Isolation, culture and identification of pulmonary arterial smooth muscle cells from rat distal pulmonary arteries

Gongyong Peng · Juan Xu · Rongmin Liu · Zhenli Fu ·
Shaoxing Li · Wei Hong · Jinglong Chen · Bing Li ·
Pixin Ran

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Abstract The culture of pulmonary arterial smooth muscle cells (PASMCs) is one of the most powerful tools for exploring the mechanisms of pulmonary hypertension (PH). Both pulmonary vasoconstriction and remodeling occur predominantly in distal pulmonary arteries (PA). In this study, we provide our detailed and standardized protocol for easy isolation and culture of PASMCs from rat distal PA to supply every investigator with a simple, economical and useful method in studying PH. The protocol can be divided into four stages: isolation of distal PA, isolation of cells, growth in culture and passage of cells. Rat distal PASMCs were characterized by morphological activity and by immunostaining for

smooth muscle α -actin and smooth muscle myosin heavy chain, but not for CD90/Thy-1 or von Willebrand factor. Furthermore, functional assessments were performed, confirming the presence of voltage-dependent Ca^{2+} channels and physiological characteristic of response to hypoxia. In conclusion, we have developed a detailed and simple protocol for obtaining rat distal PASMCs. These PASMCs exhibit features consistent with vascular smooth muscle cells, and they could subsequently be used to further explore the pathophysiological mechanisms of PH.

Keywords Cell culture · Smooth muscle cells · Pulmonary artery · Rat

G. Peng (✉) · J. Xu · R. Liu · Z. Fu ·
S. Li · J. Chen · P. Ran (✉)

Guangzhou Institute of Respiratory Disease, State Key Laboratory of Respiratory Disease, The First Affiliated Hospital, Guangzhou Medical University, 151 Yanjiang Road, Guangzhou 510120, Guangdong, People's Republic of China
e-mail: gongyong19761@163.com

P. Ran
e-mail: pxran@vip.163.com

S. Li
Intensive Care Unit, Guangzhou Panyu Central Hospital, Guangzhou, Guangdong, People's Republic of China

W. Hong · B. Li
The Research Center of Experiment Medicine, Guangzhou Medical University, Guangzhou, Guangdong, People's Republic of China

Introduction

Pulmonary hypertension (PH) is a complex and progressive complication of a variety of disorders which affect the heart and lungs, such as left ventricular dysfunction, chronic obstructive pulmonary disease, interstitial lung disease, lymphangioleiomyomatosis and sarcoidosis. It is widely accepted that the hallmarks of various forms of PH are sustained pulmonary vasoconstriction and pulmonary vascular remodeling. Remodeling of pulmonary arteries (PA) is characterized by thickening of the medial layer of the vessels resulting from proliferation and hypertrophy of pulmonary arterial smooth muscle cells (PASMCs).

Studies reveal that proliferation of PASMCs makes a greater contribution than hypertrophy of PASMCs in the smaller, more distal PA, whereas hypertrophy of PASMCs is more prevalent in the larger, more proximal PA (Meyrick and Reid 1980; McKenzie et al. 1984). Furthermore, the available functional and anatomical evidences indicate that both pulmonary vasoconstriction and pulmonary vascular remodeling occur predominantly in small PA (Gan et al. 2007; Mahapatra et al. 2006; Vanderpool et al. 2011; Wharton et al. 2000). Therefore, obtaining a pure population of PASMCs from distal PA is highly desirable for in vitro experiments to explore the mechanisms of PH.

