ORIGINAL RESEARCH

Nur77 Suppresses Pulmonary Artery Smooth Muscle Cell Proliferation through Inhibition of the STAT3/Pim-1/NFAT Pathway

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Abstract

The orphan nuclear receptor 4A (NR4A) family plays critical roles in the regulation of cell proliferation, differentiation, and survival in the cardiovascular system. However, the molecular mechanisms underlying the regulation of NR4A receptor expression and its role in pulmonary artery smooth muscle cell (PASMC) function remain unclear. Here, we investigated whether the NR4A family regulates PASMC proliferation, and if so, which mechanisms are involved. By using quantitative real-time RT-PCR, we showed that the orphan nuclear receptor Nur77 was the most abundant member of NR4A family expressed in rat PASMCs, as compared with the two other members, NOR-1 and Nurr1. In rat PASMCs, expression of Nur77 was robustly induced in response to several pathologic stimuli of pulmonary arterial hypertension (PAH), such as hypoxia, 5-hydroxytryptamine (5-HT), platelet-derived growth factor, and endothelin-1. Importantly, Nur77 was also significantly increased in lungs of rats with monocrotaline-induced PAH. Furthermore, we demonstrated that 5-HT markedly up-regulated Nur77 expression through the mitogen-activated protein kinases/extracellular signal–regulated kinase 1/2 pathway. Overexpression of Nur77 inhibited 5-HT–induced PASMC proliferation, as well as the expression of cyclin D1 and proliferating cell nuclear antigen. Mechanistically, we demonstrated that Nur77 specifically interacts with signal transducer and activator of transcription 3, thus inhibiting its phosphorylation and expression of its target genes, such as Pim-1, nuclear factor of activated T cells c2, and survivin in PASMCs. These results indicate that Nur77 is a novel negative-feedback regulator of PASMC proliferation through inhibition of the signal transducer and activator of transcription 3/Pim-1/nuclear factor of activated T cells axis. Modulation of Nur77 activity may potentially represent a novel therapeutic strategy for the treatment of PAH.

Keywords: orphan nuclear receptor Nur77; pulmonary artery hypertension; smooth muscle cells; proliferation; signal transducer and activator of transcription 3

Clinical Relevance

Using *in vitro* and *in vivo* models of pulmonary arterial hypertension (PAH), we demonstrated that the orphan nuclear receptor, Nur77, is substantially increased in proliferative pulmonary artery smooth muscle cells (PASMCs) and the lungs of rats with experimental PAH. Overexpression of Nur77 markedly inhibited 5-hydroxytryptamine–induced PASMC proliferation, phosphorylation of signal transducer and activator of transcription 3, and expression of Pim-1, nuclear factor of activated T cells c2, and survivin. These results implicate critical roles of Nur77 in suppressing PASMC proliferation and the development of experimental PAH.

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Pulmonary arterial hypertension (PAH) is a devastating and life-threatening vascular disease that is characterized by sustained pulmonary artery constriction and obstructive pulmonary vascular remodeling, leading to elevated vascular resistance and subsequent right heart failure and death (1). Accumulating evidence suggests that aberrant proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) is an important pathogenic feature that contributes significantly to the development of PAH (2, 3). Indeed, several growth factors and neurotransmitters, such as platelet-derived growth factor (PDGF)-BB (4, 5), epidermal growth factor (EGF) (6), serotonin (7), and endothelin-1 (8), have been shown to induce PASMC proliferation and promote the development and progression of PAH. Serotonin (also known as 5-hydroxytryptamine [5-HT]) is one of the most potent, naturally occurring vasoconstrictors in pulmonary artery remodeling and pulmonary hypertension (7, 9, 10). Indeed, through the 5-HT transporter (5-HTT) and 5-HT receptors, 5-HT has been shown to trigger activation of PDGF receptor-β and mitogen-activated protein kinase (MAPK), thus increasing proliferation of PASMCs (11, 12). Accordingly, inhibition of the 5-HT pathway, by using either 5-HTT inhibitors or a combination of 5-HTT inhibitors and 5-HT receptor antagonists, has been shown to effectively attenuate the development of experimental PAH (7, 13). Although several molecular mechanisms have been proposed to contribute to the 5-HT–induced proliferation of PASMCs (14, 15), further identification of novel molecular mechanisms, particularly novel inhibitors controlling the proliferation of PASMCs, is of considerable scientific and therapeutic interest.

The members of the nuclear receptor 4A (NR4A) family—namely, Nur77 (NR4A1), Nurr1 (NR4A2), and NOR1 (NR4A3)—are immediate-early genes that are activated by many physiological stimuli, including hormones, inflammatory signals, and growth factors (16, 17). Accumulating evidence suggests that these receptors play an essential role in the regulation of several key cellular processes, including proliferation, differentiation, and cell survival (18, 19). In particular, their biological effects in the cardiovascular system have recently gained considerable attention (20, 21). For instance, in vascular smooth muscle cells (VSMCs), the expressions of Nur77 and NOR-1 were significantly induced by mitogenic stimuli, such as PDGF-BB, EGF, and α-thrombin, and overexpression of Nur77 has been shown to inhibit cell proliferation and attenuate vascular injury–induced neointimal formation *in vivo* (22, 23). Importantly, our recent study has implicated Nur77 as a potent negative regulator of the proinflammatory response in vascular endothelial cells via selective inhibition of NF-κB activation (24). Although the NR4A family is highly expressed in the lung (25, 26), the specific role of NR4A members in pulmonary biology remains elusive. Thus, the purpose of this study was to investigate whether the NR4A family is involved in PASMC proliferation and the development of PAH, and, if so, to determine the mechanism(s) involved.

Materials and Methods

Cell Culture

Rat PASMCs (RPASMCs) were obtained from Cell Biologics, Inc. (Chicago, IL) and cultured in SmGM-2 (Lonza, Walkersville, MD) in 5% fetal bovine serum at 37°C in a humidified 5% CO2 incubator.

