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Upregulated expression of STIM2, TRPC6, and Orai2 contributes to the transition of pulmonary arterial smooth muscle cells from a contractile to proliferative phenotype

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Elevated pulmonary arterial pressure (PAP) in patients with PAH is a progressive disease that, if left untreated, eventually leads to right heart failure and death. Elevated pulmonary arterial pressure (PAP) in patients with PAH is mainly caused by an increase in pulmonary vascular resistance (PVR). Sustained vasoconstriction and excessive pulmonary vascular remodeling are two major causes for elevated PVR in patients with PAH. Excessive pulmonary vascular remodeling is mediated by increased proliferation of pulmonary arterial smooth muscle cells (PASMC) due to PASMC dedifferentiation from a contractile or quiescent phenotype to a proliferative or synthetic phenotype. Increased cytosolic Ca2+ concentration ([Ca2+]cyt) in PASMC is a key stimulus for cell proliferation and this phenotypic transition. Voltage-dependent Ca2+ entry (VDCE) and store-operated Ca2+ entry (SOCE) are important mechanisms for controlling [Ca2+]cyt. Stromal interacting molecule proteins (e.g., STIM2) and Orai2 both contribute to SOCE and we have previously shown that STIM2 and Orai2, specifically, are upregulated in PASMC from patients with idiopathic PAH and from animals with experimental pulmonary hypertension in comparison to normal controls. In this study, we show that STIM2 and Orai2 are upregulated in proliferating PASMC compared with contractile phenotype of PASMC. Additionally, a switch in Ca2+ regulation is observed in correlation with a phenotypic transition from contractile PASMC to proliferative PASMC. PASMC in a contractile phenotype or state have increased VDCE, while in the proliferative phenotype or state PASMC have increased SOCE. The data from this study indicate that upregulation of STIM2 and Orai2 is involved in the phenotypic transition of PASMC from a contractile state to a proliferative state; the enhanced SOCE due to upregulation of STIM2 and Orai2 plays an important role in PASMC proliferation.

PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive and fatal disease that predominantly affects women. In patients with PAH, the elevated pulmonary arterial pressure (PAP) is mainly caused by increased pulmonary vascular resistance (PVR), which results in an increase in the afterload for the right ventricle and, if untreated, leads to right heart failure and eventually death. Elevated PVR in patients with PAH is primarily caused by sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling (including pulmonary vascular wall thickening, small vessel obliteration, and formation of plexiform lesions). In pediatric patients, those with persistent pulmonary hypertension in the newborn (PPHN) or hypoxia-induced pulmonary hypertension, inhalation of nitric oxide (NO) and infusion of vasodilator (e.g., adenosine, prostacyclin) significantly reduces PAP and PVR (2). In adult patients with idiopathic PAH, however, only a minority of the patients respond to vasodilators, i.e., acute treatment of the patients with inhaled NO or infused vasodilators can decrease PAP and PVR to the normal level in ~15% patients (34). These data indicate that, at early stage, sustained pulmonary vasoconstriction plays an important role in the initiation and development of pulmonary hypertension in patients with PAH; while at late stage, a gradual transition from sustained vasoconstriction to vascular remodeling may play a critical role in the progression of pulmonary hypertension. Then, at the stage when patients develop physiologic right ventricular hypertrophy, excessive pulmonary vascular remodeling characterized by the adventitial, medial, and intimal hypertrophy of the pulmonary arteries, the intraluminal obliteration and occlusion of small pulmonary arteries and arterioles, the neointimal and plexiform lesions in the pulmonary vasculature are the predominant causes for maintaining the increase in PVR and PAP. Sustained increase in PVR and PAP, or the elevated afterload, would eventually cause pathological right ventricular hypertrophy and, if untreated, right heart failure and death.

These assumptions and observations are also in good agreement with the clinical manifestation of the disease. In pediatric patients, for example, intravenous administration of vasodilators or inhalation of NO are very effective to reduce PVR and PAP, while in adult patients there are only a few patients (15%)
who are defined as “responders” (23, 31, 33, 34). Administration of vasodilators (e.g., adenosine, prostacyclin) or inhalation of NO cause significant reduction of PVR and PAP only in “responders.” Most of the adult patients with PAH, however, are defined as “nonresponders”; conventional treatment with Ca\(^{2+}\) channel blockers like nifedipine and verapamil or vasodilators are no longer effective and useful for the patients. Other newly developed drugs which have antiproliferative effects are commonly used to treat nonresponder patients (11, 12).

Both basic and clinical research data indicate that, at the early stage of PAH, sustained pulmonary vasoconstriction is an important contributor to the development or initiation of the disease manifestation such as the elevated PVR and PAP (24, 29, 40). However, at the progression and/or late stage of the disease, the transition from a sustained pulmonary vasoconstrictive phenotype to an excessive pulmonary vascular remodeling phenotype is an important pathogenic process in the progression of PAH.

Smooth muscle cells including pulmonary arterial smooth muscle cells (PASMC) are extremely plastic and can dedifferentiate in response to various environmental stimuli from a contractile or quiescent phenotype to a proliferative or synthetic phenotype (28, 30). In addition, a phenotypic switch of PASMC from a contractile to proliferative phenotype is inevitable for any pathological and physiological vascular remodeling process to occur. Contractile PASMC represent the majority of PASMC in the in vivo functional pulmonary vessels, and they are responsible for maintenance and regulation of vascular tone that is required for maintaining PVR to the blood flow. Normal pulmonary vasoconstriction and vasodilatation, due primarily to PASMC contraction and relaxation, respectively, play an important role in regulating and maintaining a normal pulmonary arterial pressure and blood flow.

Enhanced PASMC proliferation has been demonstrated to play an important role in the development and progression of pulmonary vascular remodeling in patients with idiopathic PAH and animals with experimental pulmonary hypertension. The increased PASMC proliferation is due at least in part to an increased cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\textsubscript{cyt}]) resulting from augmented store-operated Ca\(^{2+}\) entry (SOCE) and upregulated expression of STIM1/STIM2, Orai1/Orai2, and TRPC6 is required for the enhanced SOCE.

**MATERIALS AND METHODS**

**Isolation of rat pulmonary artery smooth muscle tissue.** Protocols involving the use of experimental animals for all experiments were reviewed and approved by the Ethics/Animal Care Committee of the University of Illinois at Chicago and The University of Arizona. Sprague-Dawley male rats (150–200 g) were decapitated, and the whole lung and heart were removed en bloc and placed in warm Hanks’ balanced salt solution (HBSS, Life Technologies, Carlsbad, CA) supplemented with 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, Sigma Aldrich, St. Louis, MO). The right and left branches of the main pulmonary as well as the intrapulmonary arteries were first isolated from the main lung with fine forceps under a dissecting microscope. The fat and connective tissues were then removed gently from the isolated pulmonary artery (PA) under sterile conditions. The isolated PA was incubated in HBSS containing 1.7 mg/ml collagenase type II (Worthington Biochemical; Lakewood Township, NJ) for 20 min at 37°C. Then, the short-digested PA ring was rinsed with HBSS to remove residual collagenase, the adventitia of the PA ring was carefully stripped off with fine forceps, and the endothelium was demuded with a sterile cotton swab. The remaining PA smooth muscle tissue was then used to prepare single PASMC and to extract total protein for Western blot experiments.

