Santa Cruz Biotechnology, siRNA against *BDNF* (Santa Cruz Biotechnology), control siRNA from Ambion. Knockdown efficiency was assessed 24 hours (*Egr-1*) or 72 hours (*TrkB* and *BDNF*) post transfection by real-time PCR.

Statistical Analysis

Bar charts show mean $\pm$ SEM. Differences between more than two groups were assessed by analysis of variance followed by Dunnett's multiple-to-one comparison post hoc tests. All data that were subjected to statistical analysis were checked for normal distribution by quantile-quantile plots. Where appropriate, the paired Student's *t*-test was performed. All experiments were designed with matched control conditions to enable statistical comparison. An asterisk indicates $P \leq 0.05$.

Results

Hypoxia Array Data

RNA expression profiles were compared from lung homogenates from mice exposed to normoxia or hypoxia for 24 hours using whole-genome microarray analysis. Under hypoxia, 918 genes were up-regulated and 648 genes were down-regulated (10% false discovery rate). Among these genes, 99 were up- and 27 down-regulated by fold change ≥ 2 . Genes previously reported to be involved in hypoxic remodeling [ie, *PDGFb*, vascular endothelial growth factor (*VEGF*) c, and endothelin1 (*Edn1*)] revealed strong up-regulation following hypoxia exposure (Figure 1A). Several gene pathways were overrepresented in the candidate lists (Figure 1B). Of interest, genes involved in the MAPK signaling pathway revealed strong regulation, where the strongest induction was observed for *Ntrk2/TrkB* (~sevenfold up). Because tyrosine kinases have previously been implicated in the develop-

ment of PH, we therefore studied in more detail the role of TrkB in vascular remodeling.

We subsequently investigated the expression of TrkB at more time points by real-time PCR. Short exposure to hypoxia (24 hours) strongly induced TrkB expression, confirming the array data (Figure 1C). High levels of TrkB expression were maintained during prolonged exposure of mice to hypoxia (7 and 21 days). To localize the expression of TrkB and its ligands, BDNF and neurotrophin 4 (NT-4) in the lung parenchyma, immunohistochemical staining was performed. All three proteins localized to epithelial cells in the bronchial tree; however, TrkB and BDNF were also localized to the pulmonary vasculature as indicated by smooth muscle actin (SMA) staining of consecutive sections (Figure 1D; see also Supplemental Figure S2 at <http://ajp.amjpathol.org>). Antibody specificity was assessed on *BDNF* and *TrkB* knockout mice tissues (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). To determine TrkB- and BDNF-specific expression in PH, we isolated major pulmonary arteries from mice exposed to either 21 days hypoxia or normoxia. Similar to the induction observed in whole lungs, increased expression of TrkB was observed in pulmonary arteries (Figure 1E). Interestingly, increased BDNF expression was also detected (Figure 1E). Laser-microdissection of intrapulmonary arteries in combination with real-time PCR showed similar induction of TrkB and BDNF expression under hypoxic conditions (Figure 1F). Expression of *TrkB* and *BDNF* by SMC was confirmed by real-time PCR (Figure 1G), and immunofluorescence staining of isolated mouse PASMC (Figure 1H) and microvascular pulmonary SMCs (see Supplemental Figure S4A at <http://ajp.amjpathol.org>). Of note, in normoxic conditions in mouse primary PASMC *TrkB* expression levels were on the limit of detection, whereas *BDNF* was abundantly expressed. Hypoxia exposure led to increased expression of *TrkB* in mouse PASMC (see Supplemental Figure S4B at <http://ajp.amjpathol.org>). Specificity of immunofluorescence staining was assessed by using siRNA to knockdown *TrkB* and *BDNF* expression (see Supplemental Figure S5, A and B, at <http://ajp.amjpathol.org>).

Regulation of TrkB and BDNF in Monocrotaline and Su5416-Treated Rats

To determine whether induction of TrkB and BDNF expression was unique to the hypoxic mouse model of PH, we used monocrotaline-treated rats as an alternative PH model. Monocrotaline treatment up-regulated TrkB and BDNF mRNA in rat lung homogenate compared to control animals (Figure 2A). Induction was observed at later time points (28 days), correlating with progression of PH. As observed in mouse lung sections, expression of TrkB and BDNF was detected in the pulmonary vasculature, whereas NT-4 was restricted to the bronchial epithelial layers (Figure 2B; see also Supplemental Figure S6 at <http://ajp.amjpathol.org>). Additionally, elevated expression of TrkB and BDNF was also observed in Su5416 rat model (Figure 2C).

