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The Mechanosensitive Ion Channel TRPV4 is a Regulator of Lung Development and Pulmonary Vasculature Stabilization

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Abstract

Introduction—Clinical observations and animal models suggest a critical role for the dynamic regulation of transmural pressure and peristaltic airway smooth muscle contractions for proper lung development. However, it is currently unclear how such mechanical signals are transduced into molecular and transcriptional changes at the cell level. To connect these physical findings to a mechanotransduction mechanism, we identified a known mechanosensor, TRPV4, as a component of this pathway.

Methods—Embryonic mouse lung explants were cultured on membranes and in submersion culture to modulate explant transmural pressure. Time-lapse imaging was used to capture active changes in lung biology, and whole-mount images were used to visualize the organization of the epithelial, smooth muscle, and vascular compartments. TRPV4 activity was modulated by pharmacological agonism and inhibition.

Results—TRPV4 expression is present in the murine lung with strong localization to the epithelium and major pulmonary blood vessels. TRPV4 agonism and inhibition resulted in hyper- and hypoplastic airway branching, smooth muscle differentiation, and lung growth, respectively. Smooth muscle contractions also doubled in frequency with agonism and were reduced by 60% with inhibition demonstrating a functional role consistent with levels of smooth muscle differentiation. Activation of TRPV4 increased the vascular capillary density around the distal airways, and inhibition resulted in a near complete loss of the vasculature.

Conclusions—These studies have identified TRPV4 as a potential mechanosensor involved in transducing mechanical forces on the airways to molecular and transcriptional events that regulate the morphogenesis of the three essential tissue compartments in the lung.

Keywords—Lung morphogenesis, Mechanotransduction, Mechanics of morphogenesis, Airway smooth muscle, Lung reciprocal signaling.

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Jason P. Gleghorn is an Assistant Professor at the University of Delaware in the Department of Biomedical Engineering. Gleghorn received his Ph.D. from Cornell University under the mentorship of Lawrence Bonassar. He then completed postdoctoral fellowships at Princeton University with Celeste Nelson and Cornell University with Brian Kirby. During his postdoctoral training, Gleghorn applied microfluidic and microfabrication techniques to identify new physical mechanisms that regulate organ development and he created novel microfluidic systems to isolate rare circulating tumor cells from patient blood samples respectively. His lab, started in 2014 at the University of Delaware, develops and uses microfluidic and microfabrication technologies to determine how cells behave and communicate within multicellular populations to form complex 3D tissues and organs. The long-term goals of this research are to develop techniques to engineer physiologically relevant 3D culture systems with well-defined structure, flows, and cell-cell interactions to study tissue-scale biology and disease. These techniques in combination with what they learn in studies of the native cellular behaviors and interactions in the embryo are used to define new therapeutic approaches for regenerative medicine. Gleghorn's honors include the ORAU Powe Junior Faculty Award, the March of Dimes Basil O'Connor Award, the UD Bernard Canavan Faculty Research Award, and the BMES CMBE Rising Star Award.

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ABBREVIATIONS

BPD	Bronchopulmonary dysplasia
TRPV4	Transient receptor potential cation channel subfamily V member 4
Pa	Pascal
PV	Pulmonary vasculature
PH	Pulmonary hypertension
HIF1α	Hypoxia-inducible factor 1 alpha
VEGF	Vascular endothelial growth factor
ASM	Airway smooth muscle

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease and the leading cause of perinatal mortality and morbidity, affecting over 30% of extremely preterm infants.[8] The malformed nature of the pulmonary vasculature (PV)[36,56] predisposes children suffering from BPD to pulmonary hypertension (PH), a severe complication with a mortality of 40–50% in their first year of life.[11,15,36,57,64] The PH mortality rate has remained largely unchanged since the 1980s, a staggering realization for a disease that lacks an agreed-upon definition in the context of congenital defects and BPD.[4,6,11,19,47] Whereas much of the etiology remains unclear, there is a recognized alteration in the PV, with a suggestion of both a decrease in pulmonary vasculature density early in the disease and compensatory dysmorphic PV at later disease stages.[5] The decrease in pulmonary vessel density, or rarefication, is thought to be a precursor to development of PH.[58,62] Although some advances have been made in symptomatic treatment of a specific subset of PH, pulmonary arterial hypertension,[7,21,53] the ongoing mortality of PH underscores the need to identify novel regulators of the PV and overall lung growth.

