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Yong-Xiao Wang *Editor*

Pulmonary Vasculature Redox Signaling in Health and Disease

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Preface

This book is composed of 26 chapters contributed by numerous outstanding basic, translational, and physician scientists in the fields of pulmonary vasculature redox signaling in health and disease; thus, it offers a widespread and comprehensive overview for academic and industrial scientists, postdoctoral fellows, and graduate students who are interested in redox signaling in health and disease and/or normal and pathological functions of the pulmonary vasculature. The book may also be very valuable for clinicians, medical students, and allied health professionals.

Redox signaling is a major molecular process involved in almost every physiologic cellular response in the pulmonary vasculature including energy metabolism, host defense, gene expression, contraction, proliferation, and migration. Aberrancy in this important signaling pathway leads to a critical role in the development of nearly all pulmonary diseases, such as pulmonary hypertension, cor pulmonale, pulmonary edema, and vasculitis, among others. These well-recognized concepts with recent advances have been comprehensively described by Prof. Jeremy Ward in his chapter "From Physiological Redox Signalling to Oxidant Stress."

Key members of redox signaling are reactive oxygen species (ROS), e.g., superoxide and hydrogen peroxide. These well-known molecules usually participate in specific oxidation or reduction modifications of one or more targeted molecules in order to mediate pulmonary vasculature cellular responses. ROS can be produced by mitochondria, NADPH oxidase (Nox), or other sources. In the chapter "Crosstalk Between Mitochondrial Reactive Oxygen Species and Sarcoplasmic Reticulum Calcium in Pulmonary Arterial Smooth Muscle Cells," Dr. Tengyao Song et al. have delivered detailed information with respect to the contribution and crosstalk of mitochondria and Nox in hypoxia-induced ROS production in pulmonary artery smooth muscle cells (PASMCs). These authors have further reported new data demonstrating that reciprocal interplays between mitochondrial ROS and sarcoplasmic reticulum Ca^{2+} signaling are important to hypoxic cellular responses in PASMCs and hypoxic pulmonary hypertension. In support, Drs. Qiujun Yu and Stephen Chan have highlighted the importance of mitochondrial ROS in pulmonary vascular endothelial cells to mediate the development of pulmonary hypertension in their chapter titled "Mitochondrial and Metabolic Drivers of Pulmonary Vascular Endothelial Dysfunction in Pulmonary Hypertension." In the chapter "Adventitial Fibroblast Nox4 Expression and ROS Signaling in Pulmonary Arterial Hypertension," Profs. Scott Barman

and David Fulton have further provided emerging evidence that Nox4-mediated ROS signaling in adventitial fibroblasts plays an important role in pulmonary hypertension. Similarly, Drs. Megha Sharma and Adeleye Afolayan have meticulously presented convincing evidence for the critical roles of mitochondrial and Nox-mediated redox in the development of pulmonary hypertension of newborns in their chapter "Redox Signaling and Persistent Pulmonary Hypertension of the Newborn."

Typically, ROS are metabolized and degraded by superoxide dismutase (SOD), glutathione peroxidase, catalase, or other antioxidant enzymes. This fact, together with the view that increased ROS production may cause cardiovascular disease development and progression, has led to preclinical and clinical trials of antioxidant interventions for cardiovascular disorders including pulmonary hypertension. Prof. Nozik-Grayck and her associates have provided a thorough overview of the roles of different SOD isoforms in the physiological and pathological cellular responses in the pulmonary circulation in their chapter titled "Redox regulation of the superoxide dismutases SOD3 and SOD2 in the pulmonary circulation." Consistent with the therapeutic roles of endogenous antioxidant enzymes, Dr. Gerald Maarman has reported new and ample data to draw notable attention to the potential effectiveness of melatonin as a natural antioxidant pulmonary hypertension therapy in his chapter "Natural Antioxidants as Potential Therapy, and a Promising Role for Melatonin Against Pulmonary Hypertension."

The potential roles of sophisticated redox signaling in the pulmonary vasculature are further reinforced by the recent experimental indications that reactive nitrogen species (e.g., NO) and reactive sulfur species (e.g., H_2S) are likely to be important for a number of physiological and pathological pulmonary vascular cellular responses. In the chapter "A Brief Overview of Nitric Oxide and Reactive Oxygen Species Signaling in Hypoxia-Induced Pulmonary Hypertension," Profs. Ariel Jaitovich and David Jourd'heuil have provided a systemic summary of the interactive roles of NO and ROS in the development of pulmonary hypertension. Largely based on their own research, Prof. You-Yang Zhao and his colleague have articulated new molecular mechanisms for the roles of NO and relevant nitrative stress in pulmonary hypertension in their chapter "Molecular Basis of Nitrative Stress in the Pathogenesis of Pulmonary Hypertension." In complement, the chapter "Redox Mechanisms Influencing cGMP Signaling in Pulmonary Vascular Physiology and Pathophysiology" from Prof. Michael Wolin's group has systematically elaborated the functional importance of redox-mediated, NO-dependent cGMP signaling in physiological and pathological cellular responses in the pulmonary vasculature. Equally interestingly, Drs. Jesus Prieto-Lloret and Philip I Aaronson have contributed an excellent chapter, "Hydrogen Sulfide as an O_2 Sensor: A Critical Analysis," that provides a comprehensive indication that H_2S may function as an O_2 sensor and play an important role in hypoxic responses in pulmonary vascular cells.

