A novel secreted-cAMP pathway inhibits pulmonary hypertension via a feed-forward mechanism

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Abstract

Aims

Cyclic adenosine monophosphate (cAMP) is the predominant intracellular second messenger that transduces signals from Gs-coupled receptors. Intriguingly, there is evidence from various cell types that an extracellular cAMP pathway is active in the extracellular space. Herein, we investigated the role of extracellular cAMP in the lung and examined whether it may act on pulmonary vascular cell proliferation and pulmonary vasculature remodeling in the pathogenesis of pulmonary hypertension (PH).

Methods and Results

The expression of cyclic AMP-metabolizing enzymes was increased in lungs from patients with PH as well as in rats treated with monocrotaline and mice exposed to Sugen/hypoxia. We report that inhibition of the endogenous extracellular cAMP pathway exacerbated Sugen/hypoxia-induced lung remodeling. We found that application of extracellular cAMP induced an increase in intracellular cAMP levels and inhibited proliferation and migration of pulmonary vascular cells *in vitro*. Extracellular cAMP infusion in two *in vivo* pulmonary hypertension models prevented and reversed pulmonary and cardiac remodeling associated with PH. Using protein expression analysis along with luciferase assays, we found that extracellular cAMP acts via the A_2R/PKA/CREB/p53/Cyclin D1 pathway.

Conclusions

Taken together, our data reveal the presence of an extracellular cAMP pathway in pulmonary arteries that attempts to protect the lung during PH, and suggest targeting of the extracellular cAMP signaling pathway to limit pulmonary vascular remodeling and PH.

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Key words: Pulmonary hypertension, cyclic nucleotides, extracellular cAMP, vascular signaling

Translational Perspective

Lungs samples from patients with clinical PAH and from animals with PH display increased cyclic AMP-metabolizing enzymes expression levels. Our results indicate that an endogenous extracellular cAMP pathway is activated during PH and attempts to counteract vascular remodeling. Additionally, our study demonstrates that extracellular-cAMP inhibits chronic hypoxia-induced PH in mice and MCT-induced PH in rats by activating the A_2R/PKA/CREB/p53/Cyclin D1 pathway. Importantly, PAH patients display an inactive PKA/CREB/p53/Cyclin D1 pathway that could be stimulated by extracellular-cAMP. Targeting the extracellular cAMP pathway may represent a novel therapeutic approach for the treatment of pulmonary arterial hypertension.

Introduction

Pulmonary arterial hypertension (PAH), a rare disease with an estimated prevalence of 15 to 50 per million population [1], is usually progressive, leading to right heart failure and ultimately death [2, 3]. Despite advances in clinical management of PAH, there is no cure for PAH, highlighting the need for new directions and therapies.

Pulmonary artery smooth muscle cell (PASMC) proliferation and vascular endothelial dysfunction are crucial features of the vascular remodeling process that occurs in PAH [4]. In vascular SMC, the canonical activation of G-protein-coupled receptors stimulates adenylyl cyclase, leading to an increase in intracellular cyclic adenosine monophosphate (cAMP) formation and reduced cell proliferation *in vitro* [5] and *in vivo* [6, 7]. Prostacyclin, the main product of arachidonic acid in the endothelium, increases intracellular cAMP formation via adenylyl cyclase stimulation. Prostacyclin and its analogs have proved to be beneficial for patients with PAH. However, the main limitations of prostacyclin drugs are their side effects that impact quality of life in patients with PAH. Therefore, new therapies that can offer higher long-term efficacy and improved tolerability are needed.

Cyclic AMP levels are also regulated by cyclic nucleotide phosphodiesterases (PDEs), enzymes that hydrolyze cyclic nucleotides, and PDE5 inhibitors have been approved for the treatment of

PAH [8, 9]. Moreover, besides hydrolysis by PDEs, cAMP can also be transported outside the cells by members of the ATP-binding cassette transporters (ABCC, also termed MRP) [10-12]. We recently reported cardiomyocyte-derived cAMP to be secreted into the extracellular space and to be stepwise metabolized by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) and ectonucleotide 5′-nucleotidase (NT5E) to adenosine (ENPP1 and NT5E convert cAMP to AMP and AMP to adenosine, respectively). This extracellular cAMP (□-cAMP) pool protects the heart from adrenergically induced hypertrophy and fibrosis in a process that is mediated through its metabolite adenosine acting mainly on adenosine receptors in cardiomyocytes and cardiac fibroblasts [13]. Interestingly, cAMP transporters [14] and enzymes that metabolize cAMP to adenosine [15-17] are also expressed in the lung, suggesting an analogous mechanism may be at play. Together, these observations prompted us to ask whether the extracellular cAMP pathway exists in pulmonary vascular cells, and whether extracellular cAMP prevents and/or reverses pathological vascular remodeling in pulmonary hypertension (PH).

We addressed these issues by combining *in vitro* and *in vivo* experiments to examine the role of extracellular cAMP in PH. In this report, we provide the first evidence for the antiproliferative activity of extracellular cAMP in human pulmonary vascular cells and in two PH animal models.

Methods

Human lung samples

Lung specimens were obtained at the time of lung transplantation from 7 patients with PAH, and from 5 non-PAH patients. Mount Sinai Institutional Review Board approved procurement of the human tissue samples. Human lung tissues were obtained from the University of South Paris (Le Plessis Robinson) and from the University General Consortium Hospital of Valencia. All usage was done as per Mount Sinai approved guidelines. This study was also approved by the local ethics committee (Comité de Protection des Personnes, CPP Ile de France VII, Le Kremlin

Bicêtre, France, and by the local research and independent ethics committee of the University General Consortium Hospital of Valencia (CEIC/2013)), and patients gave written informed consent. No human subjects were involved. Investigation of human tissues conformed to the principles outlined in the Declaration of Helsinki.

Cell Culture

Human pulmonary artery endothelial cells (hPAECs) and pulmonary artery smooth muscle cells (hPASMCs) from at least 3 different healthy donors were purchased from Lonza, Inc. PAECs were grown in EBM-2 medium supplemented with 5% fetal bovine serum (FBS) supplemented with EGM-2 SingleQuots (Lonza). PASMCs were cultured in SmBM medium supplemented with 5% FBS and SmGM-2 SingleQuots (Lonza). Normal Healthy Lung Fibroblasts (NHLF) were purchased from Lonza, Inc. NHLF, isolated from 3 different healthy donors, were grown in FBM medium supplemented with 2% FBS. Cells were grown in 5% CO_2 at 37°C and passaged at confluence. The cells were studied between passages 2 through 7.

Rat Monocrotaline-PH Model

All animal experiments were approved by the Icahn School of Medicine at Mount Sinai institutional animal use and care committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In the prevention protocol, adult male Sprague-Dawley rats (Charles River), weighing 300 to 350 g, received a single injection of MCT (60 mg/kg) and miniosmotic pumps (Alzet) containing cAMP (delivering 30 mg/kg/day, Sigma-Aldrich) that were implanted subcutaneously into the animals at the same time. Twenty-one days after the administration of MCT or vehicle control, hemodynamic measurements were performed and rats were euthanized for tissue collection. In the curative protocol, 3 weeks after MCT injection, rats were randomly assigned to receive miniosmotic pumps (Alzet) containing cAMP (delivering 30 mg/kg/day, Sigma-Aldrich) or a saline solution for 2 weeks. Hemodynamics

and morphometric measurements were performed 5 weeks after MCT injection. Animals were anaesthetized with ketamine (75-100 mg/kg) and xylazine (4-8 mg/kg) and sacrificed by exsanguination via PBS cardiac perfusion.

Mouse Sugen/hypoxia-PH model

For the prevention protocol, ten-week-old C57B6 WT mice subcutaneously received 20 mg/kg of Sugen (SU5416, Cayman Chemical), and miniosmotic pumps containing cAMP (30 mg/kg/day, Sigma-Aldrich) or a saline solution were implanted. Mice were then exposed to chronic hypoxia (10%O_2) in a ventilated chamber for 21 days. Sugen was injected once a week during the next 2 weeks. The end point for hemodynamic measurements and sacrifice was 14 weeks of age.

For the curative protocol, ten-week-old C57B6 WT mice subcutaneously received 20 mg/kg of Sugen and were exposed to hypoxia (10%O_2) in a ventilated chamber for 21 days. Sugen was injected once a week during the next 2 weeks. Mice were then randomized to receive miniosmotic pumps (Alzet) containing cAMP (30 mg/kg/day, Sigma-Aldrich), adenosine (30 mg/kg/day, Sigma-Aldrich) or a saline solution. Hemodynamic measurements and sacrifices were performed 3 weeks after miniosmotic pumps implantation. Control mice (normoxic group) were housed in room air at ambient atmospheric pressure. Animals were fed standard chow and water ad libitum.