It is well established that the development of the mammalian lung relies on reciprocal signaling: the complex interplay of soluble factors that are secreted and sensed between neighboring tissues to regulate morphogenesis. This cross-talk between tissue compartments guides the formation of new airway branches into the surrounding mesenchyme[45,65] and ensures tight coordination of vascular morphogenesis around the airway tree.[14,20,24,26,30,39,48,55] Whereas several signaling pathways important to vascular morphogenesis have been identified, notably HIF1α-mediated expression of VEGF in the airway epithelium,[14,24,55] little is known about the upstream regulators of these pathways. As the PV develops in concert with the airways, it is likely both tissue compartments are influenced by shared regulators.

It has been long understood that in addition to the genetic and molecular regulators of lung development, physical forces also sculpt lung architecture. Clinical and *in vivo* observations going back decades have observed the build-up of fluid with the developing lung, resulting in a pressure of approximately 200–400 Pa during development.[25,51,54] This differential pressure acting on the developing airways, termed transmural pressure, has been long suspected to be essential for lung growth. Increasing fluid retention or transmural pressure results in airway elaboration,[3,50,63] and relieving fluid pressure results in reduced airway growth.[3,18] This quasi-static pressure is coupled to active contractility at the cellular level.[37,43,44] Most notably, airway smooth muscle (ASM), which wraps circumferentially around the epithelial lumen of the lung undergoes peristaltic contractions that have been shown to correlate with lung growth,[31–35] possibly due to cyclic distension of the airway tips.[38] Further, pharmacological activation or inhibition of ASM contractility leads to parallel acceleration or inhibition of lung branching.[17,65] Additionally, in the nitrofen-induced rat model of congenital diaphragmatic hernia, a precursor of BPD, peristalsis is dysregulated and leads to disease progression through lung hypoplasia,[16,35] suggesting that altered ASM contractions play a key role in the dysregulated mechanical environment present in BPD and PH.

Despite the long history demonstrating the profound importance of both quasi-static and dynamic mechanical forces on airway development, it remains to be elucidated how the cells of the tissue sense these forces. Whereas specific mechanisms remain unknown, a reasonable hypothesis is that there exists an unidentified mechanotransducer that regulates key signaling pathways, such as calcium (Ca^{2+}) signaling. Ca^{2+} signaling has been shown to be essential for overall lung growth with signaling occurring in epithelium and smooth muscle compartments.[12] As a prominent example of this, Ca^{2+} entry into the ASM is an essential part of peristalsis control,[17] consistent with other smooth muscle tissues. For these reasons, we were interested in testing for the expression and function of a mechanosensitive Ca^{2+} channel as a putative mechanosensor molecule in lung development. One such candidate is TRPV4, a calcium permeable cation channel that has been previously implicated in stretch mechanotransduction in multiple cell and tissue systems.[40,66] Due to its expression in the adult lung, TRPV4 has been implicated in mediating the mechanosensitive response to ventilator-induced barotrauma.[22,23] While this response has been minimally investigated in late fetal lungs,[49] TRPV4's direct correlation to early lung development remains unstudied.