Redox signaling may mediate cellular responses in a temporally and spatially dynamic manner. Redox molecular processes can also occur in a specific fashion, dependent on cell type. The pulmonary vasculature is composed of different types of cells including smooth muscle cells, endothelial cells,

adventitial cells, fibroblasts, neutrophils, macrophages, lymphocytes, and stem/progenitor cells. Thus, each of these distinctive types of cells may produce its own specific redox signaling in response to distinctive stimuli. The functional crosstalks within and among individual cells are likely to further make redox signaling more effective and specific in the pulmonary vasculature. In addition to the aforementioned chapters, Dr. Karthik Suresh and Larissa Shimoda elegantly review the reciprocal roles of ROS and Ca^{2+} signaling in endothelial cells as key players in mediating pulmonary hypertension in their chapter "Endothelial Cell Reactive Oxygen Species and Ca^{2+} Signaling in Pulmonary Hypertension." Another well-written chapter, "Metabolic Reprogramming and Redox Signaling in Pulmonary Hypertension" by Dr. Lydie Plecitá-Hlavatá et al., has provided a systemic overview of redox-dependent metabolic reprogramming in almost all types of cells in the pulmonary vasculature and their contributions in the development of pulmonary hypertension.

Redox molecules have their own intrinsic physicochemical properties (e.g., redox potential, life time, and diffusive ability), diverse physiological functions, unique spatial and temporal profiles, and also distinctive metabolic products. The contribution of specific redox molecules and relevant cellular processes may vary with the development and progression of different pulmonary vascular diseases. The complexity and diversity of redox systems indicate that general antioxidants may not have sufficient accessibility to target molecules to produce specific actions. These may well explain the relatively low efficiency of generalized antioxidants in clinical trials. In the chapter titled "Subcellular Redox Signaling," Prof. Qinghua Hu's team has provided broad and detailed discussions on the subcellular ROS signaling in PASMCs, with particular focus on the mechanisms of subcellular ROS production and potential use of exogenous mitochondria in the treatment of pulmonary hypertension. Compatibly, Drs. Ryota Hashimoto and Sachin Gupte have further dedicatedly depicted the significance of interactive roles of redox molecules between the cytosol and mitochondria in pulmonary hypertension in their chapter "Pentose Shunt, Glucose-6-Phosphate Dehydrogenase and NADPH Redox, and Stem cells in Pulmonary Hypertension."

Indubitably, innovative state-of-the-art methods and techniques are very helpful in the elucidation of redox functions and processes in the cell. For instance, the use of the powerful electron paramagnetic resonance (EPR) spectrometry makes in vitro and in vivo studies of previously indescribable redox molecules possible. Recently identified redox biosensors may specifically and clearly outline changes of redox molecule levels in cells and even in separate cellular compartments, collecting important data that would clarify previously conflicting results. Similarly, novel and highly selective labeling agents are now available to investigators to help detect uncommon redox modifications within and outside the cell. Furthermore, innovative proteomic, gene mapping and other methods have been introduced to monitor posttranslational modifications of redox proteins and enzymes as well as complex redox responses. These modern approaches and techniques have opened the door to entirely new areas of redox studies, and more importantly, will pave the way for life-saving interventions. Very appreciatorily, Prof. Steven Qian

and his associate have updated the current techniques and methods for measurements of intracellular ROS in their chapter "Techniques for Detecting Reactive Oxygen Species in Pulmonary Vasculature Redox Signaling."

The precise functional roles, signaling processes, and molecular mechanisms of redox molecules, particularly in the formation and progression, are very complex and still remain mostly elusive. Further basic and translational research on redox signaling in the pulmonary vasculature will significantly promote discoveries of new and more effective antioxidants and redox-regulatory drugs for treatment of pulmonary vascular diseases. Dr. Annarita Di Mise along with Prof. Yun-Min Zheng has detailed the current knowledge on the possible essential roles of transcription factors for the initiation and progression of pulmonary hypertension in the chapter "Role of Transcription Factors in Pulmonary Artery Smooth Muscle Cells: Focus on Pathogenesis of Hypoxia Pulmonary Artery Hypertension." The chapter that Prof. Laura Bosc and his colleagues have written, "Altered Redox Balance in the Development of Chronic Hypoxia-Induced Pulmonary Hypertension," expounds the significant contribution of redox-dependent activation of the nuclear factor activated T-cells in pulmonary hypertension. Moreover, my own group has composed a chapter titled "Emerging Role of MicroRNAs and Long Noncoding RNAs in Health and Disease Pulmonary Vasculature" to further elucidate the functional impacts of the transcriptional and nontranscriptional regulation of various key molecules by microRNAs and long noncoding RNAs in physiological and pathological responses in pulmonary vascular cells.

Recent studies reveal that redox signaling is necessary for the normal function and development of the pulmonary vasculature. Prof. Christina Pabelick and her associates fully explicate the importance of redox-mediated hyperoxic signaling in postnatal vascular and alveolar development in their chapter titled "Effects of Hyperoxia on the Developing Airway and Pulmonary Vasculature." In complement, the chapter by Dr. Michael Thompson et al. with a title "Hypoxia and Local Inflammation in Pulmonary Artery Structure and Function" exquisitely delineates whether and how redox signaling mediates the effects of hypoxia and inflammation on pulmonary structure and function.

