Original Research

Smurf1 ubiquitin ligase causes downregulation of BMP receptors and is induced in monocrotaline and hypoxia models of pulmonary arterial hypertension

Koko Murakami[1,2], Rajamma Mathew[1,3], Jing Huang[3], Reza Farahani[2], Hong Peng[2], Susan C Olson[1,4] and Joseph D Etlinger[2]

[1]The Center for Pulmonary Hypertension Translational Research; [2]Department of Cell Biology and Anatomy; [3]Department of Pediatrics; [4]Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York, USA
Corresponding author: Koko Murakami, Department of Cell Biology and Anatomy, New York Medical College, Valhalla, NY 10595, USA. Email: koko_murakami@nymc.edu

Abstract

Reduced bone morphogenetic protein (BMP) receptor (BMPR) expression and BMP signaling have been implicated in vascular cell proliferation and remodeling associated with pulmonary arterial hypertension (PAH). The low penetrance of the *BMPR II* disease gene in familial PAH suggests that additional genetic or environmental factors are involved in clinical manifestation of PAH. Smurf1 ubiquitin ligase, together with inhibitory SMAD 6/7, forms a negative feedback loop for the attenuation of BMP signals by downregulating BMPR and signaling molecules and, in addition, functions in the integration of MAPK/Ras mitogenic pathways. The present study found that Smurf1 was significantly elevated in pulmonary arteries of monocrotaline and hypoxia-induced PAH rats. In the pulmonary artery of hypoxia-exposed mice, elevation of Smurf1 and SMAD7 was correlated with reduced expression of BMPR II protein. Over-expression of Smurf1 in cultured cells induced ubiquitination and degradation of BMPR I and II whereas ligase-inactive Smurf1 reduced ubiquitination and elevated their protein levels, thus serving a dominant-negative function. Smurf1-induced receptor degradation was inhibited by both proteasomal and lysosomal inhibitors. Thus, Smurf1 reduces steady-state levels of BMPRs by ubiquitination and subsequent degradation involving proteasomes and lysosomes. Therefore, these results show that Smurf1 induction could be a key event for triggering downregulation of BMP signaling and causing vascular cell proliferation and remodeling in PAH and that abrogating Smurf1 function could be a strategy for PAH therapeutics.

Keywords: Smurf1 ubiquitin ligase, BMPR II, SMAD7, vascular remodeling, pulmonary arterial hypertension, animal model

Experimental Biology and Medicine 2010; **235**: 805–813. DOI: 10.1258/ebm.2010.009383

Introduction

Pulmonary arterial hypertension (PAH) displays proliferation of endothelial and smooth muscle cells and remodeling/obliteration of peripheral arteries, resulting in right ventricular hypertrophy (RVH) and right heart failure. Bone morphogenetic protein (BMP) receptor (BMPR) II (of the transforming growth factor [TGF]-β receptor family) is reduced in the lungs of patients with severe PAH, most notably in patients with heterozygous germline mutations in the *BMPR II* gene.[1] Disruption of BMPR signaling, associated with a reduction in BMPR, is also identified in vascular cells of pulmonary arteries in secondary PAH[2] and hypoxia and monocrotaline (MCT)-induced PAH in animals.[3–6] Thus, reduced BMPR-mediated signaling, which causes removal of the BMP-apoptotic/cytostatic effect, is suggested to be involved in the pathogenesis of not only familial PAH but also of mutation-negative cases including secondary PAH. Due to the low penetrance of *BMPR II* gene mutations in familial PAH, the second-hit theory has been proposed, suggesting an involvement of other genetic/environmental factors in PAH.[7,8] Regulatory mechanisms responsible for downregulation of BMPR II and abnormal BMP signaling could thus be critical for PAH pathogenesis.

Smurf1 HECT-domain ubiquitin ligase was originally discovered by yeast hybrid screening as an interacting protein with SMAD1, a canonical signaling molecule downstream of BMPR.[9] Smurf1 induces ubiquitination and subsequent proteasome-dependent degradation of BMP-regulated R-SMADs as well as TβRs (TGF-β receptor), thus inactivating BMP/TGF-β signaling.[9–11] In a negative feedback loop

for the TGF-β/BMP-signaling pathway, Smurfs induce nuclear export of SMAD6/7 and, upon ligand activation, translocate them to the plasma membrane, inducing binding of Smurfs to TβRs and receptor degradation.[10–13] Therefore, we hypothesized that Smurf1, a negative regulator of BMP signaling pathway, might be a factor contributing to down-regulation of BMPR II and abnormal BMP signaling in pulmonary vascular cells in PAH.

In the present study, we examined expression of Smurf1 in the lungs of animals with MCT and hypoxia-induced PAH. In both cases, Smurf1 was significantly elevated. In addition, inhibitory SMAD7 increased while BMPR II was downregulated. Over-expression of Smurf1 in cultured cells induced degradation of BMPRs, and dominant-negative Smurf1 resulted in accumulation of receptors. These results show that induction of Smurf1 in pro-PAH conditions can lead to downregulation of BMPR and BMP signaling in PAH. Therefore, suppression of Smurf1 function, by dominant-negative-Smurf1 or siRNA, could be a therapeutic strategy for recovering BMP-responsiveness and treating PAH.

Materials and methods

Materials

MCT (Trans World Chemicals, MD, USA) was dissolved in 1 N HCl and adjusted to pH 7.3 at the final concentration of 20 mg/mL. MCT solution was freshly prepared immediately before injection. Protease inhibitor cocktail and chloroquine were obtained from Sigma (St Louis, MO, USA). Lactacystin and MG132 were obtained from BostonBiochem (Cambridge, MA, USA). Antibodies used were anti-Smurf1 (H-60, Santa Cruz, CA, USA; Abnova, Taiwan) for Western blotting, anti-Smurf1 (Zymed Laboratories, CA, USA) for immunohistochemistry, anti-BMPR II (H-300, Santa Cruz, CA, USA), anti-SMAD7 (H-79, Santa Cruz), anti-β-actin (Santa Cruz), anti-HA (Cell Signaling, MA, USA), anti-Flag (Sigma, MI, USA), anti-His (Cell Signaling) and horse radish peroxidase (HRP)-conjugated goat-anti-rabbit and mouse IgGs (Chemicon International, CA, USA). Histo-stain IHC Detection kit (Invitrogen, CA, USA) was used for immunohisochemical analyses of paraffin sections. Prestained *Rec* Protein Ladder (Fisher Scientific, PA, USA) and Western Lighting Chemiluminescence Reagent Plus (Perkin Elmer, CT, USA) were used for Western blotting. NiNTA gel (Qiagen, CA, USA) was used for isolation of His-tag proteins.

