Noggin Inhibits Hypoxia-induced Proliferation by Targeting Store-operated Calcium Entry and Transient Receptor Potential Cation Channels

Kai Yang\textsuperscript{1, 2}, Jing Jia\textsuperscript{1}, Jie Zhang\textsuperscript{1}, Mingming Zhao\textsuperscript{3}, Sabrina Wang\textsuperscript{2}, Haiyang Jiang\textsuperscript{2}, Lei Xu\textsuperscript{1, 2}, Wenju Lu\textsuperscript{1} and Jian Wang\textsuperscript{1, 2}

\textsuperscript{1}Guangzhou Institute of Respiratory Disease, State Key Laboratory of Respiratory Diseases, The 1st Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, 510120; \textsuperscript{2}Division of Pulmonary & Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States, 21224; \textsuperscript{3}Laboratory of Molecular Biology and Immunology, National Institute on Aging, Baltimore, Maryland, United States, 21224.

Corresponding Authors:

Jian Wang, MD or Wenju Lu, MD
Guangzhou Institute of Respiratory Diseases
State Key Laboratory of Respiratory Diseases
The First Affiliated Hospital
Guangzhou Medical University
151 Yanjiang Road, Guangzhou, Guangdong, 510120
People’s Republic of China
E-mail: jwang31@jhmi.edu or wlu92@yahoo.com

Or

Jian Wang, MD
Division of Pulmonary and Critical Care Medicine
Johns Hopkins University School of Medicine
5501 Hopkins Bayview Circle, Rm. 4B30
Baltimore, MD 21224
Email: jwang31@jhmi.edu

Running title: Noggin inhibits TRPC-SOCE signaling in PASMC
ABSTRACT

Abnormally elevated bone morphogenetic protein 4 (BMP4) expression and mediated signaling play critical role in the pathogenesis of chronic hypoxia-induced pulmonary hypertension (CHPH). In this study, we investigated the expression level and functional significance of four reported natural existed BMP4 antagonists: noggin, follistatin, gremlin1 and matrix gla protein (MGP), in the lung and distal pulmonary arterial smooth muscle cell (PASMC). A 21-day chronic hypoxic (10% O2) exposure rat model was utilized, which has been previously shown to successfully establish experimental CHPH. Among the four antagonists, noggin, but not the other three, was selectively down-regulated by hypoxic exposure in both the lung tissue and PASMC, in correlation with markedly elevated BMP4 expression, suggesting the loss of noggin might account for the hypoxia-triggered BMP4 signaling transduction. Then, by using treatment of extrogenous recombinant noggin protein, we further found that noggin significantly normalized 1) BMP4-induced phosphorylation of cellular p38 and ERK1/2; 2) BMP4-induced phosphorylation of cellular JAK2 and STAT3; 3) hypoxia-induced PASMC proliferation; 4) hypoxia-induced store-operated calcium entry (SOCE) and 5) hypoxia-increased expression of transient receptor potential cation channels (TRPC1 and TRPC6) in PASMC. Combinedly, these data strongly indicated that the hypoxia-suppressed noggin accounts, at least partially, for the excessive PASMC proliferation, while restoration of noggin may be an effective way to inhibit cell proliferation by suppressing SOCE and TRPC expression.

KEY WORDS: noggin, TRPC, store-operated calcium entry, pulmonary hypertension
INTRODUCTION

Pulmonary hypertension (PH) is a disease characterized by a list of functional and structural changes in the pulmonary vasculature that leads to enhanced distal PA contraction and remodeling, eventually causes heart failure. During the disease process, remodeling of small vessels in the lung, due to abnormal proliferation and migration of vascular smooth muscle cell and endothelium cell is well studied and accepted (10).