In addition to the well-documented pulmonary hypertension as described above, a series of recent studies demonstrate that redox signaling plays a key role in several other acute and chronic pulmonary diseases; the role of redox singling in each disease is mediated by one or more unique molecular mechanisms. Prof. Stephen Black's team has provided a full review highlighting how the Nox4-relied ROS signaling in endothelial cells and fibroblasts contributes to acute lung injury and respiratory distress syndrome, two of the most common and severe pulmonary illnesses, in the chapter titled "ROS Signaling in the Pathogenesis of Acute Lung injury (ALI) and Acute Respiratory Distress Syndrome." The chapter "Lung Ischemia/Reperfusion Injury: The Role of Reactive Oxygen Species," written by Prof. Norbert Weissmann and his colleagues, includes an extensive review about the actions of ROS and reactive nitrogen species with Nox, xanthine oxidases, nitric oxide synthases, and mitochondria to involve in ischemia/reperfusion-mediated

lung injury. The two excellent laboratories led by Prof. Yunchao Su and Li Zuo, respectively, contribute the chapter "Redox-Dependent Calpain Signaling in Airway and Pulmonary Vascular and Remodeling in COPD" and "Reactive Oxygen Species in COPD-Related Vascular Remodeling." The former chapter focuses on the role of reactive oxygen and nitrogen species-reliant calpain signaling in the development of airway and pulmonary vascular remodeling in COPD, while the latter chapter emphasizes the potential utilization of anti-inflammatory and antioxidative agents against ROS-mediated impairments of pulmonary functions in treatments of COPD.

A major cause of death in pulmonary diseases, particularly COPD, is right ventricular failure. It is not surprising that Prof. Yuichiro Suzuki's laboratory has conducted a series of excellent investigations with respect to the functional significance of right ventricular redox signaling for many years. As such, he and his associate have written an elegant chapter "Redox Signaling in the Right Ventricle," in which they thoughtfully deliberate the current compelling molecular and biochemical understandings of redox signaling-mediated right ventricular functional abnormalities and failure in pulmonary hypertension.

Finally, I sincerely express my wholehearted gratitude to all of the authors for their dedicated and diligent contributions. Many of the authors of this book have played a unique role as well as the reviewers; as such, their additional efforts are further highly appreciated. I also wish to thank Ms. Dana Bigelow, Associate Editor at Springer Nature in New York, and Mr. Silembarasan Panneerselvam, Project Coordinator (Books) for Springer Nature, for their kind patience and assistance.

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Dr. Yong-Xiao Wang is a Full Professor in the Department of Molecular and Cellular Physiology at Albany Medical College, Albany, New York, USA. He received his M.D. at the Wannan Medical College (China), Ph.D. at the Fourth Military Medical University (China), and postdoctoral training at the Technical University of Munich (Germany) as well as the University of Pennsylvania (USA). Dr. Wang's primary research interests have focused on basic, translational, and drug discovery research concerning cardiac arrhythmias and hypertrophy, pulmonary hypertension, asthma, and diabetes for over 30 years. In particular, he has had extensive research experience in the studies of the molecular geneses, regulatory mechanisms, network pathways, physiological functions, and the critical roles of calcium, redox, inflammatory, and other signaling in the aforementioned illnesses. Dr. Wang has been the corresponding author, first author, and key contributor in numerous publications in highly peer-reviewed journals including ***Antioxid Redox Signal***, ***Proc Natl Acad Sci USA***, ***Free Radic Biol Med***, ***Cell Calcium***, ***J Gen Physiol***, ***J Biol Chem***, ***FASEB J***, ***Nature***, ***Circ Res***, and more. He has been the editor of several academic books in the field including a recent one entitled ***Calcium Signaling in Airway Smooth Muscle Cells*** that was published by Springer in 2014. Dr. Wang has been the principal investigator on multiple research grants and awards from the National Institutes of Health, American Heart Association, American Diabetes Association, and other agencies. He has collaborated with numerous well-known scientists, served on various grant review panels, been the Editor-In-Chief and Editorial Board Member for several scientific journals, and trained a number of scholars, some of whom have become well-known independent investigators in the field.

Adventitial Fibroblast Nox4 Expression and ROS Signaling in Pulmonary Arterial Hypertension

Scott A. Barman and David Fulton

1 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a progressive disease of the lung vasculature, which is characterized by sustained pulmonary arterial pressure, resulting in increased pulmonary vascular resistance, with eventual right heart failure [1]. Vascular remodeling caused by the medial hyperplasia of pulmonary artery (PA) smooth muscle cells is a hallmark feature of PAH [2], which causes occlusion of the vessels [2]. In most forms of PAH, muscularization of small distal PA occurs [3], and is further characterized by excessive vascular cell proliferation, inward remodeling, rarefaction, and a loss of compliance of the pulmonary blood vessels [3–5]. Increased resistance to blood flow and more rigid blood vessels (loss of vascular compliance) leads to failure of the right ventricle and eventual death. PAH is more frequent in women than men, and left untreated has a survival time of 5–7 years post diagnosis [6]. From a therapeutic standpoint, there are a number of vasodilator drugs that are indicated for the treatment of PAH, but none of the current therapeutics offers long-term success for survival due to limited effectiveness and unwanted side effects [7], and more importantly, do not address the underlying causes of the disease [1].