**Isolation and preparation of rat PASMC.** Following removal of the adventitia and endothelium, the rat PA was further digested in HBSS 1.7 mg/ml collagenase type II, 0.5 mg/ml elastase (Sigma), and 1 mg/ml bovine serum albumin (BSA, Sigma) at 37°C for 50 min. The PA tissue was agitated every 15–20 min to speed digestion. The dispersed PA tissue was then triturated approximately 8–10 times with a fire-polished Pasteur pipette, to further dissociate the cells. Ten milliliters of Dulbecco’s modified Eagle’s medium (DMEM; Corning, Herndon, VA) supplemented with 7 mM NaH\(_2\)CO\(_3\), 10 mM HEPES (pH 7.2), 20% fetal bovine serum (FBS, Corning), and 1% penicillin and streptomycin (Pen/Strep, Corning) was then added to the enzymatic solution to stop digestion. The cell suspension was centrifuged for 5 min at 1,500 rpm at room temperature (22–24°C). The supernatant was aspirated off and the resulting pellet was resuspended in 2 ml of fresh 10% FBS-DMEM and triturated to separate the cells.

For experiments using freshly dissociated PASMC, aliquots of the cell suspension were plated directly onto glass coverslips coated with 5% gelatin (porcine, Sigma Aldrich) or in six-well dishes (Corning) with 2.5 ml of 10% FBS-DMEM. These freshly dissociated rat PASMC were allowed to attach to the coverslips for 3–4 h before loading with fura-2 AM (10 nM) for measurement of cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\textsubscript{cyt}]). The freshly dissociated cells plated directly into the six-well dishes were used for immunocytochemistry and immunoblotting experiments to determine protein expression levels of various ion channels.

To prepare primary cultured rat PASMC, aliquots of the cell suspension were plated onto gelatin-coated coverslips in 25-mm petri dishes or directly onto 10-cm petri dishes with 10% FBS-DMEM and incubated in a humidified atmosphere of 5% CO\(_2\) in air at 37°C. Twenty-four hours later, the culture media were changed to Medium 199 (M199) supplemented with 10% FBS, 100 μg/ml cell growth supplement, and antibiotics (penicillin and streptomycin). The medium was changed 24 h after initial seeding and every 48 h subsequently. When the cells reached 80–90% confluency, they were gently washed with phosphate-buffered saline (PBS), incubated briefly with 1 ml of 0.025% trypsin-EDTA solution until detachment (3–5 min), and then 9 ml 10% FBS-M199 was added to the plate. The cell suspension was then transferred to a sterile 15-ml round-bottom tube, centrifuged at room temperature for 5 min at 200 × g (1,500 rpm), resuspended in the appropriate growth media and seeded onto...
coverslips or petri dishes, and used for Western blot and \([Ca^{2+}]_{cyt}\) measurement experiments.

**Mouse PASMC isolation.** PASMC were isolated from mouse lungs, as described previously (37). Briefly, a mixture of 5 ml of M199 growth medium containing 5 g/l low-melting-point agarose type VII (Sigma), 5 g/l iron beads (diameter <10 μM; Sigma), and antibiotics (penicillin and streptomycin) was slowly injected over a period of 60 s through the right ventricle, thereby perfusing the PA. M199 growth medium (1 ml) containing 5 g/l agarose type VII was injected in airways through the trachea. The lungs were plunged in cold PBS to cause the agarose to gel. Because of the rapidly solidifying nature of the agarose and the size of the iron particles, the likelihood of traversing the capillary space is minimized. All the lobes were then isolated and finely minced in a petri dish. The tissue was further disrupted by passing through a 16-gauge needle approximately five times. The suspension was then mixed in M199 growth medium containing 80 U/ml type IV collagenase (Sigma) and incubated at 37°C for 90 min. With the use of a magnetic column (Invitrogen), the arteries containing the iron beads were collected. The supernatant was aspirated and the arteries were washed and suspended in 5 ml M199 containing 20% FBS. Aliquots of the suspension were transferred to T25 culture flasks. Smooth muscle cell purity was determined by immunostaining with smooth muscle specific actin antibody.

**Measurement of \([Ca^{2+}]_{cyt}\) in PASMC.** \([Ca^{2+}]_{cyt}\) was measured using fura-2 AM (Invitrogen-Molecular Probes, Eugene, OR), a membrane-permeable Ca2+-sensitive fluorescent indicator, and a Nikon digital imaging fluorescent microscope system. Cells on 25-mm coverslips were loaded with 4 μM fura-2 AM in normal physiological salt solution (PSS) for 60 min at room temperature (22–24°C) in the dark. The PSS solution contained (in mM) 137 NaCl, 5.4 KCl, 1.8 CaCl2, 1.2 MgCl2, 10 HEPES, and 10 glucose (pH adjusted to 7.4 with 1 N NaOH). The fura-2 AM-loaded cells were then placed in a recording chamber on the stage of an inverted fluorescent microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) equipped with an objective lens (S. Plan Fluor x20/0.45 ELWD; Nikon) and Em-CCD camera (Evoke; Photometrics, Tucson, AZ). The recording chamber was continuously perfused with PSS at a flow rate of 2 ml/min using a mini pump (model 3385; Control, Friendswood, TX). The fura-2 AM-loaded cells were then washed by perfusion with normal PSS for 20 min to remove excess extracellular fura-2 AM and allow sufficient time for intracellular esterase to cleave fura-2 AM to active fura-2. The cells were excited at 340- and 380-nm wavelengths (D340x2, 380x2 filters, respectively; Chroma Technology, Bellows Falls, VT) by a xenon arc lamp (Lambda LS; Sutter Instrument, Novato, CA) and an optical filter changer (Lambda 10-B). Emission of fura-2 fluorescence was collected through a dichroic mirror (S. Plan Fluor x40) and an Eppendorf tube on ice and lysed with cold RIPA buffer (Millipore, Billerica, MA) supplemented with protease inhibitor cocktail (Roche; Basel, Switzerland) by sonication three times for 30 s each time. Cultured rat PASMC and HEK cells were washed with ice-cold PBS, scraped, placed into an Eppendorf tube, and centrifuged. The pellet cells were resuspended in 20–50 μl of RIPA buffer supplemented with protease inhibitor cocktail. Lysed tissues and cells were incubated in lysis buffer for 15 min on ice. The lysates were then centrifuged at 13,300 rpm for 15 min at 4°C. The protein was then collected and the supernatant, protein concentration was determined by Bradford Protein Assay (Bio-Rad, Hercules, CA) with BSA as a standard. Proteins (10–20 μg) were mixed and boiled in Laemmli Apparatus). The resting passive tension was set and maintained at an optimal tension of 300 mg (43), and the rings were allowed to stabilize at resting tension for ~1 h before experimentation. The isometric tension was continuously measured and recorded, and data were acquired using DATATQ software (DATATQ Instruments). Isolated PA rings were perfused with modified Krebs solution (MKS: at 37°C) consisting of the following chemicals (in mM): 138 NaCl, 1.8 CaCl2, 4.7 KCl, 1.2 MgSO4, 1.2 Na2HPO4, 5 HEPES, and 10 glucose (pH 7.4). The active tension (or the absolute amount of force) relative to the basal tension was measured and expressed as the net increase in tension (mg). The endothelium was removed by repeatedly passing a rough-surface stainless steel wire through the intralumen of the PA ring; functional removal of the endothelium was confirmed by the loss of acetylcholine-mediated dilatation of the isolated PA ring. To stabilize the vessel and obtain a stable contractile response, the isolated PA rings were challenged by superfusion with 60 mM K+-containing solution (60K) three times before experimentation. The amplitude of the 60K-induced PA contraction (or the 60K-induced increase in active tension in isolated PA rings) and the baseline tension of the isolated PA rings are usually stable after three times of challenges with 60K-containing solution.

