Prostacyclin receptor agonists induce DUSP1 to inhibit pulmonary artery smooth muscle cell proliferation

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Abstract

Aims: Upregulated $p38^{MAPK}$ signaling is implicated in the accelerated proliferation of pulmonary artery smooth muscle cells (PA-SMCs) and the pathogenesis of pulmonary artery remodeling observed in pulmonary arterial hypertension (PAH). Previously, we reported that after endothelin-1 (ET-1) pretreatment, bone morphogenetic protein 2 (BMP2) activates $p38^{MAPK}$ signaling and accelerates PA-SMC proliferation. The activity of $p38^{MAPK}$ signaling is tightly regulated by the inactivation of dual-specificity phosphatase 1 (DUSP1). Activated $p38^{MAPK}$ induces DUSP1 expression, forming a negative feedback loop. Prostacyclin IP receptor agonists (prostacyclin and selexipag) are used to treat PAH. In this study, we aimed to verify whether IP receptor agonists affect DUSP1 expression and accelerate the proliferation of PA-SMCs.

Main Methods: PA-SMCs were treated with BMP2, ET-1, prostacyclin, and MRE-269, an active metabolite of selexipag, either alone or in combination. We quantified mRNA expressions using real-time quantitative polymerase chain reaction. Pulmonary artery specimens and PA-SMCs were obtained during lung transplantation in patients with PAH.

Key Findings: Both prostacyclin and MRE-269 increased DUSP1 expression. Combined treatment with BMP2 and ET-1 induced cyclin D1 and DUSP1 expression and increased PA-SMC proliferation. MRE-269 attenuated BMP2/ET-1-induced cell proliferation. ET-1 increased DUSP1 expression in PA-SMCs from control patients but not in PA-SMCs from patients with PAH.

Significance: This study showed that the $p38^{MAPK}$/DUSP1 negative feedback loop is impaired in PAH, contributing to unregulated $p38^{MAPK}$ activation and PA-SMC hyperplasia. IP receptor agonist MRE-269 increases DUSP1 expression and inhibit $p38^{MAPK}$-mediated PA-SMC proliferation. Future elucidation of the detailed mechanism underlying reduced DUSP1 expression would be informative for PAH treatment.

Key Words: pulmonary arterial hypertension; smooth muscle cells; DUSP1; IP receptor; MAPK

Abbreviations

BMP2=bone morphogenetic protein 2
CCND1=cyclin D1
CREB=cAMP-response element-binding protein
DUSP1=dual-specificity phosphatase 1
ET-1=endothelin-1
ID1=inhibitor of DNA binding 1
MSK1=mitogen-and stress-activated protein kinase 1
PAH=pulmonary arterial hypertension
PA-SMC=pulmonary artery smooth muscle cell
PKA=protein kinase A

Introduction

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease characterized by exacerbated pulmonary artery remodeling and vasoconstriction, leading to elevated pulmonary vascular resistance, right ventricular failure, and ultimately death [1]. Excessive proliferation of pulmonary artery smooth muscle cells (PA-SMCs) has been observed in patients with PAH [2]. In patients with PAH, increased expression of endothelin-1 (ET-1) in remodeled pulmonary arterioles [3] and mutations in genes associated with bone morphogenetic protein (BMP) signaling [4] have been discovered. Although both the ET-1 and BMP signaling pathways are considered to be involved in the development of this disease, the detailed pathobiology underlying vascular remodeling remains incompletely understood.

$p38^{MAPK}$ is known to play a major role in cell growth and differentiation in response to growth factors and inflammation [5]. Activation of $p38^{MAPK}$ has been previously implicated in the pathogenesis of pulmonary artery remodeling observed in experimental models of pulmonary hypertension [6] and in patients with PAH [7,8]. We previously reported that after ET-1 pretreatment, BMP2 activates $p38^{MAPK}$ signaling and accelerates PA-SMC proliferation [9], and this accelerated proliferation is abrogated by selective $p38^{MAPK}$ inhibitor [10]. Phosphorylated/activated mitogen-activated protein kinases (MAPKs), including $p38^{MAPK}$, induce the expression of dual-specificity protein phosphatase 1 (DUSP1) through mitogen- and stress-activated protein kinase 1 (MSK1) [11]. DUSP1 dephosphorylates/deactivates phosphorylated $p38^{MAPK}$, forming a negative feedback loop [12,13]. Protein kinase A (PKA) is also known to induce DUSP1 expression by phosphorylating cyclic AMP (cAMP)-response element-binding protein (CREB) [14]. A previous study showed that DUSP1-deficient mice developed severe pulmonary hypertension after exposure to chronic hypoxia [15].

Prostacyclin (also called prostaglandin I2 or PGI2) is widely used in the treatment of PAH, and selexipag, a non-prostanoid prostacyclin receptor (IP receptor) agonist, was recently authorized for application in PAH treatment [16]. IP receptor binds to its agonists, resulting in the conversion of ATP to cAMP, with a subsequent increase in PKA activity [17]. Selexipag is metabolized to MRE-269, which binds to IP receptors with an affinity higher than that of selexipag [18]. Although many stimulatory pathways affecting $p38^{MAPK}$ have been delineated, the role of IP receptor agonists in the $p38^{MAPK}$ pathway remains unexplored. In this context, we aimed to verify whether IP receptor agonists affect DUSP1 expression and inhibit the abnormal proliferation of PA-SMCs observed in the lungs of patients with PAH.

Methods

Lung tissue sampling

For immunohistochemistry and *in vitro* experiments, we used lung and pulmonary artery specimens obtained during lung transplantation in patients with PAH (n=7) and during lobectomy or pneumectomy for localized lung cancer in control patients (n=14). In the control group, preoperative transthoracic echocardiography was performed to rule out pulmonary hypertension, and the lung specimens were sampled at a distance from the tumor foci. The demographic and clinical characteristics of patients with PAH are: 2 women and 2 men, age 58.5 ± 2.4 years, mean pulmonary artery pressure 57.5 ± 6.3 mmHg and PVR 12.1 ± 1.5 WU. The control patients (6 women, 1 men; age 61.3 ± 1.6 years) underwent transthoracic echocardiography pre-operatively to rule out pulmonary hypertension. None of the patients harbored *BMPR2* or *ALK1* mutations. The experimental procedures conformed to the principles outlined in the Declaration of Helsinki. This study was approved by the local institutional ethics committee at Erasmus University Hospital, Brussels, Belgium. All the patients provided written informed consent before participating in the study.

Lung immunolocalization of phospho-p38^{MAPK} and DUSP1

Lung specimens were fixed, embedded in paraffin, and immunohistochemically stained as previously described [19]. Briefly, lung sections (5 µm) were deparaffinized, incubated with a target retrieval buffer (Dako, Glostrup, Denmark), and heated in a bath for 20 min at 95°C. Endogenous peroxidase activity was quenched with hydrogen peroxide and sections were saturated with bovine serum albumin (3% in PBS) for 30 min. The sections were then incubated overnight at 4°C with primary antibodies against phosphorylated p38^{MAPK} (Cell Signaling Technology, Danvers, MA) and DUSP1 (Sigma-Aldrich, Saint Louis, MO). Antibody binding was detected using an LSAB staining system with HRP-DAB (Santa Cruz, CA, USA). Nuclei were counterstained with hematoxylin. Negative controls lacked the primary antibody.