2 Reactive Oxygen Species, NADPH Oxidase and PAH

The generation of reactive oxygen species (ROS) is well recognized as a pathophysiological mechanism underlying the vascular remodeling and proliferation that occurs in PAH. The major ROS that are produced in the pulmonary vasculature are superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and hydroperoxyl radical ($HO^{\cdot}{}_2$) [8]. Of these ROS, both O_2^{-} and H_2O_2 activate multiple signaling pathways that cause cell proliferation and apoptosis, elevated vascular tone, fibrosis, and inflammation, which are all hallmark signs of PAH [8]. However, the cellular origin and functional significance of ROS in PAH remain poorly delineated. Elevated levels of ROS in PAH occur because there is increased production and decreased enzymatic degradation of the ROS moieties, of which, evidence exists for both phenomena in the underlying etiology of elevated

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pulmonary arterial pressure and pulmonary arterial resistance [9–13]. The major intracellular sources of ROS include the mitochondrial electron transport chain, abnormal oxygenase activity, and the NADPH oxidase family (Noxes) [14, 15]. The human genome encodes five Nox isoforms, and four of these isoforms, Nox1, Nox2, Nox4, and Nox5 are expressed in vascular cells. In relation to other sources of ROS, Nox enzymes are regarded as professional ROS generators and are capable of generating high levels of ROS in a spatial and temporal manner. Nox1, Nox2, Nox3, and Nox4 are bound to p22phox, and Nox1 and Nox2 are activated by binding numerous cytosolic subunits, including p47phox, p67phox or NOXO1 and NOXA1. In contrast, Nox4 is regarded as a constitutively active enzyme with ROS levels primarily controlled by changes in gene expression [16, 17]. In mice, genetic deletion of Nox2 has been shown to reverse hypoxia-initiated PAH [10], and Nox1 has been shown to be an important signaling mediator for both systemic and pulmonary arterial hypertension [18, 19].

Specific to the scope of this review, increased expression of Nox4 has been reported in human PAH [20]. However, despite these observations, the functional significance of Nox4 in the development of PAH is poorly understood. Nox4 is expressed in all three layers of the vascular wall [21, 22], is constitutively active [23], and whose expression is increased by a plethora of diverse stimuli including angiotensin II, TGF-β, TNF-α, γ and hypoxia [24, 25]. In addition to human PAH, Nox4 expression is upregulated in the pulmonary vasculature of hypoxia-exposed mice, and in rat models of PAH [20, 22]. Evidence suggests that Nox2 may be involved in the induction of Nox4 to cause ROS production and subsequent pulmonary arterial smooth muscle cell (PASMC) proliferation that is characteristic of PAH [20]. Specifically, it is thought that initial activation of Nox2 induces the production of Nox4 in pulmonary endothelium to initiate events that cause pulmonary arterial remodeling [20].

Evidence also suggests that Nox4-derived ROS mediates both rodent and human PASMC proliferation under hypoxic conditions [25, 26]. In particular, during hypoxia, Nox4 is induced by TGF-β, which promotes smooth muscle cell proliferation in pulmonary arteries, a major cause of pulmonary arterial remodeling [27], and specific growth factors such as insulin-like growth factor binding protein (IGFBP-3) increases Nox4 gene expression, resulting in PASMC proliferation and subsequent medial thickening [27]. Further, Nox4 has been shown to be important in hypoxia-inducible factor 2α (HIF-2α) expression and transcription, which suggests an important relationship exists between Nox4 and HIF-2α, as well as TGF-β in pulmonary vascular remodeling in PAH [8].

3 Nox4 Expression in PAH

As stated above, reactive oxygen species (ROS) are important regulators of pulmonary vascular remodeling, and abundant evidence supports a prominent role for Nox4 in the pathogenesis of PAH [20, 28]. Nox4 is the major NADPH oxidase homolog expressed in human PASMCs [29], and its expression both at the mRNA and protein level is significantly increased in lungs from patients with idiopathic pulmonary arterial hypertension (IPAH) compared to healthy lungs [20], which suggests a correlation between Nox4 and the onset of PAH. In experimental rodent models of PAH, Nox4 expression is increased. Specifically, Nox4 is upregulated in chronic hypoxia-induced PAH in mice [20, 28, 30], and monocrotaline (MCT)-treated rats [22, 31]. Nox4 mediates the hypoxia-induced growth of human PASMCs [27], and silencing Nox4 expression by RNA interference decreases human PASMC and fibroblast proliferation [30, 32, 33]. Severe forms of PAH are associated with plexiform lesions, which are comprised of proliferating endothelial cells and elevated levels of angiogenic factors such as VEGF [34, 35]. Pneumonectomy increases the severity of PAH in animals treated with MCT, and has been shown to stimulate the formation of lesions that are morphologically similar to plexiform lesions [36]. Pneumonectomy also further increases the expression of Nox4 in MCT-treated animals [31] but it is not currently known whether Nox4 expression contributes to the formation of these lesions. Collectively, these findings support the premise for Nox4 expression being inherently involved in pulmonary vascular

remodeling by promoting arterial medial smooth muscle proliferation and adventitial fibroblast activation in PAH. Reports on the location of Nox4 expression in pulmonary arteries varies as Nox4 has been observed in the media of both normotensive and hypertensive pulmonary arteries [20], as well as in endothelial cells and fibroblasts [21, 22, 37, 38].

4 Enzymatic Properties of Nox4

Nox4 is unique in that it is the only Nox enzyme which is constitutively active. Nox4 colocalizes with and directly binds the integral membrane protein p22phox, which is essential for the Nox4 activity [39] to stabilize p22phox expression [40]. The binding and activation of Nox4 by p22phox does not depend on the proline rich region of p22phox, which is important in the regulation of Nox1, Nox2, and Nox3 [41]. A further distinction is that Nox4 does not require the binding of cytosolic proteins for ROS production [40], and instead produces ROS constitutively. This is due to unique characteristics of the C-terminus of Nox4 that facilitates the constitutive transfer of electrons from NADPH to FAD [16]. Another distinguishing feature of Nox4 compared to the other Nox enzymes is that robust production of H_2O_2 can be detected which contrasts from a mixture of superoxide and H_2O_2 from Nox1, Nox2, Nox3, and Nox5 [40, 42, 43]. The mechanism underlying the preferential production of H_2O_2 versus superoxide is related to the presence of a highly conserved histidine residue in the E-loop of Nox4 that promotes the rapid dismutation of superoxide before it leaves the enzyme [44].