**Measurement of pulmonary arterial pressure in isolated and perfused/ventilated lungs.** Experiments using the mice in this study were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago and The University of Arizona. C57BL/6 mice (22–25 g) were anesthetized with ketamine (100 mg/kg)-xylazine (26 mg/kg) via intra-abdominal injection. After a tracheostomy was performed, mice were ventilated with a gas mixture of 21% O2, 5% CO2 via a rodent ventilator (minivent type 845, Harvard Apparatus). Respiratory rate was maintained at 80 breaths/min and tidal volume was 10 ml/kg (~250 ml). Positive end-expiratory pressure was maintained at 2 mmH2O. End-inspiratory plateau pressure (EIP) was measured with a pressure transducer (MPX type 399/2, Hugo Sachs Elektronik-Harvard Apparatus, Germany). To prevent blood coagulation, 20 IU heparin was injected into the right ventricle. A stainless steel catheter was inserted into the main pulmonary artery (PA) after a right ventriculotomy was performed, and the PA and ascending aorta were tied together. Pulmonary arterial pressure (PAP) was measured using a pressure sensor (P75 Type 379, Hugo Sachs Elektronik-Harvard Apparatus, Germany), which was connected to the PA catheter. The PAP, left atrial pressure, and EIP were monitored continuously. For data acquisition and data storage, Powerlab 8/30, Quad Bridge Amp, and LabChart (AD Instruments, Australia) were used. After basal PAP was stabilized for 40–60 min, the experiments were performed. The physiological salt solution (PSS) used for the perfusate consisted of the following composition (mM): 120 NaCl 120, 4.3 KCl, 19 NaHCO3, 1.1 KH2PO4, 10 glucose, 1.8 CaCl2, and 1.2 MgCl2 (pH 7.4). To protect prostaglandin synthesis, 3.1 mM sodium mcellofenamate was added to the perfusate. For isotonic high-K+ solutions (40 mM), NaCl was replaced by an equimolar amount of KCl. To demonstrate the role of Ca2+ on contraction, voltage-dependent Ca2+ channel blocker (1 μM nifedipine) was added to the perfusate. Full methods were described previously by Yoo et al. (44).

**Western blot experiments.** PA smooth muscle tissue was placed in an Eppendorf tube on ice and lysed with cold RIPA lysis buffer (Millipore, Billerica, MA) supplemented with protease inhibitor cocktail (Roche; Basel, Switzerland) by sonication three times for 30 s each time. Cultured rat PASMC and HEK cells were washed with ice-cold PBS, scraped, placed into an Eppendorf tube, and centrifuged. The pellet cells were resuspended in 20–50 μl of RIPA buffer supplemented with protease inhibitor cocktail. Lysed tissues and cells were incubated in lysis buffer for 15 min on ice. The lysates were then centrifuged at 13,300 rpm for 15 min at 4°C. The pellet was discarded and from the supernatant, protein concentration was determined by Bradford Protein Assay (Bio-Rad, Hercules, CA) with BSA as a standard. Proteins (10–20 μg) were mixed and boiled in Laemmli AJP-Cell Physiol • doi:10.1152/ajpcell.00202.2014 • www.ajpcell.org

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sample buffer supplemented with 2-mercaptoethanol (BME, Sigma) reducing agent. Protein lysates were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.45-μm nitrocellulose membranes (Bio-Rad). Membranes were incubated for 1 h at 22–24°C in a blocking buffer [0.1% Tween 20 in TBS (TBST)] containing 5% nonfat dry milk powder. The membranes were then incubated with primary antibodies diluted in TBST containing 5% BSA, with shaking overnight at 4°C. Membranes were washed three times in TBST for 5 min each, followed by incubation in secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature in TBST containing 5% milk. Membranes were washed three times for 5 min each, and peroxide activity was visualized with enhanced chemiluminescence substrate (Pierce, Rockford, IL). Primary antibodies included anti-calponin (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), myosin heavy chain (MYH, 1:1,200, Santa Cruz), smooth muscle 22-α (SM22α; 1:1,000, Santa Cruz), proliferating cellular nuclear antigen (PCNA, 1:1,000, Santa Cruz), STIM1 (1:1,000, Pro-Sci), STIM2 (1:1,000, Sigma), Orai1 (1:500, Pro-Sci), Orai2 (1:500, Alomone, Israel), Orai3 (1:500, Alomone), TRPC6 (1:500, Sigma), and β-actin (1:2,000, Santa Cruz). Band intensity was quantified with ImageJ (National Institutes of Health, Bethesda, MD), normalized to β-actin control, and is expressed as arbitrary units.

Transfection of PASMC. Twenty-four hours after seeding, HEK293 cells at 60–80% confluency were transiently transfected with STIM2 and Orai2 expression constructs using Lipofectamine 2000 transfection reagent, based on the manufacturer’s protocol. Transfection was performed at 37°C in serum-free Opti-MEM medium (Gibco) with 0.5 μg/ml DNA and transfection reagent (μl). After 5–6 h of incubation at 37°C with transfection medium, the medium was changed to serum-containing DMEM and cells were incubated for 24–72 h before experiments. Rat PASMC were transiently transfected with STIM2 and Orai2 expression constructs and siRNA targeting STIM2 and Orai2 using Amaxa Basic Nucleofector kit (Lonza, Walkersville, MD) via electroporation according to the manufacturer’s instructions. Serum-free Opti-MEM medium (Gibco) was used to dilute cells, transfection reagent, DNA, and siRNA. After 5–6 h of incubation at 37°C with transfection medium, the medium was changed to serum-containing M199 and cells were incubated for 24–72 h. [Ca2+]i, measurements and Western blotting using DNA- and siRNA-transfected cells were performed 48–72 h after transient transfection.

cDNA vectors used for upregulation were as follows: STIM2 [plasmid 18868: pEX-CMV-SP-STIM2 (15-746)], Orai2 [plasmid 16369: pcDNA3.1 Orai2]. Small interfering RNA agents used for downregulation were as follows: STIM2 (Ambion Life Technology, Carlsbad, CA), Orai2 (siGENOME Thermo Scientific), and scrambled siRNA (siGENOME Thermo Scientific).

