CREB depletion in smooth muscle cells promotes medial thickening, adventitial fibrosis and elicits pulmonary hypertension

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Abstract

Levels of the cAMP-responsive transcription factor, CREB, are reduced in medial smooth muscle cells in remodeled pulmonary arteries from hypertensive calves and rats with chronic hypoxia-induced pulmonary hypertension. Here, we show that chronic hypoxia fails to promote CREB depletion in pulmonary artery smooth muscle cells or elicit significant remodeling of the pulmonary arteries in mice, suggesting that sustained CREB expression prevents hypoxia-induced pulmonary artery remodeling. This hypothesis was tested by generating mice, in which CREB was ablated in smooth muscle cells. Loss of CREB in smooth muscle cells stimulated pulmonary artery thickening, right ventricular hypertrophy, profound adventitial collagen deposition, recruitment of myeloid cells to the adventitia, and elevated right ventricular systolic pressure without exposure to chronic hypoxia. Isolated murine CREB-null smooth muscle cells exhibited serum-independent proliferation and hypertrophy in vitro and medium conditioned by CREB-null smooth muscle cells stimulated proliferation and expression of extracellular matrix proteins by adventitial fibroblasts. We conclude that CREB governs the pathologic switch from homeostatic, quiescent smooth muscle cells to proliferative, synthetic cells that drive arterial remodeling contributing to the development or pulmonary hypertension.

Keywords
smooth muscle cell, pulmonary hypertension, CREB, adventitia, fibrosis

Introduction

Hypoxia-induced pulmonary hypertension (PH) observed in chronic obstructive pulmonary diseases such as emphysema and chronic bronchitis, and sleep-related hypoventilation disorders¹ is a major cause of morbidity and mortality. Despite major advances in the treatment of severe cardio-pulmonary conditions, PH remains a deadly disease that is largely unresponsive to current treatment regimens.

PH is characterized by thickening of the pulmonary artery (PA) wall and persistent vasoconstriction of the pulmonary vasculature.² Thickening of the PA wall is the result of medial smooth muscle cell (SMC) proliferation and hypertrophy, and extracellular matrix production. In addition, contractile SMCs appear around previously nonmuscularized distal arterioles, transforming them into...
“resistance vessels.” Often the earliest and most significant structural changes occur in the adventitia of the PA. These changes include proliferation of adventitial fibroblasts, their acquisition of myofibroblast characteristics, and deposition of extracellular matrix components. These processes are associated with narrowing of the arterial lumen and progressive reduction in vessel wall distensibility.

Phenotypic changes in medial SMCs and adventitial cell populations are triggered by the expression of growth factors including platelet-derived growth factor-BB (PDGF-BB), vascular endothelial growth factor, transforming growth factor-β, endothelin-1, and thrombospondin-1. These growth factors stimulate several intracellular signaling systems including mitogen-activated protein kinases, phosphatidylinositol-3-kinase/Akt pathway. These growth-promoting and chemotactic pathways are normally restrained in healthy arteries due to the low level of growth factor and cytokine production. In addition, vasodilatory agents such as prostacyclins and nitric oxide exert antiproliferative effects on SMCs by increasing intracellular cyclic nucleotides, which stimulate the activity of protein kinases A and G (PKA and PKG).

Cyclic-AMP response element binding protein (CREB) is the primary regulator of gene transcription in response to elevated intracellular cAMP levels. Given the potent proliferation-suppressing action of cAMP on SMCs, and CREB's role as a mediator of cAMP signaling, we reasoned that CREB might participate in controlling SMC proliferation. In testing this hypothesis, we previously demonstrated that CREB levels are reduced in medial SMCs in remodeled PAs from hypertensive calves and rats with chronic hypoxia-induced PH. In vitro studies demonstrated that forced depletion of CREB with siRNA promotes SMC proliferation and migration, leads to decreased expression of differentiated SMC markers like SM-myosin and calponin, as well as increased collagen and elastin production. These changes are consistent with those observed in the PA as it remodels, wherein SMC dedifferentiation, proliferation, migration, and extracellular matrix synthesis are upregulated and contribute to vascular remodeling, thickening, and stiffening.

To directly assess the role of CREB in pulmonary hypertension, McLoughlin et al. measured a variety of pulmonary vascular endpoints in CREB hypomorphic mice (CREB α/Δ knockout mice) exposed to normoxic and hypoxic conditions. They found that the loss of CREBs α and Δ upregulates RNA for the β isoform of CREB in lung lysates, while the CREB protein levels for all three isoforms remain low. Moreover, while hypoxia changed pulmonary vascular resistance, pulmonary vessel wall thickness, and left lung volume, the loss of CREB α/Δ isoforms had minimal effect alone or with hypoxia. Since these results ran counter to observations we reported in models of chronic hypoxic PH in neonatal calf and rat lungs or isolated SMCs we tested whether complete ablation of CREB in SMCs regulates homeostasis and transition to disease of the pulmonary vasculature. Ablation of CREB α, β and Δ isoforms in medial SMCs was achieved using the Sm22 gene promoter-driven CRE recombinase to excise loxP-floxed introns common to all three isoforms of CREB. Ablation of CREB in SMCs promoted structural and hemodynamic changes consistent with PH in vivo. CREB-deficient SMCs exhibited serum-independent proliferation and hypertrophy in vitro and secreted soluble factors that stimulated proliferation and expression of extracellular matrix proteins by adventitial fibroblasts. We conclude that CREB is the full molecule responsible for regulating the pathologic switch from homeostatic, quiescent SMCs to proliferative, synthetic cells that drive arterial remodeling contributing to the development of PH.