For the assessment of the effects of endogenous extracellular cAMP in PH, ten-week-old C57B6 WT mice subcutaneously received 20 mg/kg of Sugen and were exposed to chronic hypoxia (10%O_2) in a ventilated chamber for 14 days. Mice received an additional Sugen injection on day 7. Mice that were used as control were maintained under normoxic conditions. Normoxia and Sugen/hypoxia exposed mice were intraperitoneally injected with either saline or SYL-001 (7.5 mg/kg, every two days for 2 weeks. SYL-001 is a potent and highly selective inhibitor of ENPP1 that was previously developed [13]. The end point for hemodynamic

measurements and sacrifice was 12 weeks of age. Animals were anaesthetized with ketamine (75-100 mg/kg) and xylazine (4-8 mg/kg) and sacrificed by exsanguination via PBS cardiac perfusion.

Right Ventricle and Pulmonary artery Hemodynamic Studies

Animals were anaesthetized with 3-4% isoflurane, intubated via a tracheotomy, and mechanically ventilated. Next, the thoracic cavity was opened and a catheter (Transonic Systems Inc.) was inserted directly into the right ventricle or into the pulmonary artery. One to two animals died in each group during the hemodynamic measurements, independently from the disease or the treatment, mainly because of bleeding when the cavities were opened or during the tracheotomy. The heart rate, pulmonary artery systolic pressure, pulmonary artery diastolic pressure, right ventricular end-systolic and diastolic pressures were measured directly. Hemodynamic data were recorded using an ADVantage P–V Control Unit (Transonic Systems).

Blood collection and cAMP assay

Miniosmotic pumps containing cAMP (delivering 30 mg/kg/day) or a saline solution were implanted into mice exposed to 3 weeks of Sugen/Hypoxia. Three weeks later, blood samples were collected from the pulmonary artery and plasma was retrieved after centrifugation. Circulating e-cAMP levels in the pulmonary artery were then determined by an enzymatic immunoassay, as recommended by the manufacturer (Cayman Chemical).

Statistical analyses

All quantitative data are reported as means ± SEM. Statistical analysis was performed with the Prism software package (GraphPad Version 7). Differences between two means were assessed by a 2-tailed paired or unpaired *t* test. Differences among multiple means were assessed by one-way Anova followed by Bonferroni correction or followed by Holm-Sidak's test analysis. P-

values <0.05 were considered significant (corresponding symbols in figures are * for P<0.05, ** for P<0.01 and *** for P<0.001).

Further details on the methods, as well as supplementary figures are available in the Supplementary material online.

Results

Upregulation of ENPP1 and NT5E expression in human PAH and experimental PH animal models

First, to determine whether the enzymes that metabolize extracellular cAMP to adenosine are expressed and active during PAH, we compared the expression profiles of ENPP1 and NT5E in lung samples from patients with clinical PAH (for patient characteristics, *see* Supplementary material Online, Table S1) and from healthy lung samples from non-PAH human subjects (Supplementary material Online, Table S2). Remarkably, mRNA and protein expression levels of ENPP1 and NT5E were upregulated in the lungs of diseased patients (Figure 1A,B). Next, we explored ENPP1 and NT5E expression profiles in two different *in vivo* PH animal models: Monocrotaline (MCT)-induced PH in rats and Sugen/hypoxia-induced PH in mice. In line with the human data, quantitative PCR analysis revealed significant increases in ENPP1 and NT5E mRNAs levels in diseased lungs from both *in vivo* models (Figure 1C,E). These findings were confirmed by immunoblot analyses of total lung extracts from diseased animals. Western blot analyses revealed a marked upregulation of ENPP1 and NT5E in diseased lungs from both *in vivo* animal models (Figure 1D,F). Additionally, a consistent upregulation of the cAMP transporter (ABCC4) and adenosine receptors 2 ($A_{2A}R$ and $A_{2B}R$) was detected in humans, mice and rats with PH (Supplementary material Online, Figure S1). These results indicate that the enzymes that metabolize extracellular cAMP to adenosine are expressed in the lung and are

upregulated during PH.

Extracellular cAMP induces intracellular cAMP formation in pulmonary vascular cells *in vitro*

We next sought to identify the primary source of secreted cAMP in pulmonary arteries. Major cells present in the arterial wall (hPASMC, hPAEC and human lung fibroblasts (NHLF)) were incubated with Forskolin (Fsk, an adenylate cyclase activator), and extracellular cAMP was measured using a colorimetric assay (Figure 2A). Fsk treatment increased extracellular cAMP levels in all cell types (Figure 2B). Fibroblasts showed substantially more cAMP efflux under Fsk treatment compared to hPASMC and hPAEC (Figure 2B). In line with these results, a preferential expression of ABCC4 in fibroblasts was detected (Figure 2C). These results point towards fibroblasts being the primary human pulmonary source of secreted cAMP.

In order to determine the effects that exogenous extracellular cAMP exerts on intracellular cAMP formation in pulmonary vascular cells, quantitation of intracellular cAMP was performed by fluorescence resonance energy transfer (FRET) measurements. Human PASMC and PAEC were transfected with a vector expressing a FRET-based cAMP sensor [18] (Figure 2D), and FRET was measured in real time. Addition of non-cell permeable extracellular cAMP in hPASMC led to an increase in the donor emission (Figure 2E left, green tracings) and a decrease in the acceptor emission (Figure 2E left, blue tracings). An increase in the FRET ratio was detected in hPASMC after extracellular cAMP addition, reflecting a rise in intracellular cAMP (Figure 2E right). Similarly, extracellular cAMP enhanced intracellular cAMP formation in hPAEC (Figure 2F). These experiments demonstrate that extracellular cAMP induces intracellular cAMP formation in pulmonary vascular cells (Figure 2G).

Extracellular cAMP inhibits pulmonary vascular cells proliferation and migration *in vitro*

Since an increase in intracellular cAMP levels has been shown to modulate vascular cell proliferation [19], we expected extracellular cAMP to regulate hPASMC proliferation. Indeed, treatment with extracellular cAMP inhibited serum-induced hPASMC proliferation (Figure 3A). We then applied adenosine receptor antagonists and found $A_{2B}R$ blockade to significantly inhibit the antiproliferative effect of extracellular cAMP (Figure 3A). Additionally, extracellular cAMP treatment led to a marked decrease in serum-induced migration of hPASMCs, an effect that was abrogated by a specific $A_{2B}R$ antagonist (Figure 3B), consistent with a high expression of this receptor in hPASMC (Figure 3C). Since endothelial cell dysfunction has a key role in the initiation and progression of PH, we therefore determined the extracellular cAMP effect on hPAEC proliferation and migration. We found that extracellular cAMP inhibits serum-induced hPAEC proliferation and migration (Figure 3D,E). $A_{2A}R$ antagonist reversed the antiproliferative and antimigratory effects of extracellular cAMP (Figure 3D,E). These results are in agreement with a lower expression of A_1R, $A_{2B}R$ and A_3R in hPAEC than of $A_{2A}R$ (Figure 3F). These experiments demonstrate that extracellular cAMP inhibits pulmonary vascular cells proliferation and migration through $A_{2A}R$ and $A_{2B}R$ activation.

Since cAMP leads to the activation of Protein Kinase A (PKA), which phosphorylates key regulators of cells proliferation and migration, we then assessed whether the extracellular cAMP effects involve the PKA pathway. Markedly, a specific inhibitor of protein kinase A (PKI) completely reversed the inhibitory effects of extracellular cAMP on hPASMC and hPAEC migration (Figure 3G), suggesting that extracellular cAMP inhibits pulmonary vascular cells migration by activating the PKA-dependent signaling pathway.

Since PKA regulates the activity and phosphorylation of the cAMP-responsive element binding protein (CREB), and since CREB is involved in the inhibition of proliferation and migration of vascular cells [20, 21], CREB activity was assessed in cells infected with an adenoviral vector expressing a CREB Responsive Element-luciferase (CRE-luciferase) reporter gene. Extracellular cAMP treatment increased CREB activity in both pulmonary vascular cell types,

whereas PKI inhibited the extracellular cAMP effects (Figure 3H). In addition, we found antagonism of the $A_{2B}R$ in hPASMC to efficiently block the effect of extracellular cAMP on CREB activity, whereas blockade of the $A_{2A}R$ abolishes the extracellular cAMP effect in hPAEC (Supplementary material Online, Figure S2A,B).

Since CREB has been shown to activate the tumor protein 53 (p53) [22] and since p53 activation has been shown to prevent and reverse PAH [23], we then tested whether extracellular cAMP is able to modulate p53 activity. Human PASMC and PAEC treated with extracellular cAMP displayed increased levels of p53 reporter activity, suggesting that extracellular cAMP activates p53 (Figure 3I).