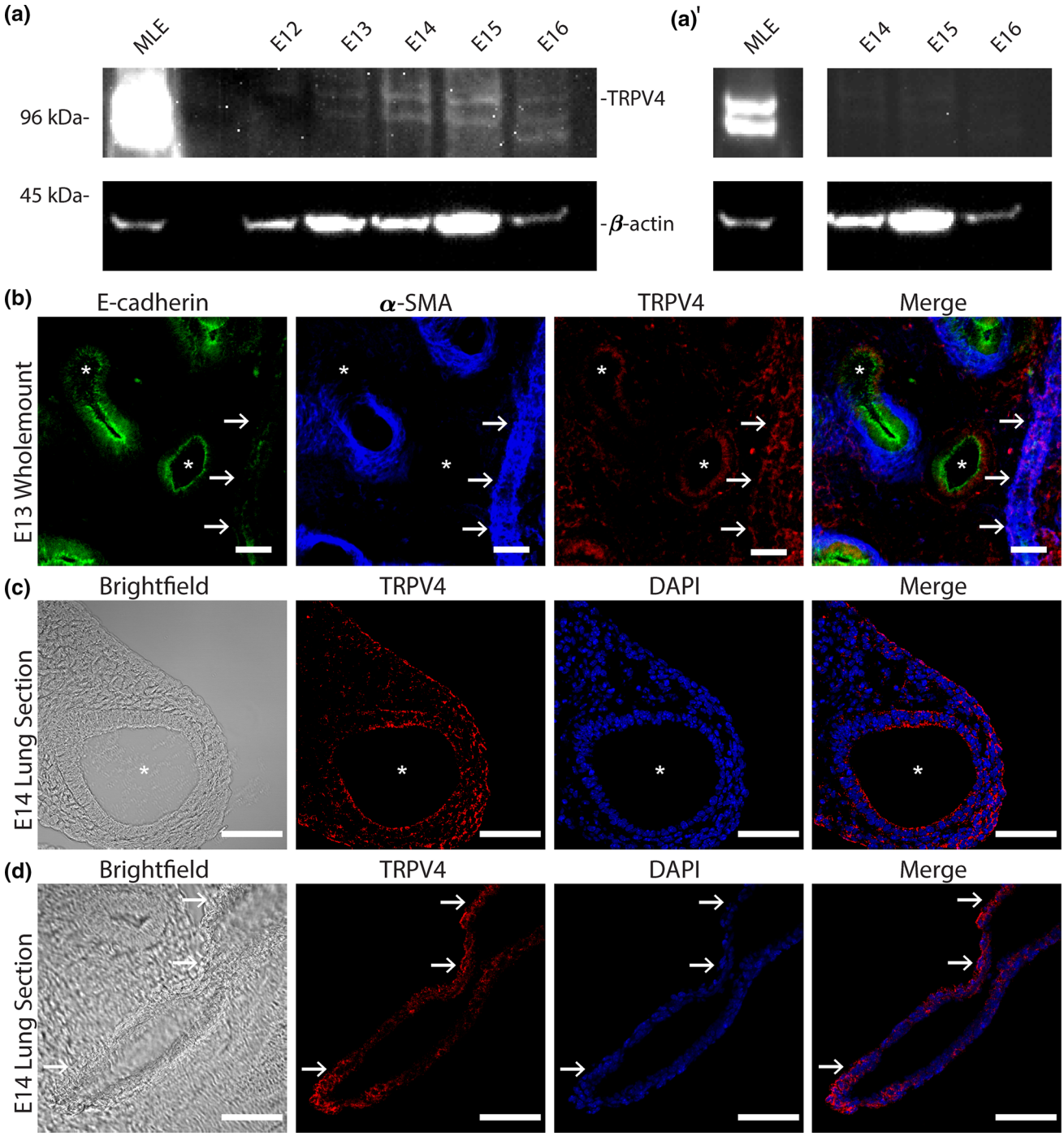


FIGURE 1. TRPV4 is expressed in embryonic murine lung during pseudoglandular development. (a) Mouse lung explants at embryonic day (E) 12–16 were homogenized, and TRPV4 expression was assessed *via* Western blotting. Positive expression at ~ 98 kDa was detected in E12–E16 lung lysates as well as in the positive control (MLE12 lysate). Blotting against β-actin was used as a loading control. Positions of molecular weight markers indicated to the left. (a′) The underexposed blot shows a conserved band morphology in the positive control as the lung lysate (E14 to E16). (b) E13 mouse lung was fixed and whole-mount stained for TRPV4 (red) along with counterstains for airway epithelium (E-cadherin, green) and smooth muscle (α-smooth muscle actin; αSMA, blue). Images are maximum projections of 286 × 286 × 15 μm confocal volume, median filtered for clarity. (c) Cryosections of E14 explants showing epithelial and (d) vascular structures were stained for TRPV4 and DAPI. TRPV4 primarily localizes to airway (white *) epithelium, the subepithelial mesenchyme, and major blood vessels (white arrows). Scale bars 50 μm.

In the present work, we provide evidence that TRPV4 is present during fetal lung development and is a positive regulator of lung growth, airway smooth muscle activity, and early PV stability. Identification of TRPV4 as a positive regulator of lung growth reveals a novel signaling axis that offers new insight into morphogenesis and a potential therapeutic avenue for BPD and PH.

METHODS

Explant Cultures

Briefly, timed pregnant CD-1 mice were euthanized at E13.5 by CO_2 asphyxiation confirmed by cervical dislocation. Uteri were removed from timed pregnant females and temporarily stored in cold (4 °C) Phosphate Buffered Saline (PBS) with 1% penicillin–streptomycin (PS). Lungs were explanted from embryos in cold (4 °C) PBS with 1% PS. Tracheas of the explants were sutured closed, and then whole lungs were transferred to Matrigel (Corning) thin gels in the base of 24 well tissue culture plates. Transmural pressure was varied *via* the addition of different volumes (200 and 500 μL) of Dulbecco's Modified Eagle Medium/Ham's F12 nutrient mixture (DMEM/F12) with 5% Fetal Bovine Serum (FBS) and 1% PS. Lung explants were cultured for 24 and 48 h at 37 °C and 5% CO_2. TRPV4 expression was pharmacologically activated with 100 nM GSK1016790A and inhibited with 10 μM GSK205 in DMEM/F12 with 5% FBS and 1% PS. Vehicle control was achieved by adding equivolume dimethyl sulfoxide (DMSO) to the culture medium.