Materials and methods

Materials

Fetal bovine serum, glutamine, and penicillin/streptomycin were purchased from Gemini-Bio products (Sacramento, CA). Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F-12 50/50 mix was from Corning CellGro (Manassas, VA). CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI). QCM 24-well colorimetric cell migration assay was purchased from EMD Millipore (Temecula, CA). Cell culture media was analyzed using a mouse cytokine antibody array C2000 (RayBiotech; Norcross, GA) designed to analyze 144 mouse proteins simultaneously. Polyclonal antibody to α-smooth muscle (SM) actin was purchased from Sigma (St. Louis, MO), and polyclonal antibody to smooth muscle (SM)-myosin heavy chain 2 was from Abcam (Cambridge, MA). Rabbit polyclonal antibody to CREB was from Cell Signaling Technology, Inc. (Beverly, MA) and rabbit polyclonal anti-actin (C-11) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NE-PER™ Nuclear and Cytoplasmic Extraction reagents were from Life Technologies (Grand Island, NY). Picosirius red stain was obtained from Electron Microscopy Sciences (Hatfield, PA). Alexa Fluor 594 and Alexa Fluor 488-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). VectaShield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA). Single-stranded DNA PCR primers for fibronectin (forward-CCGGGTTCTTGAGTACACAGTC and reverse-GGAGGGTCTCTTCACCAGGGA), collagen I (forward-TGTTCGTGGTTCTCAGGGTAG and reverse-TTGTCGTAGCAGGGTTCTTTC), and GAPDH (forward-CTCATGACACAGTCCATGC and reverse-CACATTGGGGTGATGACAC) were obtained from Integrated DNA Technologies, Inc. (Coralville, CA).
**SMC-targeted CREB knockout mouse model**

SM22cre mice (Tg(Tagln-cre)1Her/J, cat. No. 004746) were obtained from The Jackson Laboratory (Bar Harbor, Maine). Floxed CREB mice were graciously provided by Dr. Gunter Schutz (German Cancer Research Center, Heidelberg, Germany). SM22cre mice were bred to floxed CREB mice to produce SMC-specific CREB-knockout mice (SM22creCREBfl/fl, designated SMC CREB KO hereafter) carrying a single SM22cre allele and two floxed CREB alleles. Littermates bearing two floxed CREB alleles but having no SM22cre gene (CREBfl/fl, designated Control (Cntrl) hereafter) were used as controls. Mouse genotypes from tail biopsies were determined using real-time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN). Only male mice were used in these studies as co-lateral studies in female mice revealed substantial variability in certain endpoints related not only to CREB depletion or exposure to hypoxia, but also to age and ovarian cycle timing.

**Hypoxia exposure**

All animal studies were performed with approval and in accordance with the guidelines of the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee. Eight-week-old male mice were exposed to ambient normoxia (Denver altitude, ~5200 ft, 1600 m, 630 mmHg) or hypobaric hypoxia (18,000 ft, 5500 m, 410 mmHg) as indicated in the figures for up to five weeks with interruptions of <1 h every three to four days for animal maintenance. Animals were allowed ad libitum access to food and water, and kept on a 12-h day–night cycle.

**Verification of hypoxia in animals**

Hematocrit was measured at the end of each study in capillary tubes as the ratio of packed red blood cell volume to total blood sample volume.

**In vivo hemodynamic measurements and tissue procurement**

Mice were anesthetized with ketamine and xylazine (100 and 15 mg/kg, respectively) and placed supine while spontaneously breathing room air; a 26-gauge needle was introduced percutaneously into the thorax via a subxiphoid approach. Right ventricular systolic pressure (RVSP) was verified in real time and recorded. Animals from chronic hypoxia exposure were kept hypoxic until immediately before hemodynamic measurement. After hemodynamic measurements, the mice were euthanized by exsanguination. Phosphate buffered saline (PBS) containing 5 mM ethylenediamine tetracetic acid (EDTA) was perfused through the pulmonary circulation to remove red blood cells. Lungs were then inflated and fixed with 4% paraformaldehyde-PBS-5 mm EDTA. The heart and lungs were removed in bloc.

**Measurements of right ventricular hypertrophy**

The heart was resected and the atria were removed at the plane of the atrioventricular valves. The right ventricular (RV) free wall was then dissected free of the left ventricle (LV) and septum (S). The RV and LV plus S were weighed (g), and the RV/(LV + S) ratio was calculated as an index of RV hypertrophy.