Culture of human pulmonary artery smooth muscle cells (PA-SMCs)

Primary human PA-SMCs were cultured from pulmonary artery explants collected from PAH patients and control patients, as previously described [9,19]. Briefly, small pieces of freshly isolated arteries were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L l-glutamine, and antibiotics. Isolated PA-SMCs were assessed for the expression of muscle-specific contractile and cytoskeletal proteins including α-smooth muscle actin, desmin, and vinculin. Cells were used at passages less than 5.

Healthy human PA-SMCs were purchased from Lonza (Basel, Switzerland) and cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% FCS and insulin-transferrin-selenium solution (Gibco, Paisley, UK).

Effects of ET-1, BMP2, and prostacyclin analogs on p38^{MAPK} signaling activation

We evaluated p38^{MAPK}-induced gene expression, including *MSK1*, *DUSP1*, *CCND1*, and *ID1*, to confirm the activation of p38^{MAPK} signaling activation and canonical BMP signaling.

To assess the expression of DUSP1 in PA-SMCs, the cells were treated with human recombinant ET-1 (1 μmol/L; Sigma-Aldrich, St. Louis, MO) [9] for 5 h after a 48-h serum deprivation period.

To assess p38^{MAPK} activation in human control PA-SMCs, we treated the cells with human recombinant BMP2 (10 ng/mL; R&D Systems, Minneapolis, MN) for 5 h after 48-h serum starvation and 24-h pretreatment with human recombinant ET-1 (100 nmol/L) [9]. We also treated PA-SMCs with PGI2 (30 nmol/L; Cayman Chemical, Ann Arbor, MI), MRE-269 (30–300 nmol/L; Cayman Chemical) for 5 h. After treatment, cells were washed with ice-cold PBS, harvested in specific lysis buffer, and used for mRNA extraction, cDNA synthesis, and RTq-PCR experiments, as described below.

Real-time quantitative polymerase chain reaction (RTq-PCR)

Total RNA was extracted from growth-arrested human primary cultured PA-SMCs and treated human control PA-SMCs using an RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentration was determined using standard spectrophotometric techniques, and RNA integrity was assessed by visual inspection of GelRed-stained (Biotium, Hayward, CA) agarose gels. First-strand cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions.

RT-qPCR experiments were performed in duplicate using the TaqMan Probe System with Thunderbird Probe qPCR Mix (Toyobo, Osaka, Japan) and specific probes (Roche

Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Sense and anti-sense primers were designed for *Homo sapiens DUSP1*, *CCND1*, *MSK1*, *ID1*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) mRNA sequences (Table 1) using the Roche Universal Probe Library Assay Design Center. Signal detection and analysis were performed using Light Cycler 96 (Roche Diagnostics, Mannheim, Germany). For relative quantification, the comparative $2^{-\Delta\Delta Ct}$ method normalized to the housekeeping genes *HPRT1* and *GAPDH*.

Evaluation of PA-SMC proliferation

Purchased human PA-SMCs were seeded in 96-well plates and allowed to adhere in 10% FCS-supplemented DMEM. After 24-h pretreatment with ET-1 (100 nmol/L) [9], PA-SMCs were treated with the active form of selexipag, MRE-269 (300 nmol/L; Cayman Chemical), and subsequently treated with BMP2 (10 ng/mL) in 10% FCS-supplemented DMEM for 48 h. Cell proliferation was assessed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan), which quantifies the amount of formazan dye generated by dehydrogenases in living cells. The absorbance was measured using a Varioskan Lux (Thermo Fisher Scientific, Vantaa, Finland). Proliferation rates were expressed as the relative fold increase over the mean value of the proliferative rate of the basal condition corresponding to 10% FCS-treated PA-SMCs arbitrarily fixed to 1.

Statistics

All data were analyzed using Microsoft Excel and the add-in software Statcel2 (OMS Publishing, Saitama, Japan) and are presented as dot plots together with the mean ± standard error of the mean. Differences between two unpaired groups were compared using Welch's t-test, and differences between two paired groups were tested using the Wilcoxon signed-rank test. For comparisons of multiple groups, we used one-factor analysis of variance (ANOVA)

with Scheffé's post-hoc test. Statistical significance was set at $P < 0.05$.

Results

Effects of ET-1 and BMP2 on gene expression in the canonical and noncanonical BMP signal pathway

Previously, we reported that after ET-1 pretreatment, BMP2 activates $p38^{MAPK}$ signaling [9]. In healthy human PA-SMCs, combined treatment with ET-1 (100 nmol/L) and bone morphogenetic protein 2 (BMP2; 10 ng/mL) increased the mRNA expression of inhibitor of DNA binding 1 (*ID1*), cyclin D1 (*CCND1*), *MSK1*, and *DUSP1* (Figure 1A-D), suggesting both the canonical BMP signal pathway (ID1 expression) and noncanonical BMP signal pathway ($p38^{MAPK}$) were activated.

Effect of IP receptor agonists on DUSP1 expression

In healthy human PA-SMCs, treatment with PGI2 (30 nmol/L) did not affect the mRNA expression level of *MSK1* (Figure 2A) but increased *DUSP1* expression (Figure 2B). Treatment with MRE-269 decreased the mRNA expression of *CCND1* (Figure 3A) and increased *DUSP1* expression (Figure 3C). These effects were observed with 30 nmol/L MRE-269 treatment and were not augmented by higher MRE-269 concentrations (Figure 3A and 3C). mRNA expression of *MSK1* remained unchanged after MRE-269 stimulation (Figure 3B).

Effects on PA-SMC proliferation

As we previously showed [9], PA-SMCs, in the presence of 10% FCS, exhibited increased proliferation after combined ET-1 (100 nM) and BMP2 (10 ng/mL) treatment compared to that in control group. Addition of MRE-269 (300 nmol/L) completely abrogated

this accelerated proliferation (Figure 4). There is no statistical difference between the control group and group treated with BMP2, ET-1 or MRE-269 alone.

p38^{MAPK} and DUSP1 expression in the lungs of PAH patients

As illustrated in Figure 5A, immunohistochemical staining revealed that phosphorylated p38^{MAPK} was expressed in the small intra-acinar pulmonary arterioles of PAH patients, suggesting SMC localization. In the lungs of control patients, the expression of phosphorylated p38^{MAPK} was very weak or absent (Figure 5A). DUSP1 staining was strong in the medial layer of pulmonary arterioles from control patients, whereas it was weak and heterogeneous in remodeled pulmonary arterioles from PAH patients (Figure 5B).

DUSP1 expression in PA-SMCs from PAH patients

The mRNA expression of *DUSP1* was similar in primary cultured PA-SMCs from PAH patients and control patients without stimulation by ET-1 (Figure 6A). Treatment with ET-1 (1 μmol/L) increased the mRNA expression of *DUSP1* in serum-starved PA-SMCs from control patients (Figure 6B) but not in PA-SMCs from PAH patients (Figure 6C).