Cell proliferation assays. To determine rat PASMC proliferation rate in vitro, we used bromodeoxyuridine (BrdU) incorporation assay and measured the changes in cell number using a cell counter. BrdU incorporation assay (Millipore) was used according to the manufacturer’s protocol. Confluent primary cultured cells were detached with 0.05% trypsin-EDTA, seeded into 96-well plates (2.5 × 104 cells/well) in M199 containing 10% FBS, and allowed to attach onto the culture plate overnight. PASMC were then incubated in serum-free M199 for 24 h and then incubated with vehicle control or with 1, 2, or 3 ng/ml of transforming growth factor-β (TGF-β) for 24, 72, or 96 h, respectively. In addition, cells were incubated with 0.3%, 1.0%, or 10% FBS-M199 for 24, 72, or 96 h. Cells were also incubated in 10% FBS-M199 with 500 nM Ca2+ or 1.2 mM Ca2+ for 24 or 72 h. During the final 4 h of incubation, BrdU was incorporated into actively dividing cells. Cells were fixed/denatured in room temperature fixative solution. Anti-BrdU antibody was added, followed by incubation with secondary anti-mouse IgG peroxidase-conjugate. Fluorescence was measured using a spectrophotometer microplate reader at 450/540 nm.

For cell counting experiments, PASMC growth curves were examined by cell counting with the Bio-Rad TC10 automated cell counter. Subculture cells were grown to confluence overnight, after which they were collected, counted, and equally seeded into eight-well multibridishes (Nunclocn, 10.5 cm2 of culture area per well; Thermo Scientific), with 1 × 104 cells/well (0.95 × 104 cells/cm2, 3 wells/sample); this number was used as baseline (0 h). Treatment cells were counted 24, 72, and 96 h after the start of experiments. Each count was an average of three repeats, and each data point was the average of four experiments.

PCR analysis. Genomic DNA from a tail biopsy was extracted by standard procedures and subjected to PCR for genotyping. The primer sequences and anticipated band sizes are as follows: TRPC6-WT (378 bp): forward (F)-TCT TTA TGC AAT CGC TGT GG; reverse (R)-GCT AGT CTT CCT GCA ATC CA; TRPC6-Mut (175 bp): F-TCT ATT AAC ACT CAA CTG GCA CCT; R-GCC AGA GGC CAC TTG TGT AG.

The cycling parameters for the PCR were as follows: 1 cycle at 94°C for 3 min, 33 cycles of 94°C for 15 s, then 15 s of 60°C, and 72°C for 18 s. The PCR ended with 5 min at 72°C.

Statistical analysis. Data are expressed as means ± SE and were analyzed for statistical significance by the unpaired Student’s t-test or one-way ANOVA for multiple groups using SigmaPlot software. Differences were considered to be significant at P < 0.05. Significant difference is expressed in the figures or figure legends as P < 0.05, P < 0.01, and P < 0.001.

RESULTS

Upregulated expression of STIM2, TRPC6, and Orai2 in the proliferative phenotype of PASMC in culture compared with the contractile phenotype of PASMC in isolated PA. To determine the potential differences in the expression levels of STIM1, TRPC6, and Orai2 between proliferative phenotype and contractile phenotype of PASMC, we used Western blot analysis to compare protein levels of STIM1, TRPC6, and Orai2 in isolated PA rings with denuded endothelium and stripped-off adventitia (contractile PASMC phenotype) and in primary cultured PASMC (proliferative PASMC phenotype). To confirm the contractility of PASMC in isolated PA rings, we first examined the response of isolated PA rings from rat to high K+-containing solution (40 mM and 60 mM) in the presence and absence of extracellular Ca2+ or in the absence or presence of nifedipine (1 μM), a dihydropyridine Ca2+ channel blocker that potently blocks the L-type and T-type voltage-dependent Ca2+ channels in PASMC.

As shown in Fig. 1, in isolated pulmonary artery (PA) rings from rats with denuded endothelium and stripped-off adventitia (Fig. 1A), raising extracellular K+ concentration ([K+]o) from 4.7 to 60 mM caused a large vasoconstriction determined by the increase in isometric tension (Fig. 1Ba, left). Increasing [K+]o, by changing the K+ equilibrium potential, causes membrane depolarization, opens L-type voltage-dependent Ca2+ channels (VDCC), and increases [Ca2+]cyt (39, 41, 43). The high K+ (60 or 40 mM K+)-mediated PA contraction shown in Fig. 1Ba (left) was obviously due to membrane depolarization-mediated Ca2+ influx through VDCC. Removal of extracellular Ca2+ almost abolished the 60K-induced increase of tension (Fig. 1Ba, left, and Bb, left), while application of 1 μM nifedipine, a dihydropyridine L-type VDCC blocker, significantly reduced the 60 mM K+-mediated PA contraction (Fig. 1Ba, right and Bb, right) (41). In isolated perfused/ventilated mouse lung, raising [K+]o from 4.7 mM to 40 mM also
significantly increased pulmonary arterial pressure (PAP) by causing pulmonary vasoconstriction or contraction of PASMC in contractile phenotype. Extracellular application of the VDCC blocker nifedipine (Fig. 1, Cb and Eb) significantly and reversibly inhibited 40K-induced increase in PAP. These results obtained from rat and mouse lung PA demonstrate that 1) PASMC in isolated PA rings (used in these contraction experiments and the following Western blot experiments) are in contractile phenotype, and 2) high Ca\(^{2+}\)-mediated pulmonary vasoconstriction in the contractile phenotype is significantly dependent on the rise in cytosolic [Ca\(^{2+}\)]\(_{cyt}\) due to Ca\(^{2+}\) influx through VDCC.