ASM Contraction Quantification

Lungs were explanted and sutured shut as described above and then transferred to semipermeable membranes (Whatman Nuclepore Hydrophilic Membrane, 8.0 μm pore size, 25 mm circle) on raised PDMS pillars in 6 well culture dishes. DMEM/F12 with 5% FBS and 1% PS was filled under the membranes to support culture of the embryonic lungs. Pharmacological activation and inhibition of TRPV4 was achieved using the addition of 100 nM GSK1016790A or 10 μM GSK205 respectively, with DMSO acting as a vehicle control. Live imaging of explants was performed at 1 Hz for 72 h on a Zeiss Axio Observer with incubated stage (37 °C and 5% CO_2). Peristaltic contractions were quantified by a masked observer.

Immunoflourescent Imaging and Vascular Morphometrics

Following culture, lungs were fixed in 4% paraformaldehyde/0.5% Triton-X in PBS for 90 min at 4 °C, permeabilized overnight in 0.5% Triton-X in PBS at 4 °C, blocked overnight with 1% BSA/0.2% cold-fish gelatin/0.1% Tween-20 in PBS at 4 °C, and then blocked for 72 h with Mouse on Mouse IgG Blocking Reagent (Vector Labs). Lungs were rinsed 3X in PBS then incubated overnight at 4 °C with rabbit anti-ECAD (1:500) (Cell Signaling) to label the airways, rat anti-PECAM (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA) to label the vasculature, and mouse anti-α-Actin (1:250) (Santa Cruz Biotechnology) to label airway smooth muscle, or rabbit anti-TRPV4 (1:250) (Proteintech) in 1% BSA/0.2% cold-fish gelatin/0.1% Tween-20 in PBS. Specificity of the TRPV4 antibody was validated in fixed mouse lung epithelial cells (MLE12) using siRNA specific to TRPV4 (Supplemental Fig. 1). Lungs were then rinsed 3X for 20 min in 4 °C PBS and placed in appropriate secondary antibodies overnight at 4 °C and finally rinsed 3X in PBS. Lungs were initially imaged using a Zeiss Axio Observer. Lungs were then cleared *via* dehydration by rinsing 3X in 100% Methanol for 5 min and rehydrating 3X 15 min rinses with 1:1 Benzyl Alcohol: Benzyl Benzoate. For immunofluorescent staining of lung explant sections, lungs were harvest and fixed as described above. Fixed tissue was washed 3X in PBS for 5 min and placed in a 15%/sucrose PBS solution for overnight incubation at

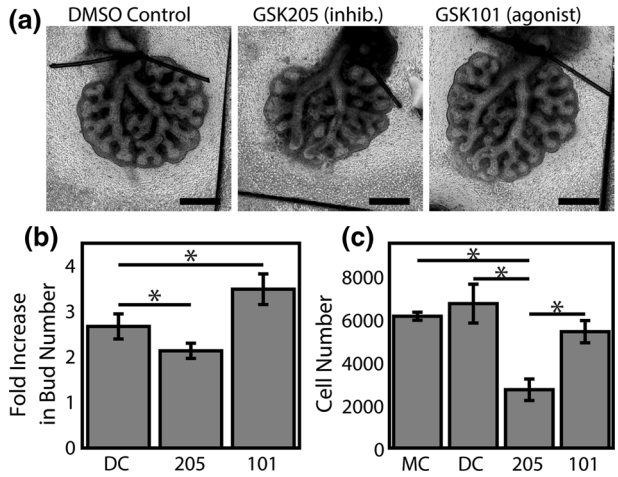


FIGURE 2. TRPV4 activity regulates murine lung branching morphogenesis. Embryonic mouse lungs were isolated at E12.5 and cultured on floating membranes for 48 h. (a) Lungs cultured with 10 μM GSK205, a TRPV4 inhibitor, were smaller with less elaborated airway structure than lungs treated with equivolume DMSO as a vehicle control. Conversely, lungs treated with 100 nM GSK1016790A (GSK101), a TRPV4 agonist, were larger and exhibited increased airway branching. Scale bars 500 μm. (b) Branch counts before and after culture by a masked observer demonstrate a significant effect of TRPV4 inhibition (205; 10 μM GSK205) and activation (101; 100 nM GSK1016790A). n = 11–12 (isolated from 6 uteri). (c) Proliferation of MLE12 cells following 24 h of culture show a significant decrease in cultured adult pulmonary epithelial cells with TRPV4 inhibition. * Indicates $p < 0.05$, Tukey's HSD post hoc test after repeated measures ANOVA.