Historically, different techniques have been employed for the isolation and culture of PASMCs. In early reports, the main and proximal segments of PA were obtained from large animals, such as bovine, ovine, swine, and patients undergoing lung or heart–lung transplantation (Lee et al. 1991; Suzuki et al. 2003; Farrow et al. 2008; Li et al. 2011; Goncharova et al. 2002; Bradbury et al. 2002; Morrell et al. 2001). The medial layer of proximal PA was peeled away and cut into thin strips, after which these explants were placed intimal side down in culture dishes. PASMCs migrated out from the explants after 1 week and grew to confluence after 2 weeks. To shorten the period of cell culture, a method for the direct isolation of PASMCs by digesting the resultant PA smooth muscle tissue with digestion enzyme would be preferred (Zhang et al. 2003; Wharton et al. 2000). Although PASMCs could be obtained by digestion within 1 week, it has been proven inconvenient, expensive and difficult to obtain PASMCs from humans and large animals. Small animals, such as mice and rats, have long been used to model PH. Some investigators have isolated and utilized PASMCs from mice. However, these methods have limitations represented by the need for complex equipments, such as iron particles and perfusion equipment (Lee et al. 2013). In addition, mouse PASMCs were insufficient and grew very slowly (Lee et al. 2013). Although some studies have described the isolation of PASMCs from rats by enzyme digestion (Rothman et al. 1992; Fouty and Rodman 2003; Yuan 1995; Zeng et al. 2015; Plomaritas et al. 2014; Lin et al. 2004), rat PAs in these reports were mainly isolated from the pulmonary truncus where vasoconstriction and vascular remodeling occur slightly (Rothman et al. 1992; Fouty and Rodman

2003; Yuan 1995; Zeng et al. 2015), as well as some digestive enzyme formulas in these reports were complex including 2–4 components (Zeng et al. 2015; Plomaritas et al. 2014; Lin et al. 2004; Yuan et al. 1996). Additionally, some groups have applied PASMCs from rat intrapulmonary PAs, but the protocols for isolation and culture of PASMCs from rat PAs were too brief to instruct new investigators, as well as the data of identification of PASMCs were absent in these researches (Plomaritas et al. 2014; Lin et al. 2004; Yuan et al. 1996).

For many years our group focused on the physiology and pathophysiology of rat pulmonary vascular myocytes including PASMCs and pulmonary venous smooth muscle cells (PVSMCs, Peng et al. 2010a, b, 2013a, 2015; Lu et al. 2009, 2010; Wang et al. 2015; Li et al. 2013). In the present paper we describe a detailed and standardized protocol for easy isolation and culture of PASMCs from rat distal PA to supply all researchers including beginners with a method that can be used to further explore the pathophysiological mechanisms of PH with minimum equipments and reagents.

Materials and methods

Rats

Healthy male Wistar rats (body weight 250–350 g) were used in all experiments. All animal care and experiments were approved and carried out in accordance with the Animal Care and Use Committee of Guangzhou Medical University and in accordance with the principles and guidelines of the National Institutes of Health.

Reagents

Unless otherwise specified, all reagents were obtained from Sigma Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle media (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic, phosphate buffered saline (PBS) and 0.25% trypsin and 0.01% EDTA were from GIBCO (Carlsbad, CA, USA), mouse monoclonal antibody to smooth muscle myosin heavy chain, rabbit monoclonal antibody to CD90/Thy-1 and rabbit polyclonal antibody to von Willebrand factor were from Abcam (Cambridge, MA,

USA), Fura-2 AM was from Invitrogen (Carlsbad, CA, USA), Cy3-conjugated goat anti-mouse IgG antibody, HRP-conjugated goat anti-mouse IgG antibody and donkey anti-rabbit IgG antibody were from Jackson ImmunoResearch (West Grove, PA, USA), rat renal fibroblasts (NRK-49F) were from American Type Culture Collection (ATCC, Manassas, VA, USA).