PAH model animals

All protocols were approved by the Institutional Animal Care and Use Committee at New York Medical College and conformed to the guiding principles for the use and care of laboratory animals of the American Physiological Society and the National Institutes of Health. Male Sprague–Dawley rats and CD-1 mice were obtained from Charles River Laboratories (Wilmington, DE, USA). After being acclimatized in the animal facility for 5 d, rats (150–175 g) were given a single subcutaneous injection of MCT (60 mg/kg). For hypoxia exposure, rats were subjected to hypobaric hypoxia (Kpa50). Exposure of mice to hypoxia was performed as described previously.[14] Briefly, a combination of nitrogen (N_2) and oxygen (O_2) was injected into the chamber to achieve $10 \pm 0.1\%$ O_2 concentration. The oxygen concentration was controlled by the Oxycycler hydraulic system (Model A44 × 0, BioSpherix, Redfield, NY, USA) and ANA-Win2 Software (Version 2.4.17, Watlow Anafaze, Watsonville, CA, USA). Generally, significant PAH and RVH developed after hypoxic exposure for two and three weeks, respectively (data not shown).

Pulmonary arterial pressure and RVH measurement

Animals were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). The trachea was cannulated with PE240 tubing, through which the animal was ventilated with room air. The chest was opened, and right ventricular systolic pressure was recorded on Grass polygraph (model 7E) through PE50 tubing inserted into the right ventricle (RV). The right ventricular systolic pressure (RVSP) was used as pulmonary arterial pressure (PAP). After pressure measurements, lungs were perfused with saline to remove blood. Lungs and hearts were removed and placed in 10% buffered formaldehyde or snap-frozen.

For assessment of RVH, hearts were removed from formaldehyde, and atria were trimmed off. The free wall of the RV was separated from the left ventricle and the septum (LV). They were weighed separately, and the ratio of RV/LV was calculated.

Immunohistochemical staining

Left lungs and pulmonary arteries preserved in 10% buffered formaldehyde were embedded in paraffin. Paraffin sections were de-paraffinated and quenched for endogenous peroxidase activity. After blocking with non-immune serum, paraffin sections were incubated with primary antibodies at the optimal concentrations, followed by incubation with biotinylated second antibody (Invitrogen). After incubation with HRP-streptavidin, visualization was performed with DAB-chromogen. Counterstaining was performed using hematoxylin. The quantification of positive signals was performed using AdobePhotoShop. Briefly, five evenly stained areas of the same size were selected for each image. Signal intensities were expressed in pixel numbers using a color range that represents positive staining and averaged. These analyses were performed by a blinded person for the experimental conditions. The two-tailed Student's *t*-test was performed between the control and hypoxia groups for each protein.

Western blotting

Whole lung tissues or cultured cells were homogenized using Tris-buffered saline, pH 7.4, containing 1% NP40 and protease inhibitors. After brief centrifugation, protein concentrations in supernatants were measured by the

Bio-Rad Bradford protein assay reagent with bovine serum albumin as a reference protein. Supernatant proteins (100 μg of lung tissues or 60 μg of cultured cells) were separated by polyacrylamide gel electrophoresis and analyzed by Western blotting using primary antibodies and HRP-conjugated second antibodies. HRP activity was visualized from chemiluminescence emission and autoradiography. Specific protein bands were confirmed from the estimated molecular weights using a prestained protein ladder. Densitometry was performed with Chemilmage 5500 (Alpha Innotech, CA, USA).

Plasmid DNA construction

HA-BMPRs, Flag-Smurf1 and HECT-H2 expression vectors were kindly provided by K Miyazono (University of Tokyo), JL Wrana (University of Toronto) and CM Pickart (Johns Hopkins University). His-BMPR expression vector was constructed by the ligation of BamHI/XhoI fragment into pcDNA4/HisMax. Smurf1-CA, a ligase-inactive Smurf1, was generated by Cys to Ala mutation at Cys743 using mutated primers in a PCR-based site mutation method. Mutations were confirmed by DNA sequencing.

Cell culture and plasmid transfection

HEK293T cells (ATCC, VA, USA) were cultured in Dulbecco's modified Eagle's medium media including 10% fetal bovine serum, penicillin, streptomycin and amphotericin B at 37°C and 5% CO_2. Plasmid transfection was performed according to the manufacturer's instructions using Superfect (Qiagen). For transfection in 35 mm culture dishes, the total DNA transfected did not exceed 2.5 μg. The DNA concentration of each expression vector was the same for all dishes. Normally, cells were harvested the next day after transfection. Proteasome and lysosome inhibitors were added for the last four hours before harvesting.

Ubiquitination assay

HEK293T cells in 100 mm culture dishes were transfected with His-BMPR (4 μg DNA) and HA-ubiquitin (2 μg DNA) expression vectors and harvested the next day. Where indicated, Smurf1 or Smurf1-CA vectors (4 μg DNA each) were included for the total 10 μg DNA concentration. The DNA concentrations of His-BMPR and HA-ubiquitin vectors were consistent for all dishes. When used, transfected cells were treated with proteasome or lysosome inhibitor for the last 30 min before harvesting. His-tagged BMPR protein was isolated using NiNTA gel (Qiagen) according to the manufacturer's instructions and subjected to Western blotting. Ubiquitinated BMPR was identified using anti-HA antibody.

Statistical analysis

Statistical analyses of hemodynamic and Western blotting data were performed by the two-tailed Student's *t*-test.

Results

Smurf1 is elevated in pulmonary arteries with MCT and hypoxia-induced PAH

Animals were treated by MCT injection or hypoxia exposure. Significant PAH and RVH were observed after two weeks following MCT injection (Table 1). In contrast, exposure to hypoxia resulted in progressive PAH and RVH after one week, followed by further increases after two weeks (Table 1). Smurf1 expression, as analyzed by Western blotting, significantly increased in lungs after both treatments (Figure 1). In hypoxia-induced PAH, increased Smurf1 expression was seen early and continued progressively (Figure 1, right panel). Thus, Smurf1 expression appears to precede PAH development (Table 1). In MCT-induced PAH, increases of Smurf1 after two weeks paralleled that of RVSP and RV/LV (Figure 1, left panel and Table 1). Immunohistochemical analyses of Smurf1 protein expression in lung tissues of rats following MCT treatment showed that Smurf1 increased early, even after two days, in pulmonary vascular cells (Figure 2).