**Lung morphometric analysis**

Five micrometer sections of paraformaldehyde-fixed, paraffin-embedded lung tissue were deparaffinized with CitriSolv, and rehydrated in a graded ethanol/water series. Sections were subjected to antigen retrieval in citrate buffer in a microwaveable pressure cooker for 20 min. Sections were blocked with PBS containing 5% horse serum for 30 min at room temperature. The sections were incubated overnight in PBS/5% horse serum at 4°C with the primary antibodies indicated in the figure legends. The sections were then washed and incubated with the indicated Alexa Fluor-conjugated or horse radish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Sections of fixed lung or heart tissue were subjected to hematoxylin-and-eosin (H&E) or trichrome staining by the University of Colorado Anschutz Histology Core Laboratory. Immunohistochemistry for Ki-67 was performed by IHC Tech (Aurora, CO). Picrosirius red staining of lung sections was performed as directed by the supplier’s instructions.

Distal pulmonary vessels (outside diameter 10–50 μm) were assessed by a blinded observer for degrees of circumferential SM actin-positive staining indicative of muscularization. Vessels smaller than 10 μm were considered capillaries and excluded from further consideration. Proximal vessels (outside diameter 50–250 μm) were analyzed for medial wall thickness by measuring the area of the SM-actin-staining and correcting for lumen area via computer-assisted image analysis (NIS Elements software, Nikon Corp.). SMC nuclei were localized by their presence within the SM-actin-expressing region of the PA wall by immunohistochemistry. CREB content of SMC nuclei was measured using the Circular Region of Interest Tool to measure the fluorescence of circular regions in individual nuclei with diameters equal to the mean width of each nucleus. The fluorescence signal of individual nuclei was recorded in arbitrary units. Medial cell number was determined by counting DAPI-stained nuclei in the SM-actin-stained region of proximal vessels, and corrected for lumen area as an index of SMC proliferation. In vivo SMC hypertrophy was determined by dividing the area of the SM-actin-positive region
by the number of DAPI-stained nuclei to calculate the average cross-sectional area.

Microscopy was performed using a Nikon TE2000-U inverted epifluorescence microscope. Brightfield, phase contrast, and fluorescent digital images were captured to a personal computer with either a color Nikon Digital Sight DS-Fi2 or a monochrome Nikon Digital Sight DS-Qi1Mc camera.

**Isolation and characterization of Cntrl and SMC CREB KO PA SMCs**

Five hundred-micrometer PAs were recovered from mice lungs. Segments of the PAs were cut open and mechanically stripped of adventitia and endothelium. The segments were then placed lumen-side-down into individual wells of a six-well plate. Tissue explants were maintained in complete DMEM/F12 supplemented with 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 10% fetal bovine serum.

Since our goal was to obtain pure subpopulations of SMCs, we selectively isolated individual cell colonies with a distinct, although uniform, morphological appearance from primary culture using cloning cylinders. Expression of SMC-specific markers (SM actin and SM-myosin heavy chains) in each isolated cell subpopulation was examined. Only cell subpopulations with uniform morphological appearance and uniform patterns of expression of SMC markers were selected for further experimentation. Individual cell colonies growing from tissue explants in primary culture were isolated by placing cloning cylinders (5 to 10 mm in diameter, greased on the bottom) over each cell colony of interest. Cells within the ring were trypsinized and transferred to a 24-multiwell plate for expansion. Cell cultures were tested for mycoplasma contamination using the Gen-Probe Mycoplasma T. C. Rapid Detection System (GenProbe Inc., San Diego, CA). All studies were carried out using cells at passages 1 to 8. For low serum conditions, cells were transferred to DMEM/F-12 containing 0.2% fetal bovine serum.

**Reverse transcriptase PCR**

cDNA was prepared from SMCs using Cells-to-cDNA II (Ambion, Austin, TX) reagents according to the manufacturer’s instructions. PCR amplification was performed with 3 μl of transcribed cDNA and 1 pmol of each primer for 30 cycles of hot start at 94°C for 1 min, denaturation at 94°C for 1 min, annealing at the appropriate temperature for 45 s, and elongation at 72°C for 2 min followed by a final elongation for 8 min at 72°C. Negative PCR controls included omission of reverse transcriptase and omission of cDNA. GAPDH primers were used to validate each batch of the template before use. PCR products were resolved on 2% agarose gels containing ethidium bromide and photographed under UV illumination.

**Subcellular fractionation and Western blots**

Nuclear lysates were prepared from SMCs using NE-PER reagents following the supplier’s instructions. After correcting for protein concentrations, cell lysates were mixed with an equal volume of Laemmli SDS loading buffer, resolved on 10% polyacrylamide-SDS gels, and transferred to PVDF membranes. The blots were blocked with phosphate-buffered saline (PBS) containing 5% dry milk and 0.1% Tween 20, and then treated with antibodies that recognize the target proteins indicated in each figure overnight at 4°C. The blots were washed and subsequently treated with appropriate secondary antibodies conjugated to horseradish peroxidase. After the blots are washed, specific immune complexes were visualized with SuperSignal West Pico Chemiluminescent Substrate (Life Technologies, Grand Island, NY).