Inhibition of the cAMP-metabolizing enzyme, ENPP1, exacerbates mouse Sugen/hypoxia-induced lung remodeling *in vivo*

We next assessed whether blocking the processing of endogenous extracellular cAMP to AMP, using a potent and highly selective inhibitor of ENPP1 (SYL-001 [13]), worsens PH. For logistical and economical reasons (mice being 10 times lighter than rats, thus needing 10-fold less SYL-001), we choose to use the PH model of mice subjected to chronic hypoxia combined with the VEGF receptor blocker Sugen (SU5416) (Figure 4A). Sugen/hypoxia-exposed wild-type mice were treated with either saline or SYL-001 every two days for 2 weeks (Figure 4A). Mice maintained under normoxic conditions and treated with SYL-001 did not show any cardiac or pulmonary phenotype (Figure 4B-D). Mice exposed to Sugen/hypoxia and treated with SYL-001 showed a marked increase in right ventricular systolic pressure (RVSP), and a moderate increase in right ventricular (RV) hypertrophy (assessed by the Fulton index and cardiomyocyte size) (Figure 4B,C). Importantly, morphometric analysis of distal pulmonary arteries demonstrated a significant increase in medial thickness of SYL-001 treated mice (Figure 4D). These experiments suggest that endogenous extracellular cAMP-derived adenosine attenuates Sugen/hypoxia-induced pulmonary hypertension, however the extracellular cAMP levels are not

sufficient to completely inhibit PH. Therefore, addition of exogenous extracellular cAMP may prevent and/or reverse PH.

Extracellular cAMP infusion prevents and reverses rat monocrotaline-induced PH *in vivo*

To evaluate the *in vivo* effect of extracellular cAMP treatment on pulmonary vascular remodeling, we used the MCT-PH rat model. We first performed a prevention model, where rats received MCT and miniosmotic pumps containing cAMP were implanted simultaneously (Supplementary material Online, Figure S3A). MCT administration resulted in a marked increase in RV hypertrophy, RVSP, and pulmonary artery pressures (Supplementary material Online, Figure S3B-D). Extracellular cAMP-treated rats displayed lower RVSP, Fulton index and pulmonary arterial pressures. Analysis of wheat-germ agglutinin (WGA)–stained RV sections revealed that extracellular cAMP prevented PH-induced cardiomyocyte hypertrophy (Supplementary material Online, Figure S3D). Morphometric analysis of distal pulmonary arteries demonstrated a significant decrease in medial thickness of extracellular cAMP-treated animals (Supplementary material Online, Figure S3E). These results indicate that extracellular cAMP protects against the development of MCT-induced PH.

We then determined whether extracellular cAMP treatment would reverse PH induced by MCT administration. Rats were randomly assigned to receive cAMP or a saline solution 3 weeks after MCT treatment (Figure 5A). Hemodynamics and morphometric measurements were performed 5 weeks after MCT injection. No significant difference in mortality was observed between the MCT and the MCT+e-cAMP groups (Supplementary material Online, Figure S4). Saline-treated rats displayed all the hallmarks of PH (i.e., increased RVSP, Fulton index, and pulmonary artery pressures) (Figure 5B,C), whereas extracellular cAMP-treated rats displayed a marked decrease in these parameters (Figure 5B,C). Remarkably, extracellular cAMP-treated rats were protected from PH-induced cardiac hypertrophy, showing lower levels of hypertrophy-associated marker gene (*Nppa)* and reduced cardiac myocyte hypertrophy (Figure 5D,F). In addition, right

ventricular and pulmonary fibrosis were reduced in the extracellular cAMP-treated group, as determined by Col1a1 and fibronectin expression (Figure 5D,E). Treatment with extracellular cAMP also reduced pulmonary arterial media wall thickness (Figure 5G). These results indicate that extracellular cAMP reverses MCT-induced PH development.

Extracellular cAMP inhibits Sugen/hypoxia-induced pulmonary hypertension in mice

To further study the role of extracellular cAMP on pulmonary artery remodeling, we used Sugen/hypoxia as another *in vivo* model of PH. Mice subcutaneously received Sugen, and miniosmotic pumps containing extracellular cAMP, or a saline solution as control, were implanted. Mice were then exposed to 3 weeks of hypoxia (10%O_2) with a weekly injection of Sugen (Supplementary material Online, Figure S5A). In saline-treated mice, hypoxia resulted in a marked increase in RV weight, Fulton index, and RVSP (Supplementary material Online, Figure S5B). In contrast, we were not able to detect significant changes in these parameters in extracellular cAMP-treated mice (Supplementary material Online, Figure S5B). Mice treated with saline developed cardiac hypertrophy and pulmonary fibrosis, whereas extracellular cAMP infusion significantly prevented these structural changes, as determined by quantification of the mRNAs that encode *Nppa, Col1a1* and *fibronectin* (Supplementary material Online, Figure S5C,D). Histological analysis of cardiac tissue confirmed that extracellular cAMP prevented PH-induced cardiomyocyte hypertrophy (Supplementary material Online, Figure S5E). In addition, extracellular cAMP-treated mice showed a significant reduction in the percentage of medial thickness in pulmonary arteries (Supplementary material Online, Figure S5F).

We next sought to determine whether extracellular cAMP is able to reverse Sugen/hypoxia-induced PH. We first used adenosine as a positive control and found that adenosine-treated mice showed display a marked decrease in RV weight, Fulton index, and RVSP (Supplementary material Online, Figure S6A,B). In addition, cardiomyocyte hypertrophy and medial thickness of pulmonary arteries were reduced in adenosine-treated group (Supplementary material Online,

Figure S6C,D). These results indicate that adenosine reverses Sugen/hypoxia-induced PH in mice. Next, mice were maintained in hypoxia for 3 weeks with a weekly injection of Sugen and were then randomized to receive miniosmotic pumps containing cAMP or a saline solution for 2 weeks, and placed back under normoxic conditions for an additional week (Figure 6A). Extracellular cAMP infusion significantly increased circulating e-cAMP levels in the pulmonary artery, confirming that cAMP, infused through miniosmotic pumps, is delivered into the pulmonary circulation (Supplementary material Online, Figure S7). Extracellular cAMP significantly decreased RV hypertrophy, RVSP, perivascular lung fibrosis and pulmonary artery remodeling as determined by morphometric measurements, quantitative real-time PCR and histological analyses (Figure 6B-G). In addition, extracellular cAMP treatment significantly reduced the number of proliferating SMCs with Ki67 positive nuclei in the pulmonary arteries of Sugen/hypoxia-treated mice and MCT-treated rats (Supplementary material Online, Figure S8). Next, we sought to analyze whether the circulating e-cAMP level in the pulmonary artery has a relationship with RV remodeling. The correlation analysis showed that circulating e-cAMP level in the pulmonary artery was negatively correlated with Fulton index and cardiac myocyte size and does not correlate with right ventricular systolic pressure (Supplementary material Online, Figure S9). These results indicate that more efficient delivery of e-cAMP reduces right ventricular remodeling. Together, these data demonstrate that extracellular cAMP delivery reverses chronic hypoxia-induced PH in mice.

Extracellular cAMP exerts its effects through the PKA/CREB/p53/Cyclin D1 pathway

Consistent with our *in vitro* findings, extracellular cAMP treatment increased phosphorylation of CREB at Ser133 (PKA site) and restored p53 expression level in MCT-PH rats (Figure 7A). This was associated with a concomitant decrease in Cyclin D1 (a key regulator of cell cycle progression) expression (Figure 7A). The beneficial effects of extracellular cAMP were associated with increased eNOS expression and restored phosphorylated eNOS level in lungs

of PH rats (Supplementary material Online, Figure S10), suggesting that extracellular cAMP improved endothelial function *in vivo*. Similarly, western blot analyses revealed a marked downregulation of phosphorylated CREB and p53, and an upregulation of Cyclin D1 in lungs of Sugen/hypoxia-treated mice (Figure 7B). These changes were significantly reversed by extracellular cAMP treatment (Figure 7B). Therefore, we can infer from these results that extracellular cAMP reverses PH through PKA/CREB/p53/Cyclin D1 pathway activation. We further investigated whether this pathway is deregulated in human PAH patients. In line with the animal models, phosphorylated CREB and p53 expression levels were decreased whereas Cyclin D1 level was increased in the lungs of PAH patients (Figure 7C). Therefore, extracellular cAMP treatment, by activating the A_2R/PKA/CREB/p53/Cyclin D1 pathway (Figure 7D), could represent a novel therapy for treating patients with PAH.