Bone morphogenetic proteins (BMPs) belong to a subgroup of the transforming growth factor β (TGF-β) superfamily, which are a group of growth factors originally discovered by their ability to induce the formation of bone and cartilage. Recently, evidence strongly indicated that dysregulated BMP signaling is involved in the pathogenesis of PH (9, 11). BMP4, a member of BMP ligands, has been found selectively up-regulated by chronic hypoxia in the lungs and plays important role during the development of chronic hypoxia-induced pulmonary hypertension (CHPH) by regulating the proliferation, migration of the pulmonary arterial smooth muscle cell (PASMC) (11, 13, 20). In mechanisms, BMP4-mediated signaling transduction is mainly regulated by two groups of molecules, the typical receptors and the extracellular soluble antagonists (3). BMP4 transduces signals by binding to type II serine–threonine kinase receptors (44), which then causes recruitment and phosphorylation of type I receptors, leading to activation of a number of cellular kinases (18).

The group of BMP antagonists belongs to natural secreted endogenous proteins that can block the BMP ligand-receptor interaction to inhibit the BMP signaling transduction. Increasing evidences demonstrated that dysfunction of these proteins leads to excessive BMP activity and signaling transduction, which is present and may account for the development of numerous diseases. In detail, gremlin1 specifically binds to BMP2, 4 and 7, and inhibits their actions on the downstream signaling (14, 23). Evidence suggests that gremlin1 is up-regulated in lungs isolated from 2-day alveolar hypoxia exposed mice (6). The increased gremlin1 expression mostly localizes in the pulmonary endothelium, but not smooth muscle (2). Deletion of gremlin1 increases cell
proliferation and migration responses in mouse embryonic fibroblasts (7). Noggin, follistatin and matrix gla protein (MGP) are all demonstrated as BMP4 antagonists, which are found to co-express with BMP4 at sites of oscillatory shear stress in the systemic vasculature (4). Noggin has long been known as a classic BMP antagonist with a high affinity binding to BMP4 (25, 26, 49). Traditionally defined as an antagonist of activin protein, follistatin could also interact with BMPs (including BMP4, 5, 6, 7 and 15), though in a lower affinity range (5, 12, 32, 35). MGP is an extracellular matrix component expressing high abundance in vascular smooth muscle cells. MGP has been defined to contribute to the development of vasculature by using MGP-deficient mice (45). MGP inhibits or activates BMPs (BMP2 and BMP4) in a concentration dependent manner (46, 48). So far, many groups have discussed the effects of BMPs antagonists on interfering BMPs signaling and participating in the development of different diseases. However, the full action of these members in CHPH remains largely unknown. We previously demonstrated that animals exposed to chronic hypoxia (CH, 10% O2) for 21-day was used as an animal model for mimicking CHPH (21, 40). Thus, in this study, we aim to figure out the changes of these antagonists upon hypoxic stresses and determine their roles on hypoxia-induced proliferation in PASMCs.

MATERIALS AND METHODS

Chronic hypoxic exposure of rats and primary culture of PASMC

All the animal experiment procedures were approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine. The surgical procedure was performed under anesthesia with sodium pentobarbital (65 mg/kg i.p.), and all efforts were made to minimize animal suffering. Adult male Wistar rats (150-250 g) were purchased from Harlan (Frederick, MD). Rats were randomly divided into two groups and exposed to normoxia or chronic hypoxia (10% O2) for 21-day as previously described (34, 40). The hypoxic chamber was continuously flushed with a mixture of room air and N2 to maintain 10 ± 0.5 % O2 and CO2 <0.5 %. Chamber O2 concentration was continuously monitored using a PRO-OX unit (RCI Hudson, Anaheim, CA). Rats were
exposed to room air for 10 minutes every day for administering food and water, or changing cage. By the end of the exposure, rats from all groups were anesthetized with pentobarbital sodium (65 mg/kg i.p.) and sacrificed for harvesting lung tissues. The blood in the vessel was gently completely washed out with PBS to eliminate the interference of the circulation. The primary culture of distal PASMC was based on an enzymatic digestion method, as previously described (39). Basically, distal (>4th generation) intralobar distal pulmonary arteries (PAs) were dissected from the lungs. Adventitia was removed from the isolated PAs, and endothelium was denuded by opening the vessel longitudinally and rubbing the luminal surface with a cotton swab.