Isolation of PSMCs from rat distal pulmonary arteries

Rats were deeply anesthetized with pentobarbital sodium (65 mg/kg^{-1} i.p.). Figure 1 shows the detailed procedure used to isolate PSMCs from rat distal PA. Under sterile conditions, anesthetized rats were placed in the supine position and the anterior and posterior legs were fixed with tapes. The skin was removed from the chest and epigastrium with pincers and scissors (Fig. 1a). The chest was opened and the heart and lungs were removed *en bloc* and transferred into a 100 mm petri dish with 10 ml of cold (4°C) PBS. The heart and lungs were rinsed twice with cold PBS to remove watery blood so that they were clearly visible in a dissection dish (Fig. 1b). Under a stereomicroscope, the heart and lungs were placed in the arteries-up position and fixed with Chinese acupuncture needles (Tianxie Acupuncture Instruments, Suzhou, Jiangsu, China) in a dissection dish contained cold PBS. The thymus gland was removed rapidly with microforceps (Jinzhong Surgical Instruments, Shanghai, China, Fig. 1c). The inferior vena cava was cut with microscissors (Jinzhong Surgical Instruments, Shanghai, China, Fig. 1d). After having been rinsed twice with cold PBS, the esophagus was removed with microforceps and microscissors (Fig. 1e). The aortic arch and connective tissue were isolated and removed with microforceps and microscissors (Fig. 1f). After having been rinsed twice with cold PBS, the trachea and main bronchus were removed with microforceps and microscissors (Fig. 1g). The extrapulmonary veins were isolated and removed carefully with microforceps and microscissors (Fig. 1h). The right and left branches of the intrapulmonary arteries were dissected from the lungs. The adventitial connective tissues were carefully removed from the arteries with microforceps (Fig. 1i). The peripheral lung tissues were removed from the remaining PA tissues (endothelial cell layer and muscular tissues). The PA tissues and the heart

were transferred into a 35 mm petri dish with 2 ml of cold PBS (Fig. 1j). The heart was removed from the PA tissues (Fig. 1k) and the left and right intrapulmonary arteries (>4 th generation) tissues were cut from the pulmonary truncus and 2–3rd generation PA tissues (Fig. 1l). The left and right distal PA tissues were cut lengthwise to expose the lumen (Fig. 1m). The endothelium was denuded by gently rubbing the luminal surface with a cotton swab. The distal PA muscular tissues were allowed to recover for 20 min in cold PBS (Fig. 1n) and then placed in the 15-ml plastic conical tube containing 2 ml of Ca^{2+} -reduced collagenase enzymatic solution (type I, 1800 U/ml, Fig. 1o). After having been incubated for 20 min at 37°C , the digested tissues were kept in the tube by carefully decanting the upper digestion solution. Ten ml of DMEM containing 20% FBS was added into the tube. The cells were dispersed by triturating the solution approximately 20 times with a wide-bore transfer pipette. The cells were plated onto culture plates and then incubated in DMEM containing 20% FBS in a 37°C , 5% CO_2 incubator. A half medium change was performed every 2 or 3 days.

Distal PSMCs passage

When the cells reached approximately 90% confluence, they were rinsed twice with PBS warmed to 37°C and digested with 0.25% trypsin and 0.01% EDTA for approximately 2 min at 37°C in a 5% CO_2 culture incubator. When most cells rounded up and came off the culture plate under a light-contrast microscope, they were neutralized with DMEM containing 10% FBS. The cells were detached by gently triturating the solution approximately 10 times with a wide-bore transfer pipette. The cell suspension was collected in a 15-ml conical tube and centrifuged for 5 min at 300 g at 20°C . The supernatant on top of the cell pellet was removed. After resuspension of the cell pellet with DMEM containing 10% FBS, the cells were passaged at a ratio of 1: 2 or 1: 3 and incubated at 37°C with 5% CO_2 . The culture medium was replaced with DMEM containing 10% FBS every 2 days.