Discussion

This study identifies secreted cAMP as an important regulator in PAH. The data presented here show an activation of the extracellular cAMP pathway in remodeled lungs from patients with clinical PAH. The upregulation of the cAMP transporter (ABCC4) and the cAMP-metabolizing enzymes (ENPP1 and NT5E) during PAH attempt to protect the lung from the detrimental consequences of pulmonary vascular remodeling and pulmonary hypertension. This study demonstrates that extracellular cAMP, through the A_2R/PKA/CREB pathway, prevents and reverses the development of pulmonary hypertension. In PAH, lung vascular remodeling is characterized by proliferation and migration of both PASMCs and PAECs. Herein, we show that extracellular cAMP treatment exerts beneficial effects by increasing intracellular cAMP formation and activating the PKA-CREB pathway, which results in decreased PASMC and PAEC proliferation and migration *in vitro* and reduced pulmonary vessel remodeling *in vivo*. Our results are the first demonstration *in vivo* that extracellular cAMP has a signaling role in the lung. Our

study is in agreement with a previous report that demonstrated the anti-proliferative effect of the cyclic AMP–Adenosine pathway in aortic vascular SMCs *in vitro* [15].

The extracellular cAMP pathway might be commonly expressed in many other mammalian tissues. Indeed, several tissues/cells have been shown to express the cAMP-Adenosine pathway, including kidney [24], heart [13], liver [25, 26], brain [27, 28], and skeletal muscle [29]. We recently reported cAMP secreted into the extracellular space to be an important paracrine factor in the myocardium [13]. After β-adrenergic stimulation, cAMP is secreted from cardiomyocytes, and via binding of its metabolite adenosine to the adenosine receptors, activates intracellular cAMP formation in cardiac fibroblasts. Infused extracellular cAMP potently represses myocardial hypertrophic and fibrotic effects of chronic catecholamine stimulation *in vivo* [13]. The data presented here suggest that the extracellular cAMP pathway is activated during PH and that fibroblasts are the primary source of secreted cAMP in pulmonary arteries. Furthermore, cAMP may promote cell-to-cell communication within the same tissue. By being released from lung fibroblasts and then acting, through its metabolite adenosine, on pulmonary SMCs and ECs, cAMP may serve as a paracrine factor in the lung between pulmonary vascular cells. Estimating the potency of fibroblast-derived secreted cAMP in inhibiting pulmonary vascular cells proliferation/migration and defining whether cAMP exerts a paracrine action in pulmonary vascular cells will be of great relevance in the analysis of PAH. In addition, other cell types, such as pericytes or immune cells, may be able to export cAMP to the extracellular space in the lung.

Is cAMP extruded to the extracellular space only through cyclic nucleotide transporters? In a recent study, cAMP has been found to be encapsulated within extracellular vesicles from pulmonary microvascular endothelial cells [30]. Extracellular vesicles from pulmonary microvascular endothelial cells and from isolated lung perfusate display an increase in their cAMP content following stimulation of intracellular cAMP formation [30]. Therefore, mechanisms other than cAMP transport might contribute to the extracellular cAMP content.

Our results demonstrate the beneficial effect that exogenous extracellular cAMP has on cardiac

and pulmonary remodeling in PH. Through these experiments, where extracellular cAMP was infused through osmotic pumps that mediate a systemic delivery of the used drug, important topics are left open: (i) Since we previously reported that extracellular cAMP prevents cardiac hypertrophy in a disease model of cardiac pressure overload [13], one can imagine that extracellular cAMP may affect PH-induced RV hypertrophy by acting directly on the heart or through its effect in the lung. (ii) The extracellular cAMP dose used in the *in vivo* experiments has been chosen in order to saturate adenosine degradation. Indeed, adenosine is rapidly degraded to inosine by the adenosine deaminase. The present study does not identify the optimal extracellular cAMP dose that can be used to treat PH. (iii) The data presented here show the beneficial cardiopulmonary effects of extracellular cAMP infusion, however extracellular cAMP might have detrimental effects in other organs.

PDE5 is an enzyme involved in the regulation of cyclic guanosine monophosphate (cGMP) by being highly specific for its hydrolysis. PDE5 inhibitors, by preventing cGMP degradation, trigger vasodilation and inhibit PASMC proliferation [31, 32], however the beneficial effects that PDE5 inhibition has on RV hypertrophy and vascular remodeling are influenced by the natriuretic peptide pathway [33]. Simultaneously increasing intracellular cAMP and cGMP levels, by combining extracellular cAMP infusion and PDE5 inhibition, could represent an efficient therapy for attenuating the development of PAH. In addition, since ABCC proteins are able to extrude cGMP from cells [34], extracellular cGMP might represent another candidate for a protective pulmonary effect. Physiological roles for extracellular cGMP have been reported in kidney Na^+ transport [35] and in intestinal motility [36]. Extracellular cGMP at physiological concentration also prevents glutamate-induced death in primary cultures of cerebellar neurons [37], and modulates intracellular calcium and CaMKII activity in Purkinje neurons [38]. Since increasing intracellular cGMP levels is a good strategy for treating PAH, extracellular cGMP may represent an efficient therapeutic approach to regulate pulmonary vascular remodeling.

Our study is subject to certain limitations: (i) Since earlier studies have indicated that extracellular cAMP inhibits T cell activation [39, 40], inflammatory cells might contribute to the beneficial effect of extracellular cAMP in PH. (ii) Our data indicate increased expression of cyclic AMP-metabolizing enzymes, ABCC4 and A_2Rs in PAH. Since tissue lysates were prepared from whole tissue homogenates, several cell types (such as airway smooth muscle cells and epithelial cells) may contribute to the observed findings. (iii) Although PAH is a disorder in which small pulmonary arteries are affected, the human PASMCs and PAECs that were used in this study were isolated from large pulmonary arteries. (iv) Our study does not elucidate whether extracellular cAMP levels are regulated in PAH. Assessing the extracellular cAMP levels in PAH patients will expand our understanding of the mechanisms involved in PAH. However, since extracellular cAMP is rapidly degraded to adenosine, assessing extracellular cAMP levels in lungs or blood from PAH patients is technically challenging.

The data presented here indicate that the endogenous extracellular cAMP pathway is active during PAH and attempts to protect the lung from the detrimental consequences of pulmonary vascular remodeling. The effect of extracellular cAMP on pulmonary vascular remodeling requires a rise in intracellular cAMP concentrations. Therefore, intracellular cAMP is essential to mediate the extracellular cAMP effects.

Our data indicate that extracellular cAMP inhibits hPASMC proliferation and migration through A_{2B}R activation. This is consistent with previous reports that observed a beneficial effect of A_{2B}R in PAH when using activators of the A_{2B}R pathway [41, 42]. However, based on the use of A_{2B}R antagonists and A_{2B}R knockout mice, A_{2B}R has been suspected to have a deleterious role in PAH [42]. The disparity between the effects of A_{2B}R activation and A_{2B}R inhibition on pulmonary vascular remodeling requires clarification. Additionally, our results demonstrate that Adora1 and Adora3 are upregulated in the lungs of MCT-treated rats but not in those of hypoxia-exposed mice or in PAH patients. The MCT molecule itself might affect Adora1 and Adora3 expression

and lead to their up-regulation, independently from PH. Another plausible explanation is that Adora1 and Adora3 upregulation is species-specific.

Our results demonstrate that extracellular cAMP treatment is associated with an increase in intracellular cAMP levels and in CREB activity, which regulates the transcriptional activity of cell cycle-related genes. CREB phosphorylation induces p53, a major regulator of cell cycle progression [22, 43]. P53 activation leads to the repression of Cyclin D1, a protein required for progression through the G1 phase of the cell cycle [44]. Another possible mechanism of cell cycle arrest upon p53 activation is Cyclin-dependent kinase (CDK) inhibition by the cyclin-dependent kinase inhibitor p21. Indeed, p21 is a transcriptional target of p53 and can inhibit the activity of CDK1 and CDK2, key players in cell cycle regulation [45, 46].

In conclusion, our results indicate that extracellular cAMP regulates intracellular cAMP levels in pulmonary artery smooth muscle cells and endothelial cells, thus inhibiting their proliferation *in vitro* and reversing pulmonary vascular remodeling *in vivo* (Figure 7D). This study demonstrates the efficacy of extracellular cAMP in the treatment of pulmonary arterial hypertension in mice and in rats.

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Disclosures

None.

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Figure legends

Figure 1: ENPP1 and NT5E upregulation in human PAH and experimental PH animal models. A, ENPP1 and NT5E mRNAs expression levels were assessed by real-time PCR in healthy lungs from non-PAH subjects (n = 5) and in lungs from PAH patients (n = 7). **B**, (Left) Western blot analysis of ENPP1 and NT5E in lung homogenates from non-PAH and PAH patients. (Right) Quantitative analysis of the data. Protein expression was normalized to GAPDH. Data are from 5 non-PAH and 7 PAH patients. **C,** Changes in mRNA levels of ENPP1 and NT5E determined by real-time PCR analysis of lung extracts from control and MCT-injected rats (n = 6 rats/group). **D**, (Left) Representative western blot analysis of ENPP1 and NT5E protein expression in total lung extracts from control and MCT-injected rats (n = 4 rats/group). (Right) Quantitative analysis of the data. **E**, Changes in mRNA levels of ENPP1 and NT5E determined by real-time PCR analysis of lung extracts from normoxic and Sugen/hypoxic mice

(n = 5 mice/group). **F**, (Left) ENPP1 and NT5E protein expression in lung homogenates from normoxic and Sugen/hypoxic mice (n = 4 mice/group). (Right) Quantitative analysis of the data. Unpaired Student's *t*-test was performed (*A*–D). * P < 0.05; ** P < 0.01.