Increase in Smurf1 expression is correlated to reduced BMPR II expression

The expression of Smurf1, BMPR II and SMAD7 proteins was analyzed immunohistochemically in pulmonary arteries of mice exposed to hypoxia for 30 d, and their positive signals were quantified. Compared with the controls, increased expressions of Smurf1 and SMAD7 were observed (Figure 3A versus B and E versus F), while BMPR II was downregulated (Figure 3C versus D).

Smurf1 induces BMPR ubiquitination and degradation which are blocked by dominant-negative Smurf1

The relationship between Smurf1-induction and BMPR degradation was examined *in vitro*. HA-tagged BMPRs were expressed in HEK293T cells with or without Smurf1 or HEC-H2. HECT-H2, a HECT-domain ubiquitin ligase,

Table 1 Hemodynamics

	MCT			
	Control ($n = 5$)	**Two days ($n = 5$)**	**One week ($n = 6$)**	**Two weeks ($n = 6$)**
RVSP (mmHg)	19 ± 0.8	20 ± 0.6	20 ± 0.95	$30 \pm 2.0^{*}$
RV/LV	0.22 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	$0.30 \pm 0.01^{*}$
	Hypoxia			
	Control ($n = 5$)	**Two days ($n = 5$)**	**One week ($n = 5$)**	**Two weeks ($n = 6$)**
RVSP (mmHg)	23 ± 1.2	26 ± 1.3	$32 \pm 1.1^{*}$	$43 \pm 2.2^{*,**}$
RV/LV	0.20 ± 0.006	0.24 ± 0.01	$0.36 \pm 0.03^{*}$	$0.41 \pm 0.02^{*,**}$

RV, right ventricle; LV, left ventricle
RVSP (right ventricular systolic pressure) and RV/LV (assessment of right ventricular hypertrophy) were measured as described in the Materials and methods section
$^{*}P < 0.05$ versus control
$^{**}P < 0.05$ versus one week

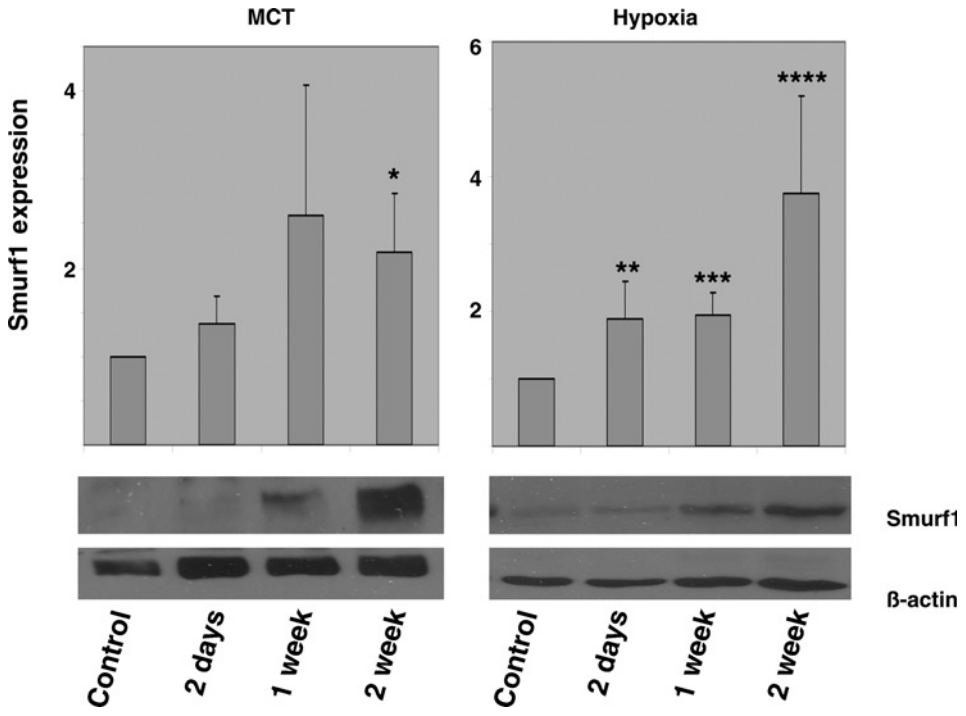

Figure 1 Smurf1 expression is upregulated in lung tissues of MCT and hypoxia-induced PAH rats. Lung tissues were isolated two days, one week and two weeks after MCT injection (60 mg/kg) or hypoxia exposure. Lower panels: Typical Western blotting of lung tissues using anti-Smurf1 and β-actin antibodies. Upper panels: Averaged densitometric quantification of specific bands (MCT $n = 5$, hypoxia $n = 6$). Smurf1 expression is standardized against β-actin band and shown in units of relative intensity to the control. $^{*}P = 0.03$ versus control, $^{**}P < 0.03$ versus control, $^{***}P < 0.01$ versus control, $^{****}P < 0.02$ versus control. Error bars: SE. MCT, monocrotaline