**Real-time quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted using TRIzol method for de-endothelialized distal PA, as previously described (21). DNA contamination in RNA preparations was removed by on-column DNase digestion using RNeasy column and RNase-free DNase (Qiagen, Valencia, CA). Reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) with reaction mixture containing 1 µg total RNA in a 20 µl volume. cDNA were amplified by RT-qPCR using QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) in an iCyclerIQ detection system (Bio-Rad, Hercules, CA). The protocol consisted of initial enzyme activation at 95°C for 3 min, followed by 40 cycles at 95°C for 5 s and at 60°C for 15 sec (22). Primer sequences are designed using Primer3 software and listed in Table 1. Relative concentration of each transcript was calculated using the Pfaffl method (33). Efficiency for each gene was determined from 5-point serial dilutions of an unknown cDNA sample (PA or PASMC). The expression of BMP antagonists were normalized to cyclophilin B as internal control.

**Western Blotting**

Lung tissue samples were sonicated or homogenized in T-PER sample buffer (Pierce, Rockford, IL) containing protease inhibitor cocktail. Total protein concentration in the homogenates
was determined by bicinehoninic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. Homogenates were denatured by adding dithiothreitol to 150 mM and heating at 95 °C for 3 min. Homogenate proteins were resolved by 10% SDS-PAGE calibrated with Precision Plus protein molecular weight markers (Bio-Rad, Hercules, CA). Separated proteins were transferred to 0.45 μM polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.2% Tween 20, and blotted with affinity-purified polyclonal antibodies specific for TRPC1 and TRPC6 (Sigma, St. Louis, Mo), MGP (Proteintech, Chicago, IL), noggin (BD Biosciences, San Jose, CA), follistatin (Abcam, Cambridge, MA), and gremlin1 (Abcam, Cambridge, MA), p-p38, t-p38, p-ERK1/2, t-ERK1/2, p-JAK2, p-STAT3 (Cell Signaling Technology, Beverly, MA) or monoclonal antibody against beta-Tubulin and beta-Actin (Sigma, St. Louis, Mo). The membranes were then washed for 10 minutes 3 times and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 1 hour. Bound antibodies were detected using an enhanced chemiluminescence system (ECL, GE healthcare, Piscataway, NJ).

Measurement of store-operated calcium entry (SOCE)

Following previously described procedures (39), cultured PASMC were seeded on coverslips, underwent 24-hour quenceent in smooth muscle basal medium (SMBM) containing 0.3% FBS and 1-hour loading with 7.5 μM fluorescent dye fura-2 AM (Molecular Probes, Eugene, OR) before calcium measurement experiments. Intracellular Ca$$^{2+}$$ concentration was calculated by the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F340/F380). The SOCE was evaluated by extracellular calcium restoration method, in which, intracellular SR calcium stores within PASMC were depleted by perfusion of 10 μM cyclopiazonic acid (CPA), in the presence of 1 mM EGTA and 5 μM voltage-dependent calcium channel (VDCC) blocker nifedipine to exclude the influence of VDCC. By restoring extracellular calcium could we measure the SOCE through store-operated calcium channel (SOCC).
Measurement of PASMC proliferation

The proliferation of PASMC was measured by the Cell Proliferation Biotrak ELISA Kit (GE Healthcare) based on a BrdU incorporation assay, as previously described (41). Briefly, PASMC were seeded in 96 well plates in SMBM at a density of $5 \times 10^3$ cell/well. After each different treatment, the cells were then labeled with BrdU for 24-hour, fixed, blocked, antibody probed, developed with TMB substrate, and stopped with sulphuric acid, following the kit instruction. The optical density of wells was measured using a microplate reader (Bio-Rad) at 450 nm.

Materials and reagents

Unless otherwise specified, all the reagents were obtained from Sigma-Aldrich. Fura-2 AM (Invitrogen) was prepared before the experiment as a 2.5 mM stock solution in 20% DMSO containing 20% pluronic F-127 (Invitrogen). Stock solutions of nifedipine and CPA were both made in DMSO at 30 mM. Human recombinant BMP4 protein was purchased from R&D Inc. and noggin recombinant protein was purchased from Invitrogen.