**Cell proliferation assay**

SMC proliferation was measured with CellTiter 96 AQ Assay Kit according to the manufacturer’s direction. Assays were performed by adding a small amount of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 1–4 h and then recording the absorbance at 490 nm with a 96-well plate reader.

**Cell migration assay**

SMC migration was measured with QCM 24-well colorimetric cell migration assay kit according to the manufacturer’s instructions. Cntrl or SMC CREB KO PA SMCs that migrated through the polycarbonate membrane were incubated with a “Cell Stain Solution” and then subsequently extracted and detected on a standard 24-well microplate at 570 nm.

**Flow cytometry**

Unstained control and SMC CREB KO PA SMCs were analyzed on a CyAn ADP flow cytometer (Beckman Coulter). The forward scatter area of singlet events was used as an index of cell size.

**Conditioned medium**

Control and SMC CREB KO cells were cultured at 2.5 × 10⁵ cells on 100 mm plates in DMEM/F-12 containing 10% FBS for 48 h. Medium was then collected and frozen down in aliquots, stored at −80°C.

**Membrane-based mouse cytokine microarray assay**

For microarray assay, we followed the directions as stated by the manufacturer. Briefly, membranes were placed in an eight-well tissue culture tray and incubated with 2 mL of a 1 x blocking buffer at room temperature for 30 min to block membranes. After decanting the blocking buffer from each container, 1 mL of undiluted cell culture media samples
collected from either control or SMC CREB KO PA SMCs were added and incubated overnight at 4°C. After decanting the samples, all membranes were washed and incubated with biotinylated antibody cocktail followed by horseradish peroxidase-streptavidin. Membranes were wrapped in plastic wrap and exposed to radiographic film (Kodak X-Omat; Kodak; Rochester, NY) for 20 min, and the signal was detected using film developer.

Statistics
Statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA). Differences among groups were assessed using one-way ANOVA or Kruskal-Wallis rank ANOVA. Differences between pairs were assessed using Mann-Whitney U-test. Results are presented as means ± SE. p ≤ 0.05 was considered significant.

Results
Chronic hypoxia fails to elicit PA remodeling or CREB loss in medial PA SMCs in mice
Our previous studies demonstrated that CREB is diminished in medial SMCs in PA of calves and rats exposed to chronic hypoxia, a phenomenon evident in Supplementary Figs. 1 and 2. In addition, forced depletion of CREB in isolated SMCs produces a proliferative, dedifferentiated and synthetic phenotype similar to the phenotype associated with pathological PA remodeling in PH. Several reports have shown that variability between species exists when developing PH upon exposure to chronic hypoxia. Mice exhibit less remodeling in response to chronic hypoxia than rats and humans, all of which respond less than neonatal calves when exposed to more severe hypoxic stimulus.

To explore the contribution of CREB to SMC phenotype in mice in vivo, nuclear CREB was visualized by immunohistochemistry in lung sections from mice following exposure to ambient normoxia or chronic hypoxia. CREB was detected throughout the PA wall and lung parenchyma in normoxic and hypoxic mouse lung sections by co-localization of the red CREB signal with the blue fluorescence of DAPI-stained nuclei resulting in a combined magenta nuclear signal (Fig. 1). Of note, there was minimal remodeling and no decrease in expression of SMC CREB in hypoxic mouse lung sections (Fig. 1a and b). Chronic hypoxia-induced PH in calf and rat models occurs concomitant with the loss of SMC CREB, whereas minimal PA remodeling is observed in chronic hypoxic mice in which CREB levels are maintained. These results suggest a possible causative relationship between CREB expression and PA remodeling.

SMC CREB depletion promotes medial thickening of the PA in normoxic mice
We generated mice lacking CREB in SMCs to directly test whether loss of CREB contributes to PA remodeling and the development of PH. To achieve targeted, and complete knockdown of CREB in SMC, we mated floxed CREB mice to a driver strain in which Cre recombinase expression was controlled by the SM22 (transgelin) gene promoter. Offspring hemizygous for SM22cre and homozygous for floxed CREB (SMC CREB KO) were selected for use in all experiments, and homozygous CREB mice lacking SM22cre were used as controls (Cntrl). Cre-mediated excision of exon 10 of the CREB gene in SMCs of the offspring results in the expression of a rapidly degraded CREB RNA molecule. Fig. 2a and b shows that CREB RNA and protein were virtually undetectable in cultured primary PA SMCs by RT-PCR or Western blot, respectively. Fig. 2c and d shows that CREB co-localizes with nuclei (red CREB signal plus blue nuclear DAPI staining yields combined magenta nuclei) including nuclei of actin-positive pulmonary artery medial SMCs of Cntrl mice. However, CREB is specifically depleted in actin-positive medial SMCs (blue nuclear DAPI signal with no overlapping red CREB signal) in SMC CREB KO mice but remains present in cells lining the vessel wall, airway epithelium, and lung parenchyma.