4 °C. Over the next 3 days lung tissue was taken through a series of overnight incubations in 30% sucrose/PBS, 1:1 30%sucrose/PBS – TFM, and finally TFM. Lung samples were then placed in fresh TFM, oriented, and frozen using copper blocks chilled in liquid nitrogen. 6 μm sections were cut using a Leica CM3050S cryostat. Sections were blocked similar to whole lungs and then labelled with Anti-TRPV4 (Proteintech) at room temperature for 1 h. Following primary incubation, sections were washed 3X for 5 min in room temperature PBS prior to be incubated in appropriate secondary for 1 h at room temperature. DAPI was added to lung sections during the final 3X PBS washes before being mounted with gelvatol. High resolution images of lungs were then collected using a Zeiss LSM880 confocal microscope. For whole mount lungs, custom MATLAB scripts were used to segment confocal volumes into airway and mesenchyme, and

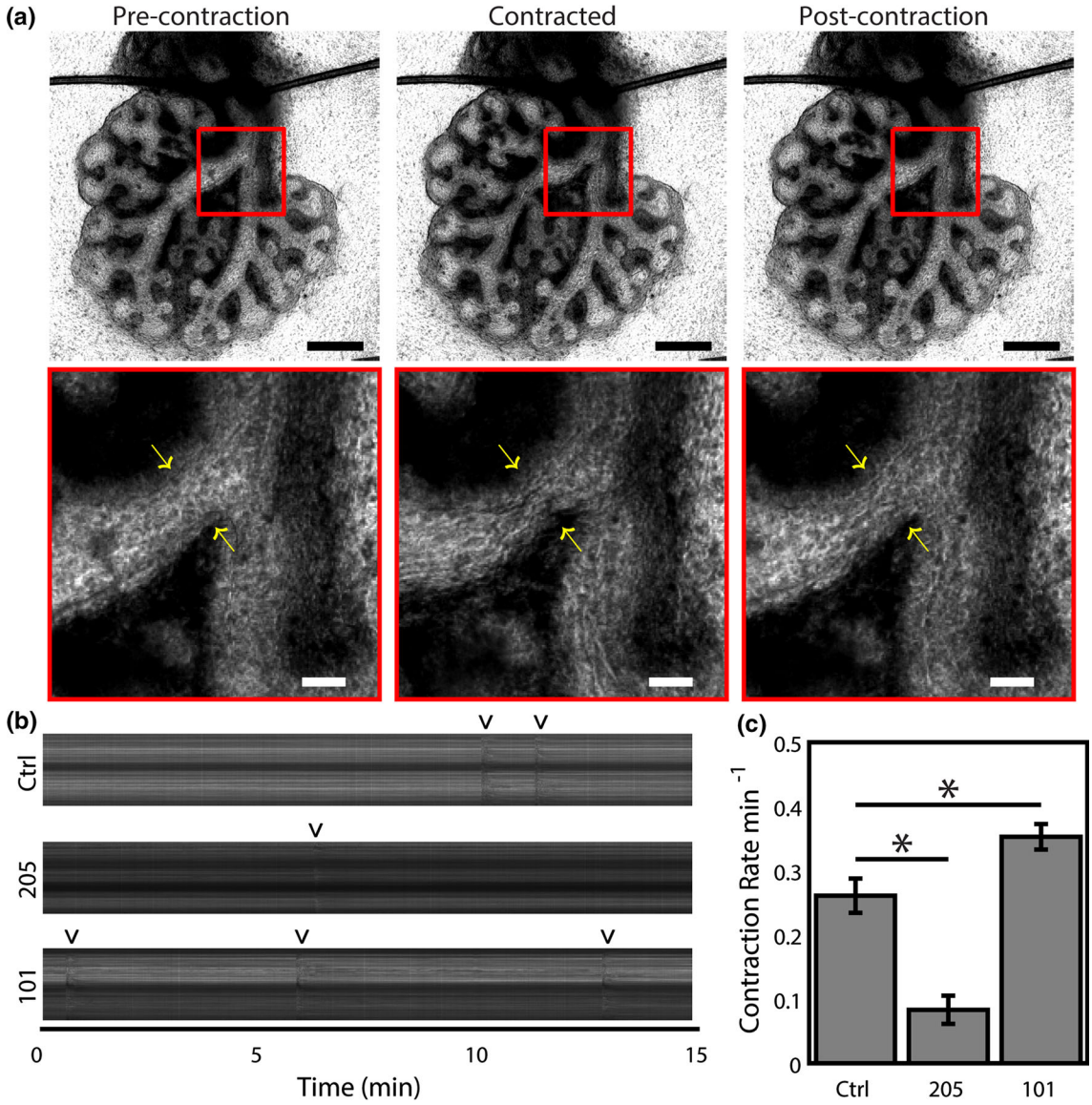
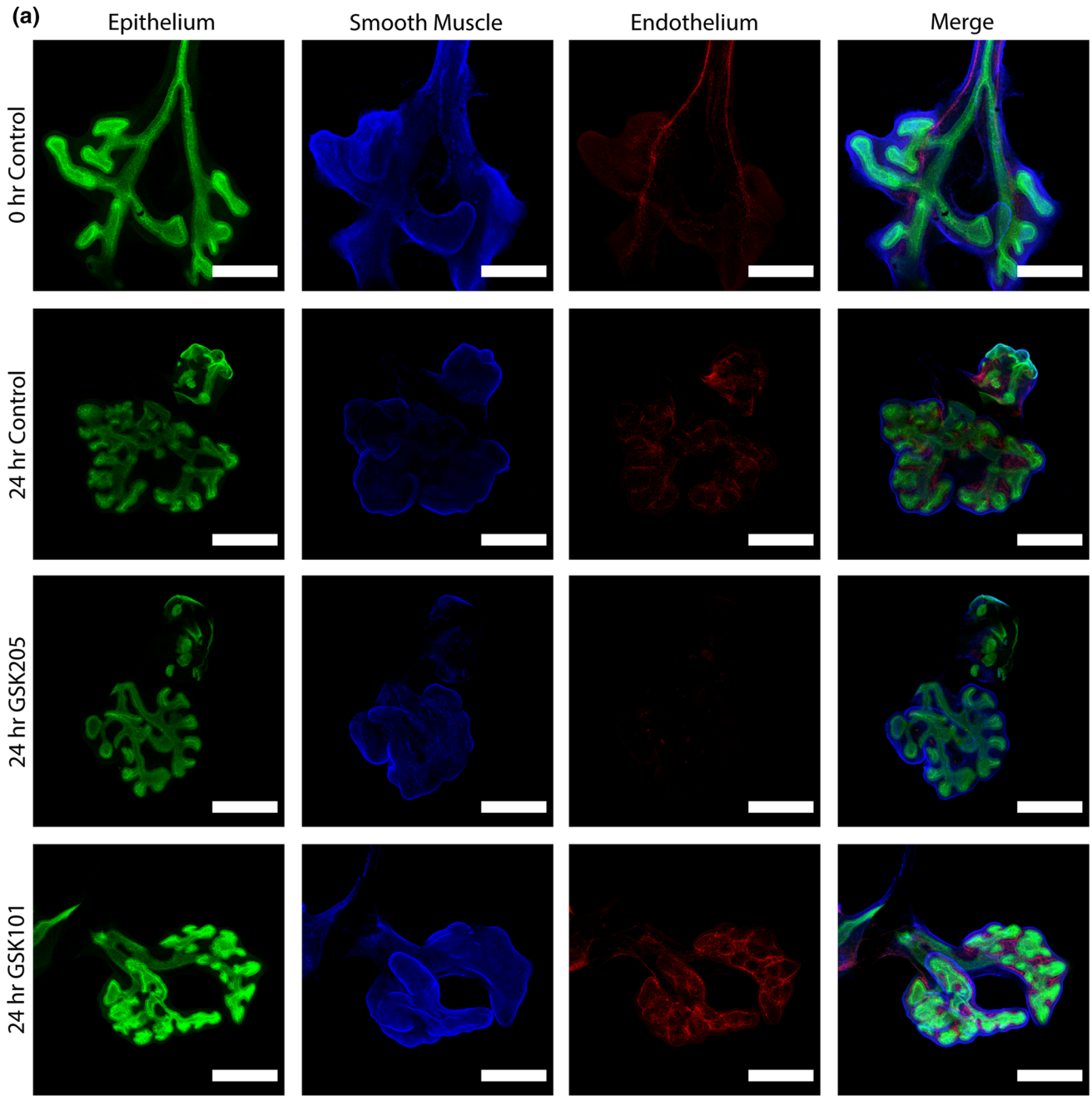
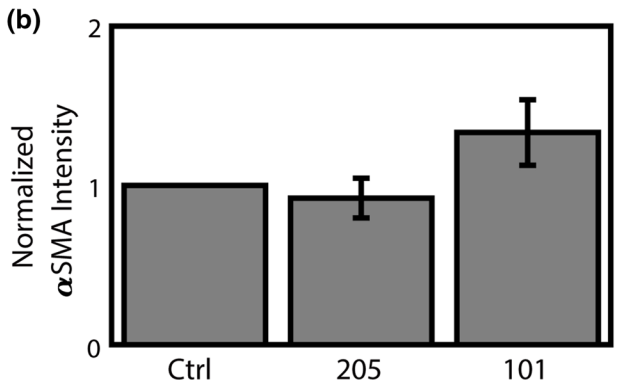