Discussion

Combined ET-1/BMP2 treatment activating p38^{MAPK} increased MSK1 and DUSP1 expression, which probably plays a role in regulating the excessive proliferation of PA-SMCs in the lungs. An impaired p38^{MAPK}/DUSP1 negative feedback loop contributes to the exaggerated proliferation of PA-SMCs in PAH patients. The present study showed that PGI2 and MRE-269 augmented DUSP1 expression in PA-SMCs, suggesting that these IP receptor agonists suppress p38^{MAPK} activity and subsequent PA-SMC proliferation by enhancing DUSP1 activity (Figure 7).

Lung immunostaining revealed strong expression of phosphorylated/activated $p38^{MAPK}$ in remodeled pulmonary arterioles from PAH patients (but not in arterioles from control patients), with PA-SMC localization (Figure 5A). This is consistent with previous studies showing activated $p38^{MAPK}$ in experimental models of pulmonary hypertension and in patients [20-22]. In rat models of chronic hypoxia-induced [23] and monocrotaline-induced [24] pulmonary hypertension, increased activation of $p38^{MAPK}$ has been reported in remodeled pulmonary arterioles and is tightly associated with exacerbated PA-SMC proliferation [25]. We observed strong DUSP1 expression in normal arterioles but not in the thickened medial layer of the PAH specimens (Figure 5B). Little is known about the contribution of DUSP1 to PAH pathogenesis. However, DUSP1 is expressed more abundantly in the lungs than in other organs [26], contributing to the maintenance of the physiological state of the lungs against constitutively activated MAPK. Chronic hypoxia-induced pulmonary hypertension has been reported to be more severe in DUSP1-deficient mice than in control mice [15], suggesting that DUSP1 exerts a protective effect against hypoxia-induced vascular remodeling.

Expression of DUSP1 was similar in cultured PA-SMCs from PAH patients and control patients without ET-1 stimulation (Figure 6A). However, ET-1 increased DUSP1 expression in control PA-SMCs, whereas it was abrogated in PA-SMCs from PAH patients (Figure 6B-C). It is known that ET-1 induces the phosphorylation/activation of MAPKs, such as $p38^{MAPK}$, in vascular smooth muscle cells [27] and PA-SMC [9]. Studies have supported the role of DUSP1 as a negative feedback regulator of the $p38^{MAPK}$ pathway and as a repressor of pro-remodeling functions in smooth muscle cells [13,28,29]; impaired DUSP1 induction results in augmented and prolonged activity of $p38^{MAPK}$ [12,30]. This enhanced $p38^{MAPK}$ activation contributes to the generation and action of several potential inflammatory mediators [31] and the exacerbation of vascular smooth muscle cell proliferation [32].

We previously reported that after ET-1 pretreatment, BMP2 accelerates $p38^{MAPK}$ signaling and causes PA-SMC proliferation [9], and that selective $p38^{MAPK}$ inhibitor abrogates this accelerated proliferation [10]. Moreover, activation of $p38^{MAPK}$ has been reported following BMP4 treatment in PA-SMCs from idiopathic PAH patients [8]. In the present study, the combination of ET-1 and BMP2 induced the expression of cyclin D1 (Figure 1B), which serves as a switch to actively regulate cell cycle progression and subsequent cell proliferation. Combined ET-1/BMP2 treatment was also associated with increased MSK1 and DUSP1 expression (Figure 1C-D). Activated $p38^{MAPK}$ mediates both the expression and phosphorylation of MSK1 [33].

Selexipag, an orally available and long-acting non-prostanoid IP receptor agonist, is metabolized to the active form MRE-269, which is highly selective for the IP receptor [18]. After IP receptor binds to its agonists, conversion of ATP to cAMP [17] activates PKA [34], which induces DUSP1 expression by phosphorylating CREB [14]. A previous study showed that prostaglandin E2 receptor agonists induce DUSP1 expression in human airway smooth muscle cells [35]. Here, we showed that IP receptor agonists, PGI2 and MRE-269, increased DUSP1 expression without increasing MSK1 expression, decreased cyclin D1 expression (Figure 2 and 3), and completely abrogated BMP2/ET-1-induced proliferation (Figure 4). These findings suggest that these agonists deactivate $p38^{MAPK}$ by inducing DUSP1 directly through the cAMP-PKA-CREB pathway but not through activated MAPK or MSK1 (Figure 7). Interestingly, the anti-proliferative effects of sildenafil, another important agent used to treat PAH, are also associated with the upregulation of DUSP1 expression and repression of activated MAPK [36].

It is well known that high-dose PGI2 is effective in patients with PAH, although the detailed mechanism underlying this effect has not been clarified. The present study highlights the unregulated $p38^{MAPK}$ following impaired expression of DUSP1 and the replenishing effect

of the IP receptor agonists on DUSP1 expression. Suppressed DUSP1 expression might be caused by unidentified mutations or desensitization following excessive and prolonged expression of ET-1 and BMP ligands commonly observed in PAH. Elucidation of the mechanism underlying reduced DUSP1 expression and identification of the pathways that effectively promote DUSP1 expression would be informative for future PAH treatment. Additionally, quantitative evaluation of DUSP1 expression following stimulation with PGI2 or selexipag may help predict the efficacy of medical treatment and determine the prognosis of PAH.

One of the limitations of the current study is that we evaluated gene expression alone. However, DUSP1 and other family members are principally regulated at the transcriptional level, as evidenced by very low to undetectable mRNA expression in quiescent cells and rapid mRNA induction after PA-SMC treatment [37].

Conclusions

The present study showed that the $p38^{MAPK}$/DUSP1 negative feedback loop is impaired in PAH, contributing to unregulated $p38^{MAPK}$ activation and PA-SMC hyperplasia. IP receptor agonist MRE-269 increase DUSP1 expression and inhibit $p38^{MAPK}$-mediated PA-SMC proliferation. This study demonstrates the potential efficacy of $p38^{MAPK}$ inhibitors and DUSP1-inducing agents for the treatment of this disease.

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Conflict of Interest

The authors declare no conflict of interest.

Figure Legends

Figure 1. Expression of genes activated by $p38^{MAPK}$ signaling in healthy human PA-SMCs.

Healthy human PA-SMCs were pretreated with ET-1 (100 nmol/L) and stimulated with bone morphogenetic protein 2 (BMP2; 10 ng/mL) (n=7). The relative expression of inhibitor of DNA binding 1 (ID1) (A), cyclin D1 (CCND1) (B), mitogen-and stress-activated protein kinase 1 (MSK1) (C), and DUSP1 (D) normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as the relative fold increase over the mean mRNA expression in the vehicle group (n=6). Quantitative data were analyzed using Welch's t-test and presented as dot plots together with the mean ± SEM. *$p < 0.05$, **$p < 0.01$ compared with the vehicle group.

Figure 2. PGI2-stimulated mRNA expression in healthy human PA-SMCs.