Figure 2: Extracellular cAMP enhances the formation of intracellular cAMP in pulmonary vascular cells. **A**, Study design to assess cAMP efflux from primary lung cells. **B**, Quantification of extracellular cAMP in supernatants from cultured hPAEC, NHLF and hPASMC treated with 10 µM Forskolin or PBS. A phosphodiesterase inhibitor (IBMX, 300 µM) was added to prevent cAMP degradation. n = 3 experiments in duplicate. Quantification represents fold changes (Fsk-treatment / basal) of extracellular cAMP level in supernatants from the indicated cells. **C**, Quantitative PCR analysis of ABCC4 mRNA by qPCR in RNA isolated from the indicated cells. n = 4 experiments in duplicate. **D**, cAMP-induced conformational switch in Epac causes a drop in FRET. **E** and **F**, (Left) Emission intensities of tdcp173V (535 nm, blue) and mTurq2d (480 nm, green) were recorded simultaneously from single cells expressing the cAMP sensor using fluorescence microscopy. Emission intensities were recorded before and after extracellular cAMP (□-cAMP, 100 µM) addition in hPASMC (**E**) and hPAEC (**F**). (Right) Representative tracings of intracellular cAMP formation in the indicated cells in the presence of extracellular cAMP. **G**, Quantitative analysis of the results. n = 3–4 experiments with 2-4 cells each. One-way ANOVA analysis with Bonferroni correction was performed. * P < 0.05; ** P < 0.01; *** P < 0.001.

Figure 3: Extracellular cAMP inhibits pulmonary vascular cells proliferation and migration. **A** and **B**, Proliferation (**A**) and migration (**B**) of hPASMC in the presence or absence of extracellular cAMP (□-cAMP, 100 µM) and antagonists against A1R (DPCPX; 100 nM), A2AR (SCH-442416; 100 nM), A2BR (PSB-1115; 500 nM), or A3R (VUF-5574; 100 nM). **C**, Quantification of endogenous adenosine receptor subtype expression in cultured hPASMC. n =

4 experiments in duplicate. **D** and **E**, Proliferation (**D**) and migration (**E**) of hPAEC in the presence or absence of extracellular cAMP and specific adenosine receptors antagonists. n = 3-4 experiments in triplicate. **F**, Quantification of endogenous adenosine receptor subtype expression in cultured hPAEC. n = 4 experiments in duplicate. **G**, Migration of hPASMC (left) and hPAEC (right) in the presence or absence of □-cAMP (100µM) and a PKA inhibitor (PKI, 10µM). n = 4 experiments in triplicate. **H**, Quantitative evaluation of the activity of the cAMP-responsive element CRE measured with the luciferase reporter CRE-Luc in proliferating hPASMC and hPAEC in the presence or absence of □-cAMP and the PKA inhibitor (PKI, 10µM). n = 4 experiments in triplicate. **I**, Quantitative evaluation of p53 activity measured with the luciferase reporter p53-Luc in hPASMC and hPAEC in the presence or absence of □-cAMP (100µM). n = 4-5 experiments in duplicate. One-way ANOVA followed by Holm-Sidak's test analysis was performed (A-H). Unpaired Mann-Whitney *U* test was performed (I). ** $P < 0.01$; *** $P < 0.001$.

Figure 4: ENPP1 inhibition exacerbates mouse Sugen/hypoxia-induced PH. A, Ten-week-old C57B6 mice subcutaneously received 20 mg/kg of Sugen (SU5416) and were then exposed to two weeks of chronic hypoxia. Sugen was injected once a week during the first 2 weeks. SYL-001 (7.5 mg/kg) or a saline solution were intraperitoneally injected every 2 days during the next 2 weeks. **B**, Fulton index and right ventricular pressure (RVSP) of the indicated groups. n = 4-8 mice/group. **C**, (Up) Representative wheat germ agglutinin (WGA)-staining of ventricular sections to assess hypertrophy of cardiac myocytes. Scale bar: 20 µm. (Down) Quantitative analysis; n = 4-6 mice/group. **D**, (Up) Representative H&E-stained sections of small pulmonary arteries from the indicated groups. Scale bar: 50 µm. (Down) Percentage of medial wall thickness. n = 5-8 mice/group. One-way ANOVA analysis with Bonferroni correction was performed (B-D). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 5: Extracellular cAMP reverses rat MCT-induced PH. A, Rats received a single injection of MCT (60 mg/kg) and miniosmotic pumps containing □-cAMP (delivering 30 mg/kg/day) were implanted 3 weeks later into the animals. Pulmonary and Right Ventricular hemodynamics were measured on week 5. **B**, (Left) RV hypertrophy reflected by the RV weight over LV plus interventricular septum (S) weight ratio (Fulton index) and (Right) Right ventricular systolic pressure (RVSP) in each group. n = 6-8 mice per group. **C**, Pulmonary artery systolic pressure (PASP), diastolic pressure (PADP), and mean pulmonary pressure (mPAP) measured in the indicated groups. n = 6 mice per group. **D**, Quantitative real-time PCR analysis of molecular markers for cardiac myocyte hypertrophy (*Nppa*) and of fibrosis-associated gene (*Col1a1*). All quantifications derive from n = 6-8 rats/group, PCR performed with 2 replicates each. **E**, Real-time PCR quantification of markers for lung fibrosis (*Col1a1* and *Fibronectin*) in right lung tissue from the indicated groups. n = 6-7 rats/group. **F**, (Up) Representative WGA-staining of ventricular sections to assess hypertrophy of cardiac myocytes. Scale bar: 50 μm. (Down) Quantitative analysis; n = 6-8 rats/group. **G**, (Up) Representative H&E-stained sections of small pulmonary arteries from the indicated groups. Scale bar: 50 μm. (Down) Percentage of the medial thickness of small arteries in relation to the cross-sectional diameter. n = 6-7 rats/group. One-way ANOVA analysis with Bonferroni correction was performed (B-G). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 6: Extracellular cAMP reverses Sugen/hypoxia-induced PAH. A, Ten-week-old C57B6 mice subcutaneously received 20 mg/kg of Sugen (SU5416), and were then exposed to three weeks of chronic hypoxia. Sugen was injected once a week during the next two weeks. Mice were then randomly assigned to receive miniosmotic pumps containing □-cAMP or a saline solution at week 13 for 3 weeks. The end point for hemodynamic measurements and sacrifice was at week 16. **B**, Right ventricular (RV) weight, left ventricular (LV) weight, Fulton index and right ventricular pressure (RVSP) of the indicated groups. n = 7-11 mice/group. **C**,

Quantitative real-time PCR analysis of molecular markers for cardiac myocyte hypertrophy (*Nppa*) and of fibrosis-associated gene (*Col1a1*) in right myocardium of the indicated groups. n = 6-7 mice/group. **D**, Quantitative real-time PCR analysis of molecular markers of fibrosis-associated genes (*Col1a1* and *Fibronectin*) in lungs of the indicated groups. Quantifications derive from n = 6-7 mice/group, PCR performed with 2 replicates each. **E**, (Up) Representative WGA-staining of ventricular sections to assess hypertrophy of cardiac myocytes. Scale bar: 50 µm. (Down) Quantitative analysis; n = 7 mice/group. **F**, (Up) Representative H&E-stained sections of small pulmonary arteries from the indicated groups. Scale bar: 50 µm. (Down) Percentage of arteries medial thickness in relation to cross-sectional diameter. n = 6-8 mice/group. **G**, (Up) Representative image sections from Masson trichrome-stained lung of the indicated groups. Scale bar: 50 µm. (Down) Quantitative analysis of perivascular fibrosis; n = 8-9 mice/group. One-way ANOVA analyses with Bonferroni correction (B, G) or followed by Holm-Sidak's test analysis were performed (C-F). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 7: Extracellular cAMP exerts its effects through the PKA/CREB/p53/Cyclin D1 pathway. A, (Left) Western immunoblotting was performed to evaluate CREB phosphorylation, total CREB, tumor protein 53 (p53), and Cyclin D1 expression. The lungs were harvested from sham and MCT-PH rats treated with □-cAMP or a saline solution. (Right) Quantitative analysis of the western blot data. Protein expression was normalized to GAPDH. Phosphorylated CREB expression was normalized to total CREB. Data are from 3 rats per group. **B**, (Left) Representative western blot of phosphorylated CREB, total CREB, p53, and Cyclin D1 protein expression in total lung extracts from mice that were exposed or not to Sugen/hypoxia and □-cAMP treatment. (Right) Quantitative analysis of the data. Data are from 3-4 mice per group. **C**, (Left) Phosphorylated CREB, total CREB, p53, and Cyclin D1 protein expression in healthy lung homogenates from non-PAH subjects and in lung homogenates from PAH patients. (Right) Quantification of the data. Data are from 5 non-PAH and 7 PAH patients. **D**, Model of

mechanism of action for the extracellular cAMP in PAH. One-way ANOVA followed by Holm-Sidak's test analysis was performed (A, B, D-F). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 1