To determine the role of STIM and SOC proteins in the phenotypic transition of PASMC, we first compared the protein expression levels of  STIM2, TRPC6, and Orai2 in proliferative PASMC and contractile PASMC. Protein expression levels of these proteins were significantly upregulated in proliferative PASMC compared to contractile PASMC. Furthermore, we investigated the role of STIM and SOC proteins in Ca\(^{2+}\)-mediated pulmonary vasoconstriction in the contractile phenotype.
expression levels of STIM2, Orai2, and TRPC6 between PASMC in contractile phenotype and proliferative phenotype. In primary cultured PASMC derived from rat PA (Fig. 1Da), addition of 10% FBS significantly increased the number of cells as determined by measuring BrdU incorporation (Fig. 1Db). Reducing extracellular Ca\(^{2+}\) concentration in the culture media from 1.8 mM to 0.5 μM (by adding the Ca\(^{2+}\) chelator, EGTA) completely abolished the 10% FBS-mediated PASMC proliferation (Fig. 1Db). The purity of PASMC in culture was determined by the positive staining with smooth muscle cell α-actin (SMαA) and the filament structure of SMαA (Fig. 1Da). These data indicate that primary cultured PASMC (used in the cell proliferation or BrdU incorporation experiments and the following Western blot experiments) are all in a proliferative phenotype.

To further confirm the phenotype of PASMC in isolated PA and in primary culture, we compared the expression levels of differentiation markers was significantly higher in the contractile phenotype of PASMC in PA rings than in the proliferative PASMC cultured in media with 10% FBS (Fig. 1, Ea and F, top). Importantly, the protein expression of STIM2, TRPC6, and Orai2 were all significantly upregulated in the proliferative phenotype of PASMC cultured in growth media in comparison to the contractile phenotype of PASMC in isolated PA rings (Fig. 1, Eb and F, bottom). These data indicate that, when contractile-phenotype PASMC are dissociated from isolated PA rings and cultured in growth media, the cells undergo phenotypical changes to become proliferative-phenotype cells, and the loss of the differentiation markers (e.g., MYH, SM22α, and calponin) is associated with a significant upregulation of STIM2, TRPC6, and Orai2.

Enhanced store-operated Ca\(^{2+}\) entry (SOCE) in the proliferative phenotype of PASMC compared with the contractile phenotype of PASMC (freshly dissociated PASMC). To determine whether the amplitude of [Ca\(^{2+}\)]\(_{cyt}\) increase due to SOCE is different between proliferative and contractile phenotypes of PASMC, we compared cyclopiazonic acid (CPA)-mediated increase in [Ca\(^{2+}\)]\(_{cyt}\) in freshly dissociated PASMC (contractile phenotype of PASMC) compared with the contractile phenotype of PASMC in isolated PA rings than in the proliferative PASMC cultured in media with 10% FBS (Fig. 1, Ea and F, top). Importantly, the protein expression of STIM2, TRPC6, and Orai2 were all significantly upregulated in the proliferative phenotype of PASMC cultured in growth media in comparison to the contractile phenotype of PASMC in isolated PA rings (Fig. 1, Eb and F, bottom). These data indicate that, when contractile-phenotype PASMC are dissociated from isolated PA rings and cultured in growth media, the cells undergo phenotypical changes to become proliferative-phenotype cells, and the loss of the differentiation markers (e.g., MYH, SM22α, and calponin) is associated with a significant upregulation of STIM2, TRPC6, and Orai2.

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tile phenotype) and primary cultured PASMC (proliferative phenotype) in media containing 10% FBS and growth factors (Fig. 2A). As shown in Fig. 2, extracellular application of 10 μM CPA, a SERCA inhibitor that induces Ca\(^{2+}\) influx due to passive depletion of Ca\(^{2+}\) in the intracellular Ca\(^{2+}\) stores or the sarcoplasmic reticulum (SR), caused a slow increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to Ca\(^{2+}\) mobilization from the SR in freshly dissociated (contractile phenotype) PASMC bathed in Ca\(^{2+}\)-free (0Ca) solution. Approximately 10 min after treatment with CPA in the absence of extracellular Ca\(^{2+}\), restoration of extracellular Ca\(^{2+}\) (to 1.8 mM) induced a rapid increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due apparently to SOCE (Fig. 2, B and C, top). The CPA-mediated increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to SOCE was significantly enhanced in proliferative PASMC compared with freshly dissociated (contractile phenotype) PASMC (Fig. 2, B and C, top), while 60 mM K\(^{+}\)-mediated increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) was significantly reduced in proliferative PASMC compared with freshly dissociated contractile PASMC (Fig. 2, B and C, bottom). The enhanced SOCE in proliferative PASMC was associated with 1) downregulated MYH, a differentiation marker, and upregulated PCNA, a proliferation marker (4), and 2) upregulated TRPC6, STIM2, Orai2, and Orai3 (Fig. 2, D and E). These data indicate that SOCE is enhanced as a result of upregulated expression of proteins that participate in forming store-operated Ca\(^{2+}\) channels (SOC), such as TRPC6, Orai2/3, and STIM2, in proliferative PASMC (in which PCNA expression is upregulated) compared with contractile or differentiated PASMC (in which MYH expression is much higher), and 2) Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels (VDCC) induced by 60 mM K\(^{+}\)-mediated membrane depolarization is significantly reduced in proliferative PASMC compared with contractile PASMC.

Expression level of STIM2 and TRPC6 is positively correlated with expression of PCNA, a cell proliferation marker, in proliferative phenotype of PASMC. Reduction of serum concentration and removal of growth factors from culture media lead to growth arrest of PASMC (47). Addition of serum or increase of serum concentration (from 1% to 10%, for example) stimulated PASMC proliferation and significantly increased protein expression level of PCNA, a cell proliferation marker. As shown in Fig. 3, A and B, the protein expression level of PCNA in PASMC cultured in media containing 10% FBS was significantly greater than in PASMC cultured in 0.1% FBS-containing media (Fig. 3, A and B). When PASMC were cultured in 10% FBS-containing media, the number of cells was increased by 2.5 times after 72 h (Fig. 3C, solid triangles); when PASMC were cultured in 0.1% FBS-containing media, however, the number of cells was not significantly changed at 72 h (Fig. 3C, open circles). While the increased PCNA expression in PASMC cultured in 10% FBS-containing media was correlated well with the increased cell proliferation (Fig. 3), the protein expression levels of the differentiation markers calponin and SM22α were actually decreased in PASMC cultured in 10% FBS-containing media compared with cells cultured in 0.1% FBS-containing media.

The increased proliferation and upregulated PCNA expression in proliferating phenotype of PASMC cultured in 10% FBS-containing media were positively associated with upregulation of STIM2, TRPC6, and Orai2 (Fig. 3, A and B). These data indicate that the change from quiescent state to proliferative state is associated with an upregulation of STIM2, TRPC6, and Orai2 in PASMC. In other words, the higher proliferation rate of PASMC is closely correlated with the higher expression level of STIM2, TRPC6, and Orai2. Given
the fact that \(Ca^{2+}\) influx through store-operated \(Ca^{2+}\) channels (SOC) is required for the enhanced PASMC proliferation (8, 26, 35, 47), it is possible that gradually upregulated STIM2, TRPC6, and Orai2 are involved in the transition of PASMC from quiescent state to the proliferative state. The upregulated STIM2, TRPC6, and Orai2 may also play an important role in maintaining a high proliferation rate in proliferating PASMC to, for example, repair vascular injury and cause pulmonary vascular medial hypertrophy.