RNA Analysis by Quantitative RT-PCR

Total RNA was extracted from RPASMCs using RNeasy mini kit (Qiagen, Valencia, CA) and quantitative RT-PCR (qRT-PCR) were performed on the cDNAs generated from 250 ng of total RNA by HotStart-IT SYBR Green qPCR Master Mix with UDG (2×) Tested User Friendly kit (USB Corporation, Santa Clara, CA). A complete list of primers used in this study is provided in Table E1 in the online supplement.

Western Blot

Western blot analysis was performed essentially as previously described (24). Briefly, cell lysates were separated by SDS-PAGE. Blots were incubated with diluted primary antibodies and followed by incubation with either IRDye 700 or 800 secondary antibodies and visualized using the Odyssey Infrared Imaging System software (Li-Cor, Lincoln, NE).

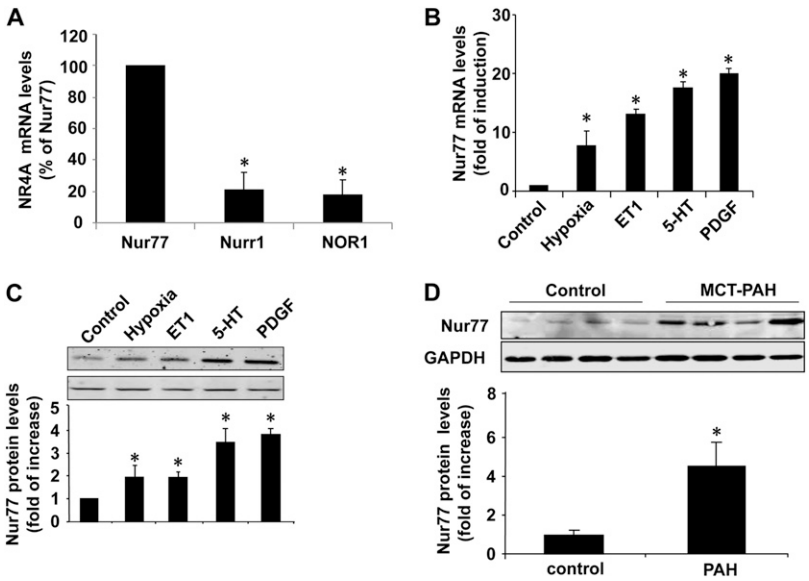

Figure 1. Expression of nuclear receptor 4A (NR4A) family members in pulmonary artery smooth muscle cells (PASMCs). (*A*) Quantitative RT-PCR (qRT-PCR) detection of NR4A family receptors in rat PASMCs (RPASMCs; $n = 5$; $^*P < 0.05$ vs. Nur77). (*B*) RPASMCs were treated with 5-hydroxytryptamine (5-HT; 1 μmol/L), platelet-derived growth factor (PDGF)-BB (20 ng/ml), or endothelin-1 (200 nmol/L) for 1 hour or hypoxia (1% O_2) for 3 hours. The expression of Nur77 was determined by qRT-PCR ($n = 5$; $^*P < 0.05$ vs. control). (*C*) RPASMCs were treated with 5-HT (1 μmol/L), PDGF-BB (20 ng/ml), or endothelin-1 (200 nmol/L) for 6 hours or hypoxia (1% O2) for 12 hours, the expression of Nur77 was determined by Western blot analysis ($n = 4$; $^*P < 0.05$ vs. control). (*D*) The expression of Nur77 was determined by Western blot analysis of lungs of rats with monocrotaline (MCT)-induced experimental pulmonary arterial hypertension (PAH; $n = 4$; $^*P < 0.05$ vs. control rats).

Coimmunoprecipitation

The supernatants were incubated with primary antibodies overnight and the Protein A/G Sepharoses (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 4 hours at 4°C. After being rinsed with lysis buffer, immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with diluted antibodies for signal transducer and activator of transcription (STAT) 3 (1:1,000) and Flag (1:1,000), followed by incubation with either IRDye 700 or 800 secondary antibodies.

Animal Model of Experimental PAH

This study was conducted in accordance with the guidelines set forth by the National Institutes of Health and the Thomas Jefferson University Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 250 and 300 g were randomly assigned to receive a single intraperitoneal injection of either 0.5 ml 0.9% NaCl or 0.5 ml 60 mg/kg monocrotaline (MCT; Sigma-Aldrich, St. Louis, MO). After 2 weeks, rats were killed and their right lungs were dissected, submerged in liquid nitrogen, and frozen at −80°C for Western blot analyses.

Adenovirus Construction

Adenoviruses harboring wild-type Flag-tagged Nur77 (Ad-Nur77) and Ad-LacZ were generated using AdMax (Microbix, Mississauga, ON, Canada), as previously described (24).

VSMC Proliferation Assay *In Vitro*

VSMC proliferation *in vitro* was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay, as previously described (27).

Luciferase Reporter Assay

RPASMCs were transfected with 500 ng plasmid containing nerve growth factor I-B response element–Luc and 50 ng of *Renilla* luciferase reporter plasmid pRL-RSV40 (Promega, Madison, WI) using FuGene 6 transfection reagent. At 24 hours after the transfection, cell lysates (20 μl) were assayed for luciferase activity with a dual-luciferase reporter assay system (Promega).

Immunofluorescence Staining

RPASMCs were transduced with Ad-LacZ or Ad-Flag-Nur77 for 48 hours and then fixed RPASMCs were incubated with Cy3-conjugated anti-Flag antibody (1:100 dilution; Sigma) and visualized using an Olympus IX70 epifluorescence microscope (Olympus, Center Valley, PA).

Knockdown of Nur77 and cAMP-Responsive Element Binding Protein Expression

Knockdown of Nur77 and cAMP-responsive element binding protein (CREB) expression was performed by using either Nur77 small interfering RNAs (siRNAs) or CREB siRNAs, with AllStars negative siRNAs (Qiagen) as a control, as previously described (27).

Statistical Analysis

Data were expressed as mean (± SD) and analyzed for statistical significance by Student's *t* test or ANOVA using SPSS software (version 18.0; Armonk, NY). A *P* value less than 0.05 was considered statistically significant in all experiments.