FIGURE 3. TRPV4 regulates active contractility of the developing lung. Embryonic mouse lungs were isolated at E12.5 and cultured on floating membranes for ~ 48 h before 1 Hz imaging. (a) Cultured lungs demonstrated active contraction. Contraction is visible throughout the lung as a decrease in brightness of the lumen as the airways contract down. Zoomed images (red boxes) show the contraction in more detail, with arrows indicating the location of the contraction. Scale bars 500 and 100 μm for the image and zooms, respectively. (b) Kymographs of contraction across the left and right bronchi. Contractions are visible as dark vertical streaks and indicated by 'v'. Lungs cultured with 10 μM GSK205, a TRPV4 inhibitor, were less mechanically active than lungs treated with equivolume DMSO as a vehicle control. Conversely, lungs treated with 100 nM GSK1016790A (GSK101), a TRPV4 agonist, exhibited more frequent contractions. (c) Contraction rate measured by a masked observer over 1 h of culture demonstrate a significant effect of TRPV4 inhibition (205; 10 μM GSK205) and activation (101; 100 nM GSK1016790A). $n = 6$ (isolated from 3 uteri). * Indicates $p < 0.05$, Tukey's HSD post hoc test after repeated measures ANOVA.


(a)
Epithelium
Smooth Muscle
Endothelium
Merge
0 hr Control
24 hr Control
24 hr GSK205
24 hr GSK101


(b)
Normalized αSMA Intensity
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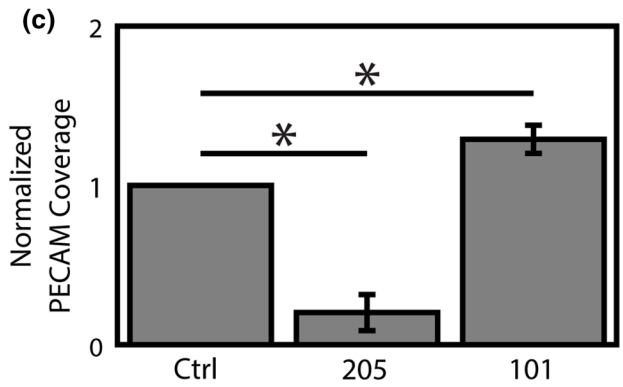
(c)
Normalized PECAM Coverage
2
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205
101

◀FIGURE 4. TRPV4 regulates the expression of αSMA and PECAM in lung morphogenesis. Embryonic mouse lungs were isolated at E12.5 and cultured for ~ 24 h before fixation, staining, and whole mount imaging. (a) At isolation (0 h Control), lungs demonstrate an intricate vascular network (Endothelium; PECAM; red) and smooth muscle around the major airways (Smooth Muscle; αSMA; blue), which expand with airway (Epithelium; E-cadherin; green) elaboration over culture (24 h Control). However, TRPV4 inhibition (24 h GSK205; 10 μM) has minimal effect on smooth muscle labeling and dramatically ablates the existing vascular network, while TRPV4 activation (24 h GSK101; 100 nM GSK1016790A) increases the labeling of both smooth muscle and vascular compartments. Scale bars 250 μm. Images median filtered for clarity. (b) Quantification of average staining intensity of αSMA around the airways, normalized to control. (c) Quantification of vascular coverage based on a set threshold of PECAM intensity in the mesenchyme, normalized to control. $n = 4$. * Indicates $p < 0.05$ for a Fisher's LSD following a significant 1-way ANOVA.

validated against a masked observer. ASM intensity and PV density were normalized to controls.

Cellular Proliferation Assay

Adult mouse lung epithelial cells (MLE12) were routinely culture in HITES medium (as per ATCC protocol) with 1% PS. At confluence cells were seeded at 3500 cells/cm^2 to 24 well culture plates where they were treated with TRPV4 inhibitor (10 μM GSK205), agonist (100 nM GSK1016790A,) vehicle control (DMSO), or standard culture media. Following 24 h of growth cells were isolated and run through a NovoCyte Flow Cytometer to quantify the total number of cells.

Western Blotting

Immediately after isolation, lungs were lysed in RIPA with 2X HALT protease and phosphatase inhibitors. As a positive control for TRPV4 expression, adult mouse lung epithelial cells (MLE12) (ATCC, Manassas, VA) were routinely cultured in HITES medium (as per formulation suggested by ATCC) with 1% PS, and similarly lysed. Protein was homogenized, quantified using a detergent compatible Lowry assay, and denatured at 95 °C in LDS sample buffer containing DTT (CBS Scientific). Protein was loaded at 20 μg per lane into a 4–12% TEO-SDS gel (CBS Scientific) and separated at 150 V for 1.5 h. Protein was transferred to Amersham™ Protran™ 0.2 μm NC membrane (GE Healthcare Life Sciences) using a G2 Fast Blotter (ThermoFisher Scientific). Membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 and probed with anti-TRPV4 (Alomone) and with anti-β-actin (Cell Signaling) as a loading control. Appropriate HRP-conjugated secondary antibodies were used and the membrane developed with Super Signal Femto ECL (ThermoFisher Scientific) and imaged with a ChemiDoc-IT[2] bioimaging system (UVP).