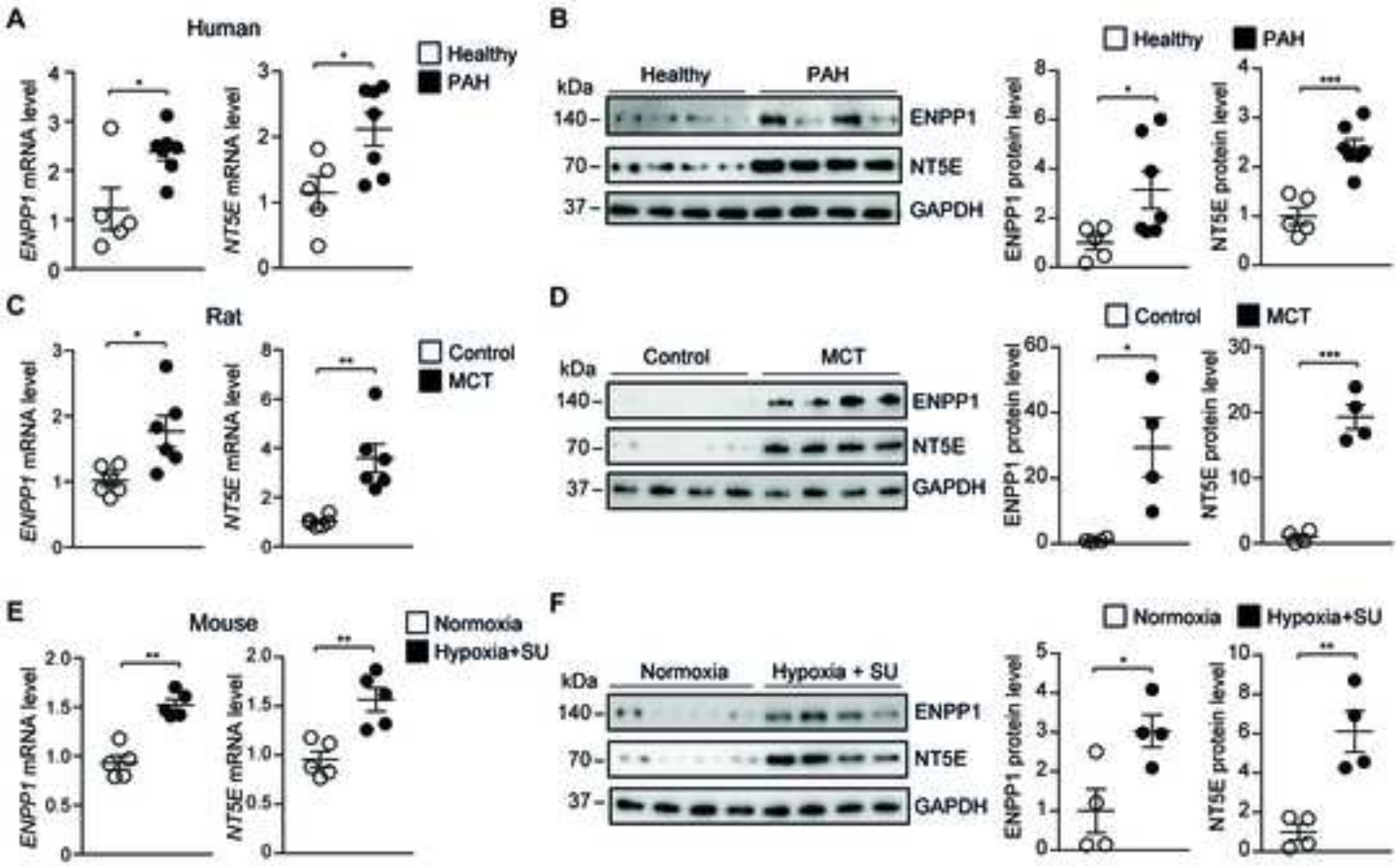


Figure 2

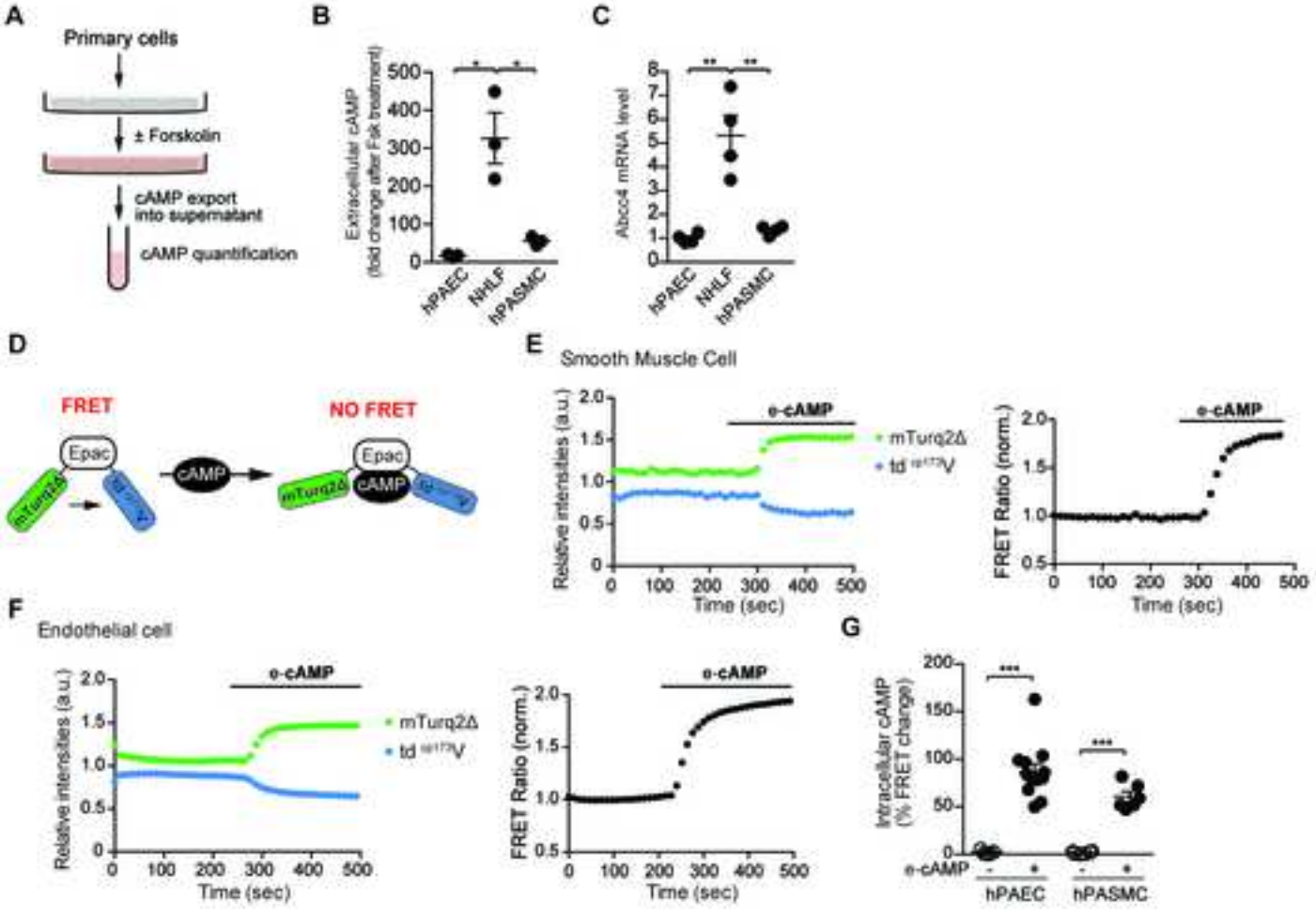


Figure 3