Results

Expression of NR4A Members in PASMCs and the Lungs of Rats with MCT-Induced Experimental PAH

To investigate the role of the NR4A family in PASMC biology, we examined the expression profile of NR4A members in RPASMCs by qRT-PCR. As shown in Figure 1A, all three members of the NR4A

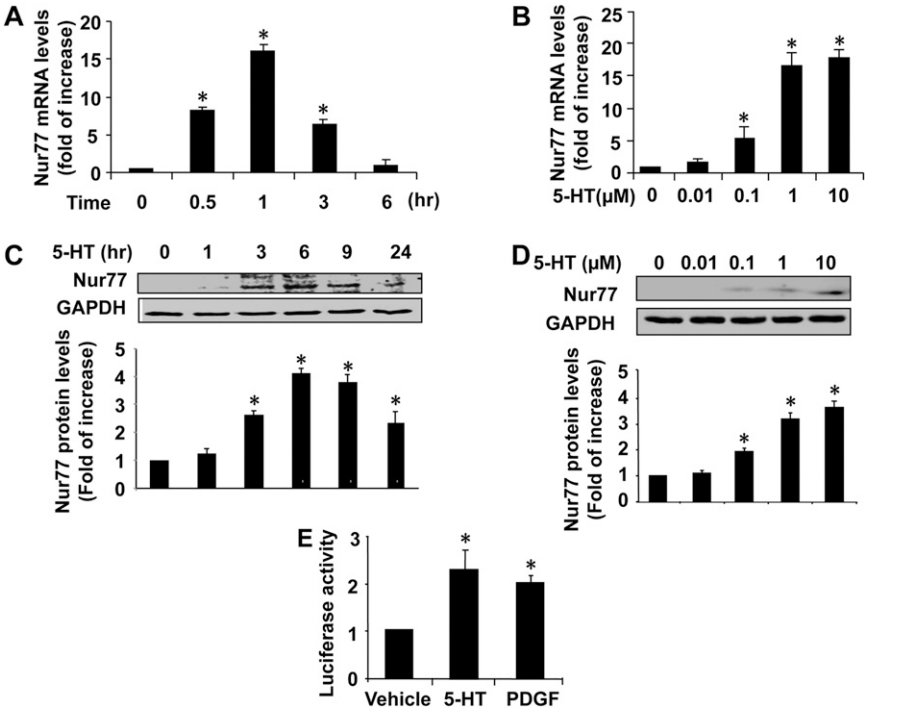

Figure 2. 5-HT induces Nur77 expression in RPASMCs. (*A*) Time-dependent effect of 5-HT (1 μmol/L) on Nur77 expression in RPASMCs, as determined by qRT-PCR ($n = 4$; $^*P < 0.05$ vs. time at 0). (*B*) RPASMCs were treated with increasing concentrations of 5-HT for 1 hour. The expression of Nur77 was determined by qRT-PCR ($n = 5$; $^*P < 0.05$ vs. treatment with vehicle). (*C*) RPASMCs were treated with 5-HT (1 μmol/L) for indicated time points. The expression of Nur77 was then determined by Western blot analysis ($n = 4$; $^*P < 0.05$ vs. time at 0). (*D*) RPASMCs were treated with increasing concentrations of 5-HT for 6 hours and the expression of Nur77 was determined by Western blot analysis ($n = 4$; $^*P < 0.05$ vs. treatment with vehicle). (*E*) RPASMCs were transfected with merve growth factor I-B (NGFI-B) response element (NBRE)–luciferase (Luc) reporter plasmid. At 48 hours after transfection, PASMCs were stimulated with either 5-HT (1 μmol/L) or PDGF (20 ng/ml) for 6 hours and the luciferase activity was then determined ($n = 5$; $^*P < 0.05$ vs. treatment with vehicle).

subfamily were expressed in RPASMCs. Among them, Nur77 was the most abundantly expressed transcript in RPASMCs, followed by Nurr1 and NOR1, respectively. Furthermore, stimulation of PASMCs with hypoxia, 5-HT, enodthelin-1, and PDGF, all of which are implicated in PASMC proliferation and the development of pulmonary hypertension, markedly increased Nur77 expression, as determined by qRT-PCR and Western blot analysis (Figures 1B and 1C). Importantly, as shown in Figure 1D, the expression of Nur77 was substantially increased by roughly fivefold in lungs of rats with MCT-induced experimental PAH as compared with normal controls, further indicating the essential role of Nur77 in PASMC proliferation and the development of experimental PAH.

5-HT Induces Nur77 Expression in RPASMCs via the MAPK Kinase 1/2/MAPK Pathway

To further substantiate the role of Nur77 in PASMC proliferation, we treated RPASMCs with 5-HT, a potent mitogen for PASMC proliferation. The time course of Nur77 expression, when incubated with 1 μmol/L 5-HT, showed a maximal induction of Nur77 expression 1 hour after 5-HT stimulation (Figure 2A). At a concentration as low as 0.1 μmol/L, 5-HT induced Nur77 mRNA expression by roughly fivefold. The maximal induction of 5-HT on Nur77 mRNA expression was observed at the concentration of 1 μmol/L, which increased Nur77 expression by approximately 15-fold (Figure 2B).

Protein expression of Nur77 was confirmed by both Western blot and Nur77-dependent luciferase assay. As shown in Figures 2C and 2D, Nur77 protein levels were up-regulated in RPASMCs, in a time and dose-dependent fashion, by 5-HT treatment. Treatment of PASMCs with 1 μmol/L 5-HT for 6 hours significantly increased Nur77 protein levels by roughly fourfold. Moreover, Nur77 transcriptional activity, as determined using the nerve growth factor I-B response element–driven luciferase reporter, was markedly increased in 5-HT–treated PASMCs (Figure 2E), indicating that 5-HT–induced Nur77 expression is predominantly localized in the nucleus of PASMCs.