Serum-starved healthy human PA-SMCs were stimulated with prostacyclin (PGI2; 30 nmol/L) for 5 h (n=7). The relative expression of mitogen-and stress-activated protein kinase 1 (MSK1) (A) and dual- specificity protein phosphatase 1 (DUSP1) (B) normalized to that of GAPDH is shown as the relative fold increase over the mean value of mRNA expression in the vehicle group (n=6) arbitrarily fixed to 1. Quantitative data are analyzed using Welch's t-test and presented in dot plots together with mean ± SEM. *$p < 0.05$ compared with the vehicle group.

Figure 3. MRE-269-stimulated mRNA expression in healthy human PA-SMCs.

Serum-starved healthy human PA-SMCs were stimulated with MRE-269 (30–300 nmol/L) for 5 h (n=5). The relative expression of cyclin D1 (CCND1) (A), mitogen-activated protein kinase (MSK1) (B), and dual- specificity protein phosphatase 1 (DUSP1) (C) normalized to

that of GAPDH is shown as the relative fold increase over the mean value of mRNA expression in the vehicle group (n=5) arbitrarily fixed to 1. Quantitative data are analyzed using one-factor ANOVA with Scheffé's post-hoc test and presented in dot plots together with mean ± SEM. **p < 0.01 compared with the vehicle group (MRE-269 0 mol/L).

Figure 4. Proliferation study of healthy human PA-SMC.

Healthy human PA-SMCs (n=6) were pretreated with ET-1 (100 nmol/L) for 24 h, treated with either MRE-269 (300 nmol/L) for 30 min, stimulated with BMP2 (10 ng/mL), and incubated for 2 days. Data were normalized to the vehicle group, which was arbitrarily fixed to 1. Quantitative data are analyzed using one-factor ANOVA with Scheffé's post-hoc test and presented in dot plots together with mean ± SEM. **p < 0.01 compared with vehicle group; ‡p < 0.05 compared with the BMP2-treated group; §§p < 0.01 compared with the ET-1+BMP2+MRE-269-treated group.

Figure 5. Immunostaining for phosphorylated $p38^{MAPK}$ and DUSP1.

Representative images of staining for (A) phosphorylated $p38^{MAPK}$ and (B) dual-specificity phosphatase 1 (DUSP1) in pulmonary arterioles from control patients (left panels) and pulmonary arterial hypertension (PAH) patients (right panels). Images in the lower row show magnified views of the area outlined in the image directly above. Phosphorylated $p38^{MAPK}$ was expressed more strongly in the medial layer of the lungs of PAH patients (red arrows).

Figure 6. Relative DUSP1 expression in primary cultured PA-SMCs.

(A) Baseline dual- specificity protein phosphatase 1 (DUSP1) expression from PA-SMCs from control patients (left; n=14) and PA-SMCs from PAH patients (right; n=7) without endothelin-1 (ET-1) stimulation or (B, C) DUSP1 expression in PA-SMCs from control

patients (B; n=7) and PA-SMCs from PAH patients (C; n=4) stimulated with ET-1 (1 μmol/L). Results were expressed as the relative fold increase over baseline mRNA expression in the corresponding cells. Quantitative data are analyzed using Welch's t-test (A) and Wilcoxon signed-rank test (B, C), and presented in dot plots together with mean ± SEM. *$p < 0.05$ compared with baseline expression.

Figure 7. Proposed mechanism of $p38^{MAPK}$ activation via BMP signaling and its inhibition by DUSP1.

After ET-1 pretreatment, BMP2 activates $p38^{MAPK}$ to enhance the proliferation of human PA-SMCs. The $p38^{MAPK}$/DUSP1 negative feedback loop is impaired in the lungs of patients with PAH. An IP receptor agonist induces DUSP1, but not via $p38^{MAPK}$ activation. IP receptor agonists induce DUSP1 through the cAMP-PKA-CREB pathway and not through activated MAPK or MSK1. CCND1, cyclin D1; CREB, cAMP-response element -binding protein; DUSP1, dual-specificity phosphatase 1; ERK, extracellular-signal regulated kinase; ETR, endothelin receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyl transferase 1; ID1, inhibitor of DNA binding 1; MSK1, mitogen-activated protein kinase; RT-qPCR, real-time quantitative polymerase chain reaction