SMC CREB depletion promotes the development of PH in normoxic mice
Hemodynamic parameters and pulmonary vascular structure were compared in adult normoxic Cntrl and SMC CREB KO male mice to determine whether the loss of SMC CREB was sufficient to elicit features of hypoxia-induced PH. Age-matched wild type mice exposed to five weeks of chronic hypobaric hypoxia to induce PH were included in the experiments, so that changes in pulmonary structure and function due to hypoxia or CREB ablation could be compared. Hemodynamic evaluation of SMC CREB KO mice revealed significantly elevated RV systolic pressure (RVSP, Fig. 3a) and RV hypertrophy by Fulton’s index (Fig. 3b), both key manifestations of PH. These endpoints were also elevated in Cntrl mice subjected to chronic hypoxia. In SMC CREB KO mice, these endpoints were accompanied by significant thickening of the PA media (Fig. 3c), which was confirmed by morphometric analysis (Fig. 3d). Medial thickening was observed in Cntrl mice subjected to chronic hypoxia, but this did not achieve statistical significance in our studies. In contrast, hypoxia stimulated profound muscularization of distal arterioles, but CREB ablation had no impact (Fig. 3e and f).

The results indicated that remodeling of conduit arteries but not arterioles was linked to SMC CREB loss. The data further suggested that increased PA pressure and RV hypertrophy in wild-type mice exposed to chronic hypoxia were primarily due to vasoconstriction alone.

Loss of CREB in SMCs promotes profound PA adventitial collagen deposition and recruitment of myeloid cells in the absence of SMC proliferation or hypertrophy in vivo
Medial thickening is frequently attributed to proliferation and/or hypertrophy of medial SMCs, extracellular matrix
deposition, and/or inflammatory infiltrate. Changes in adventitial cell populations and structure were evaluated in SMC CREB KO mice. The most notable feature was profound deposition of collagen around thickened arteries, which was absent in normoxic control animals, and minimal and variable in hypoxic wild-type mice (Fig. 4a and b). An increased number of adventitial myeloid cells (F4/80 immunostaining) were also observed adjacent to PAs in SMC CREB KO mice compared to normoxic control or hypoxic Cntrl mice (Fig. 5a and b).

To assess CREB regulation of SMC proliferation we performed immunohistochemistry to detect the interphase-associated antigen, Ki-67. Localization of Ki67 failed to reveal actively proliferating SMCs in any of the treatment groups (Fig. 6a). However, Ki-67-positive nuclei were occasionally observed in the lung parenchyma, and clusters of Ki-67-positive cells were occasionally present between adjacent airways. Because SMC proliferation may occur prior to the onset of PH, the numbers of medial cells were also quantified by counting DAPI-stained nuclei in the
Fig. 2. Phenotyping of Cntrl and SMC CREB KO mice. (a) Semiquantitative RT-PCR of RNA from Cntrl and SMC CREB KO mice for Creb and Gapdh (internal control). Images show the absence of Creb RNA in SMC CREB KO mice. (b) Western blot analysis of whole cell lysates from Cntrl and SMC CREB KO mice with antibodies to total CREB and β-actin as indicated. Image shows that CREB levels in knockout mice are dramatically decreased. (c) Lungs were retrieved from eight-week male Cntrl and SMC CREB KO mice, fixed and embedded in paraffin. Sections were stained for CREB and SM-actin as described in Fig. 1. Figure shows representative phase contrast and fluorescence microscope images of proximal PAs adjacent to airways. CREB is present in the nuclei of PA SMCs in control lungs (red CREB fluorescence plus blue DAPI fluorescence = magenta signal in overlaid images), but not in medial SMCs of SMC CREB KO mice (blue DAPI signal only). Medial thickening of the SM-actin stained region was also evident in the SMC CREB KO mice. Scale bar = 60 μm. (d) CREB immunofluorescence was measured in SMC nuclei localized within the SM-actin-positive regions of PAs located in Cntrl and SMC CREB KO lung sections. Fluorescence measurements were taken for 30 nuclei/animal, 6 mice/treatment. Fluorescence signals are in arbitrary units. *p ≤ 0.05.
Fig. 3. SMC CREB KO mice exhibit a subset of features associated with PH. Eight-week-old, male Cntrl and SMC CREB KO mice were maintained at Denver, CO ambient (Norm) or hypobaric hypoxia (Hypox) for five weeks. (a) Right ventricular systolic pressure (RVSP) was obtained by performing a closed chest puncture to directly place a pressure transducer into the right ventricle. Results show elevated RVSP in hypoxic Cntrl and normoxic SMC CREB KO mice. (n = 6 mice/group) (b) Atria were removed from freshly recovered hearts. Right ventricle wet weight was divided by left ventricle plus septum wet weight (RV/LV+S). Data show RV hypertrophy in hypoxic Cntrl and normoxic SMC CREB KO mice. (n = 6 mice/group) (c) Representative brightfield photomicrographs of hematoxylin and eosin-stained lung sections show PA medial thickening in SMC CREB KO mice, but more modest remodeling in hypoxic Cntrl mice. Scale bar = 50 µm. (d) Morphometric analysis confirms significant medial thickening in animals with SMC CREB KO. (6 mice/group, 19 vessels/mouse) (e) Representative fluorescence photomicrographs of lung parenchyma stained with primary antibodies to SM-actin and Alexa 488-conjugated secondary antibodies. Results show increased number of muscularized distal arterioles in hypoxic Cntrl mice, but not in normoxic SMC CREB KO mice. Scale bar = 100 µm. (f) Morphometric analysis of the number of SM-actin-positive micro-vessels per field confirms increased distal muscularization in hypoxic Cntrl but not SMC CREB KO mice. (6 mice/group, 10 fields/mouse) Error bars indicate standard deviation; *p < 0.05.