Figure 1. Ntrk2 is up-regulated in mouse lungs after 24 hours of hypoxia exposure ($pO_2 = 0.01$). Total RNA from mice lung homogenates was isolated ($n = 18$ per group), direct labeled, and hybridized to 44K 60mer oligonucleotide microarrays (MWG). **A:** Volcano plot: $\log_2$ -fold regulation compared to probability of regulation. Genes with log odds values ≥ 5 were considered to be regulated; black spots, exemplary genes. **B:** Functional annotation of regulated genes was performed according to Gene Ontology (GO). Ntrk2 (TrkB) belongs to MAPK signaling pathway. **C:** A time course of TrkB expression was performed using real-time PCR of lung homogenates harvested 1, 7, or 21 days after hypoxia exposure ($n = 7$ to 9, each). **D:** Immunohistochemical staining of TrkB and its ligand, BDNF in mouse lung sections. The arrow indicates positive staining. **E** and **F:** Real-time PCR analysis of TrkB and BDNF expression in (**E**) isolated major pulmonary arteries ($n = 6$), and (**F**) laser-microdissected intrapulmonary vessels (40 vessels per animal, $n = 4$), from mice exposed to 21 days of hypoxia or normoxia. **G:** Expression levels of TrkB and BDNF in mouse PASMC as indicated by real-time PCR; lower C_T values represent a more abundant transcript level. **H:** Immunofluorescence staining of TrkB and BDNF in mouse PASMC. The arrows indicate TrkB localization. Inset, negative control staining. $*P \leq 0.05$.

TrkB and BDNF Expression in IPAH Patients

We consequently examined the expression of TrkB and BDNF in IPAH patients. Similar to the hypoxia and monocrotaline models of PH, expression of TrkB and BDNF localized to the pulmonary vasculature, specifically to the SMC layer as indicated by SMA immunoreactivity in adjacent sections (see Supplemental Figure S7 at <http://ajp.amjpathol.org>) and immunofluorescence (Figure 3A). Notably, TrkB and BDNF expression was observed in remodeled vessels of IPAH patients (Figure 3A; see also Supplemental Figure S7 at <http://ajp.amjpathol.org>). Laser microdissection in combination with real-time PCR verified increased expression of TrkB and BDNF in pulmonary arteries of IPAH patients compared to donors (Figure 3B). Furthermore, expression of TrkB and BDNF by SMCs was confirmed by real-time PCR and immunofluorescence staining of isolated human PASMC from donor

lungs (Figure 3, C–E). Positive signal was also observed in PASMC from IPAH lungs (Figure 3D).

BDNF Increases Proliferation of PASMC

BDNF has previously been shown to increase the proliferation of neurons via the activation of TrkB receptor.³³ Because SMC proliferation is one of the underlying mechanisms of vascular remodeling in PH, we investigated whether BDNF can affect proliferation of SMCs. BDNF treatment resulted in a dose-dependent increase in proliferation (Figure 4A) and an abundance of the proliferation marker Ki-67 (Figure 4B). We next investigated BDNF effects on apoptosis. Interestingly, BDNF treatment did not alter PASMC apoptosis as determined by flow cytometry of Annexin V-labeled cells (Figure 4C) and by Western blot analysis of caspase-3 activation (Figure 4D).

BDNF Activates TrkB and MAPK Signaling Pathways in PASM

Next, we examined the effects of BDNF on intracellular signaling events, the activation of which could lead to proliferative response. BDNF treatment induced a rapid

phosphorylation of the TrkB receptor within 1 minute of ligand application (see [Supplemental Figure S8A](http://ajp.amjpathol.org) at <http://ajp.amjpathol.org>). TrkB phosphorylation was accompanied by a strong time-dependent activation of ERK1/2, where phosphorylation occurred by 5 minutes and peaked at 15 minutes post stimulation ([Figure 5A](#)),

Figure 4. Functional role of TrkB and its ligand BDNF in remodeling. Proliferation after BDNF stimulation was assessed by direct [3 H] thymidine incorporation (**A**) ($n = 6$), or immunofluorescence staining of Ki-67 proliferation marker (**B**). The effect of BDNF on cell apoptosis was measured by annexin V staining (**C**) and caspase-3 cleavage (**D**). 5% FCS and staurosporine were used as positive controls for proliferation and apoptosis studies, respectively. * $P \leq 0.05$.