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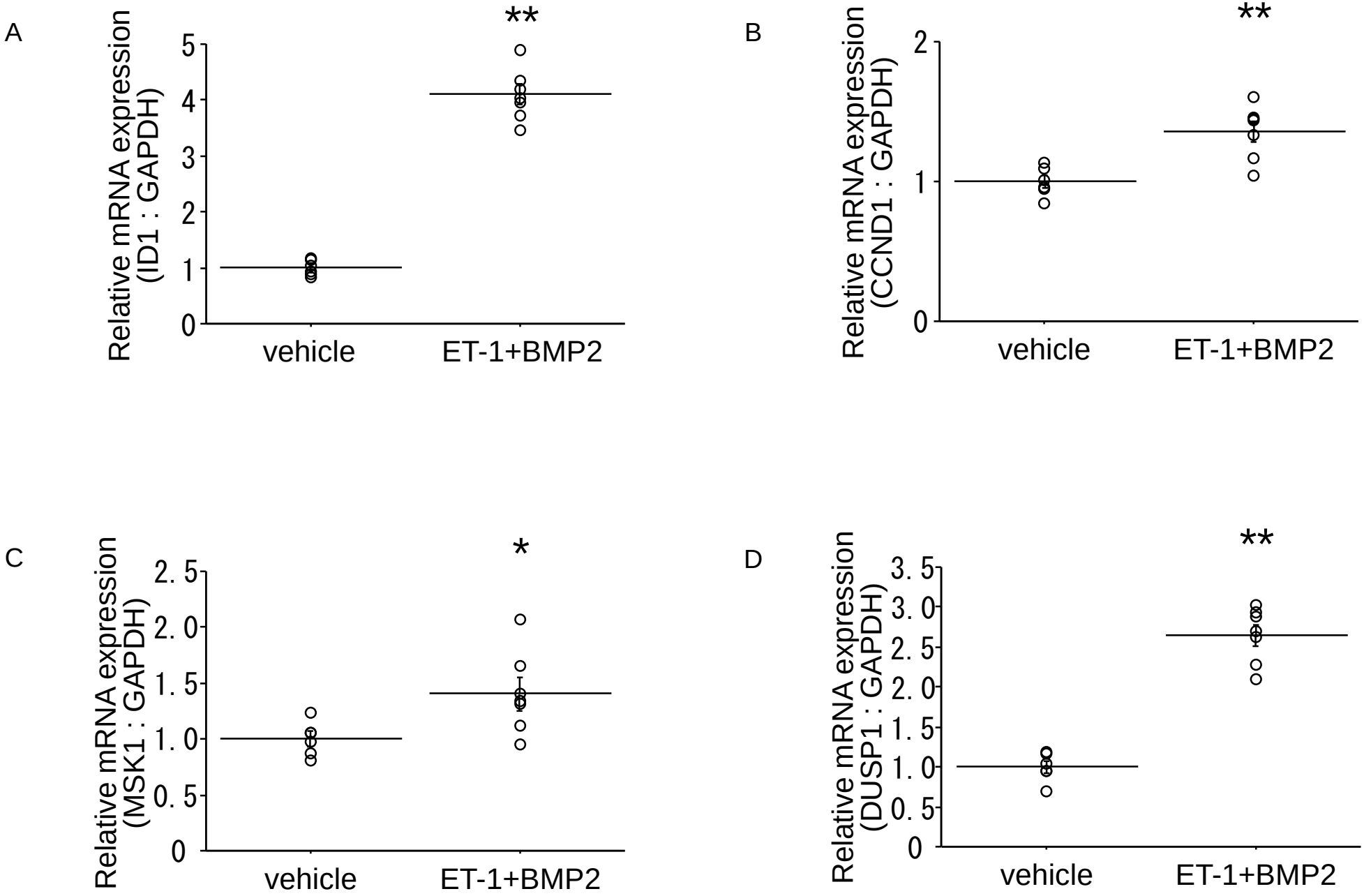
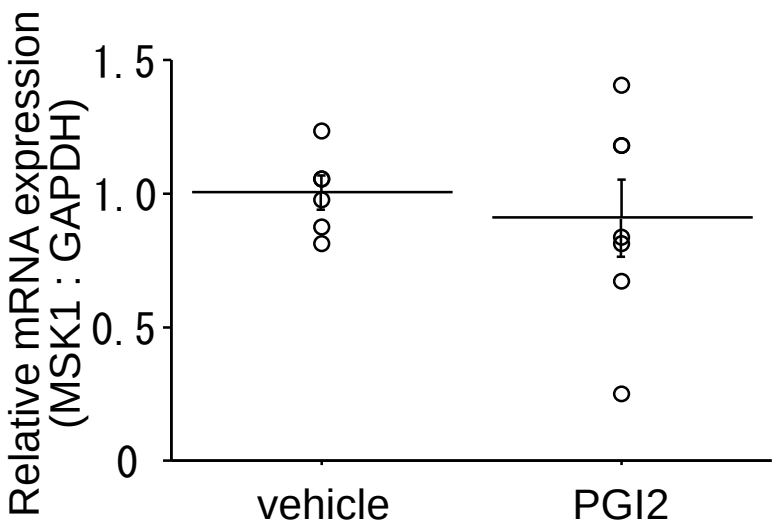
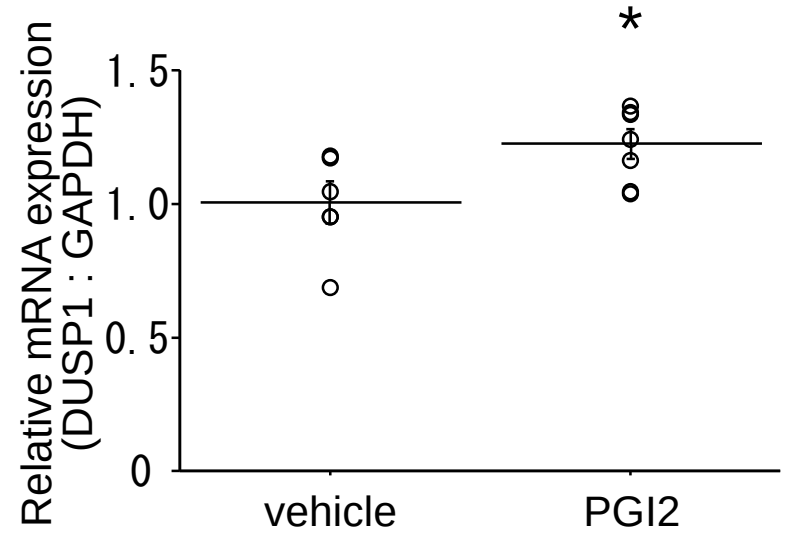

A
Relative mRNA expression
(ID1 : GAPDH)
5
4
3
2
1
0
**
vehicle
ET-1+BMP2
B
Relative mRNA expression
(CCND1 : GAPDH)
2
1
0
**
vehicle
ET-1+BMP2
C
Relative mRNA expression
(MSK1 : GAPDH)
2.5
2.0
1.5
1.0
0.5
0
*
vehicle
ET-1+BMP2
D
Relative mRNA expression
(DUSP1 : GAPDH)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
**
vehicle
ET-1+BMP2