Fig. 4. SMC CREB KO mice exhibit profound PA adventitial collagen deposition. Eight-week-old, male Cntrl and SMC CREB KO mice were maintained at Denver, CO ambient (Norm) or hypobaric hypoxia (Hypox) for five weeks. (a) Representative brightfield photomicrographs of picrosirius red-stained lung sections show PA adventitial collagen deposition in SMC CREB KO mice, but not in normoxic or hypoxic Cntrl mice. Scale bar = 50 µm. (b) Morphometric analysis confirms significant adventitial collagen levels in animals with SMC-targeted CREB loss. (6 mice/group, 19 vessels/mouse) Error bars indicate standard deviation; *p < 0.05.
SM-actin-staining region of PAs. This number was divided by arterial lumen area to correct for vessel size. Fig. 6b shows a significant increase in medial cell number between the normoxic SMC CREB KO mice and the Cntrl normoxic or hypoxic cohorts. In a similar manner, the area of the SM-actin-staining region of PAs was divided by the number of DAPI-positive nuclei as an index of cross-sectional area (Fig. 7). SMC cross-sectional area did not differ between experimental groups.

CREB-deficient PA SMCs exhibit serum-independent proliferation, and hypertrophy, but not migration in culture

Although SMC proliferation was not detected in lung sections from SMC CREB KO mice, we previously reported that siRNA-mediated depletion of CREB in primary PA SMCs stimulated their growth. Therefore, we measured the proliferation of PA SMCs isolated from Cntrl and SMC CREB KO mice to determine whether our previous results were in error. Interestingly, primary SMCs from SMC CREB KO mice were capable of proliferating in medium containing 0.2% fetal bovine serum, whereas the cells from Cntrl cells were quiescent (Fig. 8a). Medium containing 10% serum stimulated the growth of both Cntrl and SMC CREB KO SMCs, but the proliferation of SMC CREB KO SMCs was approximately three-fold greater. Migration of cells from both groups was measured in response to 0.2 versus 10% serum in Boyden chambers. No migration was evident at 4 or 8 h. After 18 h, a small number of cells from all groups had transited the membranes, but there were no significant differences in the number of migrating cells due to serum levels or CREB depletion (Fig. 8b). Visual inspection also revealed that CREB-deficient SMCs were much larger than the Cntrl SMC (Fig. 8c). Flow cytometry confirmed that the size (forward scatter area) of SMC CREB KO SMCs was greater than the SMCs from Cntrl mice (Fig. 8d). Thus, SMC CREB KO SMCs exhibit the same pro-remodeling characteristics in culture as wild-type SMCs depleted of CREB via siRNA, in spite of the absence of SMC proliferation and migration in the lungs of SMC CREB KO mice. The results suggest that the in vivo environment suppresses overt SMC proliferation and migration due to CREB loss.

CREB-deficient SMCs produce soluble factors that stimulate adventitial fibroblast proliferation and production of extracellular matrix factors

The profound adventitial collagen deposition and increased numbers of adventitial myeloid cells observed in lung sections from SMC CREB KO mice led us to explore whether CREB-null SMCs produce soluble factors that regulate adventitial cell function. Adventitial fibroblasts cultured in medium conditioned with SMC CREB KO SMCs exhibited an approximately 3.5-fold higher level of proliferation than fibroblasts exposed to medium conditioned with Cntrl SMCs (Fig. 9a). Likewise, conditioned medium from CREB-deficient cells stimulated the expression of collagen I and fibronectin gene expression in adventitial fibroblasts, while conditioned medium from control SMCs did not (Fig. 9b).

Finally, we performed a preliminary assessment of cytokines upregulated in cultured SMC CREB KO versus Cntrl SMCs using cytokine arrays. Of 144 cytokines queried by the arrays, four cytokines including macrophage chemoattractant protein-1 (MCP-1), insulin-like growth factor binding protein 6, E-cadherin and matrix metalloproteinase 2 were significantly higher in conditioned medium from CREB-deficient PA SMCs than from control cells (Fig. 10).