Statistical analysis

All the data are represented as Mean ± SEM. N is the number of experiments, which equals the number of animals providing PA or lung; number of dishes of cell for the in vitro molecular biological experiments and the intracellular calcium imaging experiments. Statistical analyses were performed using analysis of Students $t$-test and one-way ANOVA. Pairwise comparison of means was conducted with $t$-tests. For the groups containing multiple comparisons, one-way ANOVA was used for the statistical analysis. Differences were considered significant when $P < 0.05$.

RESULTS
Chronic hypoxic exposure (10% O2, 21-day) selectively down-regulates noggin expression in rat lung tissues

Firstly, we focused on the mRNA and protein expression of the four BMP antagonists noggin, follistatin, gremlin1 and MGP in the lungs isolated from both normoxic and CH-exposed rats. We designed specific primers for all of these members for Real-time PCR amplify (Table 1). By using real-time qPCR, we found that all of the four BMP antagonists were expressed in rat lung tissue and among which, noggin was selectively down-regulated for 41.9% by hypoxic exposure (Fig. 1A). Then, by using western blotting, we found that the noggin protein level was also significantly down-regulated for 45.7% (Fig. 1B-C) by CH exposure, in comparison with normoxic control. However, the other three antagonists remained unaltered by hypoxic stress in the lung.

Prolonged hypoxic exposure (4% O2, 60-hour) selectively down-regulates noggin expression in rat distal PASMC

Since previous studies strongly indicated that the excessive proliferation and migration of distal PASMC act as two major events to contribute distal PA remodeling and thickening during PH pathogenesis, we further explored if these antagonists express and also be regulated by hypoxia in cultured PASMC. Primary cultured rat distal PASMC were serum starved and exposed to prolonged hypoxia (4% O2) for up to 60-hour, the period of which has been proved to induce elevation of intracellular calcium concentration and cell proliferation (40). We used real-time PCR measuring the mRNA transcription levels and western blotting measuring the protein levels of the four BMP antagonists in PASMC upon prolonged hypoxic exposure. The RT-qPCR data showed no obvious change in the mRNA levels of all these four antagonists under different time points of hypoxic exposure (Fig. 2A-D). However, the protein level of noggin, but not the other three, was selectively reduced at the 48 and 60-hour hypoxic exposure time points, accompanied with up-regulated BMP4 protein levels (Fig. 2E-F). These results indicated the possibility that the hypoxia-suppressed noggin, acting in correlation with the hypoxia-induced BMP4, might account for the uncontrolled
proliferation and elevated intracellular calcium concentration and SOCE in PASMC.

**Treatment of exogenous recombinant noggin inhibits BMP4-induced phosphorylation of p38 and ERK1/2 in PASMC**

Our previous study indicated that among the substantial cellular signaling pathways that can be induced by BMP4, the p38 and ERK1/2 were considered to be essential for the induction of downstream SOCE process and the expression of SOCC components TRPC1 and TRPC6 (18). Thus, we applied treatment of exogenous recombinant noggin protein and detected if noggin can block BMP4-induced p38 and ERK1/2 activation. Our results showed that 200 ng/ml of noggin treatment totally blocked BMP4-induced phosphorylation of p38 and ERK1/2 (Fig. 3A-B).

**Treatment of exogenous recombinant noggin inhibits BMP4-induced phosphorylation of JAK2 and STAT3 in PASMC**

As we know, p38 and ERK are not the only kinases to initial and mediate the cellular pro-proliferation subsequences. Actually, a variety of previous publications have reported that the JAK2/STAT3 signaling axis can mediate smooth muscle cell proliferation upon activation from upstream stimulation (1, 24, 36-38). Therefore, we firstly found that BMP4 treatment (50 ng/ml) can activate the JAK2/STAT3 signaling cascade in a time dependent manner (Fig. 4A-B), then, a pre-treatment with noggin (200 ng/ml) for 30 min significantly attenuate the BMP4-induced phosphorylation of JAK2/STAT3 (Fig. 4C-D).