To further determine the molecular signaling pathways involved in 5-HT–induced Nur77 expression, RPASMCs were pretreated with either inhibitors of various kinases or inhibitors for 5-HTT and receptors for 1 hour before the 5-HT stimulation. As shown in Figure 3A, 5-HT–induced Nur77 expression was markedly inhibited by the MAPK and MAPK kinase (MEK) 1/2 inhibitors, U0126 and PD98059, the 5-HTT inhibitor, ketanserin, the 5-HT2A receptor antagonist, fluoxetine, and the 5-HT1B/1D receptor antagonist, GR127935, but not by the calcium channel blocker, BAPTA, the phospholipase C inhibitor, U73122, and the phosphoinositide 3-kinase inhibitor, LY294002. Consistent with previous reports, 5-HT transiently induced phosphorylation of MEK1/2 in RPASMCs (Figure 3B), which was markedly inhibited by the MEK1/2 inhibitor, U0126 (Figure 3C). These results suggest that 5-HT–induced Nur77 expression is MEK1/2 dependent, and is mediated by combined action of the serotonin transporter (5-HTT) and the 5-HT 1B/1D and 2A receptors.

CREB Is Involved in 5-HT–Induced Nur77 Expression in PASMCs

The transcription factor CREB has been previously shown to regulate Nur77 expression in various cell types (28, 29). To investigate whether CREB is involved in 5-HT–induced Nur77 expression in RPASMCs, we first examined CREB phosphorylation in response to 5-HT stimulation. As shown in Figure 4A, 5-HT treatment rapidly induced the phosphorylation of CREB in a time-dependent manner, with a maximal effect seen at 5 minutes Inhibition of MEK1/2 by U0126 markedly attenuated 5-HT–induced

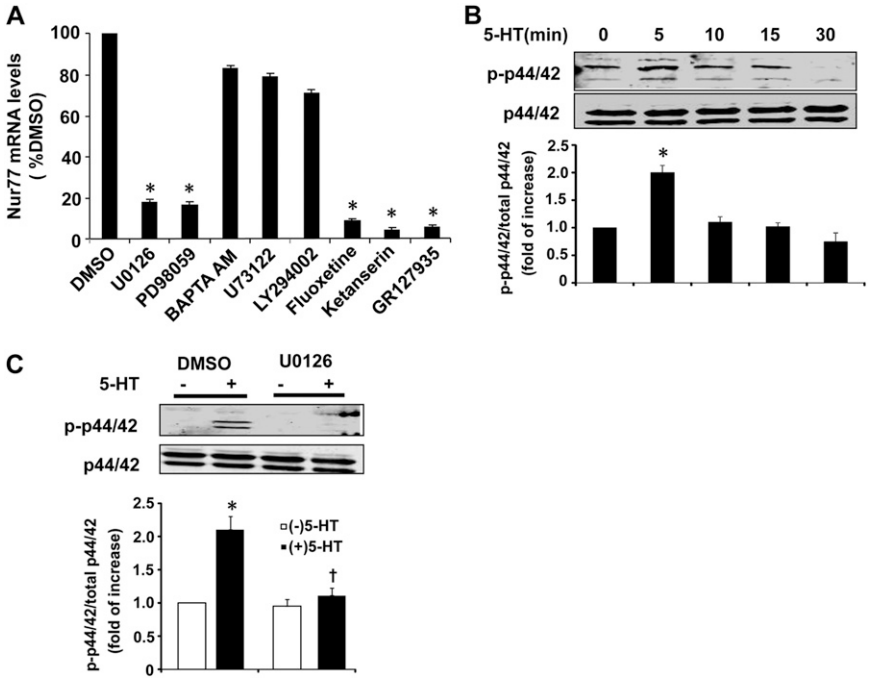

Figure 3. 5-HT induces Nur77 expression through MAPK kinase (MEK) 1/2 pathway in RPASMCs. (*A*) RPASMCs were pretreated with vehicle, the MEK inhibitor, U0126 (20 μmol/L), or PD98059 (50 μmol/L), the calcium channel blocker, BAPTA AM (10 μmol/L), the phospholipase C (PLC) inhibitor, U73122 (1 μmol/L), the phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 (1 μmol/L), the 5-HT transporter (5-HTT) inhibitor, ketanserin (10 μmol/L), the 5-HT2A receptor antagonist, fluoxetine (5 μmol/L), and the 5-HT1B/1D receptor antagonist, GR127935 (5 μmol/L), for 1 hour, and then stimulated with 5-HT (1 μmol/L) for 1 hour. The expression of Nur77 was then measured by qRT-PCR ($n = 4$; $^*P < 0.05$ vs. DMSO plus 5-HT treatment). (*B*) RPASMCs were stimulated with 5-HT (1 μmol/L) at different time points and the levels of phosphorylated p44/42 and p44/42 were determined by Western blot analysis ($n = 4$; $^*P < 0.05$ vs. 5-HT at 0 min). (*C*) PASMCs were pretreated with either vehicle or the MEK1/2 inhibitor, U0126, for 1 hour, and then stimulated with 5-HT (1 μmol/L) for 5 minutes. The levels of phosphorylated p44/42 and p44/42 were then determined by Western blot analysis ($n = 4$; $^*P < 0.05$ vs. dimethyl sulfoxide [DMSO] without 5-HT treatment; $^\dagger P < 0.05$ vs. DMSO plus 5-HT treatment).