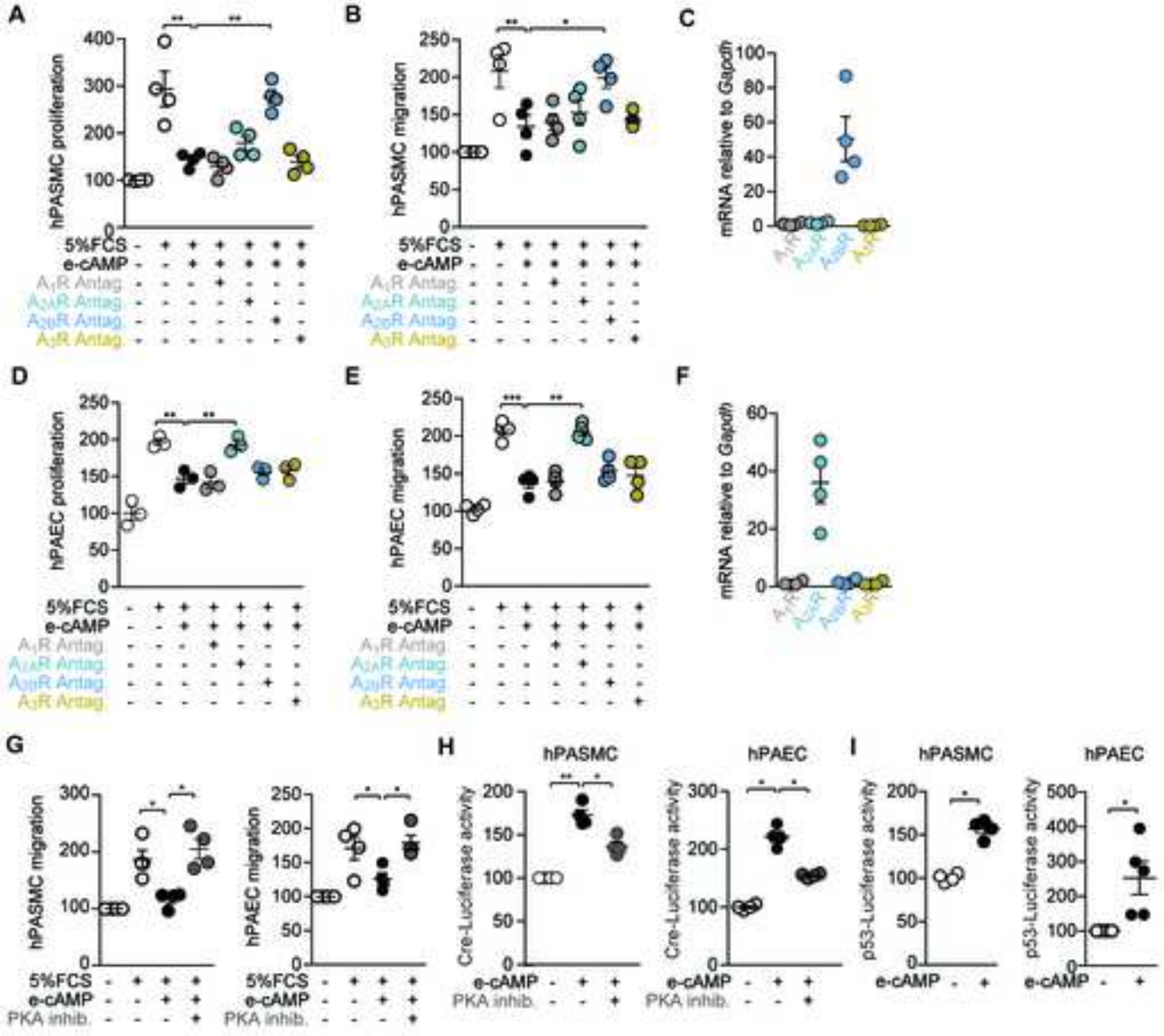


Figure 4

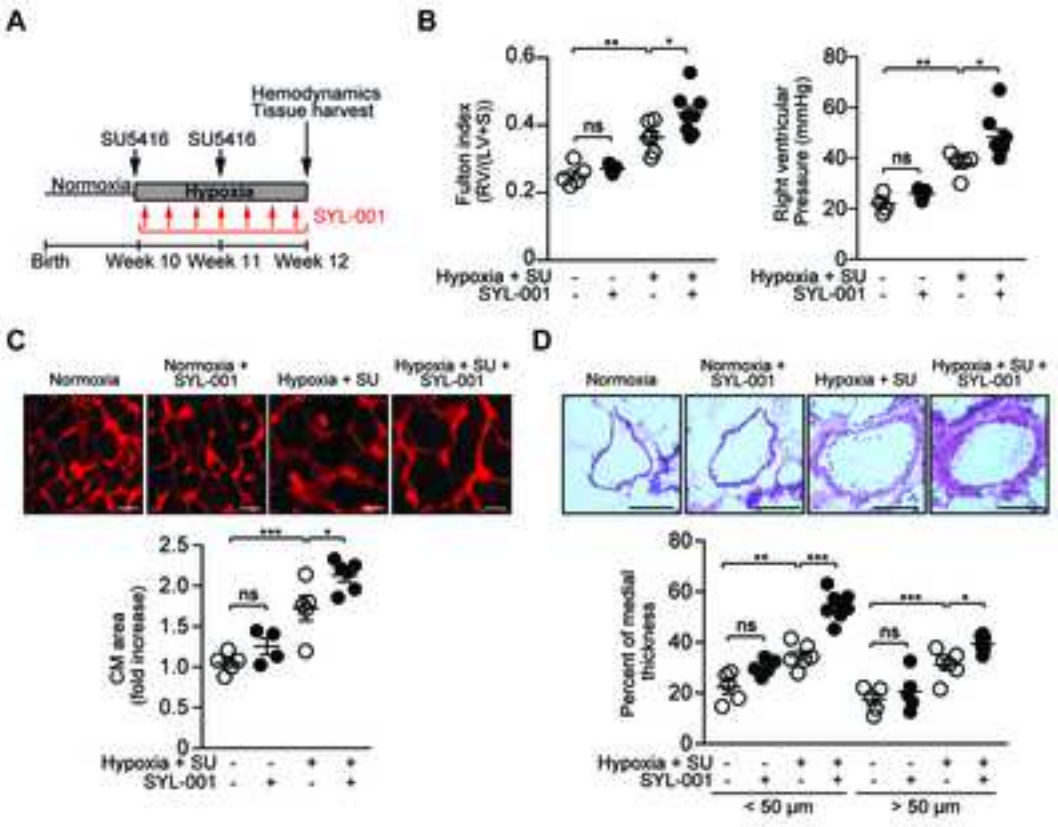


Figure 5

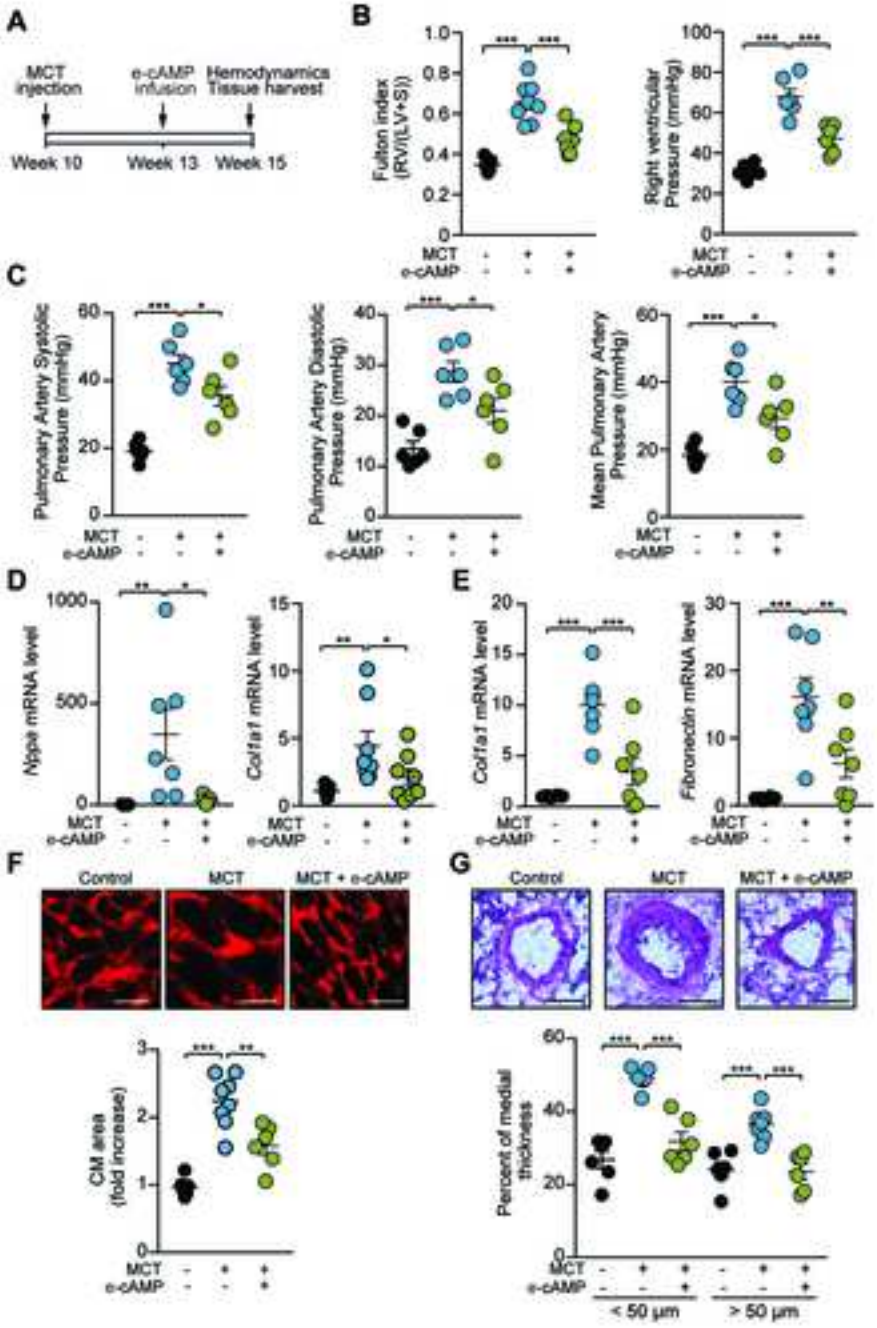