weak activation of AKT was also observed (see [Supplemental Figure S8B](#) at <http://ajp.amjpathol.org>). No significant activation of the p38 pathway was detected (see [Supplemental Figure S8C](#) at <http://ajp.amjpathol.org>). Specificity of TrkB and ERK1/2 activation was shown by pretreatment with the respective inhibitors K252a and U0126, which attenuated phosphorylation of corresponding kinases ([Figure 5](#), B and C). The specificity of BDNF/TrkB/ERK1/2 dependent signaling was validated by the use of BDNF blocking antibodies. Following BDNF stimulation, decreased ERK1/2 phosphorylation was observed as compared to control treated cells. ([Figure 5D](#)).

BDNF Induces Expression and DNA-Binding Activity of Egr-1 in Human PASC

To further investigate the effects of MAPK activation, we examined the ERK1/2 downstream signaling molecule, early growth response1 (Egr-1). Western blotting for Egr-1 revealed that after addition of BDNF to the PASC, Egr-1 was rapidly and transiently induced with peak intensity at 6 hours after treatment, which decreased over time ([Figure 6A](#)). Additionally, immunofluorescence microscopy demonstrated a strong induction of Egr-1 expression and its accumulation in the nucleus of isolated

Figure 5. BDNF stimulation leads to MAP kinase activation. **A:** Time-dependent phosphorylation in response to BDNF of the ERK1/2 kinase with densitometric analysis. **B** and **C:** Effects on BDNF signaling by pretreatment with the inhibitors (**B**) K252a (TrkB) and (**C**) U0126 (ERK1/2) with densitometric analysis. Equal protein loading was confirmed by immunoblotting against β -actin. **D:** Effects of blocking BDNF antibody on ERK1/2 phosphorylation with densitometric analysis. M(kDa), molecular weight in kilodaltons; n.s., not significant. * $P \leq 0.05$.

Figure 6. BDNF regulates and activates Egr-1. **A:** Induction of early growth-response factor 1 (Egr-1) protein in human PASMC as analyzed by Western blotting. **B:** BDNF-induced Egr-1 protein localization in PASMC as assessed by immunofluorescence analysis. **C:** Electrophoretic mobility shift assay for Egr-1 induction and DNA–protein interactions from nuclear extracts prepared from BDNF (10 ng/mL)-treated PASMC, incubated with radiolabeled oligonucleotide containing Egr-1 consensus sequence. Incubation with an excess (10-fold) of an unlabeled probe served as a control for specific binding. PDGF (10 ng/mL) treated cells served as a positive control. **D:** Egr-1 expression in lung parenchyma of donor and IPAH patients. An *Egr-1*^{−/−} mouse (KO-Egr1) served as negative control. **E:** Real-time PCR analysis for Egr-1 expression in IPAH patient and donor laser-microdissected intrapulmonary vessels ($n = 5$). n.s., not significant. * $P \leq 0.05$.

human PASMC after BDNF stimulation (Figure 6B). The increased abundance of Egr-1 also correlated with enhanced and time-dependent DNA binding as shown by electrophoretic mobility shift assay, using the consensus sequence GCGGGGCG for Egr-1 transcription factor (Figure 6C).

Egr-1 Expression in IPAH Patients

Interestingly, we could also observe intense immunohistochemical staining of Egr-1 in vessels of IPAH and donor lungs. Notably, intense staining for Egr-1 was observed in remodeled arteries of IPAH patients. Staining of adjacent sections for SMA further confirmed predominant Egr-1 expression in SMC (Figure 6D; see also Supplemental Figure S9 at <http://ajp.amjpathol.org>). In line with these results, microdissected pulmonary vessels from IPAH lungs revealed significantly elevated Egr-1 mRNA levels compared to donor lungs (Figure 6E).

Egr-1 Regulates Expression of Downstream Genes

Subsequently, to determine whether the BDNF-induced activation of Egr-1 leads to downstream gene activation,

we investigated expression levels of genes known to contain Egr-1 binding sites and have a potential role in remodeling processes. Real-time PCR analysis of cyclin D1 and fibronectin expression showed up-regulation in response to BDNF stimulation (Figure 7A). Moreover, BDNF induced the mRNA expression of Egr-1 transcription factor. Addition of either U0126 or K252a inhibitors, abolished the BDNF-induced up-regulation, and in the case of Egr-1, decreased expression to unstimulated levels (Figure 7A).