5 Role of the Adventitial Fibroblast and Nox4 in PAH

Remodeled pulmonary arteries in PAH are characterized by increased stiffness [45, 46], secondary to collagen and elastin deposition, a process which is regulated by the adventitial fibroblast. The fibroblast, a primary cell type of the adventitia, contributes to the perpetual reorganizing of the extracellular matrix, secretion of growth factors and chemokines, as well as inflammatory cytokines. Studies show that TGF-β1 induces Nox4 expression in pulmonary fibroblasts and adventitial fibroblasts surrounding pulmonary vessels, suggesting that Nox4 is a component of the TGF-β1 signaling pathway [29, 47, 48]. TGF-β1 via Nox4 may contribute toward disease pathology through transcriptional activation of extracellular matrix components such as collagen and through a number of mediators known to exacerbate the extent of fibrosis and vascular remodeling, including PAI-1 and HIF-1α [49]. TGF-β1 is known to induce PAI-1 expression in fibroblasts through Nox4-dependent ROS production and increased activation of p38 MAPK and JNK [50]. In addition, NOX4 promotes myofibroblast secretion of extracellular matrix (ECM) proteins and production of fibronectin via TGF-β1 signaling, which promotes cellular fibrogenesis [47]. In vascular diseases such as PAH, an increase in collagen and ECM matrix proteins would promote increased tissue fibrosis as well as vascular stiffness through a decrease in tissue and vessel compliance [45, 46]. Recent studies show that the pulmonary arterial remodeling that occurs in hypoxia-induced PAH is characterized by the emergence of adventitial fibroblasts, which recruit inflammatory cells and adhesion proteins, promoting a pro-inflammatory and proliferative environment, leading to vascular remodeling [51]. Fibroblasts also influence and promote the inflammatory response by manipulating leukocyte recruitment, survival, and behavior [52], a phenomenon that appears to be regulated by Nox [53]. In addition, a subset of circulating bone marrow derived cells termed fibrocytes that possess genetic markers and behaviors consistent with both fibroblasts and macrophages can also be found in the adventitia [54, 55], and fibrocytes have the capability to differentiate into collagen-producing fibroblasts and myofibroblasts [56–58]. It has been shown that Nox enzymes and elevated ROS stimulate fibroblast proliferation [30, 47, 59]; however, the contribution of specific Nox isoforms to adventitial fibroblast proliferation and the development of PAH is still poorly understood.

Cultured pulmonary adventitial fibroblasts express Nox4 [30], and Nox4 expression is upregulated by many different stimuli including

hypoxia, inflammatory mediators, and fibrotic moieties such as TGF-β, and TNF-α, which contribute to pulmonary fibroblast activation and proliferation [8]. Further, Nox4-induced ROS production by TGF-β in human fibroblasts occurs extracellularly, which is in contrast to that observed in human PASMC, where Nox4 activation by TGF-β leads to intracellular ROS production [29]. The cellular effects and mechanisms of extracellular versus intracellular generation of Nox4 are poorly understood in PAH. In many cell types, Nox4 generates low-level, predominantly intracellular ROS constitutively and in response to a variety of stimuli [60]. For example, in vascular smooth muscle cells (VSMC), ROS generation is predominantly intracellular [61], and in vascular endothelial cells (VEC), many NAD(P)H oxidase subunits are located in the nucleus, with ROS production occurring in a "nucleus-rich" fraction [62]. This prominent labeling of Nox4 in the nucleus provides a source of ROS that can potentially activate many downstream targets, which include transcription factors such as AP-1 proteins c-Fos and c-Jun, implicated in growth and differentiation processes, and NF-κB, which is involved in inflammatory reactions and apoptosis [63]. Extracellularly, TGF-β1 increases ROS release (presumably through Nox4) by human fetal lung fibroblasts in a transcriptionally mediated manner, and the ROS produced by this NADPH oxidase activity is H_2O_2 [15, 64]. In addition, ROS production in human pulmonary arterial smooth muscle cells (HPASMC) via TGF-β1 is mediated by transcriptional induction of Nox4 expression [29]. However, in contrast to lung fibroblasts [15, 64], TGF-β1 stimulation in HPASMC induces intracellular generation of ROS derived from Nox4 localized to the endoplasmic reticulum, which does not result in extracellular release of H_2O_2 [29].

Studies done with siRNA to knockdown Nox4 has lead to the conclusions that Nox4-mediated ROS production stimulates cellular proliferation and inhibits apoptosis in pulmonary fibroblasts [30]. Interestingly, Nox4 upregulation by TGF-β is inhibited by N-acetylcysteine and DPI, which suggests that Nox4-induced ROS production regulates Nox4 gene expression [48]. Further, specific inhibition of Nox4 prevents pulmonary fibroblasts from mediating TGF-β-induced myofibroblast differentiation as well as producing collagen [48].

In a recent study, Barman et al. [22] did immunofluorescence staining of lung sections from MCT-treated rats and in lungs from normal (control) and human PAH (Fig. 1). In control rat pulmonary arteries (PA), Nox4 was primarily detected in the adventitial cells with secondary labeling on the intima (endothelial cells) (Fig. 1; top panel). In 4-week MCT-treated rats, using two different antibodies that are selective for Nox4 (Epitomics and Abcam), there was a dramatic increase in Nox4-positive cells in the adventitia (Fig. 1; MCT, left panels). In PA from MCT-treated rats, Nox4-positive cells were also detected in the remodeled medial layer agreeing with previous studies [20]. Nox4 also exhibited a staining pattern in sections of human lung from normal individuals and PAH (Fig. 1, lower panels) that was consistent with Nox4 expression in the MCT-treated rat lungs. In lung sections from animals treated with the specific Nox4 inhibitor (VCC202273; C) [22], MCT-stimulated pulmonary arterial remodeling and Nox4 adventitial expression were significantly attenuated (Fig. 1; MCT + C).