Discussion

There is currently no effective treatment for either primary or secondary PH. This is in part due to its complex pathogenesis involving persistent inflammation and activation of a number of cell types in the pulmonary vasculature. For these studies, we were able to exploit the lack of downregulation of CREB in mouse hypoxic PH to probe the phenotypic
consequences of SMC CREB deletion in the pulmonary circulation. These studies demonstrate that the removal of CREB in PA SMC triggers a switch to the development of PH. SM22 gene promoter-targeted depletion of CREB in mice promoted robust PA remodeling, greater than that observed in CREB-expressing cohorts exposed to hypobaric hypoxia, in which remodeling did not achieve statistical significance. While hypoxia-induced medial thickening has often been reported in mice, some laboratories have reported only mild\textsuperscript{25} or even no remodeling\textsuperscript{26} of PAs, or only thickening and muscularization of arterioles in mice in response to hypoxia.\textsuperscript{27} We propose that the mild and variable remodeling of the murine PA in response to hypoxia is due to the protective effect of sustained CREB expression in the vasculature since remodeling is readily apparent in SMC CREB KO mice. In our studies, PA remodeling in SMC CREB-deficient mice included adventitial collagen deposition, recruitment of macrophages to the PA wall, and adventitial fibroblast proliferation. However, muscularization of distal arterioles, which was evident in mice exposed to chronic hypoxia, was not evident in the lungs of SMC CREB KO mice. Muscularization of small arterioles may instead be regulated by collateral transcriptional mechanisms that control migration of proximal SMC precursors to distal sites and their differentiation to mature SMCs. For example, Sheikh et al.\textsuperscript{28,29} have implicated a HIF-1/PDGF-B signaling axis as a potential co-lateral pathway for regulating these events.

The profound adventitial collagen deposition and recruitment of macrophages to the adventitia of SMC CREB KO mice are particularly interesting. These data suggest potential cross-talk between the CREB-deficient medial SMCs and adventitial cell populations responsible for fibrotic adventitial remodeling and inflammation. While changes in the adventitia such as the conversion of fibroblasts to myofibroblasts drive changes in the arterial media and intima that contribute to vessel stiffening and vasoconstriction,\textsuperscript{14,15} the reciprocal impact of SMCs on adventitial cells is poorly understood. Our observations suggest that communication between cells in different arterial layers is required for progressive pathological remodeling of the PA wall.

The transcription factor CREB is a ubiquitously expressed basic leucine zipper transcription factor, in which phosphorylation in the N-terminal kinase inducible domain recruits transcriptional co-activators to activate CREB-targeted gene transcription. Regulation of gene transcription by CREB is a convergence point for multiple

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**Fig. 6.** Proliferation of medial SMCs in Cntrl and SMC CREB KO mice. (a) Eight-week-old, male Cntrl and SMC CREB KO mice were maintained at Denver, CO ambient (Norm) or hypobaric hypoxia (Hypox) for five weeks as indicated. Lung sections were subjected to immunohistochemistry for Ki-67, and immune complexes visualized with diaminobenzidine (dark brown/black). Sections were counterstained with hematoxylin. Representative brightfield images show occasional Ki-67-positive cells in the parenchyma and cluster of stained cells between adjacent airways. However, no Ki-67 staining was observed in the PA wall in any treatment group. Scale bar = 50 μm. (b) PA medial cell number was estimated by counting DAPI-positive nuclei in the SM-actin-stained region of the vessel wall. Cell number was corrected for vessel size (lumen area) and plotted as an index of SMC proliferation. Data indicate proliferation of SMCs in SMC CREB KO mice, but not in normoxic or hypoxic Cntrl animals. (n = 4 mice/group, 10 vessels/mouse) Error bars indicate standard deviation; *p ≤ 0.05.

**Fig. 7.** No evidence of hypertrophy of medial SMCs in Cntrl and SMC CREB KO mice. Eight-week-old, male Cntrl and SMC CREB KO mice were maintained at Denver, CO ambient (Norm) or hypobaric hypoxia (Hypox) for five weeks as indicated. Lung sections were subjected to immunohistochemistry for SM-actin and nuclei were visualized with DAPI as described elsewhere. Average size of medial SMCs were determined by dividing the area of the SM-actin-stained region by the number of nuclei within that region. The plot shows no significant difference in SMC size (cross-sectional area) between the treatment groups. (n = 4 mice/group, 10 vessels/mouse).
signaling pathways involved in regulating the proliferation, survival, metabolism, differentiation, and inflammatory status in many cell types. The signaling cascades upstream of CREB phosphorylation are cAMP/protein kinase pathways, growth factor/receptor tyrosine kinase pathways, and phospholipase C/PKC pathways. The role of CREB on vascular SMC phenotype in response to various extracellular stimuli has, however, proven to be a complex area of investigation. Various stimulants such as PDGF-BB and Angiotensin II have been shown to stimulate CREB in vascular SMC. Alternately, phosphorylation of CREB by cAMP-dependent protein kinase in response to cAMP mimetics, adenylate cyclase activators, and phosphodiesterase inhibitors decreases proliferation, protein synthesis, and promotes vascular SMC differentiation. At least some of the anti-remodeling effects of prostanoids are attributed to
prostacyclin receptor-mediated inhibition of phosphodiesterase activity leading to the activation of CREB.  