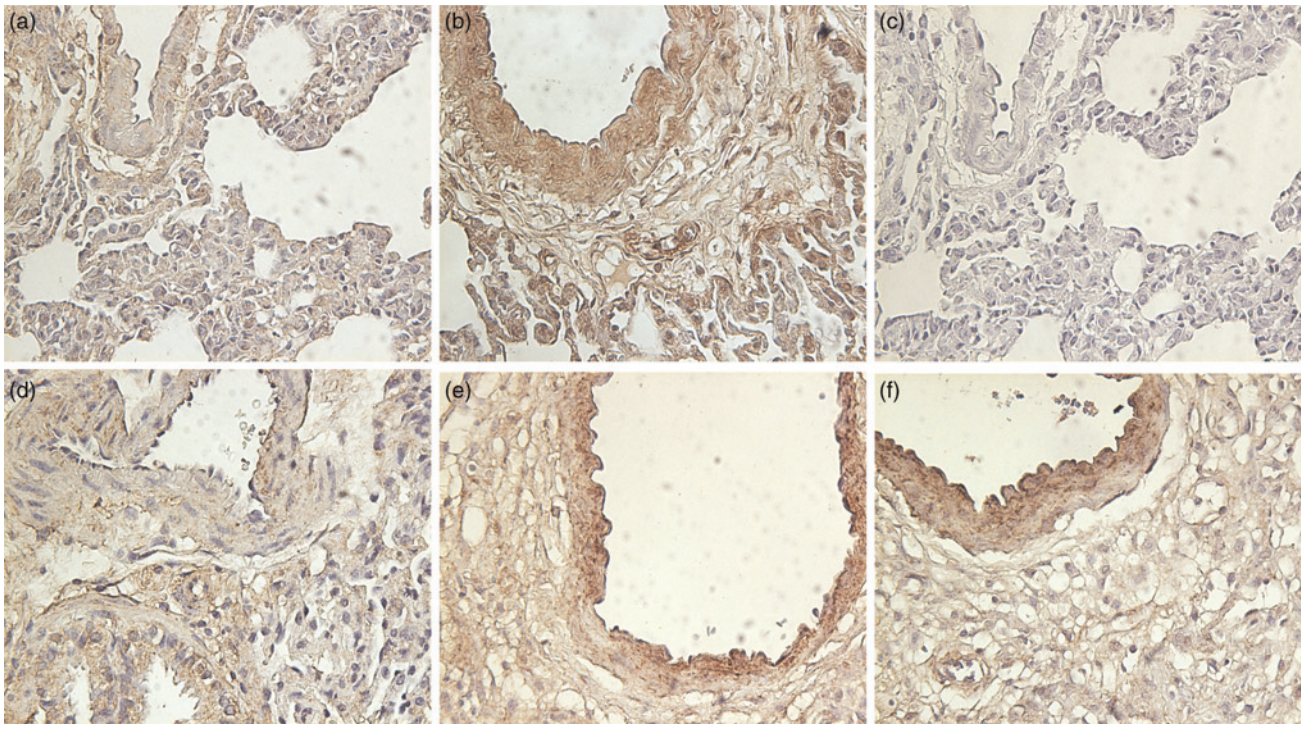

Figure 2 Smurf1 is rapidly upregulated in pulmonary arteries of MCT-induced PAH rats. Lung tissues were fixed two days, one week and two weeks after MCT injection (60 mg/kg). Immunohistochemical analyses of lung tissues for Smurf1 expression using anti-Smurf1 antibody: (a) no treatment, (b) two days after MCT injection, (c) control rabbit IgG, (d) no treatment, (e) one week and (f) two weeks after MCT treatment. (a)–(c) and (d)–(f) were from different sets of experiments. The data shown are a typical set from three animals for each time point. Magnification: 40 $\times$. Counterstaining: hematoxylin. MCT, monocrotaline; PAH, pulmonary arterial hypertension

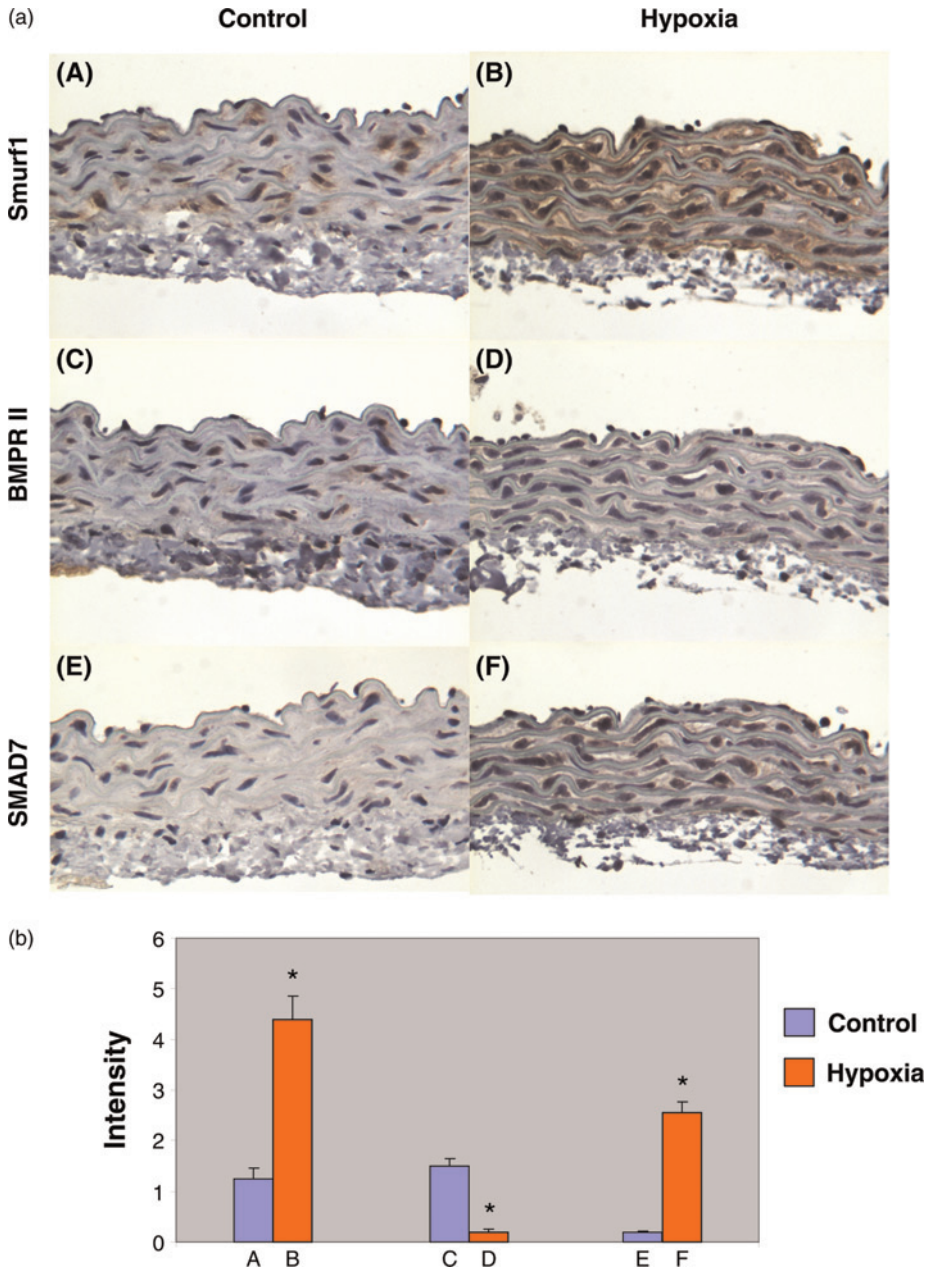

Figure 3 Smurf1 and SMAD7 proteins are elevated while BMPR II is downregulated in the pulmonary artery of hypoxic mice. Mice were exposed to 10% oxygen for 30 d. Immunohistochemical analyses of pulmonary arteries of hypoxic mice for Smurf1 (A and B), BMPR II (C and D) and SMAD7 (E and F) proteins using specific antibodies. A, C and E: control. B, D and F: hypoxia. Species-matched normal serum of each antibody showed no positive staining (not shown). (a) Immunohistochemical microscopy images; (b) quantification of positive staining expressed as a relative intensity. Error bars: SE. $^{*}P < 0.05$. The data shown are a typical set from two mice for each condition. Magnification: 40 ×. Counterstaining: hematoxylin