BDNF Leads to Proliferation of Human PASMC via ERK and Egr-1

Next, we examined whether inhibition of TrkB or MAPK phosphorylation and its downstream transcription factor, Egr-1, can inhibit the proliferative effect of BDNF. Pre incubation with K252a or U0126 inhibitors attenuated the BDNF-induced proliferation of PASMC (Figure 7B). Moreover, knockdown of *Egr-1* by the application of *Egr-1*-specific siRNA blocked the BDNF proliferative effects (Figure 7C). The efficiency of siRNA knockdown was assessed by real-time PCR analysis (see Supplemental Figure S10 at <http://ajp.amjpathol.org>).

Figure 7. BDNF-induced proliferation in human PASMC associates with an activation of MAP kinases and Egr-1. **A:** Consequence of BDNF (10 ng/mL) treatment on expression of Egr-1, cyclin D1, and fibronectin in the presence of K252a (TrkB inhibitor) and U0126 (ERK inhibitor), as assessed by real-time PCR ($n = 3$). Effects of **(B)** K252a and U0126 and **(C)** Egr-1 knockdown, on BDNF-induced proliferation in human primary PASMC as determined by direct [³H] thymidine or bromodeoxyuridine incorporation ($n = 3$). n.s., not significant. * $P \leq 0.05$.

Discussion

In this study, we initially investigated changes in the mRNA expression profiles arising from short exposure of mice to hypoxia (24 hours). Analysis of this time point, when remodeling processes are instigated but not fully established, could potentially identify key triggers of vascular remodeling. Already at this early time point, the majority of regulated genes were involved in processes related to differentiation and matrix remodeling (focal adhesion, cell adhesion, regulation of actin cytoskeleton, and extracellular matrix receptor interaction). Other significantly up-regulated genes included several that encode growth factors known to be involved in vascular remodeling, namely PDGFB and Edn1, confirming previous studies.^{34,35} Together, this demonstrates that already after a short exposure to hypoxia, many genes involved in vascular remodeling are regulated. In addition to growth factors, altered expression of different tyrosine receptors has also been documented to play an important role in hypoxia-induced and nonhypoxic models of vascular remodeling.^{7,10} In our study, the strongest up-regulated tyrosine kinase receptor was TrkB, encoded by the gene *Ntrk2*. High TrkB expression was maintained throughout prolonged hypoxia (7 and 21 days), even when vascular remodeling processes are considered to be fully established.²² Moreover, on re-exposure to normoxia (reverse remodeling), TrkB expression was attenuated (unpublished observations). Oxygen-dependent regulation of TrkB has been described in neuroblastoma cells; transcription of the *Ntrk2* gene is stimulated at low oxygen tension through a HIF1-dependent mechanism.³⁶ Similarly in our study, hypoxia exposure led to an up-regulation of TrkB in mouse lung homogenates and isolated pulmonary arteries. However, this is the

first report to describe the role of TrkB in association with PH.

The Trk receptor tyrosine kinase family is selectively activated by distinct growth factors: nerve growth factor (NGF) activates TrkA; BDNF and NT-4, TrkB; and NT-3, TrkC.³³ TrkB and its two ligands, BDNF and NT-4, were initially characterized as factors that regulate development, differentiation and survival of neurons.³³ Their expression has since been detected in nonneuronal cells, particularly in human and rat vascular SMCs, endothelial cells, skeletal muscle cells, and great vessels from late gestation through to adulthood.^{23,24} In human lung tissue, we localized TrkB and BDNF protein expression to the bronchial epithelial layer and SMCs of pulmonary arteries. Expression of TrkB and BDNF by SMCs was further validated by immunofluorescence staining of isolated human PASMC from donor and IPAH patients. By contrast, NT-4 was mostly restricted to bronchial and alveolar epithelial cells, and therefore, we concluded a subordinate role of this ligand in vascular remodeling. Accordingly, we have recently shown the importance of NT-4 in the pathogenesis of pulmonary fibrosis, and its critical role in alveolar epithelial cells and fibroblasts proliferation,³⁷ supporting the importance of neurotrophins and their receptors in pulmonary diseases.