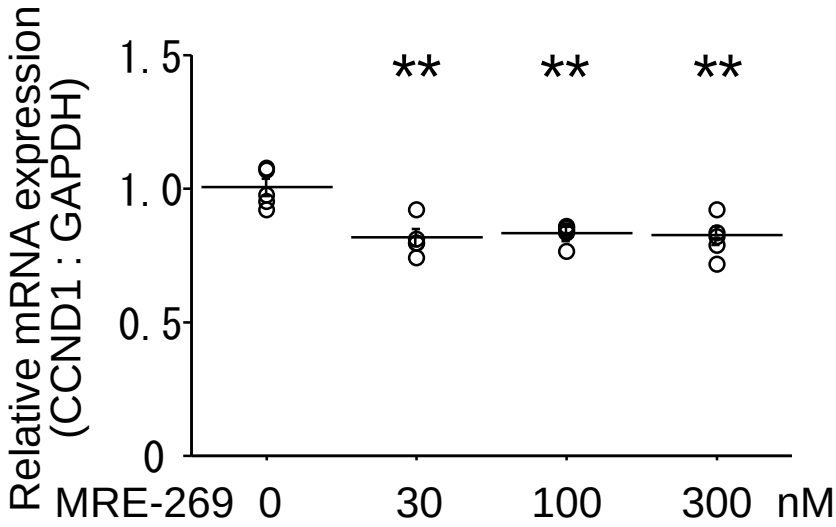
A


Relative mRNA expression
(MSK1 : GAPDH)
1.5
1.0
0.5
0
vehicle
PGI2

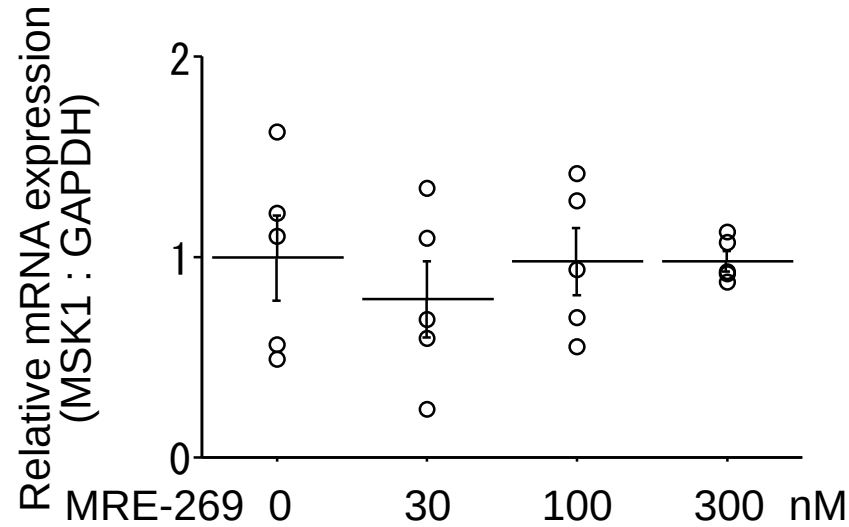
B


*
Relative mRNA expression
(DUSP1 : GAPDH)
1.5
1.0
0.5
0
vehicle
PGI2

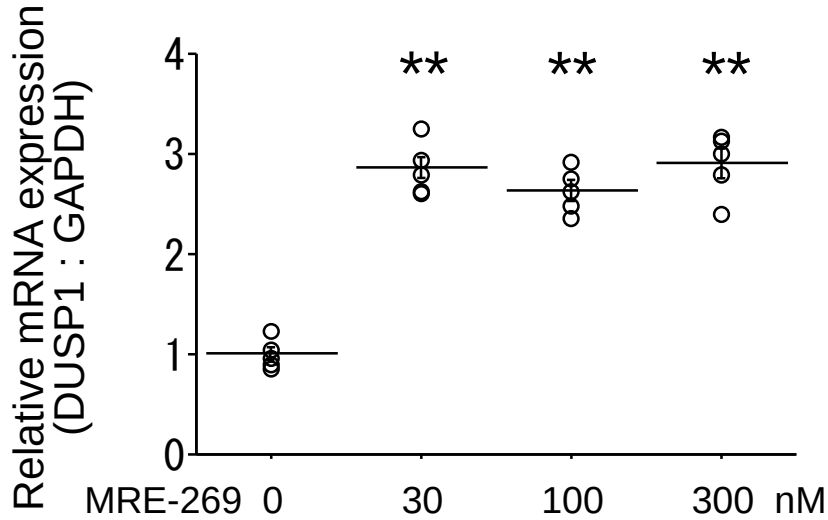
A

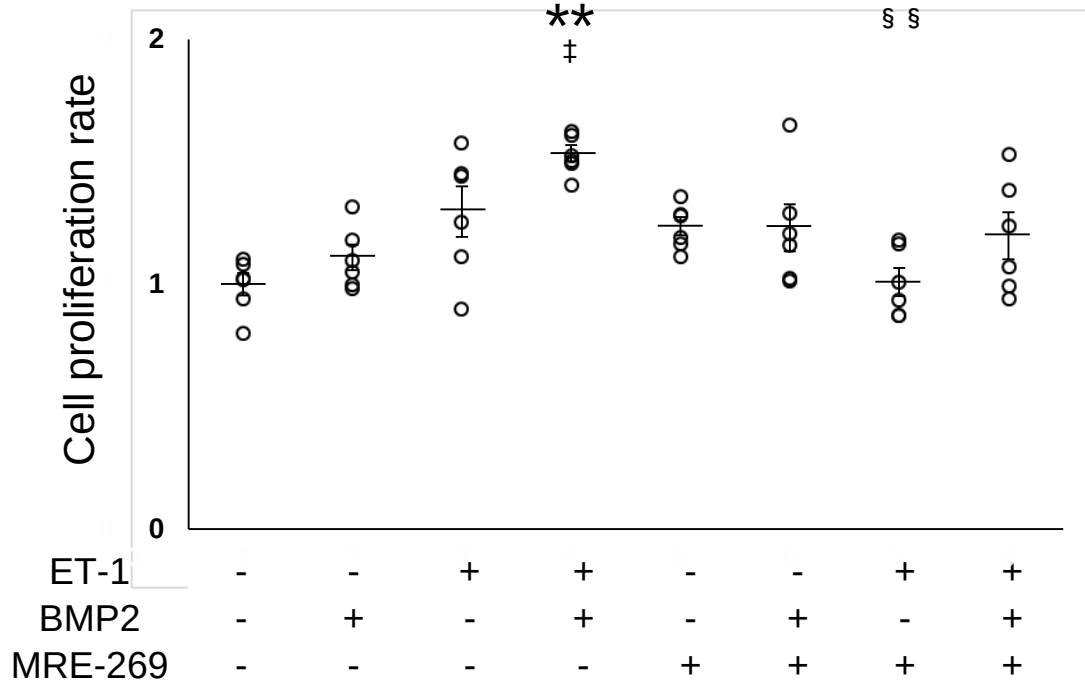

Relative mRNA expression
(CCND1 : GAPDH)
1.5
1.0
0.5
0
**
**
**
MRE-269 0
30
100
300 nM