Barman and colleagues [22] also determined the location of ROS production in hypertensive PA using immunofluorescence imaging for 8-hydroxydeoxyguanosine (8-OHdG), a DNA nucleoside that is generated by ROS. As shown in Fig. 2, the highest signal for ROS was observed in the adventitia, which overlapped significantly with the fibroblast marker, fibroblast activating protein (FAP) (Fig. 2a; MCT). ROS levels in the adventitial layer was decreased in lung sections treated with the Nox4 inhibitor VCC202273 (C) (Fig. 2a; MCT + C), suggesting that the elevated adventitial ROS production in hypertensive PA derives from increased Nox4 expression. In addition, there was significant overlap between Nox4-positive cells in the adventitia and cells expressing fibroblast markers (cellular fibronectin, CD90) as well as the monocytic cell marker CD11B (Fig. 2b).

Nox4 also modifies fibroblast function in human lung. Using an adenovirus that expresses Nox4, Barman et al. [22] showed that Nox4-transduced

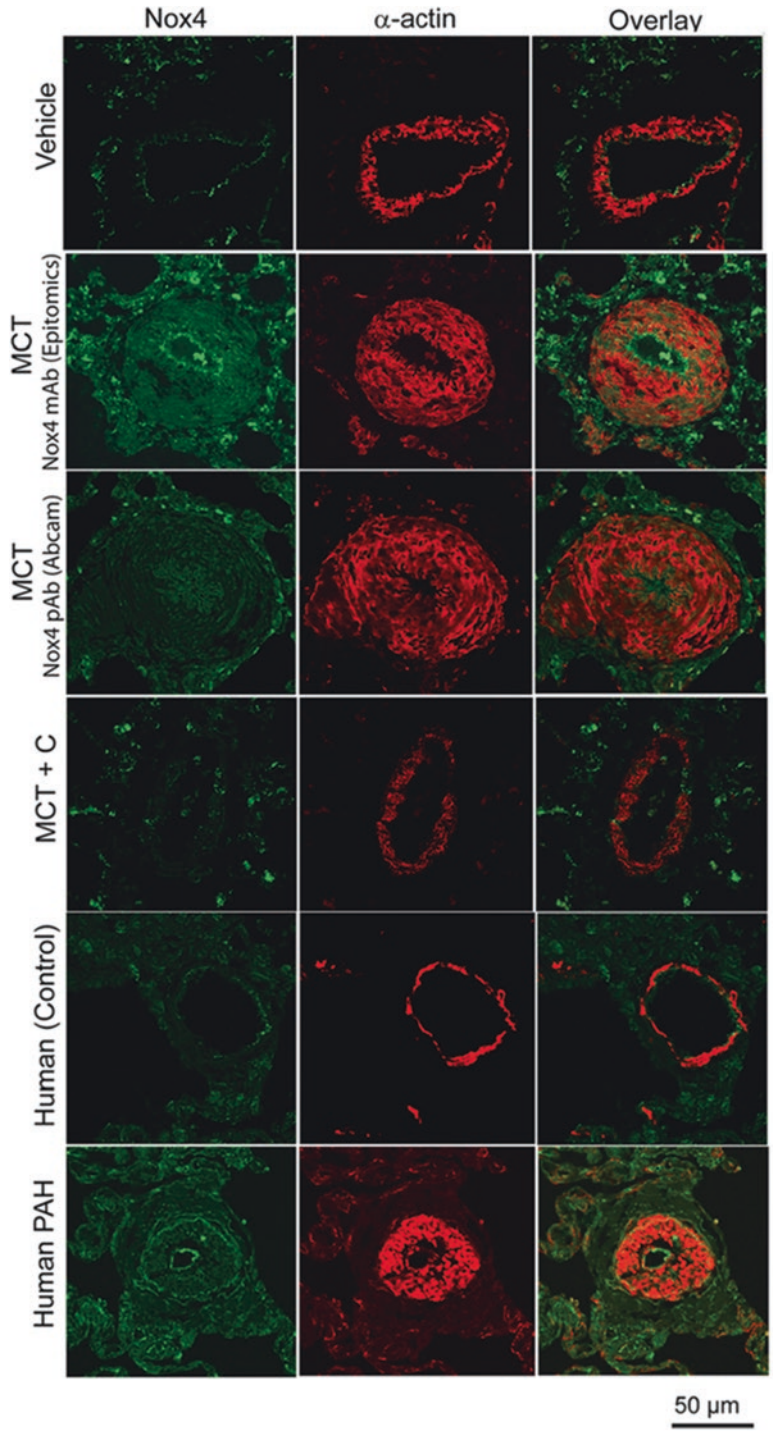

Fig. 1 Nox4 expression is upregulated in the adventitia in rat and human PAH. Confocal images of lung sections from control, experimental PAH (4-week MCT), and human PAH (IPAH undergoing lung transplant). Sections were stained with Nox4 and α-actin antibodies. Nox4 is highly expressed in cells of the adventitia (and intima) in 4-week MCT-treated rats and human PAH. There is also an abundance of Nox4-expressed cells present in the remodeled PA medial layer but devoid of α-actin expression in the MCT and human PAH. In the presence of Nox4 VCC202273 (C), (**MCT + C**), Nox4 expression is similar to vehicle-treated PA in the MCT-treated group. (Reproduced from Barman et al. (2014) Arterioscler Thromb Vasc Biol 34:1704–1715)

fibroblasts exhibited a robust increase in cellular proliferation as demonstrated by real-time changes in electrical impedance using electric cell-substrate impedance Sensing arrays (ECIS) (Fig. 3a). In addition, fibroblasts transduced with the Nox4-adenovirus displayed increased cell proliferation (total cell number, Fig. 3b), and the number of viable cells as measured via the 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyletrazolium bromide (MTT) assay (Fig. 3c).