Immunofluorescence staining

The primary cells grown on the coverslips for 4–5 days were used for immunofluorescence staining. After having been rinsed three times with PBS, cells

Fig. 1 Isolation of PASMCs from rat distal pulmonary arteries. **a** The skin was removed from the chest and epigastrium of an adult rat. **b** The heart and lungs were removed *en bloc* and transferred into a 100 mm petri dish with 10 ml of cold PBS and then rinsed twice with cold PBS to remove watery blood. **c** The thymus gland was removed with microforceps. **d** The inferior vena cava was cut with microscissors. **e** The esophagus was removed with microforceps and microscissors. **f** The aortic arch and connective tissue were isolated and removed. **g** The trachea and main bronchus were cut with microscissors. **h** The extrapulmonary veins were isolated and removed with microforceps and microscissors. **i** The right and left branches of the intrapulmonary arteries were dissected from the lungs. The

adventitial tissues were carefully removed from the arteries with microforceps. **j** The PA tissues (endothelial cell layer and muscular tissues) connected with the heart were placed into a 35 mm petri dish with 2 ml of cold PBS. **k** The heart was removed from the PA tissues. **l** The left and right intrapulmonary arteries (>4th generation) tissues were cut from the pulmonary truncus and 2-3rd generation PA tissues. **m** The left and right distal PA tissues were cut lengthwise to expose the lumen. **n** The deendothelialized distal PA tissues were transferred into a new 35 mm petri dish with 2 ml of cold PBS on ice to recover for 20 min. **o** The distal PA muscular tissues were placed into a 15 ml plastic conical tube with 2 ml of collagenase solution and then incubated for 20 min at 37 °C in a thermostatic water bath

were fixed with 4% paraformaldehyde for 15 min at RT. Following extensive washes with PBS, cells were treated with 0.25% Triton X-100 for 15 min at RT. Cells were then rinsed three times with PBS and incubated with 1% BSA for 30 min at RT. After having been incubated with mouse monoclonal antibody to smooth muscle α -actin (1:200) for 60 min at RT, cells were treated for 2 h at RT in dark with Cy3-conjugated secondary antibody goat anti-mouse IgG

antibody (1:100), which resulted in a red fluorescence. Cells were rinsed and incubated for 5 min at RT in dark with 4',6-diamidino-2-phenylindole (DAPI), which produced blue fluorescence. The coverslips were mounted with mounting medium and examined under an inverted laserscanning confocal microscope. Rat pulmonary arterial endothelial cells (PAECs), isolated as described earlier (Peng et al. 2013b), were used as a negative control for smooth muscle α -actin.

Immunocytochemistry

The fifth-to-seventh passage cells grown to 40–50% confluence were used for immunocytochemistry. As described above in Immunofluorescence staining, cells were fixed with 4% paraformaldehyde and then permeabilized with 0.25% Triton X-100 and blocked with 1% BSA. After three washes in PBS, cells were incubated for 90 min at RT with 200 μ L of the following primary antibodies: mouse monoclonal antibody to smooth muscle α -actin (1:200), mouse monoclonal antibody to smooth muscle myosin heavy chain (1:200), mouse monoclonal antibody to CD90/Thy-1 (1:100) and rabbit polyclonal antibody to von Willebrand Factor (1:200). Following repeated washes in PBS, cells were then incubated for 30 min at RT with the appropriate HRP-conjugated secondary antibody [goat anti-mouse IgG (1:100) or donkey anti-rabbit IgG (1:100)]. 3,3'-diaminobenzidine tetrahydrochloride was added to visualize the antibody complexes. Hematoxylin was added for nuclei staining. NRK-49F cells were used as a positive control for CD90/Thy-1, as well as rat PAECs were used as a positive control for von Willebrand factor.