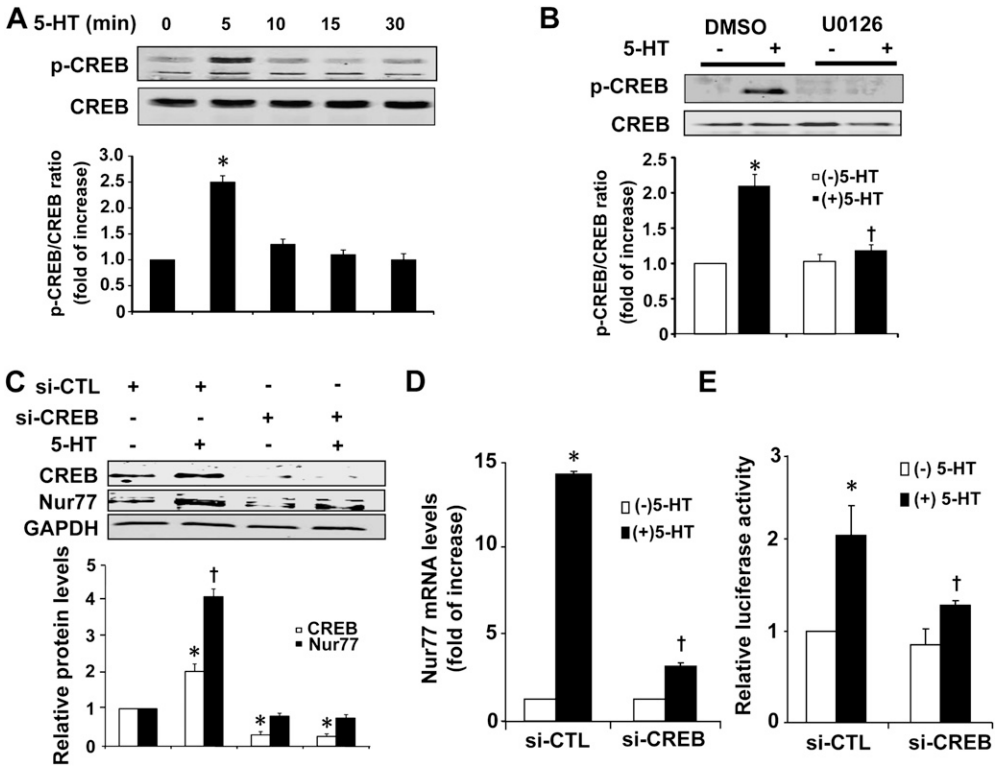

Figure 4. The transcription factor CREB mediates the induction of Nur77 expression by 5-HT in RPASMCs. (*A*) RPASMCs were stimulated with 5-HT (1 mmol/L) at different time points and the levels of phosphorylated CREB and CREB were determined by Western blot analysis. (*B*) RPASMCs were pretreated with the MEK inhibitor U0126 (20 mmol/L) for 1 hour and then stimulated with 5-HT (1 mmol/L) for 5 minutes and the levels of phosphorylated CREB and CREB were determined by Western blot analysis. (*C*) RPASMCs were transfected with either control small interfering RNAs (siRNAs [si-CTL]) or CREB specific siRNAs (si-CREB). At 72 hours after transfection, PASMCs were stimulated with 5-HT (1 mmol/L) for 6 hours and the expression of CREB and Nur77 was measured by Western blot analysis ($n = 4$; $^{*}P < 0.05$ vs. either si-CTL without 5-HT treatment or si-CREB with and without 5-HT treatment; $^{\dagger}P < 0.05$ vs. si-CREB plus 5-HT treatment). (*D*) RPASMCs were transfected with either si-CTL or si-CREB. At 72 hours after transfection, PASMCs were stimulated with 5-HT (1 mmol/L) for 1 hour, and the expression of Nur77 was measured by qRT-PCR ($n = 5$; $^{*}P < 0.05$ vs. control siRNA without 5-HT treatment; $^{\dagger}P < 0.05$ vs. control siRNA plus 5-HT treatment). (*E*) RPASMCs were transfected with either si-CTL or si-CREB. Forty-eight hours after transfection, rat PASMCs were transfected with NBRE-Luc reporter plasmid. Forty-eight hours after transfection with the reporter plasmid, PASMCs were stimulated with 5-HT (1 mmol/L) for 6 hours and the luciferase activity was determined ($n = 5$; $^{*}P < 0.05$ vs. si-CTL without 5-HT treatment; $^{\dagger}P < 0.05$ vs. si-CTL plus 5-HT treatment).

phosphorylation of CREB (Figure 4B), thus indicating that CREB may be a downstream target of the MEK1/2 pathway. To further define the role of CREB in 5-HT–induced Nur77 expression, we performed loss-of-function studies by using CREB-specific siRNAs. As shown in Figure 4C, transfection with CREB siRNAs markedly inhibited the expression of CREB in PASMCs, as determined by Western blot. Accordingly, the 5-HT–induced Nur77 expression was markedly attenuated in CREB siRNA transfected cells, as measured by both Western blot (Figure 4C) and qRT-PCR analysis (Figure 4D). In addition, 5-HT–induced Nur77 transcriptional activity was attenuated by knockdown of CREB expression in RPASMCs (Figure 4E). Taken together, our findings suggest that the transcription factor, CREB, is mainly responsible for the 5-HT–induced Nur77 expression in PASMCs.

Nur77 Is a Negative Regulator of PASMC Proliferation

To study the functional significance of Nur77 in PASMC function, we transduced RPASMCs with adenoviruses expressing Nur77 (Ad-Nur77) or LacZ (Ad-LacZ). As shown in Figure 5A, transduction of PASMCs with Ad-Nur77 resulted in a roughly sixfold increase in Nur77 levels. Increased Nur77 expression predominantly occurred in the nucleus of PASMCs, as determined by immunofluorescent staining (Figure 5B). Importantly, Nur77 overexpression markedly attenuated both 5-HT– and PDGF-BB–induced PASMC proliferation, as determined by MTT assays (Figure 5C). The effect of Nur77 on PASMC proliferation was further confirmed by the expression of a well known cell proliferation marker, proliferating cell nuclear antigen. As shown in Figure 5D, 5-HT stimulation significantly increased the expression of proliferating cell nuclear antigen, which was substantially attenuated in Ad-Nur77 transduced cells. Cyclin D1 is a critical regulator for VSMC migration and proliferation (18). Western blot analysis of cyclin D1 protein levels showed that overexpression of Nur77 decreased both basal and 5-HT–induced cyclin D1 expression in RPASMCs (Figure 5D), which is consistent with the effect of Nur77 on PASMC proliferation.