B

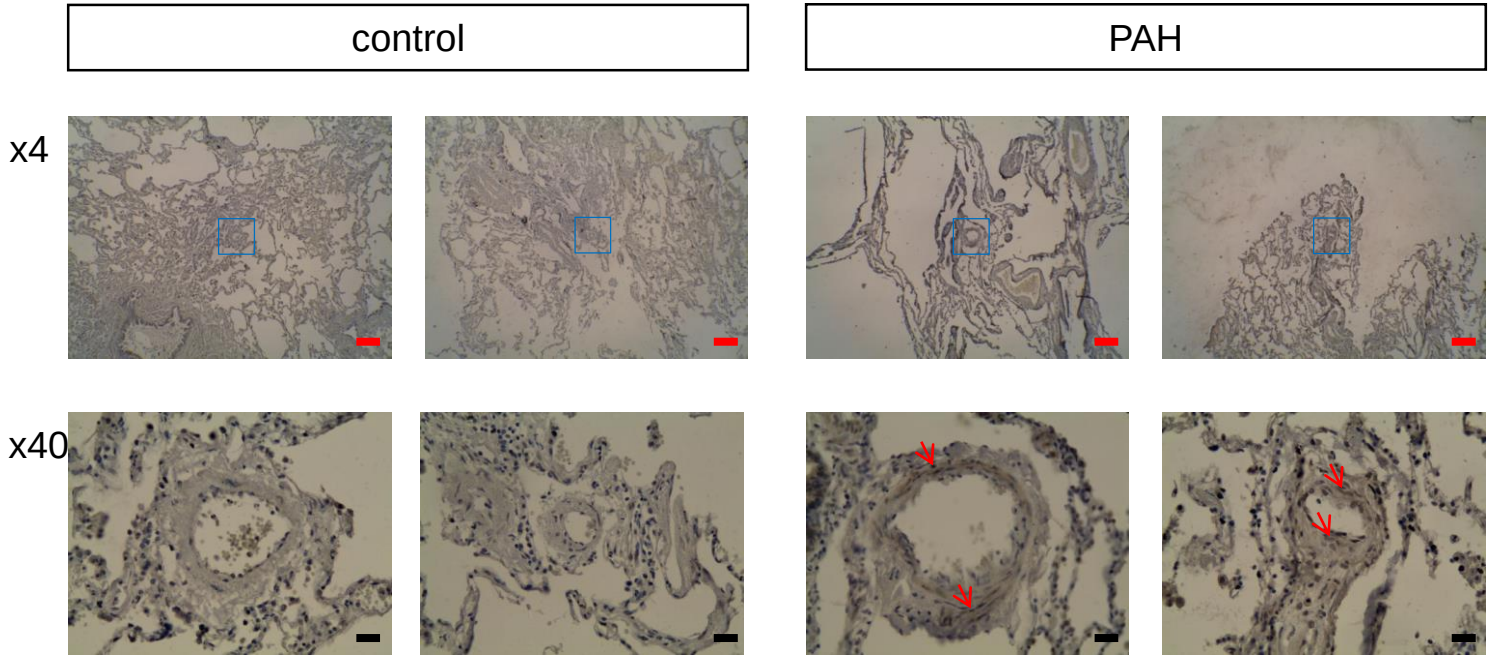

Relative mRNA expression
(MSK1 : GAPDH)
2
1
0
MRE-269 0
30
100
300 nM

C

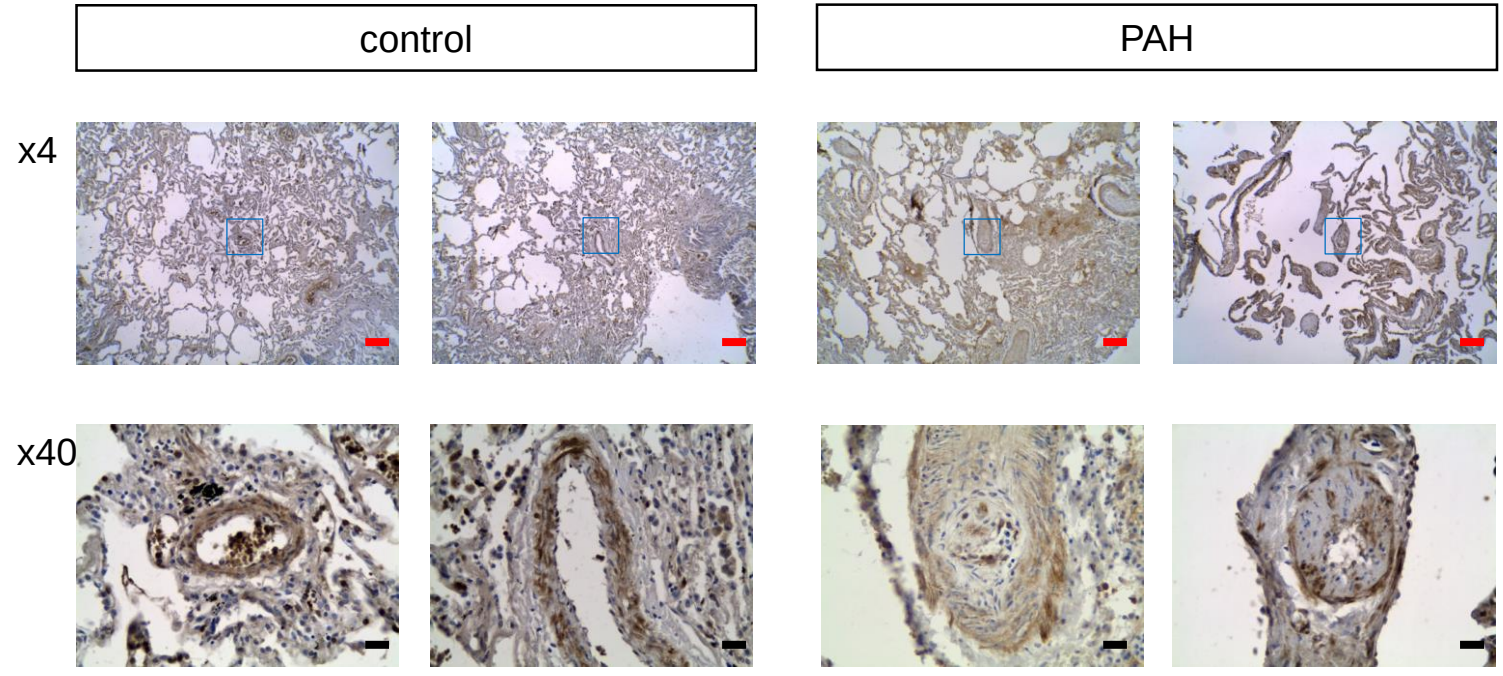

Relative mRNA expression
(DUSP1 : GAPDH)
4
3
2
1
0
**
**
**
MRE-269 0
30
100
300 nM


Cell proliferation rate
2
1
0
**
‡
§ §
ET-1 - - + + - - + +
BMP2 - + - + - + - +
MRE-269 - - - - + + + +