**Treatment of proliferative PASMC with TGF-\(\beta\)** upregulates expression of smooth muscle cell differentiation markers and downregulates expression of TRPC6 and STIM1. TGF-\(\beta\) is a growth factor that enhances smooth muscle progenitor cell differentiation (6, 17) by activating SMAD signaling cascades (15, 38). In proliferative PASMC cultured in media containing 10% FBS, treatment of the cells with TGF-\(\beta\) (1 to 2 ng/ml) significantly decreased expression of PCNA, a cell proliferation marker, and inhibited cell proliferation, but significantly increased expression levels of the differentiation markers, such as calponin and SM22\(\alpha\) (Fig. 4A). Furthermore, TGF-\(\beta\)-mediated PASMC differentiation was associated with a significant downregulation of TRPC6 and STIM2 (Fig. 4B) as well as Orai1 and Orai2 (Fig. 4C). In PASMC cultured in 10% FBS-containing media, TGF-\(\beta\) at concentrations of 1 ng/ml, 2 ng/ml, and 3 ng/ml (for 24–72 h) significantly inhibited the cell growth (Fig. 4D). These observations are consistent with the data showing that STIM2, Orai1, and TRPC6 are upregulated when PASMC underwent the transition from the quiescent (more differentiated state) to the highly proliferative state (see Fig. 3). The results shown in Fig. 4 also imply that PASMC in highly proliferative state (e.g., cultured in media containing 10% FBS and growth factors) tend to have higher expression of STIM2, Orai1/2, and TRPC6 than PASMC in more differentiated state (e.g., treated with TGF-\(\beta\) or cultured in media without serum and growth factors).

**Treatment of proliferative PASMC with heparin induces cell differentiation (by upregulating smooth muscle cell differentiation markers) and downregulates expression of STIM, TRPC6, and Orai.** To further investigate the relationship between the phenotypic state of PASMC and the expression level of STIM, TRPC, and Orai, we examined the effect of heparin, an anticoagulant that has been shown to inhibit PASMC proliferation and enhance PASMC differentiation (13, 14, 25), on the protein expression levels of STIM1/2, TRPC6, and Orai1/2/3. As shown in Fig. 5, incubation of proliferative PASMC in media containing 10% FBS and heparin (30 \(\mu\)g/ml) for 72 h significantly downregulated the cell proliferation marker PCNA but markedly upregulated the smooth muscle cell diff-

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**Fig. 4. Transforming growth factor-\(\beta\) (TGF-\(\beta\)) upregulates the cell differentiation markers, downregulates the cell proliferation markers, and downregulates protein expression of TRPC6, STIM2, and Orai1/2 in proliferative PASMC.** A: Western blot analyses on PCNA, calponin, and SM22\(\alpha\) in control proliferative PASMC (Control) and PASMC treated with 1 and 2 ng/ml TGF-\(\beta\) (left). \(\beta\)-Actin was used as a loading control. Summarized data (means \(\pm\) SE, right) showing the protein expression levels of PCNA, calponin, and SM22\(\alpha\) in control rat PASMC (Control, treated with vehicle) and PASMC treated with 1 ng/ml (1 ng) and 2 ng/ml (2 ng). \(\ast\) \(P < 0.05, \ast\ast\) \(P < 0.01\), and \(\ast\ast\ast\) \(P < 0.001\) vs. control. B and C: Western blot analyses on TRPC6, STIM1, and STIM2 (B) as well as Orai1 and Orai2 (C) in control proliferative PASMC (Control) and PASMC treated with 1 and 2 ng/ml TGF-\(\beta\) (left). \(\beta\)-Actin was used as a loading control. Summarized data (means \(\pm\) SE, right) showing the protein expression levels of TRPC6, STIM1, and STIM2 (B) as well as Orai1 and Orai2 (C) in control rat PASMC (Control, treated with vehicle) and PASMC treated with 1 ng/ml (1 ng) and 2 ng/ml (2 ng). \(\ast\) \(P < 0.05\) vs. control. D: time course of cell growth (or changes in cell numbers) in PASMC cultured in media (10% FBS-M199) containing vehicle (\(\bullet\)) and TGF-\(\beta\) (1 ng/ml; \(\blacksquare\); 2 ng/ml, \(\bullet\); or 3 ng/ml, \(\blacktriangle\)). \(\ast\) \(P < 0.05\) vs. TGF-\(\beta\)-treated groups.
Fig. 5. Heparin downregulates the cell proliferation markers, upregulates the cell differentiation markers, and downregulates protein expression of STIM1/2, TRPC6, and Orai1/2/3 in proliferating PASMC. A: Western blot analyses on PCNA, calponin, and SM22α in control proliferative PASMC (Cont) and PASMC treated with 30 μg/ml heparin (Hep) (left). β-Actin was used as a loading control. Summarized data (means ± SE) (right) showing the protein levels of PCNA, calponin, and SM22α in control PASMC (Cont) and PASMC treated with heparin. B and C: Western blot analyses on STIM1, STIM2, and TRPC6 (B) as well as Orai1, Orai2, and Orai3 (C) in control proliferative PASMC (Cont) and PASMC treated with 30 μg/ml of heparin (Hep) (left). β-Actin was used as a loading control. Summarized data (means ± SE) (right) showing the protein expression levels of STIM1, STIM2, and TRPC6 (B) as well as Orai1, Orai2, and Orai3 (C) in control rat PASMC (Cont) and PASMC treated with heparin (Hep). *P < 0.05 vs. control (Cont) bars.

Overexpression of STIM2 enhances the increase in [Ca^{2+}]_{cys} due to SOCE in PASMC. To test whether STIM2 is sufficient to enhance SOCE, we compared the CPA-induced increases in [Ca^{2+}]_{cys} in control PASMC (transfected with an empty vector) and PASMC transfected with STIM2. Overexpression of STIM2 in human PASMC (hPASMC) resulted in a marked increase in protein expression of STIM2 (Fig. 6A) and a significant increase in the amplitude of CPA-mediated increase in [Ca^{2+}]_{cys} due to SOCE (Fig. 6B). In the absence of extracellular Ca^{2+}, extracellular application of 10 μM CPA, a SERCA inhibitor that depletes Ca^{2+} from the SR, caused a small and slow increase in [Ca^{2+}]_{cys} due to Ca^{2+} leakage from the SR to the cytosol in proliferative (Fig. 6B, top). When the CPA-mediated increase in [Ca^{2+}]_{cys} in the absence of extracellular Ca^{2+} returns to the baseline level, restoration of extracellular Ca^{2+} caused a large and rapid increase in [Ca^{2+}]_{cys} due to obviously SOCE. Overexpression of STIM2 in proliferative human PASMC (Fig. 6A) significantly enhanced 1) the CPA-induced increase in [Ca^{2+}]_{cys} due to Ca^{2+} leakage from the SR to the cytosol and 2) the CPA-induced increase in [Ca^{2+}]_{cys} due to Ca^{2+} influx through store-operated Ca^{2+} channels activated by the passive depletion of Ca^{2+} from the SR (Fig. 6B). These data demonstrate that increased expression of STIM2 is sufficient to enhance SOCE in PASMC.