was used as control.[15] As shown in Figure 4a, Smurf1, but not HECT-H2, induced downregulation of all types of BMPR I. Therefore, Smurf1 targets BMPRs. Smurf1 expression induced downregulation of both BMPR I and II, while Smurf1-CA, a ligase-inactivated Smurf1, did not and instead elevated their expression levels, showing a dominant-negative effect (Figure 4b). Although Flag-Smurf1 and Flag-Smurf1-CA expression vectors were transfected at the same concentration, Flag-Smurf1 protein level was much lower than that of Flag-Smurf1-CA, most likely due to autodigestion, as described previously.[10,13] Smurf1-induced downregulation of both BMPR I and II was inhibited by both proteasome and lysosome inhibitors (Lactacystin and MG132 for the former and chloroquine for the latter) (Figure 4c). Flag-Smurf1 protein appears to be stabilized by chloroquine, but not by proteasome inhibitors (Figure 4c). Smurf1 induced ubiquitination of BMPR (Figure 4d, lane 1 versus 2). The level of ubiquitination was elevated by MG132 (Figure 4d, lane 2 versus 3), but not chloroquine. Smurf1 induced ubiquitination of BMPR, whereas Smurf1-CA reduced it below the control level, being consistent with its dominant-negative function (Figure 4e). Therefore, Smurf1-mediated ubiquitination of BMPR is associated with its downregulation. It is interesting

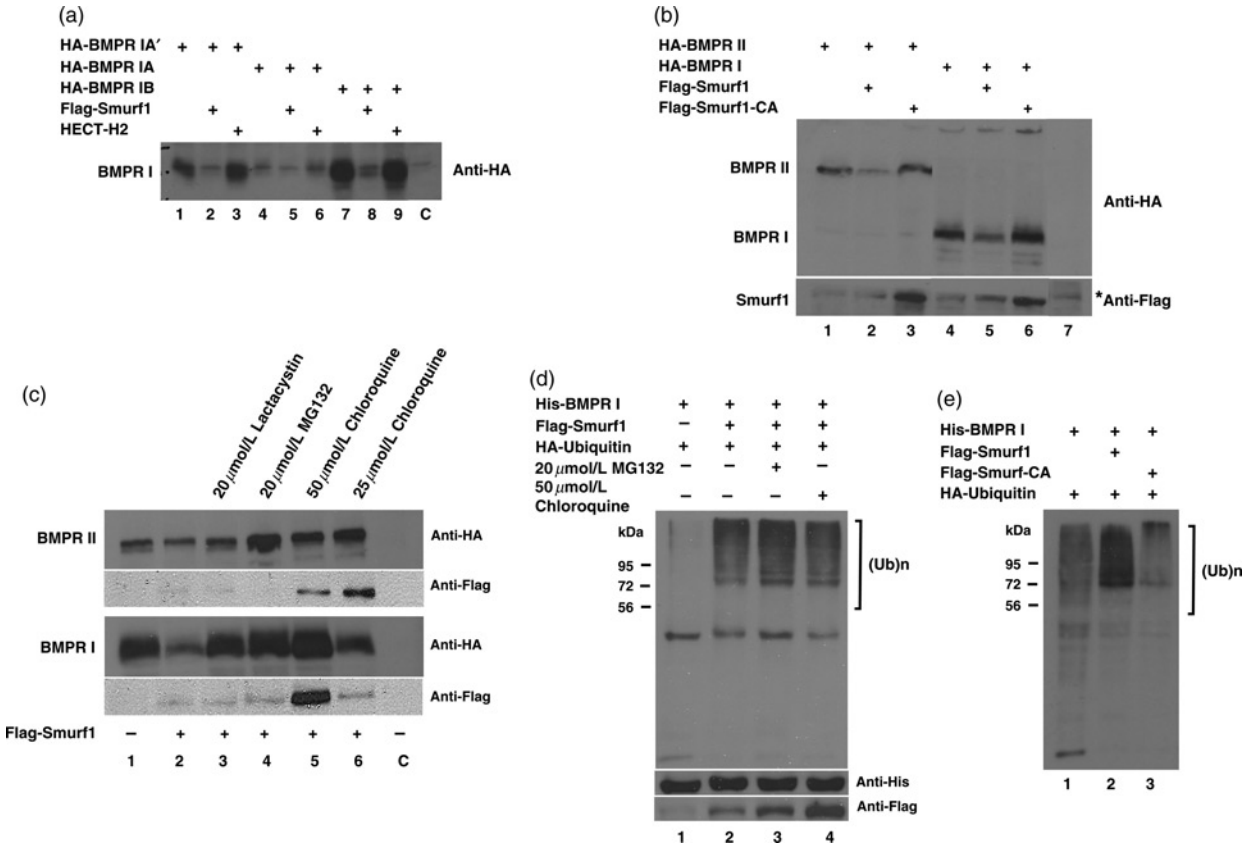

Figure 4 Smurf1 induces ubiquitination and degradation of BMPR. (a) HEK293T cells were transiently transfected with each HA-BMPR I expression vector at the same DNA concentration (1.25 μg). Where indicated, Flag-Smurf1 or HECT-H2 was expressed by transfection of a respective expression vector (1.25 μgDNA each). Protein levels of HA-BMPR Is were analyzed by Western blotting using anti-HA antibody. (b) HA-BMPR IB and II were expressed without or with expression of Flag-Smurf1 or Smurf1-CA. Their protein levels were identified by Western blotting using anti-HA and Flag antibodies. Anti-Flag antibody detects a nonspecific band (marked by *) that migrates slightly slower than the Flag-Smurf1 band. Although the same concentrations of vectors were transfected, the expression of Smurf1 protein was lower than that of Smurf1-CA, most likely due to auto-digestion. (c) HA-BMPR IB and II were expressed without (lane 1) or with Flag-Smurf1 (lanes 2–6). HEK293T cells expressing HA-BMPR and Flag-Smurf1 were treated with inhibitors for the proteasome (Lactacystin and MG132) and the lysosome (chloroquine), where indicated, for the last four hours before harvesting. Protein levels of BMPRs and Smurf1 were estimated by Western blotting using anti-HA and anti-Flag antibodies, respectively. Smurf1 protein appears to be stabilized by chloroquine. (d) His-BMPR IB and HA-ubiquitin were expressed without or with Flag-Smurf. 20 μmol/L MG132 (lane 3) or 50 μmol/L chloroquine (lane 4) was added for the last 30 min before harvesting. His-tagged protein was isolated by NiNTA gel (Qiagen, CA, USA) and subjected to Western blotting. The Western blotting of lysate proteins confirmed expression levels of Flag-Smurf1. (e) His-BMPR IB and HA-ubiquitin were expressed without or with Flag-Smurf1 or Smurf1-CA. Transfected cells were treated with 20 μmol/L MG132 for the last 30 min before harvesting to stabilize ubiquitinated proteins. His-tagged protein was isolated by NiNTA gel (Qiagen) and subjected to Western blotting