Functional assessments

As previously described (Peng et al. 2013a, 2015), coverslips with PSMCs were incubated with 5 μ M fura-2 AM and then mounted in a polycarbonate chamber clamped in a heated platform (PH-2; Warner Instrument, Hamden, CT, USA) on the stage of a Nikon TSE 100 Ellipse inverted microscope (Nikon, Melville, NY, USA). The chamber was perfused at 1 mL/min with Krebs–Ringer bicarbonate (KRB) solution, which contained (in mM) 118 NaCl, 4.7 KCl, 0.57 MgSO₄, 1.18 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, and 10 glucose. The KRB solution was equilibrated with 16% O₂–5% CO₂. After perfusion for 10 min to remove extracellular dye, intracellular Ca²⁺ concentration ([Ca²⁺]_i) was assessed from the ratio of fura-2 fluorescence emitted at 510 nm after excitation at 340 nm to that after excitation at 380 nm (F₃₄₀/F₃₈₀) measured in 20–30 cells using a xenon arc lamp, interference filters, electronic shutter, $\times$ 20 fluorescence objective, and a cooled charge-coupled device imaging camera. Protocols were executed and data were collected online with InCyte software (Intracellular Imaging, Cincinnati, OH, USA). [Ca²⁺]_i was

estimated from F₃₄₀/F₃₈₀ measured in calibration solutions with [Ca²⁺]_i of 0–1350 nM. Cells' responses to high K⁺ Solution (60 mM) or hypoxia (4% O₂) were determined in separate experiments.

Results

Cell morphology observation

The cultured cells were observed under a phase-contrast microscope. Within 2 days after the initial culture, spindle-shaped cells started to divide and extend on the culture plate in a dispersed pattern (Fig. 2a). Some colonies forming clusters were observed after an additional 1–2 days of culture (Fig. 2b). Typically, the cells grew in a “hill-and-valley”-like pattern as soon as they became confluent. They grew to complete confluence in 6–7 days. (Fig. 2c).

Immunofluorescence staining

As shown in Fig. 3a, the cultured cells stained strongly for smooth muscle α -actin (shown in red). The cytoplasm of primary cells were filled with red cable-like fibers that were typical straight and non-interrupted (Fig. 3b). The red immunofluorescence of smooth muscle α -actin was not observed in rat PAECs that were treated similarly to our cultured cells (Fig. 3c). However, the von Willebrand Factor was strongly positive in rat PAECs (shown in red) which received the polyclonal antibody raised against von Willebrand factor (Fig. 3d). The immunofluorescence staining confirmed that our cultured cells were indeed smooth muscle cells.

Immunocytochemistry

To further confirm the phenotype and the purity of our cultured smooth muscle cells, the subcultured cells were examined for the presence of smooth-muscle-cell-specific markers and the absence of fibroblast and endothelial-cell-specific markers by immunocytochemical analysis. As shown in Fig. 4, all spindle-shaped smooth muscle cells at passage 5 (Fig. 4a) and passage 7 (Fig. 4b) were both homogeneously positive for smooth muscle α -actin (shown in brown). Similarly, the subcultured cells at passage 5 (Fig. 4c) and passage 7 (Fig. 4d) were both stained strongly for

Fig. 2 Phase-contrast microscopy images of rat distal PSMCs plated on culture dishes. **a** Cultured for 2 days, rat distal PSMCs were of typical spindle-shaped morphology. **b** Cultured for 3–4 days, rat distal PSMCs clustered and formed

longitudinal bands of parallel cells. **c** Rat distal PSMCs grew in a “hill-and-valley”-like pattern as soon as they became confluent. They grow to complete confluence in 6–7 days

Fig. 3 **a** Fluorescence immunocytochemical identification of smooth muscle α -actin antibody was shown in red in rat distal PSMCs. The nuclear marker DAPI was shown in blue. **b** Rat distal PSMCs showed typical straight, noninterrupted and cable-like fibers. **c** Immunofluorescence of smooth muscle α -actin did not appear in rat PAECs which were treated similarly to PSMCs. **d** Rat PAECs strongly expressed von Willebrand Factor (shown in red). (Color figure online)