**Treatment of exogenous recombinant noggin inhibits hypoxia-elevated proliferation of PASMC**

Since that at the dosage of 200 ng/ml of noggin treatment totally blocks BMP4-induced p38 and ERK1/2 activation, we further determined the effect of 200 ng/ml noggin on the proliferation in PASMC under both normoxic and prolonged hypoxic conditions. Our results showed that firstly,
hypoxia markedly increased the proliferation rate for 41.6% comparing to normoxic control. Then, 200 ng/ml noggin significantly normalized hypoxia-elevated proliferation to basal level, without altering the proliferation under normoxia (Fig. 5).

Noggin attenuates hypoxia-elevated SOCE in PASMC

Furthermore, we also detected the role of noggin treatment on the SOCE and intracellular calcium regulation in PASMC, since hypoxia-enhanced SOCE is considered a key regulator to promote PASMC proliferation and PA remodeling (40). Our results indicated that prolonged hypoxia significantly enhanced SOCE from 295.9 nM to 372.7 nM, while noggin dramatically normalized hypoxia-enhanced SOCE to 275.7 nM, without markedly altering the SOCE under normoxia (Fig. 6).

Noggin normalizes hypoxia-induced up-regulation of TRPC1 and TRPC6 in PASMC

Given the fact that noggin significantly normalized hypoxia-induced cell proliferation and SOCE in PASMC, we therefore investigated the role of noggin on the expression of the main SOCC components TRPC1 and TRPC6, which mediate SOCE. As seen in Fig. 7, noggin treatment also attenuates the up-regulated TRPC1 and TRPC6 protein expression under hypoxic condition, but not under normoxia.

DISCUSSION

Previous evidences strongly indicated the essential contribution of increased BMP4 expression and elevated BMP4-mediated signaling transduction in CHPH pathogenesis. In this study, we investigated the expression and role of a group of four reported endogenous BMP4 antagonists in response to hypoxic stress in rat lung and distal PASMC. Our results indicated that among these four antagonists, noggin was selectively down-regulated by hypoxic exposure in both lung and PASMC, in correlation with hypoxia-elevated BMP4 expression and activity. Based on the
previous data, we hypothesized that hypoxia-suppressed noggin lacks the ability as an antagonist to inhibit the hypoxia-evoked BMP4 activity, leading to promoted BMP4 signaling transduction, which results in the transcription of numerous proliferative genes that triggers the cellular pro-proliferative subsequences. We further observed that exogenous noggin treatment significantly normalized hypoxia-triggered proliferation, SOCE and TRPC expression, suggesting that suppressed noggin contributes to the elevated BMP4 signaling and activity under hypoxia.

BMPs belong to the TGF-β superfamily, which are a group of factors originally found to induce ectopic bone formation when implanted subcutaneously (42). Then, considerable evidences demonstrated that BMPs also act key multifunctional regulators in regulating cell proliferation, differentiation, and apoptosis in different tissues (27). BMPs exert their signals via binding with type II transmembrane serine/threonine kinase receptors, thus recruit with type 1 transmembrane serine/threonine kinase receptors, then activates the intracellular Smad-dependent and Smad-independent (e.g., ERK, JNK, and p38 MAP kinase pathways) signaling pathway in regulating substantial downstream gene expression (43). Basically, three type II receptors (BMPRII, ActRIIa and ActRIIb) and three type I receptors (ALK2, ALK3 and ALK6) have already been indicated to be able to interfere with the BMP ligands and transduce signaling (27, 28). Recently, a number of studies suggest that the abnormal BMP signaling is participating in the disease pathogenesis of PH. Many works have been done based on the finding of large chances of BMPRII mutation in the idiopathic PAH (IPAH) and familial PAH (FPAH) patients (8, 17).