Because CREB is regulated by multiple pathways, CREB may have pleiotropic effects on vascular SMC functions, depending on the source and duration of stimulation. The consensus is that transient or low-dose exposure to agonists increases CREB phosphorylation, whereas long-term or high-dose stimulation causes downregulation of CREB phosphorylation.  

We have reported rapid CREB phosphorylation when SMCs are treated with mitogens like PDGF-BB for periods from 10 min to 24 h. However, prolonged exposure to PDGF stimulated CREB depletion via the PI3K/Akt pathway, which promotes nuclear export, ubiquitination, and proteasomal degradation of CREB.  

We propose that the rapid and transient activation of CREB by various mitogenic stimuli is a braking mechanism by which cells attempt to prevent uncontrolled proliferation. In this regard, it is worth noting that while agents that elevate intracellular cAMP block basal SMC proliferation in culture, the use of prostacyclin analogs (that increase intracellular cAMP) has limited benefit in clinical situations because of the heterogeneity of PH etiology in patients.  

Fig. 9. Conditioned medium prepared with SMC CREB KO SMCs stimulates proliferation of adventitial fibroblasts, and their production of collagen I and fibronectin. (a) DMEM/F12 containing 10% FBS was conditioned by culturing with either Cntrl or SMC CREB KO primary SMCs for 48 h. Adventitial fibroblasts were plated on 96-well plates and exposed to either Cntrl or SMC CREB KO conditioned media as indicated. After 72 h, cell numbers were determined with CellTiter96Aq reagents. Results indicated that medium conditioned with CREB-null SMCs stimulates fibroblast proliferation. (n = 4 replicates/group) Error bars indicate standard deviation; *p ≤ 0.05. (b) Adventitial fibroblasts were cultured in Cntrl or SMC CREB KO condition medium for 72 h and then RNA was extracted to make cDNA using Cell-to-cDNA reagents. PCR was performed for collagen I, fibronectin, and Gapdh (internal control), and duplicate reactions for Cntrl and SMC CREB KO samples are shown. Data indicated elevated production of both collagen I and fibronectin by fibroblasts exposed to medium conditioned by CREB-deficient SMCs.
Based on our data, it seems likely that cAMP-elevating agents are ineffectual therapeutic agents because of the absence or reduced levels of CREB in the vessel wall.

The findings in this article align with the published studies on the development of PH in mice with SMC-targeted ablation of phosphatase and tensin homolog (PTEN). In this study, Horita et al. demonstrated that sections of lungs from pulmonary hypertensive patients showed marked increases in phospho-PTEN staining largely localized to medial SMCs and that selective inactivation of PTEN in SMCs induced PH and hypersensitivity to hypoxia using PTEN inducible knockout (PTEN iKO) mice. In addition, these authors reported that rats exposed to chronic hypoxia demonstrated chronic PTEN inactivation selectively in SMCs and associated with increased Akt phosphorylation. SMC-specific ablation of PTEN elicited tonic activation of PI3K signaling and produced vascular remodeling and hemodynamic and histopathologic changes consistent with PH under normoxic conditions. Interestingly, the authors reported that normoxic PTEN iKO mice exhibited increased lung expression of the chemokine SDF-1α promoting recruitment of macrophages. The overlap between our observations and those published by Horita et al. may suggest that CREB may be a primary target, albeit not the only target, of PTEN/PI3K signaling in PH. However, PTEN iKO mice exhibited muscularization of distal arterioles and overt medial SMC proliferation in vivo, which were not present in our SMC CREB KO mice. It is likely that CREB is one of the several molecules targeted by PI3K signaling that together generate the full repertoire of features of PH. We propose that in healthy arteries, PI3K signaling is held in check by PTEN. Consecutive to hypoxia, PI3K-stimulated pathways elicit proteasomal CREB degradation, which promotes SMC proliferation and hypertrophy, and the release of soluble factors that stimulate proliferation of adventitial fibroblasts and their production of ECM components. Also, other resident recruited cells may be playing a significant role in fostering and maintaining a pro-inflammatory environment.

In summary, SMC phenotypic plasticity has been described in multi-organ development as well as various adult diseases characterized by vascular remodeling. However, our understanding of the molecular mechanisms that control SMC phenotype is far from complete. Distinguishing specific SMC phenotypes and cell subsets in vivo has complicated the analysis of specific cell contributions to PA remodeling. Our studies used a SMC-restricted CREB ablation system, and by selectively targeting SMC in vivo, we demonstrated that CREB directly regulated a SMC phenotypic switch that governs SMC proliferation and influences collagen production and macrophage recruitment to the adventitia. Taken together with our previous observations, CREB appears to be a key transcriptional nexus for signaling events that normally support vascular homeostasis, but the loss of CREB in rodent models is a permissive step that promotes pathological vascular remodeling.

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Conflict of interest
The author(s) declare that there is no conflict of interest.

Contributorship
JEBR, SMM and DJK – conceived, designed and directed project; wrote article with input from all authors.
CVG, TMS, JTC and DJK – planned and performed the experiments.

Ethical approval
All animal studies were performed with approval and in accordance with the guidelines of the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee.
SMC CREB loss elicits pulmonary hypertension

Garat et al.

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