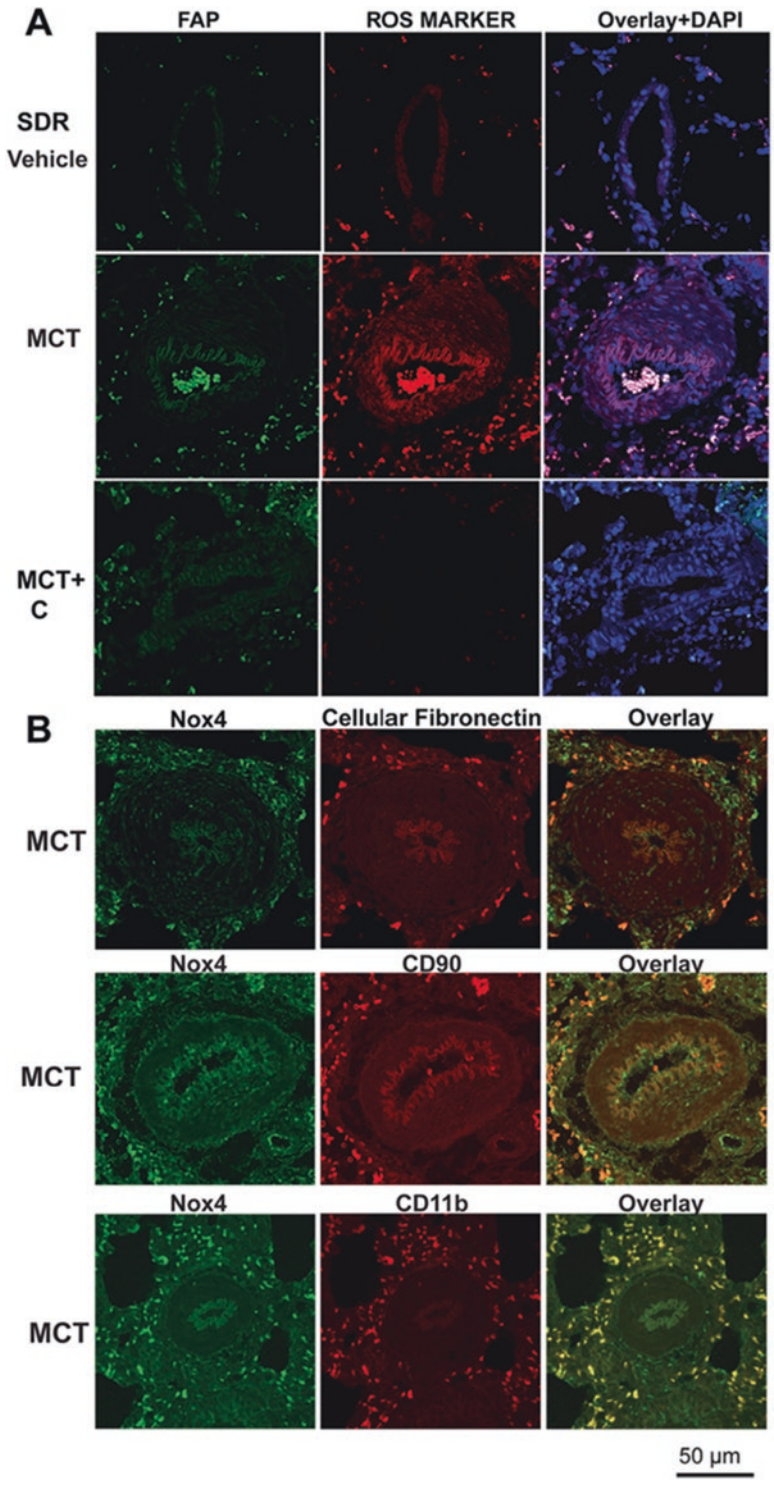

Fig. 2 Nox4 expression and reactive oxygen species (ROS) production is localized in the adventitia. (**a**) Sections of control and 4-week MCT-treated rat lungs co-stained for fibroblast activation protein (FAP), 8 Hydroxy-2'dexoyguanosine (ROS marker) and DAPI. (**b**) Co-staining for Nox4 and cellular fibronectin, CD90 and CD11b ROS production is elevated in PA adventitia from 4-week MCT-treated rats, which overlapped significantly with the fibroblast marker fibroblast activating protein (FAP) (**a; MCT**). ROS are decreased to control (vehicle) levels by the Nox4 inhibitor VCC202273 (**c**) (**A; MCT + C**). In MCT-treated PA, there is significant overlap between Nox4-positive cells in the adventitia and cells expressing fibroblast markers (cellular fibronectin, CD90) as well as the monocytic cell marker CD11B (**b**). (Reprinted from Barman et al. (2014) Arterioscler Thromb Vasc Biol 34:1704–1715)