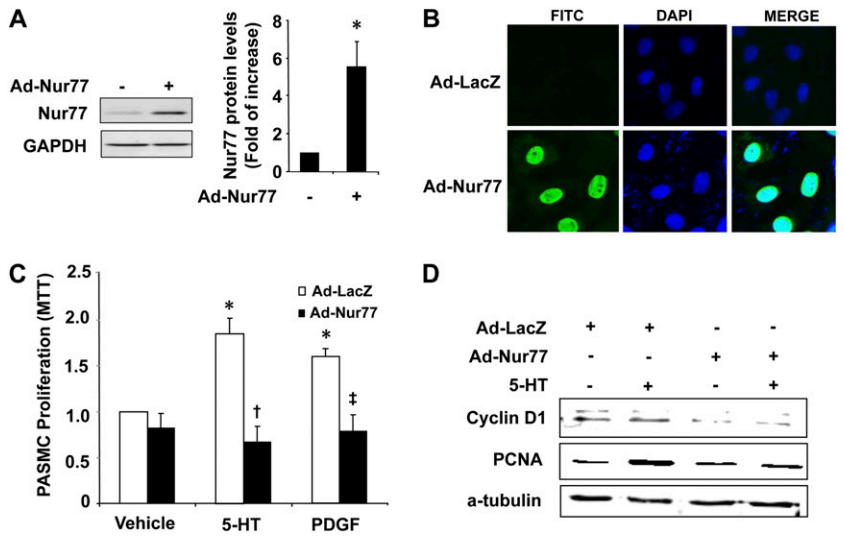

Figure 5. Nur77 suppresses PASMC proliferation. (*A*) Nur77 expression was determined by Western blot analysis after transduction with recombinant Nur77-specific adenoviruses (Ad-Nur77; multiplicity of infection (MOI) = 100) or Ad-LacZ (MOI = 100) in PAMSCs for 48 hours ($n = 4$; $^{*}P < 0.05$ vs. Ad-LacZ). (*B*) Immunofluorescent staining showing the expression of Nur77 in the nucleus of PASMCs. (*C*) At 48 hours after transduction with either Ad-LacZ or Ad-Nur77, SMCs were stimulated with 5-HT (1 μmol/L) or PDGF-BB (20 ng/ml) for 48 hours. Ad-Nur77 significantly inhibited PASMC proliferation stimulated by 5-HT and PDGF-BB (20 ng/ml), as determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays ($n = 5$; $^{*}P < 0.05$ vs. Ad-LacZ plus vehicle; $^{\dagger}P < 0.05$ vs. Ad-LacZ plus 5-HT treatment; $^{\ddagger}P < 0.05$ vs. Ad-LacZ plus PDGF treatment). (*D*) At 48 hours after transfection with either Ad-LacZ (MOI = 100) or Ad-Nur77 (MOI = 100), SMCs were stimulated with or without 5-HT (1 μmol/L) for 48 hours The expression of cyclin D1 and proliferating cell nuclear antigen (PCNA) was measured by Western blot analysis; α-tubulin was used as a loading control.

To further evaluate the functional role of endogenous Nur77 in PASMC proliferation, we performed loss-of-function studies using Nur77 siRNAs. Transduction of PASMCs with Nur77 siRNAs markedly inhibited both basal and 5-HT–induced Nur77 expression by roughly 70%, as determined by qRT-PCR (Figure 6A). Furthermore, transfection of PASMCs with Nur77 siRNAs significantly enhanced both 5-HT– and PDGF-BB–induced PASMC proliferation, as determined by MTT assays (Figure 6B). Taken together, these findings further suggest that Nur77 is a negative regulator of PASMC proliferation.

Nur77 Suppresses PASMC Proliferation through Inhibition of the STAT3 Signaling Pathway

The activation of the Src/STAT3 has been reported to play critical roles in PASMC proliferation and the development of pulmonary hypertension (30, 31). Therefore, to investigate the mechanism(s) responsible for the inhibitory effects of Nur77 on 5-HT–induced PASMC proliferation, we first examined whether Nur77 affects STAT3 activation. As shown in Figure 7A, adenovirus-mediated overexpression of Nur77 in RPASMCs markedly inhibited both basal and 5-HT–induced phosphorylation of STAT3 on tyrosine 705, as determined by Western blot, whereas the total protein levels of STAT3 were barely affected, suggesting that Nur77 can inhibit the STAT3 pathway. To further investigate the molecular mechanisms underlying the inhibition of STAT3 phosphorylation by Nur77, we examined whether there was a functional interaction between Nur77 and STAT3. To this end, we performed immunoprecipitation with anti-Flag antibodies using lysates obtained from RPASMCs transduced with recombinant adenovirus-expressing Flag-tagged Nur77. As shown in Figure 7B, STAT3 coprecipitated with the anti-Flag antibody, but not with the nonimmune control antibody, indicating that Nur77 can specifically interact with STAT3 in PAMSCs. Furthermore, we examined whether Nur77 impacted the expression of STAT3 downstream target genes, such as Pim-1, nuclear factor of activated T cells (NFAT) c2, and survivin, all of which have been implicated in PASMC proliferation and the development of pulmonary hypertension (30, 32, 33). Consistent with the 5-HT–induced phosphorylation of STAT3 in RPASMCs, 5-HT stimulation significantly induced the expression levels of Pim-1, NFATc2, and survivin, as determined by qRT-PCR (Figure 7C). Indeed, knockdown of either NFATc2 or STAT3 by specific siRNAs markedly inhibited 5-HT–induced PASMC proliferation (Figure E1). Moreover, overexpression of Nur77 markedly attenuated 5-HT–induced expression of Pim-1, NFATc2, and survivin. To investigate whether Nur77 affects the activation of NFATc2, PASMCs were transduced with adenovirus bearing NFAT-luciferase reporter (Ad-NFAT-Luc). As shown in Figure 7D, 5-HT stimulation significantly induced activation of NFAT, which was markedly inhibited by Nur77 overexpression. Together, these results suggest that Nur77 may suppress PASMC proliferation through an inhibitory interaction with the transcription factor, STAT3.