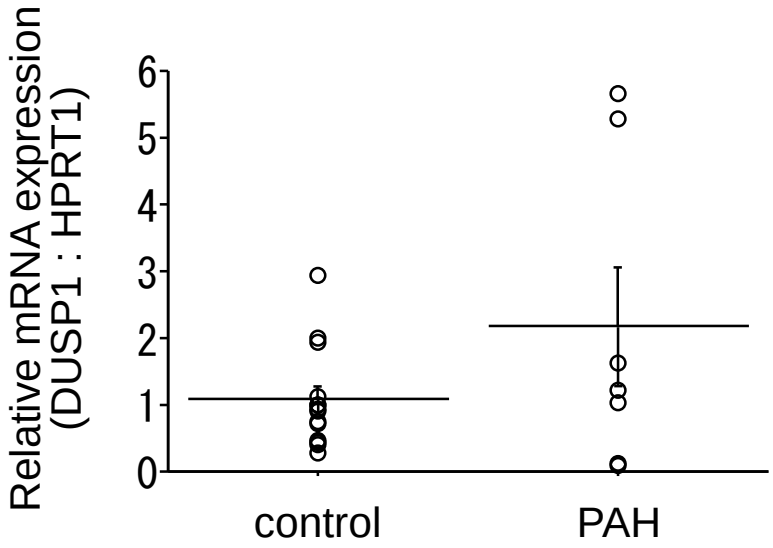
A



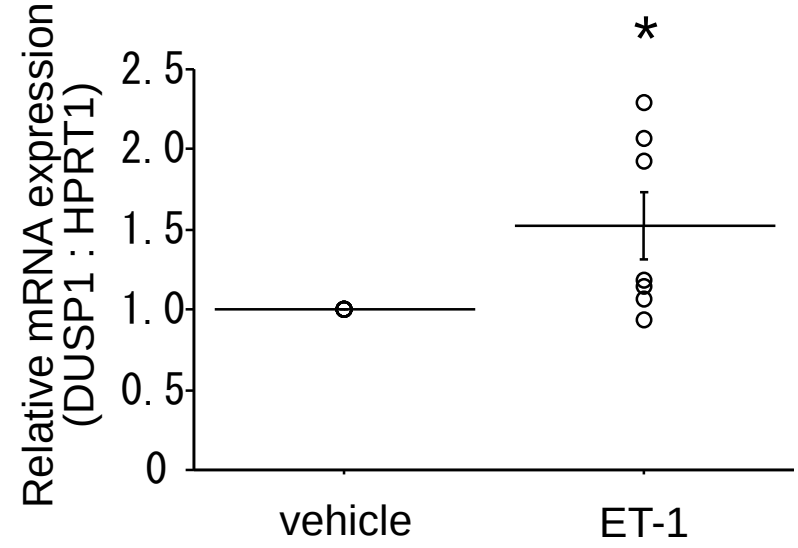
B



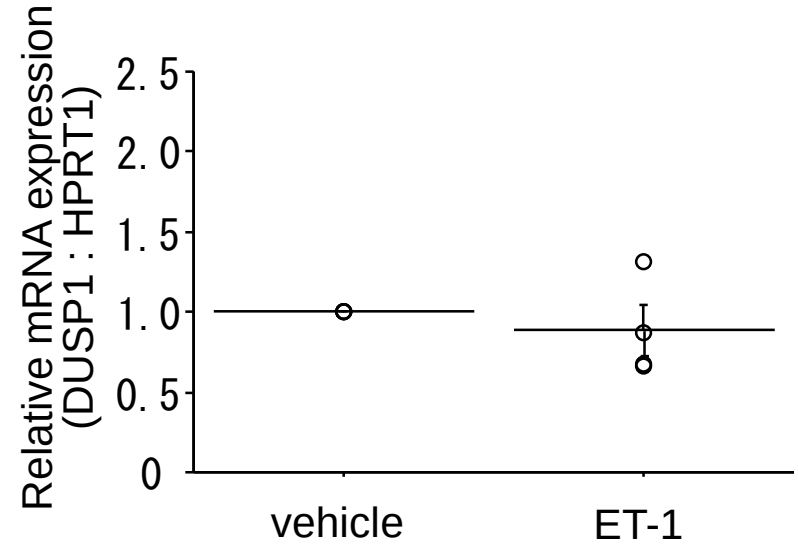
A


Relative mRNA expression
(DUSP1 : HPRT1)
6
5
4
3
2
1
0
control
PAH

B


Relative mRNA expression
(DUSP1 : HPRT1)
2.5
2.0
1.5
1.0
0.5
0
*
vehicle
ET-1

C


Relative mRNA expression
(DUSP1 : HPRT1)
2.5
2.0
1.5
1.0
0.5
0
vehicle
ET-1

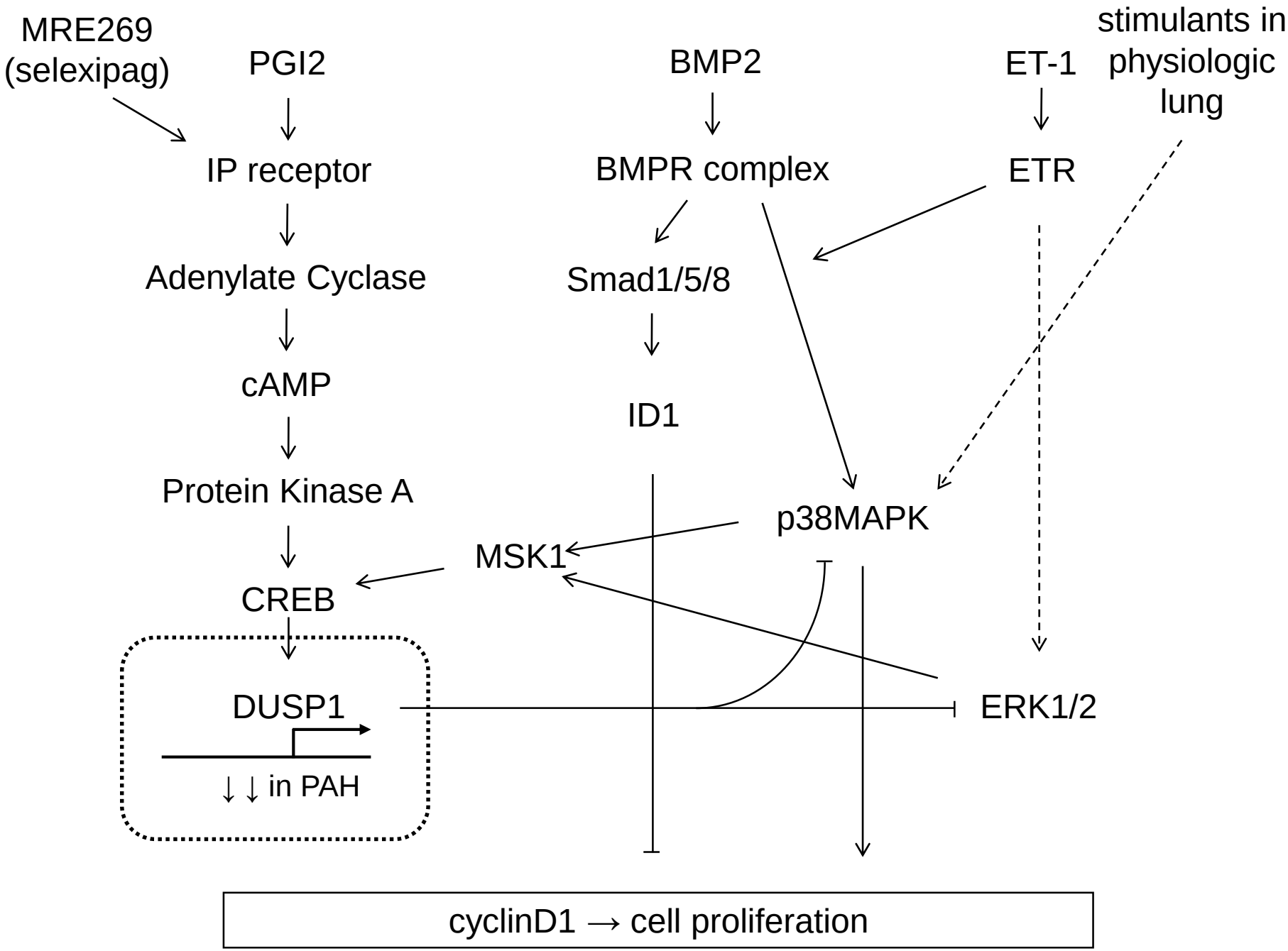

MRE269
(selexipag)
PGI2
BMP2
ET-1
stimulants in
physiologic
lung
IP receptor
BMPR complex
ETR
Adenylate Cyclase
Smad1/5/8
cAMP
ID1
Protein Kinase A
p38MAPK
MSK1
CREB
DUSP1
ERK1/2
↓↓ in PAH
cyclinD1 → cell proliferation

Table 1. Primers for *RTq-PCR*

Gene	Sense	Anti-sense
CCND1	gctgtgcatctacaccgaca	ttgagcttgttcaccaggag
DUSP1	ttcaacgaggccattgactt	cctggcagtggacaaacac
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc
HPRT1	ctttgctttccttggtcagg	acacttcgtggggtcctttt
ID1	ccagaaccgcaaggtgag	ggtccctgatgtagtcgatga
MSK1	ccaggagtgcaatgatgaag	ttatgcacacactttcgacaaa

CCND1 = Cyclin D1; DUSP1 = Dual specificity protein phosphatase 1; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; HPRT1 = Hypoxanthine phosphoribosyl transferase 1; ID1 = Inhibitor of DNA binding 1; MSK1 = Mitogen- and stress-activated protein kinase; RTq-PCR = real-time quantitative polymerase chain reaction.