smooth muscle myosin heavy chain (shown in brown). All subcultured smooth muscle cells exhibited absence of the fibroblast marker CD90/Thy-1 (Fig. 4e for passage 5 and Fig. 4f for passage 7) and the endothelial cell marker von Willebrand factor (Fig. 4g for passage 5 and Fig. 4h for passage 7). However, NRK-49F cells showed positivity for CD90/Thy-1 (Fig. 4i, shown in brown), as well as rat PAECs showed positivity for von Willebrand Factor (Fig. 4j, shown in brown). Respective negative controls for smooth muscle α -actin and smooth muscle myosin

heavy chain (data not shown), CD90/Thy-1 (Fig. 4k), and von Willebrand Factor (Fig. 4l), which were performed by omitting the primary antibody, were unstained.

Functional assessments

To confirm the physiological function of smooth muscle cells, we determined the effect of 60 mM KCl and acute hypoxia (4% O_2) on $[Ca^{2+}]_i$ in the cultured cells derived from rat distal pulmonary arteries. As shown in Fig. 5a, 60 mM KCl induced a marked increase in $[Ca^{2+}]_i$ that rose quickly to a peak of $\Delta[Ca^{2+}]_i = 164 \pm 38$ nM ($n = 5$), followed by a lower plateau before it returned to baseline by the perfusion of cells with KRB solution. Similarly, as shown in Fig. 5b, acute hypoxia (4% O_2) also caused a marked increase in $[Ca^{2+}]_i$ that rose quickly to a peak of $[Ca^{2+}]_i = 80 \pm 11$ nM ($n = 5$), followed by a plateau before it returned to baseline by the perfusion of cells with normoxia (16% O_2).

Discussion

In this study, we describe a detailed and standardized protocol for easy isolation and culture of PSMCs from rat pulmonary arterial branches for in vitro studies of pulmonary artery-related diseases. Through using this protocol, the researchers can easily obtain enough distal PSMCs with very good purity from commonly used rats in a short period of time.

It has been suggested that all vascular smooth muscle cells contain characteristic structural similarities that can be used to identify them. Therefore, cell morphology, immunological properties and functional assessments were used to identify our isolated cells

Fig. 4 Immunocytochemical analysis of subcultured PASMCs at passage 5 (**a**) and passage 7 (**b**). At both passages they were positive for smooth muscle α -actin (brown). Similarly, the subcultured cells at passage 5 (**c**) and passage 7 (**d**) were both positive for smooth muscle myosin heavy chain (brown). Passaged PASMCs were negative for the fibroblast marker CD90/Thy-1 (**e** for passage 5, **f** for passage 7) and the

endothelial cell marker von Willebrand Factor (**g** for passage 5, **h** for passage 7). **i** NRK-49F cells were positive for CD90/Thy-1 (brown). **j** Rat PAECs were positive for von Willebrand factor (brown). Respective negative controls for CD90/Thy-1 (**k**) and von Willebrand Factor (**l**) had no staining. The nuclei were counterstained with hematoxylin (blue). (Color figure online)

Fig. 5 Functional assessments of rat distal PASMCs responses to high K^+ solution (60 mM KCl) and acute hypoxia (4% O_2). Both high K^+ solution (**a**) and acute hypoxia (**b**) caused a

marked increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PASMCs that rose quickly to a peak followed by a lower plateau

from rat distal PA as vascular smooth muscle cells. The rat PASMCs that we have obtained exhibited characteristic morphological features, immunoreactivity to smooth muscle cell markers as well as functional physiological characteristics that were consistent with observations by other investigators

(Rothman et al. 1992; Yuan 1995; Yuan et al. 1996; Fouty and Rodman 2003; Lin et al. 2004; Plomaritis et al. 2014; Zeng et al. 2015).