Discussion

In the present study, we have found that Nur77, an orphan nuclear receptor, has antiproliferative activity in RPASMCs. We demonstrated that, in response to a wide range of mitogenic stimuli, such as hypoxia, PDGF-BB, 5-HT, and endothelin-1, Nur77 expression is markedly up-regulated in RPASMCs. Most importantly, the expression of Nur77 is significantly increased in the lungs of rats with MCT-induced experimental PAH, further suggesting an involvement of Nur77 in the pathogenesis of experimental PAH. Overexpression of Nur77 markedly inhibits both 5-HT– and PDGF-BB–induced PASMC proliferation. Intriguingly, we demonstrated that Nur77 interacts with STAT3, and inhibits the expression of its target genes by suppressing the phosphorylation of STAT3 in RPASMCs. Therefore, we, for the first time, provide compelling evidence suggesting that the orphan nuclear receptor, Nur77, is rapidly induced by proliferative stimuli in PASMCs, and acts as a negative-feedback regulator for limiting the activation of

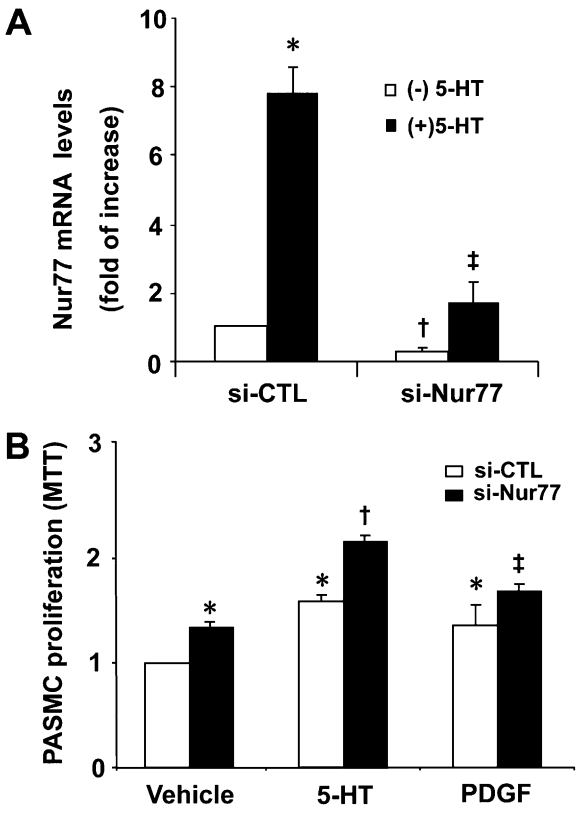

Figure 6. Knockdown of Nur77 enhances 5-HT–induced PASMC proliferation. (*A*) RPASMCs were transfected with either control siRNAs (si-CTL) or Nur77-specific siRNAs (si-Nur77). At 72 hours after transfection, PASMCs were stimulated with 5-HT (1 μmol/L) for 1 hour, and the expression of Nur77 was measured by qRT-PCR ($n = 5$; $^{*}P < 0.05$ vs. control siRNA without 5-HT treatment; $^{\dagger}P < 0.05$ vs. with si-CTL without 5-HT treatment; $^{\ddagger}P < 0.05$ vs. si-CTL plus 5-HT treatment plus 5-HT treatment). (*B*) RPASMCs were transfected with either control siRNAs (si-CTL) or Nur77-specific siRNAs (si-Nur77). At 48 hours after transfection, PASMCs were stimulated with 5-HT (1 μmol/L) or PDGF-BB (20 ng/ml) for 48 hours. PASMC proliferation stimulated by 5-HT and PDGF-BB was determined by MTT assays ($n = 5$; $^{*}P < 0.05$ vs. si-CTL plus vehicle; $^{\dagger}P < 0.05$ vs. si-CTL plus 5-HT treatment; $^{\ddagger}P < 0.05$ vs. si-CTL plus PDGF treatment).

STAT3/NFAT pathway. Similar to the negative inhibitors in cardiac hypertrophy, the negative regulators for PASMC proliferation may have two categories. The first group of negative inhibitors, such as SMAD6 and SMAD7 (34), is constitutively expressed or active in unstimulated cells, and their negative effect at baseline is manifested when the activity of the molecule is inhibited. The second group of inhibitors, such as SOCS3 (35), is activated and up-regulated only upon stimulation of PASMCs with stimuli. In this regard, Nur77 belongs to the second group of inhibitors for the PASMC proliferation.

Accumulating evidence suggests that NR4A receptors play essential roles in the pathogenesis of cardiovascular diseases, such as atherosclerosis, restenosis, and angiogenesis (17, 21). In human VSMCs, Nur77 expression is significantly increased by multiple mitogenic stimuli, including PDGF-BB, EGF, thrombin, and ox-LDL (22). Overexpression of Nur77 has been shown to inhibit SMC proliferation through a mechanism not fully understood (22, 23). Thus far, the functional role of NR4A receptor in lung biology remains largely obscure, although the members of NR4A family are highly expressed in the lung (25, 26). In the present study, we sought to examine the role of NR4A receptors in PASMC proliferation and the pathogenesis of PAH. We found that, among the three members of the NR4A family, Nur77 is the most abundant one expressed in PASMCs. Because of critical roles of the 5-HT pathway in PAH, we then examined the expression of Nur77 in response to 5-HT stimulation. We demonstrated that 5-HT treatment caused a rapid and robust induction of Nur77 in RPASMCs, in a dose- and time-dependent manner. Inhibition of the MAPK/MEK1/2 pathway by a specific inhibitor (U0126) significantly attenuated the 5-HT–induced Nur77 expression, demonstrating a direct link between MEK1/2 activation and 5-HT–induced Nur77 expression in PASMCs. Depending on the cell type, several transcription factors, including CREB, AP-1, MEF-2, and NF-κB, have been previously implicated in the expression of NR4A family (36–39). In vascular cells, CREB, however, appears to be a key factor for the induction of NR4A receptor. In accordance with these observations, we demonstrated that 5-HT stimulation led to the rapid phosphorylation of CREB at serine 133, which is a critical marker of activation. Furthermore, knockdown of CREB expression, by specific siRNAs, significantly attenuated the expression of Nur77, as induced by 5-HT in RPASMCs. Furthermore, we found that 5-HT–induced CREB phosphorylation was blocked by the MEK1/2–specific inhibitor, U0126 (data not shown), further suggesting that the MEK1/2–dependent activation of CREB contributes significantly to the up-regulation of Nur77 by 5-HT in RPASMCs.