that the proteasome inhibitor (MG132) elevated BMPR ubiquitination, but the lysosomal inhibitor (chloroquine) did not, although both inhibited degradation. Thus, Smurf1-catalyzed ubiquitination of BMPR may be required for some steps of endocytic transport to lysosomes, but not for degradation by lysosomal hydrolases and ubiquitination and deubiquitination are involved in cargo transport between the membranes. In addition, proteasomal degradation may be involved as a regulatory step in lysosomal degradation[16,17] or in an independent pathway for BMPR degradation, that is, ER-associated degradation.[18]

Discussion

The present study showed a significant increase of Smurf1 HECT-domain ubiquitin ligase in pulmonary arteries in two animal models of PAH. The discovery of *BMPR II* gene mutations underlying familial PAH[19–21] has led to the hypothesis that downregulation of BMPR and subsequent abnormal BMP signaling, in both familial and secondary PAH, cause removal of the BMP-cytostatic effect, contributing to vascular remodeling and PAH.[1,8] In support of this, BMPR II over-expression in *BMPR II* +/− mice attenuates hypoxic PAH.[22] Since Smurfs are primarily responsible for the degradation of BMPRs and their downstream signaling molecules, the elevated expression of Smurf1 in pulmonary arteries may have a significant role in the pathogenesis of PAH. We propose that Smurf1 is induced by pro-PAH factors, such as hypoxia and pro-inflammatory cytokines, leading to downregulation of BMPR and SMAD1/5 (Figure 5).

In the hypoxia-induced PAH model, Smurf1 increase was seen early and progressively and appeared to precede PAH progression. A significant increase in Smurf1 expression was correlated with PAH appearance in MCT-induced PAH, although Smurf1 induction appeared to occur early as seen by histochemical analyses (Figure 2). PAH develops slower in MCT-treated rats, whereas PAP is significantly elevated after exposure to hypoxia for one week, followed

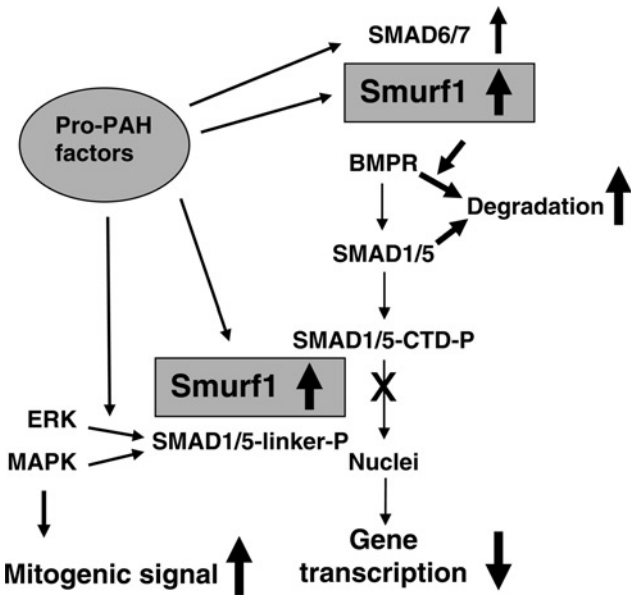

Figure 5 A role of Smurf1 for vascular remodeling in PAH: Smurf1, induced by pro-PAH factors such as hypoxia and proinflammatory cytokines, promotes downregulation of BMPRs and BMP signaling and integration of MAPK-mitogenic signals, leading to vascular cell proliferation and remodeling

by a progressive increase. The kinetics of Smurf1 and PAH expression are consistent with a causative role of Smurf1 in PAH development.

Smurfs and inhibitory SMADs (I-SMADs) cooperatively induce degradation of TβRs and inhibit TGF-β signaling in the negative-regulatory network of TGF-β signaling.[9–13] In this system, I-SMADs are induced by TGF-β family proteins and other factors, while Smurfs have been regarded as constitutive factors that cooperate with I-SMADs. Recently, Smurf1 was shown to be induced by TNF and to mediate TNF-induced bone loss.[23,24] Our study is consistent with a regulatory role of Smurf1 expression in a variety of physiological and pathological conditions. Transcription of the *Smurf1* gene is not dependent on nuclear factor-kappa B[23] and the regulatory mechanism for the *Smurf1* gene remains unknown. Since Smurf1 expression was induced by hypoxic exposure in the present study, HIF transcription factors could be involved in transcriptional control of the *Smurf1* gene.

Downregulation of BMPR has been suggested to be responsible for abnormal BMP signaling and subsequent vascular remodeling in familial and secondary PAH.[1,8] In our model (Figure 5), Smurf1 is induced by a variety of factors including MCT/inflammation and hypoxia. As shown in Figure 4, Smurf1-CA, a dominant-negative Smurf1, elevates BMPR. Therefore, these results show a potential for therapeutic application of tools that abrogate Smurf1 function such as dominant-negative Smurf1 and siRNA for the *Smurf1* gene.