Besides the specific BMP receptors, there are also several extracellular modulators that can interfere the BMPs ligand-receptor binding to block the BMPs signaling. Noggin, MGP, gremlin1 and follistatin belong to such members. These soluble antagonists dampen the BMPs signaling either by competitively interacting with the BMP ligands and protect them from binding with their specific receptors; or by blocking the intracellular signal transduction without affecting ligand-receptor reaction (3, 15, 49). Previous publications addressed the different localization of these antagonists in the lung. Gremlin1 is identified in the endothelium, proximal airway epithelium and
alveolar epithelium (19, 29). Follistatin is widely expressed in bronchial epithelial cells, alveolar
macrophages and vascular smooth muscle cells (47). MGP is excessively and broadly expressed in
the lung tissue (48). As it is well accepted that dysfunction of BMP4 signaling participates in and
contributes to the excessive proliferation of PASMC in PH, we wander the triggered BMP4
signaling is possibly, to some extent, due to the loss of expression and function of the antagonists.
Hence, we investigated the expression changes of these antagonists in response to hypoxic
exposure in both the lung and PASMC. Our results showed that among these four antagonists,
noggin was selectively down-regulated by hypoxia in both the lung and PASMC. However, by
applying the extrogenous noggin recombinant protein treatment, we further demonstrated that under
hypoxia, the restoration of noggin effectively normalized hypoxia-induced proliferation, SOCE and
TRPC expression. It is important to compare the blockage efficiency between noggin treatment and
direct BMP4 inhibition strategy (BMP4 knockdown). Actually, in one of our previous publication
(20), we reported that knockdown of BMP4 by using specific siRNA effectively leads to dramatic
decrease in the hypoxia-elevated TRPC-SOCE-[Ca^{2+}]_{i} signaling axis. Given the fact that the
intracellular free calcium concentration acts a major factor to act a second messenger, enter the
nucleus, facilitate the transcription of a number of pro-proliferative genes and promote cell
proliferation (16, 30, 31), we can conclude that in PASMC, increasing direct evidences all suggest
BMP4 is responsible for the proliferation and intracellular calcium homeostasis, especially the
SOCE process. Moreover in this study, we further showed that noggin exerts similar inhibitory
roles, in compare with direct BMP4 knock down, on the TRPC-SOCE-proliferation signaling axis.
Therefore, our data suggest that a decrease in the naturally endogenous expressing BMP4 antagonist
noggin is potentially a main reason accounting for the elevated BMP4 signaling pathway. The
hypoxia-inhibited noggin accounts for, at least partly, the hypoxia-triggered BMP4 activity and
signaling transduction. Due to the lack of noggin’s protective role, evoked BMP4 signaling leads to
up-regulated TRPC-SOCE axis, which eventually results in excessive PASMC proliferation in PH
development. While specific noggin-targeted restoration might act a potential novel strategy to
modulate BMP4 expression and activity, which thus mediate an attenuation of hypoxia-elevated proliferation in PASMC. Moreover, we also found that beside the p38/ERK-TRPC-SOCE-[Ca\textsuperscript{2+}]; signaling axis, BMP4 can also induce PASMC proliferation by inducing activation of JAK2/STAT3 signaling cascade. Our results strongly indicated that noggin can effectively attenuate BMP4-mediated proliferation by inhibiting both p38/ERK-TRPC-SOCE targeted calcium dependent signaling axis and JAK2/STAT3 targeted calcium independent signaling axis.

In summary, this study presents initial evidence for the expression pattern of the four BMPs antagonists in response to hypoxic stress. We observed that the hypoxia-inhibited noggin level acts a key element to result in the excessive PASMC proliferation due to the uncontrolled elevated BMP4 signaling, as well as the downstream triggered p38/ERK-mediated calcium dependent and JAK2/STAT3-mediated calcium independent pro-proliferative signaling pathways, while strategies targeting noggin restoration might be a useful way to attenuate the hypoxia-elevated proliferation of PASMC. This study provides evidence of a novel potential target noggin, which can potentially attenuate the hypoxia-elevated proliferation of PASMC and deserves more researches in the future study.
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CONTRIBUTORS

W. Lu and J. Wang initiated and designed the project, analyzed data, and edited the paper; K. Yang performed the animal, functional and molecular experiments, initialed the writing of the paper; L. Xu and H. Jiang contributed to the animal experiments; J. Jia and J. Zhang contributed to the molecular experiments; M. Zhao and S. Wang contributed to the editing of the manuscript.
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).