Figure 6

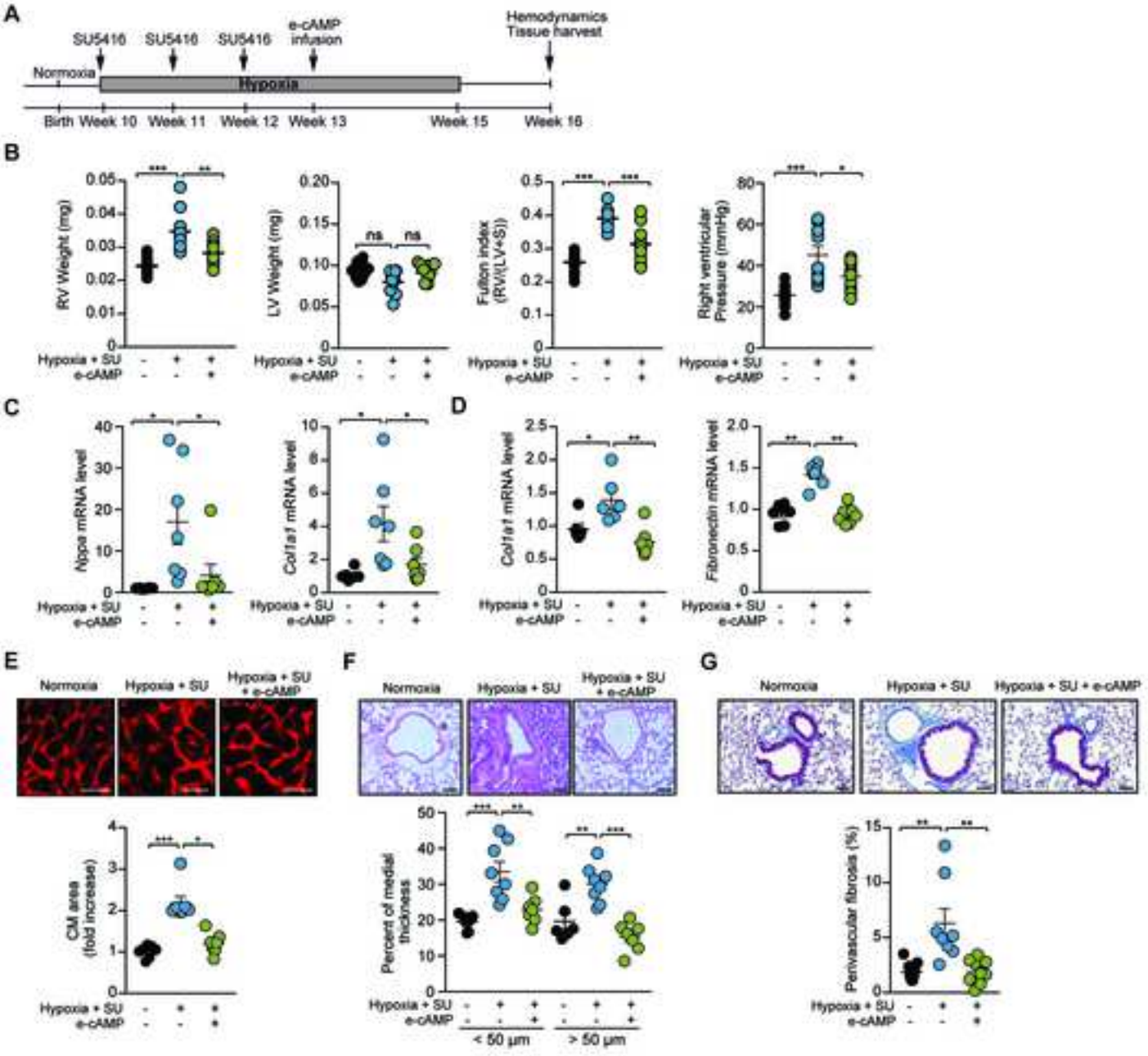
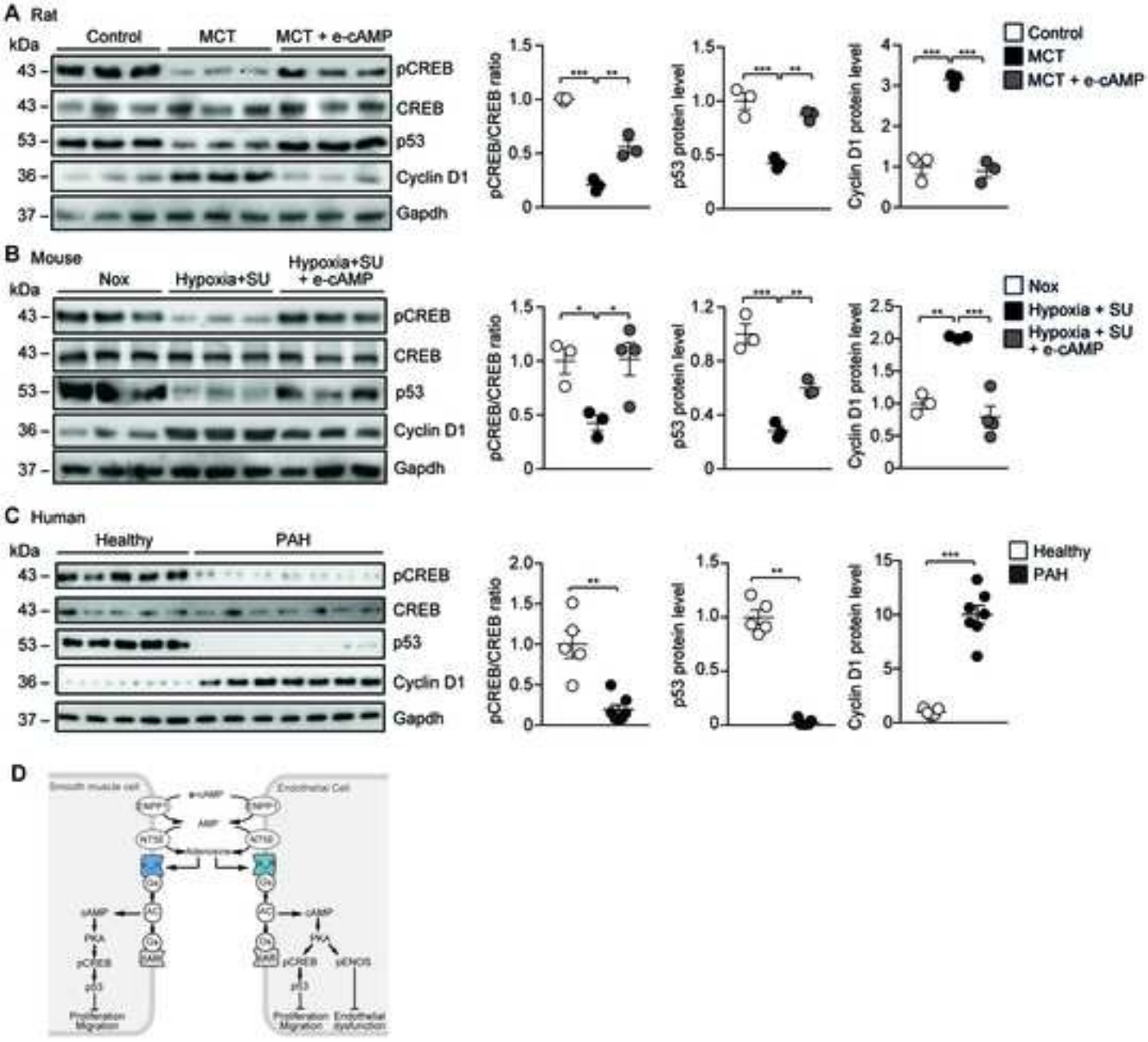


Figure 7



Graphical Abstract