Mechanistically, our studies provide evidence that Nur77 can inhibit PASMC proliferation, at least in part, through inhibition of the STAT3 transcriptional pathway. The activation of Src/STAT3 pathway in PASMCs has been well established to contribute significantly to the development of pulmonary hypertension (30, 40). Indeed, increased phosphorylation and activation of STAT3 has been documented in PAH, and mediates the expression of several key signaling molecules implicated in PASMC proliferation and survival. For instance, in PASMCs, the activation of STAT3 has been shown to increase both NFATc2 and Pim-1 expression by directly binding to their promoter regions to sustain PASMC proliferation and promote resistance to apoptosis. Pim-1 is a proto-oncogene that is regulated by STAT3, and its expression is significantly increased in PAH-SMCs (30, 41). In addition, Pim-1 can interact with NFATc2 to promote NFATc2 activation (42). Thus, STAT3 activation not only increases NFAT expression, but also promotes NFAT activation by up-regulating Pim-1 expression, hence,

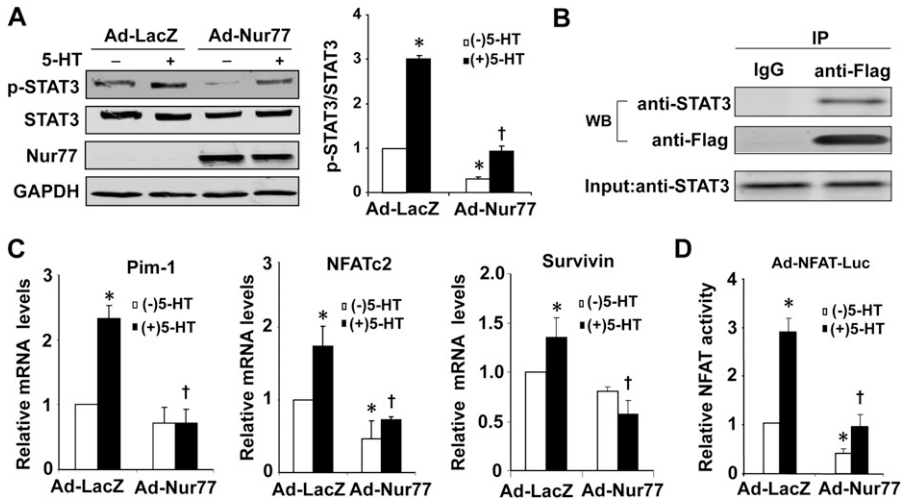

Figure 7. Nur77 inhibits the signal transducer and activator of transcription (STAT) 3/Pim-1/nuclear factor of activated T cells (NFAT) axis. (*A*) At 48 hours after transduction with either Ad-LacZ (MOI = 100) or Ad-Nur77 (MOI = 100), PASMCs were stimulated with 5-HT (1 μmol/L) for 10 minutes. The levels of phosphorylated STAT3, STAT3, and Nur77 were determined by Western blot analysis ($n = 5$; $^{*}P < 0.05$ vs. Ad-LacZ without 5-HT; $^{\dagger}P<0.05$ vs. Ad-LacZ plus 5-HT treatment). (*B*) PASMCs were transduced with Ad-Nur77 (MOI = 100). At 48 hours after transduction, cell lysates were subjected to immunoprecipitation by using either normal IgG or anti-Flag antibody. The immunocomplexes were then separated by 12% SDS-PAGE, and transferred membranes were immunoblotted with either anti-STAT3 or anti-Flag antibody. (*C*) PASMCs were transduced with either Ad-LacZ (MOI = 100) or Ad-Nur77 (MOI = 100). At 48 hours after transduction, PASMCs were stimulated with 5-HT (1 μmol/L) for 24 hours. The expression of Pim-1, NFATc2, and survivin was then determined by qRT-PCR ($n = 5$; $^{*}P < 0.05$ vs. Ad-LacZ without 5-HT treatment; $^{\dagger}P < 0.05$ vs. Ad-LacZ with 5-HT treatment). (*D*) PASMCs were transduced with Ad-NFAT-Luc (MOI = 50) together with either Ad-LacZ or Ad-Nur77 (MOI = 100). At 48 hours after transduction, PASMCs were stimulated with 5-HT (1 μmol/L) for 24 hours and luciferase activity was determined ($n = 5$; $^{*}P < 0.05$ vs. Ad-LacZ without 5-HT treatment; $^{\dagger}P < 0.05$ vs. Ad-LacZ with 5-HT treatment).

highlighting the critical importance of the STAT3 pathway in the pathogenesis of pulmonary hypertension. Depending on the stimuli and its cellular localization, Nur77 can exert different or even opposite effects through both genomic and nongenomic effects. For instance, in response to certain apoptosis-inducing agents, Nur77 expression is induced in some cancer cells, and subsequently translocates from the nucleus to mitochondria, where it causes the conformational change of Bcl-2 to promote apoptosis (43, 44). While in the nucleus, Nur77 can function as a transcription factor by binding to its DNA response elements on target genes to promote cancer cell growth and survival (45, 46). Our data suggest that neither a proapoptotic effect nor a genomic action of Nur77 is likely involved in the Nur77-mediated inhibition on PASMC proliferation, as overexpression of Nur77 is predominantly localized in the nucleus of PASMCs, and 5-HT did not cause translocation of Nur77 to the mitochondria (data not shown). Importantly, we found that Nur77 specifically interacts with STAT3 in the nucleus of RPASMCs, which leads to a marked attenuation of STAT3 phosphorylation and inhibition of its transcriptional activity. Accordingly, 5-HT–induced expression of STAT3 target genes, such as NFATc2, Pim-1, and survivin, which have been implicated in pulmonary vascular remodeling, was also substantially inhibited. In this regard, our study provides significant novel insights into the molecular mechanisms underlying the inhibitory effects of Nur77 on PASMC proliferation, presumably through a nongenomic action.

Overall, the data reported herein provide the evidence that the orphan nuclear receptor, Nur77, is a novel negative regulator of PASMC proliferation in response to 5-HT stimulation via inhibition of the STAT3/Pim-1/NFAT axis. Aberrant activation of the 5-HT pathway has been shown to contribute significantly to the development and progression of PAH in patients (47). Understanding the molecular mechanisms, particularly identifying the negative regulators governing pulmonary vascular remodeling, will be of critical importance for a better treatment of patients with PAH. Future studies, using genetically modified mouse models of Nur77, are necessary to further substantiate the *in vivo* pathophysiological significance of Nur77 in the development of experimental PAH. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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