Sidis Y, Mukherjee A, Keutmann H, Delbaere A, Sadatsuki M, and Schneyer A. Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin, and bone morphogenetic proteins.


FIGURE LEGENDS

Figure 1: Chronic hypoxia (10% O₂, 21-day) down-regulates noggin expression at both mRNA and protein levels in the lung tissue. A: bar graph represents mRNA fold induction of noggin, follistatin, gremlin1 and MGP in the lungs isolated from 21-day chronic hypoxic exposed rats normalized to the levels from normoxic control rats. Cyclophilin B (CpB) served as housekeeping internal control. B and C: western blots (B) and bar graph (C) showing the protein expression levels of the four BMP antagonists in rat lung tissues under normoxic and hypoxic conditions. Beta-actin serves as housekeeping protein. The bar graph represents the Mean ± SEM, n = 6-12 in each group; *P <0.05 vs. normoxic control.

Figure 2: Prolonged hypoxia (4% O₂) inhibits noggin protein expression in rat distal pulmonary arterial smooth muscle cell (PASMC). A-D: bar graph represents mRNA fold induction of noggin, follistatin, gremlin1 and MGP in rat distal PASMC underwent hypoxic exposure for 6-60 hours, as relevant to CpB. E and F: western blots (E) and line chart (F) showing the protein expression levels of the four BMP antagonists and BMP4 in cultured rat distal PASMC under different time points of hypoxic exposure. The bar graph represents the Mean ± SEM, n = 3-6 in each group; *P <0.05 vs. normoxic control (BMP4 line); #P <0.05 vs. normoxic control (noggin line).

Figure 3: Noggin blocks BMP4-induced p38 and ERK1/2 phosphorylation in PASMC. A: western blots (A) showing the protein expression levels of p-p38, t-p38, p-ERK1/2, t-ERK1/2 and beta-tubulin in cultured rat distal PASMC upon BMP4 (50 ng/ml) for 15-min after pre-treatment with noggin (50 and 200 ng/ml) for 30-min. B: bar graphs representing the phosphorylation rates of p-p38 (left) and p-ERK1/2 (right) as normalized to t-p38 and t-ERK1/2. The bar graph represents
Figure 4: Noggin attenuates BMP4-induced JAK2 and STAT3 phosphorylation in PASMC. A-B: BMP4 time dependently induces JAK2 and STAT3 phosphorylation in PASMC. Western blots (A) showing the protein expression levels of p-JAK2, p-STAT3 and beta-tubulin in cultured rat distal PASMC upon BMP4 (50 ng/ml) treatments for 5-120 minutes. B: bar graphs representing the phosphorylation rates of p-JAK2 (left) and p-STAT3 (right) as normalized to beta-tubulin. C-D: Pre-treatment of noggin (200 ng/ml) for 30-min attenuates BMP4 (50 ng/ml, 30-min)-induced phosphorylation of JAK2/STAT3 in PASMC. Western blots (C) showing the protein expression levels of p-JAK2, p-STAT3 and beta-tubulin in cultured rat distal PASMC upon BMP4 and noggin treatments. D: bar graphs representing the phosphorylation rates of p-JAK2 (left) and p-STAT3 (right) as normalized to beta-tubulin. The bar graph represents the Mean ± SEM, n = 3-5 in each group; *P <0.05 vs. control; #P <0.05 vs. BMP4 (50 ng/ml) treated group.