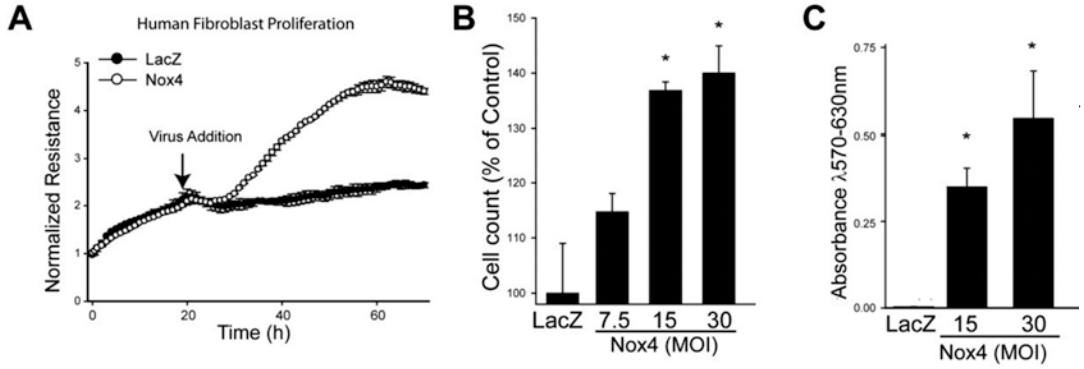

Fig. 3 Nox4 stimulates fibroblast proliferation and migration (**a**) Electrical impedance (ECIS) of human lung fibroblasts in the presence/absence of Nox4 adenovirus. Nox4-transduced fibroblasts exhibit a robust increase in cellular proliferation in real time using ECIS. (**b**) Nox4 increases fibroblast cell number, and (**c**) the number of viable cells using MTT assay. * Significantly different from Lac Z, $p < 0.05$ ($n = 3$–6 per group). (Reprinted from Barman et al. (2014) Arterioscler Thromb Vasc Biol 34:1704–1715)

robust than those observed in fibroblasts.

The functional relevance of Nox4 in adventitial cells is not well described especially in the realm of PAH. The *tunica externa* or adventitia is a loosely defined collection of cells including fibroblasts and immune cells, collagen, and elastic fibers that encircle the tunica media and intima layers of the blood vessel [66]. The adventitia orchestrates inflammation and vascular proliferation in response to injury, atherosclerosis and both pulmonary and systemic hypertension [67]. The fibroblast is a primary cell type of the adventitia, responsible for the continual reorganization of the extracellular matrix via matrix deposition and secretion of growth factors, chemokines and inflammatory cytokines [68]. Aberrant vascular remodeling in PAH occurs through increased inflammation, proliferation, and fibrosis, processes that collectively yield more muscular and less compliant pulmonary blood vessels [69]. Fibroblasts promote PAH by actively secreting matrix proteins, growth factors as well as promoting the inflammatory response by manipulating leukocyte recruitment and behavior [52, 70]. Supportive of this phenomenon, Barman et al. [22] observed that increased expression of Nox4, in the absence of other stimuli, was sufficient to increase fibroblast migration and proliferation. Similarly, it has been shown that silencing Nox4 in fibroblasts decreases the ability of stimuli such as TGF-β to increase matrix and induce contractile gene expression, which is consistent the number and behavior of adventitial fibroblasts that are inherently involved in PAH.

TGF-β1, a proliferative autocrine growth factor implicated in the pathophysiological vascular remodeling in PAH [73] robustly increases both Nox4 mRNA and protein levels in human lung fibroblasts. When comparing the ability of TGF-β1 to drive Nox4 expression in intimal cells (endothelial), medial cells (smooth muscle), or adventitial cells (fibroblasts), the greatest expression of Nox4 occurs in fibroblasts [22]. This observation by Barman and colleagues [22] is in agreement with previous studies [29, 74], and others have shown that TGF-β1 can upregulate Nox4 expression in other cell types including human cardiac fibroblasts, airway smooth muscle and vascular smooth muscle [29, 65, 75]. A role for fibroblasts in pathologic remodeling in PAH is further supported by studies in transgenic mice with fibroblast specific activation by TGF-β1 signaling, which develop mild PAH with medial hypertrophy, inflammation and fibrosis [76]. While studies strongly support a role for fibroblast TGF-β1 signaling in aberrant pulmonary vascular remodeling, PAH can be further exacerbated with additional stress on the endothelium and reflects the important contributions of multiple cell types in the development of PAH in addition to adventitial signaling.

In summary, Nox4 has gained considerable attention as a primary source of ROS, and cellular proliferation in the pathogenesis of both idiopathic pulmonary fibrosis and PAH, and connects Nox4

as a common variable in fibroblasts (and other perivascular cells) that contributes to the proliferation and remodeling of hypertensive pulmonary arterioles. The remodeling of pulmonary blood vessels requires the "joint effort" of all three vascular layers and while numerous studies have proposed a central role for endothelial cells ("inside out" remodeling), it is also readily apparent that vascular remodeling can be driven by changes in the adventitia (i.e., "outside in" remodeling). In both humans and animal models of PAH, prominent inflammation, activation and restructuring of the adventitia is observed [58]. The adventitial location of Nox4 is therefore highly suited to orchestrate the changes in vascular inflammation, matrix deposition, and subsequent vascular remodeling that occur in PAH. From a therapeutic standpoint, current treatments for PAH are ineffective in the long term, and merely prolong the disease, with a focus on ameliorating increased pulmonary vascular tone and improving quality of life. The development of more efficacious Nox4 inhibitors may be a viable direction to pursue in the quest to halt the morphological progression of PAH. It remains to be determined whether modalities that target adventitial Nox4 and the production of ROS in combination with current therapeutic approaches will have superior efficacy, as well as newly found success in the continuing battle against PAH and other pulmonary vascular diseases.

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