Downregulation of Orai2 with siRNA attenuates the amplitude of [Ca^{2+}]_{cys} increase due to SOCE in proliferative PASMC. To investigate whether Orai2 is necessary for enhanced SOCE in proliferative PASMC, we compared the rise in [Ca^{2+}]_{cys} due to SOCE in control PASMC (transfected with scrambled siRNA) and PASMC treated with siRNA specifically targeted against Orai2. Downregulation of Orai2 with siRNA significantly decreased the protein expression level of Orai2 in proliferative PASMC cultured in 10% FBS-containing media (Fig. 7A). When proliferative PASMC were bathed in Ca^{2+}-free solution, inhibition of SERCA by extracellular application of CPA induced a small increase in [Ca^{2+}]_{cys} as a result of Ca^{2+} mobilization from the SR to the cytosol. Restoration of extracellular Ca^{2+} (to 1.8 mM) in the presence of...
In this study, we demonstrate that 1) upregulated expression of STIM2, TRPC6, and Orai2 is associated with a proliferative phenotype of PASM and may play an important role in the transition of PASM from the contractile phenotype to the proliferative phenotype, 2) SOCE is enhanced in the proliferative phenotype of PASM compared with the contractile phenotype of PASM, 3) overexpression of STIM2 is sufficient to enhance SOCE in PASM in proliferative PASM, 4) differentiation of proliferative PASM (by treatment with TGF-β or heparin) results in downregulation of STIM2 and TRPC6, and 5) downregulation of Orai2 or TRPC6 attenuates SOCE in the proliferative phenotype of PASM. Our data indicate that the transition of PASM from a contractile phenotype to a proliferative phenotype is associated with enhanced SOCE which requires upregulation of STIM2, TRPC6, and Orai2. This study.

Deletion of TRPC6 significantly attenuates the increase in [Ca^{2+}]_{cyt} due to SOCE in proliferative PASM. Many TRPC channels contribute to the formation of receptor-operated Ca^{2+} channels (ROC) and store-operated Ca^{2+} channels (SOC) in vascular smooth muscle cells including PASM (45L–47). To confirm that TRPC6 is involved in forming SOC responsible for enhanced SOCE in proliferative PASM, we isolated PASM from wild-type (WT) mice and Trpc6^{-/-} mice (Fig. 8A) and cultured the cells in media containing 10% FBS and growth factors. The mice were genotyped using the standard PCR procedure and confirmed downregulation and absence of Trpc6 mRNA expression in the Trpc6^{+/+} and Trpc6^{-/-} mice, respectively (Fig. 8A). In proliferative PASM isolated and prepared from Trpc6^{-/-} mice, the CPA-mediated increase in [Ca^{2+}]_{cyt} due to SOCE was significantly lower than that in proliferative PASM isolated from WT mice (Fig. 8, B and C). In human proliferative PASM, we previously reported that downregulation of TRPC6 with antisense oligonucleotides (46) significantly reduced SOCE, while upregulated TRPC6 was associated with enhanced SOCE in PASM isolated from patients with idiopathic PAH and animals with experimental pulmonary hypertension (42, 47). These results indicate that upregulated TRPC6 is involved in the enhanced SOCE in proliferative PASM compared with PASM in a contractile or differentiated phenotype.

**DISCUSSION**

Fig. 7. Downregulation of Orai2 attenuates SOCE in proliferative PASM. A: Western blot analyses on Orai2 and PCNA in proliferative PASM treated with scrambled siRNA (Scram-siRNA) and Orai2-targeted siRNA (Orai2-siRNA). β-Actin was used as a loading control. B: representative records (top) of the 10 μM CPA-induced changes in [Ca^{2+}]_{cyt} in Scram-siRNA-treated PASM and Orai2-siRNA-treated PASM superfused with Ca^{2+}-free (0Ca) or 1.8 mM Ca^{2+}-containing solution. Summarized data (means ± SE, bottom) showing the 10 μM CPA-induced increases in [Ca^{2+}]_{cyt} (amplitude and area under the curve) due to Ca^{2+} mobilization (Release) form the SR to the cytosol and Ca^{2+} influx through store-operated Ca^{2+} channels (SOC) in PASM treated with Scram-siRNA or Orai2-siRNA. *P < 0.05 vs. Scram-siRNA.

CPA then induced a large increase in [Ca^{2+}]_{cyt} due to SOCE (Fig. 7B, top). In proliferating PASM treated with siRNA for Orai2, the amplitude of the CPA-mediated increase in [Ca^{2+}]_{cyt} due to SOCE was significantly attenuated in comparison to PASM treated with scrambled (Scram) siRNA, whereas the CPA-mediated increase in [Ca^{2+}]_{cyt} due to Ca^{2+} mobilization was not significantly changed (Fig. 7B). Downregulation of Orai2 also significantly decreased expression of PCNA (Fig. 7A), suggesting that Orai2 expression also contributes to PASM proliferation. These results indicate that Orai2 is necessary for the enhanced SOCE in proliferative PASM. It is possible that upregulated STIM2 and Orai2 functionally interact with each other to enhance SOCE in proliferative PASM.

**Fig. 8.** Deletion of TRPC6 significantly attenuates CPA-induced increase in [Ca^{2+}]_{cyt} due to store-operated Ca^{2+} entry (SOCE). A: genotyping characterization of Trpc6^{+/+} and Trpc6^{−/−}, and wild-type (WT) mice demonstrates the presence of only knockout alleles in the homozygous mice, while heterozygotes have both knockout (KO) and WT alleles. PASM were isolated and prepared from Trpc6^{−/−} WT and WT mice for the fluorescence microscopy experiments in B. B: representative records of the 10 μM CPA-induced changes in [Ca^{2+}]_{cyt} in WT-PASM and Trpc6^{−/−}-PASM superfused with Ca^{2+}-free (0Ca) or 1.8 mM Ca^{2+}-containing solution. C: summarized data (means ± SE) showing the 10 μM CPA-induced increase in [Ca^{2+}]_{cyt} due to Ca^{2+} influx through store-operated Ca^{2+} channels (SOC) in PASM isolated from WT and Trpc6^{−/−} WT mice. *P < 0.05 vs. WT.
identifies a potential benefit for targeting STIM2, TRPC6, and/or Orai2 to prevent PASMC proliferation in the development of pulmonary hypertension.