We showed that BMPR basal degradation was mediated by both proteasomes and lysosomes, as shown previously for Smurf2-induced TβR degradation.[12] Smurf2/Smad7-mediated TβR basal turnover is suggested to occur via a raft-caveolar internalization pathway.[25] Smurf1, along with SMAD7, was shown to mediate TβR transport to the plasma membrane.[13] Smurf1 and Smurf1-CA, respectively, induced BMPR internalization from and accumulation in the plasma membrane (K Murakami, JD Etlinger, unpublished observation). The present study showed that BMPR was ubiquitinated directly by Smurf1. Therefore, Smurf1-mediated ubiquitination of BMPRs appears to serve as a signal for endocytic trafficking of BMPRs, as ubiquitination is a major signal for endocytosis of membrane proteins.[26]

Recent studies suggest an essential role of Smurf1 for integrating TGF-β/BMP signals with mitogenic and stress signaling pathways.[27] The SMAD proteins consist of two conserved domains, MH1 and MH2, connected by a linker domain. The canonical BMP signal is mediated through phosphorylation of the C-terminal domain (CTD) of SMAD1/5/8 and subsequent nuclear localization. The activation of the MAPK/Ras mitogenic pathway counterbalances CTD phosphorylation by phosphorylation of the SMAD1 linker region.[28,29] This interplay has been shown to be a critical step in integrating a variety of signals during embryogenesis, development and cell fate determination.[30–33] The linker phosphorylation recruits Smurf1 on SMAD1, inducing SMAD1 ubiquitination/degradation and inhibiting nuclear localization/BMP signaling.[27] CTD-phosphorylated SMAD is excluded from the nuclei, only when Smurf1 is co-expressed.[27] Therefore, Smurf1 recruitment to linker phosphorylated SMADs integrates suppression of BMP signals. Our results support a pivotal role for Smurf1 in PAH development whereby elevated Smurf1 downregulates BMP signaling molecules and suppresses BMP-dependent gene transcription, thus facilitating the MAPK/Ras mitogenic pathway and leading to vascular cell proliferation and remodeling (Figure 5).

Vascular lesions manifesting extensive remodeling are limited to small pulmonary arteries in PAH. Phenotypic diversity has been shown between pulmonary artery smooth muscle cells isolated from proximal and peripheral human pulmonary arteries, involving differential activation of SMAD-dependent and MAPK signaling pathways.[6,34] This difference was attributed to different developmental origins of these cells, the proximal (main) pulmonary artery from neural crest and peripheral arteries by vasculogenesis within the developing lung mesenchyme.[35,36] Neural differentiation is induced by antagonizing the BMP signal by over-expression of Smurf1,[9] whereas the mesenchymal origin is dependent on BMP signaling. While Smurf1 protein was elevated in both main and peripheral pulmonary arteries in the present study, its elevation can thus be expected to have differential consequences on BMP signaling.

In our study, expression of both Smurf1 and SMAD7 was increased while BMPR II was downregulated. SMAD6 and 7 are inhibitory SMADs that are induced by TGF-β in a feedback mechanism inhibiting TGF-β-mediated transcriptional activation[37–39] and facilitating downregulation of TβR signaling.[10–13] SMAD 6 and 7 were originally discovered as the genes that were specifically induced in endothelial cells by steady laminar shear stress and suggested to function as integrators of humoral and mechanical stimuli in these cells.[40] Therefore, expression of SMAD7 may be cooperatively regulated with Smurf1 to facilitate suppression of BMP signaling and vascular proliferation in the lung.

The studies implicating SMURF 1/2 in inflammation-associated bone loss may provide insight into PAH. For example, TNF induces degradation of SMAD1, inhibition of SMAD-mediated transcription as well as increase in Smurf1/2 ubiquitin ligase expression in osteoblasts. This inhibitory effect of BMP signaling by TNF is blocked by Smurf1-deletion,[23] and bone loss in TNF-overexpressing transgenic mice (TNF-Tg) is rescued in TNF-Tg/*Smurf1*−/− mice,[24] thus showing that Smurf1 induction mediates these TNF actions. Inflammation plays a significant role in the pathogenesis of PAH[41] and MCT-induced PAH.[42] PAH is not an uncommon complication with inflammatory diseases. In primary cultures of bovine pulmonary endothelial cells, Smurf1 protein is induced by TNF-alpha (Murakami K and Olson SC, unpublished data). Thus, induction of Smurf1 could be a key event in promoting PAH by proinflammatory cytokines (Figure 5).

In conclusion, our observations suggest that Smurf1, elevated in experimental models of PAH, could play a primary role for reducing BMP signaling and integrating mitogenic signals, leading to vascular remodeling in PAH. Since Smurf1 plays multiple functions, including downregulation of BMPRs and integration of BMP and MAPK signaling, suppression of Smurf1 function, as shown here using dominant-negative Smurf1, may have therapeutic potential in preventing PAH progression in both familial and secondary PAH.

Author contributions: KM conducted Western blotting, expression vector constructions, cultured cell studies, data analyses and manuscript writing. RM and JH generated PAH rats and measured RVSP and RVH. RF generated hypoxia PAH mice. HP conducted histochemical analysis. SCO conducted cultured cell studies. JDE was involved in experimental designs and manuscript review/editing.

ACKNOWLEDGEMENTS

Dr Michael Wolin is greatly appreciated for his critical reading and constructive comments on our manuscript. We thank Ms Joanne Abrahams for her professional help on the illustrations. This work was supported by grants from the Astellas USA Foundation and the intramural funding from New York Medical College (KM).