The rat distal PASMCs started to divide within 2 days and showed a spindle-shaped appearance. Similar to other vascular smooth muscle cells

described by other investigators (Seidel et al. 1991; Heimli et al. 1997), the primary culture of distal PSMCs became confluent within 1 week and grew in a ‘hill-and-valley’-like pattern, which is characteristic of smooth muscle cell growth. From one rat, it is easy to obtain 12 confluent 35-mm dishes of primary culture of distal PSMCs within 1 week. The typical yield at passage one is between 1 and 2 million cells. This is sufficient for multiple experiments at low passage, such as gene and protein expression profiling and functional assessments. If larger amounts of cells are required for other experiments, distal PSMCs can be passaged at a ratio of 1:2–1:3 every 4–5 days with medium changes every 2–3 days.

To identify the origin of the cultured cells, we used a specific marker of vascular smooth muscle cells, smooth muscle α -actin, to analyse primary cells using immunofluorescence. The presence of smooth muscle α -actin is the most widely used criterion for identification of smooth muscle cells. We found that smooth muscle α -actin expression was strongly positive and cable-like myofilaments filled in our cultured cells, consistent with observations from other investigators (Dufresne and Warocquier-Clérout 2001; Lu et al. 2013; Crosas-Molist et al. 2015). However, the immunofluorescence of smooth muscle α -actin was negative in rat PAECs which were stained strongly for von Willebrand Factor. These data suggest clearly that the cells that we acquired from rat distal PA were, in fact, vascular smooth muscle cells.

To further confirm the phenotype of rat distal PSMCs, in our subsequent experiments, we used one additional smooth muscle cell marker, smooth muscle myosin heavy chain as well as two other fibroblast or endothelial cell markers, CD90/Thy-1 and von Willebrand Factor, in the immunocytochemical analysis of the fifth-to-seventh passage cells. Smooth muscle myosin heavy chain is one kind of smooth muscle cell myofilamentous proteins and is also widely used for identification of smooth muscle cell phenotype (Teng et al. 2006; Cairrão et al. 2009; Kennedy et al. 2014). All fusiform cells at passage 7, similar to those cells at passage 5, expressed smooth muscle α -actin and smooth muscle myosin heavy chain. Meanwhile, neither the fifth passage cells nor the seventh passage cells expressed CD90/Thy-1 or von Willebrand Factor. CD90/Thy-1 is a glycoposphatidylinositol-anchored protein expressed on the cell surface and is known as a fibroblast marker (Hagood et al. 2005;

Sabatini et al. 2005; Khoo et al. 2008). Von Willebrand factor is the most widely used criterion for identification of cells of endothelial origin. Therefore, the cells we obtained from rat distal PA maintained their phenotype of smooth muscle cells and were not contaminated with fibroblasts or endothelial cells between 5 and 7 passage. Usually, the large amounts of PSMCs at passage 5 are totally sufficient for multiple experiments in PH investigation.

The functional assessments of the cells’ responses to high K^+ solution and acute hypoxia were carried out to further determine whether the cells in culture exhibited physiological features of vascular smooth muscle cells, although the cell morphology and immunology indicated that the cultured cells were smooth muscle cells. Functional assessments showed that 60 mM KCl can cause a marked increase in $[Ca^{2+}]_i$ in cultured PSMCs, indicating that functional voltage-dependent Ca^{2+} channels (VDCCs) were present in rat distal PSMCs. Furthermore, we confirmed that 4% O_2 also caused a marked increase in $[Ca^{2+}]_i$ in cultured PSMCs, which is consistent with observations from other investigators (Ng et al. 2012; Weigand et al. 2011), indicating that the rat distal PSMCs that we acquired using this protocol were in good physiological condition.

In summary, we have developed a detailed and standardized protocol with simple and economical method for obtaining PSMCs from rat distal PA. Rat distal PSMCs show the morphological characteristics of vascular smooth muscle cells, express smooth muscle α -actin and smooth muscle myosin heavy chain, lack expression of CD90/Thy-1 and von Willebrand factor, possess VDCCs and respond to hypoxia consistent with their vascular smooth muscle cell origin. PSMCs could subsequently be used to further explore the pathophysiological mechanisms of PH.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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