Unlike skeletal or cardiac muscle cells, PASMC are amazingly plastic and can undergo extreme and reversible changes in phenotype in response to local environmental cues that regulate phenotype (27). In response to vascular injury, for example, smooth muscle cells exhibit a phenotypic change characterized by a dramatic increase in rate of proliferation, migration, and synthetic capacity (28). This “synthetic” or “highly proliferative” phenotype plays an active role in repair of vascular damage. Unfortunately, the significant level of plasticity of smooth muscle cells predisposes the cell to abnormal environmental signals that can lead to adverse phenotypic switching and acquisition of characteristics that can contribute to development and/or progression of vascular disease (28). Smooth muscle cell phenotype modulation contributes to the pathogenesis of numerous vascular disorders, including PAH.

There is a spectrum of different subtypes of smooth muscle cells that are present in the medial layer, which ranges from a contractile phenotype and a proliferative phenotype. The majority of healthy PASMC show a contractile phenotype, characterized by high contractile ability and low proliferation rate.

The molecular mechanisms driving smooth muscle cell differentiation/dedifferentiation in the PA media layer are not completely understood. Many studies have established that smooth muscle cells express multiple markers that are indicative of their phenotype; however, no single marker exclusively identifies a specific phenotype to the exclusion of other (28). The genes specific for smooth muscle cell differentiation include those that encode for smooth muscle α-actin (SM α-actin), smooth muscle 22-α (SM22α), smooth muscle myosin heavy chain (MYH), smooth muscle myosin light chain kinase (MLCK) and calponin, which all contain CArG elements in their promoter regions. Previous studies have shown that mutations of the conserved CArG elements in these genes promoter enhancer’s results in abolished expression of SM22α and MYH (18, 20). Furthermore, these smooth muscle cell markers are useful for assessing smooth muscle cell differentiation with a particular importance on identifying and assessing the degree of phenotypic switching in smooth muscle cells.

Loss of contractile markers (MYH, SM22α, and calponin) and increased proliferation is seen in dedifferentiated/proliferative smooth muscle cells (7). Myocardin has been shown to selectively induce the expression of all CArG-dependent smooth muscle cell marker genes (5). It has been also demonstrated that myocardin is both necessary and sufficient to activate various smooth muscle cell contractile markers.

A commonly used smooth muscle cell marker is SM α-actin, in part because it was the first known protein expressed during differentiation of smooth muscle cells during development and it is very specifically selective for smooth muscle cells (10). Additionally, SM α-actin is required for force contraction in fully differentiated smooth muscle cells, and it is the most abundant protein in differentiated smooth muscle cells making up 40% of total cell proteins (9). However, SM α-actin should not be used as a definitive smooth muscle cell lineage marker, since its expression varies in many non-smooth muscle cell types under specific situations (including, e.g., myofibroblasts and endothelial cells in response to TGF-β stimulation), which is why it was not used in these experiments to characterize smooth muscle cell differentiation.

Regardless of the methods of differentiating PASMC, our data presented here demonstrate that STIM2, TRPC6, and Orai2 are all upregulated in highly proliferative PASMC, suggesting that this upregulation is required for phenotypic switching. Indeed, it has been previously confirmed that depletion of STIM2 reduces the proliferative capabilities of dedifferentiated PASMC (35). Additionally, it has been shown that in STIM1 knockout cells there was a failure to progress to S phase of the cell cycle due to upregulation of p21 (CDK inhibitor) and reduction in Rb phosphorylation (Rb is one of the major modulators of the G1/S transition in mammalian cells) (16), as well as a reduction in cAMP response element binding protein (CREB) phosphorylation (36), and nuclear factor of activated T cells (NFAT) transcriptional activity (1), suggesting that multiple signaling pathways may be regulated by STIM2 in PASMC. Furthermore, the roles of various ion channels, specifically Ca2+ channels and K+ channels, have been established to play a role in the development of pulmonary hypertension (21, 48), and they too can potentially contribute to the development of pulmonary hypertension by increasing intracellular Ca2+ and thus cause PASMC differentiation. Alternatively, it was recently published that the Akt

![PASMC Phenotypic Transition](https://example.com/pasmc_phenotypic_transition)

Fig. 9. Schematic diagram of the proposed mechanisms in the transition of PASMC from the contractile phenotype to proliferative phenotype and its potential role in the development of pulmonary vascular remodeling.
signaling cascade is involved in regulating cell proliferation and pulmonary arterial remodeling, specifically through PTEN/ 
Akt1/mTOR signaling, suggesting a potential role of Akt within the pulmonary vasculature along with STIM proteins 
potentially (37). However, these suggestions have yet to be 
confirmed and would be necessary to complete this story. On 
the basis of previous sequence analysis, the promoter of TRPC1 
gene contains binding sequences for many transcription factors 
(such as AP-1, signal transducer and activator of transcription, 
Smad, and c-myc) that are involved in progression of the cell 
cycle and regulation of cell proliferation, differentiation, and 
apoptosis. It would be interesting to investigate whether in-
flammation mediators (such as cytokines, chemokines, and 
histamine) and growth factors, which are upregulated in pul-
monary hypertension, affect not only TRPC expression in 
normal PASMC by regulating these transcription factors and 
their DNA binding activity with the TRPC genes, but as well 
for the STIM2 and Orai2 genes. 

In summary, vascular smooth muscle cells, including 
PASMC, are extremely plastic and can dedifferentiate in re-
sponse to various environmental stimuli from a contractile/ 
quiescent to a proliferative/synthetic phenotype (28, 30). Under 
pathological conditions or vascular injury, PASMC undergo a 
change in phenotype, characterized by an increase in prolifer-
ation rate, migration, and vascular repair, due to increases in 
cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) (22). Importantly, an increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in PASMC is a major trigger for pulmo-

nary vasoconstriction and an important stimulus for cell pro-

liferation (24, 29). Here, we demonstrate that the transition 
from quiescent/contractile PASMC to proliferative/synthetic 
PASMC is associated with enhanced SOCE due to upregu-
lation of STIM2, TRPC6, and Orai2 (Fig. 9). Understanding the 
mechanical mechanisms that regulate phenotypic switching of 
PASMC and increased PASMC proliferation is critical to 
elucidation of the pathogenesis of PAH. STIM2, TRPC6, and 
Orai2 are potentially good targets for the development of new 
therapies for the treatment of PAH. 

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AUTHOR CONTRIBUTIONS

R.A.F., K.A.S., A.M., and J.X.-J.Y. conceived and designed the 
university, and S.S., K.A.S., and H.T. performed experiments; 
R.A.F., J.W., S.S., K.A.S., Y.G., M.T., and J.X.-J.Y. interpreted results of 
experiments; R.A.F., J.W., S.S., K.A.S., Y.G., M.T., H.T., A.M., D.M., and 
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DISCLOSURES

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