In our study, we found increased expression of TrkB and BDNF in three different animal models of PH, namely, the hypoxic mouse model, monocrotaline-treated rats, and Su5416-treated rats. The latter is especially important as rats treated with Su5416 in combination with hypoxia are currently regarded as the most accurate representation of human PAH and display many hallmarks of the human disease. Furthermore, we were able to independently show that the mRNA levels of both molecules were increased in iso-

lated pulmonary arteries and laser-microdissected intrapulmonary vessels. Finally, we demonstrated an *in vitro* role of BDNF/TrkB in PASMC by regulating proliferation, a process that is important in vascular remodeling. Altogether, our data strongly imply a role for the BDNF/TrkB axis in the pathogenesis of PH.

Interestingly, the higher expression of BDNF during later stages of vascular remodeling in all experimental models and also in IPAH patients indicates that disease progression could be enhanced by up-regulation of both the receptor and ligand. We cannot exclude the involvement of other factors in addition to hypoxia in regulating BDNF/TrkB, especially in inflammatory models where many different growth factors are released.

BDNF and TrkB have previously been described to be involved in other vascular diseases and disease models. Enhanced BDNF levels were observed in macrophages and SMCs of atherosclerotic coronary arteries.^{23,38} In the rat aortic balloon deendothelialization model of vascular injury, expression of BDNF and TrkB are both increased dramatically in the area of injury within 3 days and persist during the formation of the neointima.²³ Thus, local secretion of neurotrophins in the developing neointima may serve to promote vascular SMC accumulation or activation, thereby promoting atherogenesis.^{23,39} Increased neovascularization could be observed following adenoviral BDNF delivery in mice and also in the vicinity of foam plugs in rats.^{40,41} A role of BDNF in angiogenesis is further supported by observations in *BDNF*^{-/-} mice, which suffer from destabilization of the myocardial vessel wall, leading to intraventricular hemorrhage and early postnatal lethality.²⁴ Similarly, *TrkB*^{-/-} mice show a marked reduction in blood vessel formation in the subepicardial region and suffer from an early postnatal lethality.⁴² Taken together, these findings suggest a marked role of BDNF and TrkB in angiogenesis and imply that altered expression of either ligand and/or receptor can facilitate pathogenesis.

In neuroblastoma, the BDNF/TrkB signaling cascade acts in a cell-protective and anti-apoptotic (proliferative) manner.⁴³ Similarly, we could demonstrate that BDNF is an important proliferative factor for PASMC. On ligand binding, TrkB receptors dimerize and autophosphorylate their specific tyrosine residues, thereby activating intracellular signaling cascades.⁴⁴ BDNF stimulation of human PASMC activated the pro-survival ERK1/2 and AKT pathways, induced expression of downstream genes including *Egr-1*, cyclin D1, and fibronectin. The importance of extracellular matrix components, such as fibronectin, in remodeling processes has been well documented,^{22,45} whereas cyclin D1 is a well-described mediator of cell proliferation.^{25,46} As the promoter of both genes contain *Egr-1* binding sites,^{47,48} this suggests that their regulation in PASMC might be *Egr-1* dependent. This is in line with previous findings showing *Egr-1*-dependent regulation of fibronectin and cyclin D1 in mouse lung fibroblasts.^{46,49} Other stimuli known to induce *Egr-1*, fibronectin, or cyclin D1 expression include mechanical forces, reactive oxygen species, PDGF, EGF, and FGF,^{50,51} all of which have been implicated in the

pathogenesis of PH. Moreover, *Egr-1* can also regulate BDNF and PDGF expression,⁵² which could result in a positive feedback loop and potentiate pathogenesis. Strong expression of *Egr-1* was observed in the smooth muscle layer of intrapulmonary arteries in human lung sections, and was even more pronounced in remodeled arteries of IPAH patients. This observation was further validated by laser microdissection in combination with real-time PCR analysis. Our findings indicate that BDNF/TrkB signaling induces proliferation of PASMC in IPAH patients via *Egr-1*.

Furthermore, K252a and U0126 suppressed the BDNF-induced proliferation of PASMC, underlining the importance of BDNF/TrkB/ERK signaling in vascular remodeling processes. K252a has been already described as an antiproliferative factor in angiogenesis and reduces blood vessel formation in Matrigel (BD Biosciences).^{53,54} However, further studies should be performed to determine whether application of the inhibitor attenuates or even reverses PH *in vivo*.

Taken together, our results show for the first time that the BDNF/TrkB/ERK1/2 pathway is up-regulated during vascular remodeling and that elevated expression of these genes leads to a proliferative effect and correlates with disease progression. Inhibition of this signaling cascade was able to attenuate SMC proliferation and may thus present an interesting new intervention approach in pulmonary hypertension.

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