REFERENCES

1 Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* 2002;**105**:1672–8
2 Du L, Sullivan CC, Chu D, Cho AJ, Kido M, Wolf PL, Yuan JX, Deutsch R, Jamieson SW, Thistlethwaite PA. Signaling molecules in nonfamilial pulmonary hypertension. *N Engl J Med* 2003;**348**:500–9
3 Takahashi H, Goto N, Kojima Y, Tsuda Y, Morio Y, Muramatsu M, Fukuchi Y. Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension. *Am J Physiol* 2006;**290**:L450–8
4 Takahashi K, Kogaki S, Matsushita T, Nasuno S, Kurotobi S, Ozono K. Hypoxia induces alteration of bone morphogenetic protein receptor signaling in pulmonary artery endothelial cell. *Pediatr Res* 2007;**61**:392–7
5 Long L, Crosby A, Yang X, Southwood M, Upton PD, Kim DK, Morrell NW. Altered bone morphogenetic protein and transforming growth factor-{beta} signaling in rat models of pulmonary hypertension. Potential for activin receptor-like kinase-5 inhibition in prevention and progression of disease. *Circulation* 2009;**119**:566–76
6 Yang X, Long L, Southwood M, Rudarakanchana N, Upton PD, Jeffery TK, Atkinson C, Chen H, Trembath RC, Morrell NW. Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. *Circul Res* 2005;**96**:1053–63
7 De Caestecker M, Meyrick B. Bone morphogenetic proteins, genetics and the pathophysiology of primary pulmonary hypertension. *Respir Res* 2001;**2**:193–7
8 Morrell NW. Pulmonary hypertension due to BMPR2 mutation: a new paradigm for tissue remodeling? *Proc Am Thoracic Soc* 2006;**3**:680–6
9 Zhu H, Kavsak P, Abdollah S, Wrana JL, Thomsen GH. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* 1999;**400**:687–93
10 Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, Miyazono K. Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* 2001;**276**:12477–80
11 Murakami G, Watabe T, Takaoka K, Miyazono K, Imamura T. Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. *Mol Biol Cell* 2003;**14**:2809–17
12 Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 2000; **6**:1365–75
13 Suzuki C, Murakami G, Fukuchi M, Shimanuki T, Shikauchi Y, Imamura T, Miyazono K. Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. *J Biol Chem* 2002;**277**:39919–25
14 Scafidi S, Douglas RM, Farahani R, Banasiak KJ, Haddad GG. Prostaglandin transporter expression in mouse brain during development and in response to hypoxia. *Neuroscience* 2007;**146**:1150–7
15 Schwarz SE, Rosa JL, Scheffner M. Characterization of human hect domain family members and their interaction with UbcH5 and UbcH7. *J Biol Chem* 1998;**273**:12148–54
16 van Kerkhof P, Govers R, Alves dos Santos CM, Strous GJ. Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. *J Biol Chem* 2000;**275**:1575–80
17 Yu A, Malek TR. The proteasome regulates receptor-mediated endocytosis of interleukin-2. *J Biol Chem* 2001;**276**:381–5
18 Hirsch C, Jarosch E, Sommer T, Wolf DH. Endoplasmic reticulum-associated protein degradation – one model fits all? *Biochim Biophys Acta* 2004;**1695**:215–23
19 Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet* 2000;**67**:737–44
20 Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA III, Loyd JE, Nichols WC, Trembath RC. Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Int PPH Consortium, Nat Genet* 2000;**26**:81–4
21 Machado RD, Pauciulo MW, Thomson JR, Lane KB, Morgan NV, Wheeler L, Phillips JA III, Newman J, Williams D, Galie N, Manes A, McNeil K, Yacoub M, Mikhail G, Rogers P, Corris P, Humbert M, Donnai D, Martensson G, Tranebjaerg L, Loyd JE, Trembath RC, Nichols WC. BMPR2 haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet* 2001;**68**:92–102
22 Reynolds AM, Xia W, Holmes MD, Hodge SJ, Danilov S, Curiel DT, Morrell NW, Reynolds PN. Bone morphogenetic protein type 2 receptor gene therapy attenuates hypoxic pulmonary hypertension. *Am J Physiol* 2007;**292**:L1182–92
23 Kaneki H, Guo R, Chen D, Yao Z, Schwarz EM, Zhang YE, Boyce BF, Xing L. Tumor necrosis factor promotes Runx2 degradation through up-regulation of Smurf1 and Smurf2 in osteoblasts. *J Biol Chem* 2006;**281**:4326–33
24 Guo R, Yamashita M, Zhang Q, Zhou Q, Chen D, Reynolds DG, Awad HA, Yanoso L, Zhao L, Schwarz EM, Zhang YE, Boyce BF, Xing L. Ubiquitin ligase Smurf1 mediates tumor necrosis factor-induced

systemic bone loss by promoting proteasomal degradation of bone morphogenetic signaling proteins. *J Biol Chem* 2008;**283**:23084–92
25 Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* 2003;**5**:410–21
26 Hicke L, Dunn R. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Ann Rev Cell Dev Biol* 2003; **19**:141–72
27 Sapkota G, Alarcon C, Spagnoli FM, Brivanlou AH, Massague J. Balancing BMP signaling through integrated inputs into the Smad1 linker. *Mol Cell* 2007;**25**:441–54
28 Kretzschmar M, Doody J, Massague J. Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature* 1997;**389**:618–22
29 Kretzschmar M, Massague J. SMADs: mediators and regulators of TGF-beta signaling. *Current Opin Genet Dev* 1998;**8**:103–11
30 Massague J. Integration of Smad and MAPK pathways: a link and a linker revisited. *Genes Dev* 2003;**17**:2993–7
31 Grimm OH, Gurdon JB. Nuclear exclusion of Smad2 is a mechanism leading to loss of competence. *Nat Cell Biol* 2002;**4**:519–22
32 Pera EM, Ikeda A, Eivers E, De Robertis EM. Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. *Genes Dev* 2003;**17**:3023–8
33 Aubin J, Davy A, Soriano P. *In vivo* convergence of BMP and MAPK signaling pathways: impact of differential Smad1 phosphorylation on development and homeostasis. *Genes Dev* 2004;**18**:1482–94
34 Wharton J, Davie N, Upton PD, Yacoub MH, Polak JM, Morrell NW. Prostacyclin analogues differentially inhibit growth of distal and proximal human pulmonary artery smooth muscle cells. *Circulation* 2000;**102**:3130–6
35 Beall AC, Rosenquist TH. Smooth muscle cells of neural crest origin form the aorticopulmonary septum in the avian embryo. *Anatomical Rec* 1990;**226**:360–6
36 Hall SM, Hislop AA, Pierce CM, Haworth SG. Prenatal origins of human intrapulmonary arteries: formation and smooth muscle maturation. *Am J Respir Cell Mol Biol* 2000;**23**:194–203
37 Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA Jr, Wrana JL, Falb D. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 1997;**89**:1165–73
38 Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K. Smad6 inhibits signalling by the TGF-beta superfamily. *Nature* 1997;**389**:622–6
39 Nakayama T, Gardner H, Berg LK, Christian JL. Smad6 functions as an intracellular antagonist of some TGF-beta family members during Xenopus embryogenesis. *Genes Cells* 1998;**3**:387–94
40 Topper JN, Cai J, Qiu Y, Anderson KR, Xu YY, Deeds JD, Feeley R, Gimeno CJ, Woolf EA, Tayber O, Mays GG, Sampson BA, Schoen FJ, Gimbrone MA Jr, Falb D. Vascular MADs: two novel MAD-related genes selectively inducible by flow in human vascular endothelium. *Proc Natl Acad Sci USA* 1997;**94**:9314–9
41 Dorfmuller P, Perros F, Balabanian K, Humbert M. Inflammation in pulmonary arterial hypertension. *Eur Respir J* 2003;**22**:358–63
42 Bhargava A, Kumar A, Yuan N, Gewitz MH, Mathew R. Monocrotaline induces interleukin-6 mRNA expression in rat lungs. *Heart Dis (Hagerstown, Md)* 1999;**1**:126–32

(Received December 18, 2009, Accepted February 11, 2010)