Figure 5: Noggin normalized hypoxia-elevated proliferation in PASMC. Bar graph representing the fold induction of the PASMC proliferation rate in response to prolonged hypoxia (4% O2, 60-hour) exposure with or without noggin (200 ng/ml) treatment, as normalized to normoxic control value. The bar graph represents the Mean ± SEM, n = 4 in each group; *P <0.05 vs. normoxic control; #P <0.05 vs. hypoxic control.

Figure 6: Noggin attenuates hypoxia-elevated SOCE in PASMC. A: line charts showing the dynamic traces of the intracellular calcium concentration in response to intracellular calcium store depletion by cyclopiazonic acid (CPA, 10 μM), in presence of voltage-dependent calcium channel (VDCC) blocker nifedipine (5 μM) in calcium free solution and restoration of extracellular calcium concentration afterward. B: bar graphs indicated the delta changes before and after extracellular
calcium restoration was calculated as store-operated calcium entry (SOCE). The bar graph represents the Mean ± SEM, n = 3 experiments in each group. Four groups were designed as normoxic control, normoxia+noggin (200 ng/ml, 60-hour), hypoxic control and hypoxia+noggin (200 ng/ml, 60-hour). *P <0.05 vs. normoxic control; #P <0.05 vs. hypoxic control.

Figure 7: Noggin normalized hypoxic upregulation of TRPC1 and TRPC6 protein expression in PASMC. A: western blots showing the protein expression levels of TRPC1, TRPC6 and beta-tubulin in cultured rat distal PASMC upon hypoxic exposure (4% O₂, 60-hour) and noggin (200 ng/ml) treatments. B: bar graphs representing the fold induction of TRPC1 (left) and TRPC6 (right) under different treatments, as normalized to normoxic control. Beta-tubulin served as internal control. The bar graph represents the Mean ± SEM, n = 3 in each group; *P <0.05 vs. normoxic control; #P <0.05 vs. hypoxic control.
Table 1: Primers for real time-qPCR

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Protein: BMP antagonists to β-actin

Figure 1
Figure 2
Figure 3

A

Rat PASMC

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BMP4 (50 ng/ml) + Nog (ng/ml)

B

Protein: p-p38

Protein: p-ERK1/2

β-tubulin

BMP4 (50 ng/ml) + Nog (ng/ml)
Figure 4

A

BMP4 (50 ng/ml, minute)

Cont  5  15  30  60  120

P-JAK2
P-STAT3
β-tubulin

B

BMP4 (50 ng/ml)

Control  5  15  30  60  120

Protein: P-JAK2/β-tubulin

BMP4 (50 ng/ml)

Control  5  15  30  60  120

Protein: P-STAT3/β-tubulin

C

BMP4 (50 ng/ml)

Cont  0  200

P-JAK2
P-STAT3
β-tubulin

D

BMP4 (50 ng/ml) + Nog (ng/ml)

control  0  200

Protein: P-JAK2/β-tubulin

BMP4 (50 ng/ml) + Nog (ng/ml)

control  0  200

Protein: P-STAT3/β-tubulin
Figure 5

Proliferation: % normalized to Nor
**Figure 6**

A

- **Nor**
- **Hyp**
- **Nor+Nog**
- **Hyp+Nog**

B

- **Δ[Ca^{2+}]_i (nM)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Δ[Ca^{2+}]_i (nM)</th>
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</thead>
<tbody>
<tr>
<td>Nor</td>
<td>300</td>
</tr>
<tr>
<td>Nor+Nog</td>
<td>250</td>
</tr>
<tr>
<td>Hyp</td>
<td>450</td>
</tr>
<tr>
<td>Hyp+Nog</td>
<td>350</td>
</tr>
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</table>

* and # indicate statistical significance.
A

Rat PASMC

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia (4% O₂, 60 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noggin (ng/ml)</td>
<td>0</td>
<td>200</td>
</tr>
</tbody>
</table>

91 KDa

106 KDa

55 KDa

TRPC1

TRPC6

β-tubulin

B

Protein: TRPC1/β-tubulin

Protein: TRPC6/β-tubulin

Figure 7