RESULTS AND DISCUSSION

The development of the lung is a tightly coordinated process that produces an airway and vascular architecture critical for survival. Clinical observations and animal models suggest a critical role for the dynamic regulation of physical forces including transmural pressure and peristaltic airway smooth muscle contractions for proper lung development. However, it is currently unclear how such mechanical signals are transduced into reciprocal signaling cascades between tissue compartments that regulate morphogenesis of the lung. To connect these physical findings to a mechanotransduction mechanism, we identified a known mechanosensor, TRPV4, and downstream Ca^{2+} signaling as a component of this pathway.

TRPV4 is Expressed in Embryonic Murine Lung During Pseudoglandular Development

Given the known importance of Ca^{2+} signaling in ASM contractility,[17,35] the mechanosensitive role of TRPV4 in other tissues, and its presence in the mature lung,[22,23] we sought to determine if TRPV4 is expressed in the early embryonic lung. Immunoblots of lysates from lung explants at various gestational ages (E12-16) demonstrate the presence of TRPV4 in early lung development (Fig. 1a). Mature lung epithelial cells (MLES12s) serve as a positive control and we provide an adjusted contrast version of the blot (Fig. 1a′) to demonstrate the band morphology is conserved in the positive control. Similarly, whole-mount staining (Fig. 1b) and immunofluorescent imaging of cryo-sectioned E14 lung explants show that this mechanosensitive ion channel is localized to the apical and basal surfaces of airway epithelium, subepithelial mesenchyme, (Fig. 1c) and the major pulmonary vessels (Fig. 1d). Interestingly, these spatial patterns of TRPV4 correspond to key tissues that experience (epithelium) or generate (ASM and vascular smooth muscle) dynamic mechanical forces during embryonic lung development. The epithelium undergoes cyclic deformations as a result of ASM contraction-mediated fluid flows within the airway lumen. The presence of TRPV4 at the apical surface may serve to sense these deformations and regulate cellular behaviors necessary for the formation of new airways. This would be consistent with numerous studies that demonstrate increased airway branching with ASM

contraction frequency and an arrest in airway branching with the absence of ASM contractions.[16,31,50] Further, localization to the subepithelial mesenchyme and to the pulmonary vasculature, at an embryonic stage where the ASM and vasculature are rapidly assembling, suggests TRPV4 may regulate the differentiation, assembly, or function of these critical tissues.

TRPV4 Activity Regulates Murine Airway Morphogenesis

As a Ca^{2+} channel, TRPV4 is positioned to regulate a broad spectrum of cellular behaviors essential to airway morphogenesis. Ca^{2+} is a centralized second messenger signal molecule implicated in proliferation, differentiation, and contraction of cells.[9,10] Further, at the tissue scale, gap junctions allow for cell–cell communication *via* propagation of intercellular Ca^{2+} waves.[28,29] These waves propagate elevated levels of intracellular Ca^{2+} throughout cell populations, leading to coordinated proliferation, migration, and contraction of cells.[17,41] Proliferation, migration, and cell–cell communication are all critical in the formation of airway branches.[12,59] However, the molecular mechanism that propagates Ca^{2+} influx during airway development has yet to be identified. As we have demonstrated the expression of TRPV4 in the early lung, we further sought to determine if TRPV4 was a functional regulator in overall airway morphogenesis. We cultured the lung explants *ex vivo* at the air-medium interface using a floating membrane culture over 48 h. Lungs were treated with selective pharmacological modulators to activate (100 nM GSK1016790A; abbreviated GSK101)[61] or inhibit (10 μM GSK205)[52] TRVP4 (Fig. 2a), and the number of terminal airway branches were quantified at the start and end of culture. Activation of TRPV4 significantly increased airway branching with lung explants exhibiting a larger fold increase in the number of terminal branches over culture compared to controls (3.11 ± 0.22 vs. 2.49 ± 0.23; Fig. 2b). Conversely, antagonism of TRPV4 resulted in a smaller fold increase in terminal branches (1.96 ± 0.13); however, airway branching morphogenesis was not completely arrested. To determine if alterations in airway branching were the result of TRPV4-induced changes in epithelial proliferation we quantified MLE12 proliferation in culture in response to TRPV4 activation and inhibition (Fig. 2c). These studies demonstrate an approximately 60% decrease in proliferation over 24 h with TRPV4 inhibition and no appreciable change in proliferation with TRPV4 activation compared to untreated cells. These *ex vivo* and cell culture experiments provide compelling evidence that TRPV4 activity and the subsequent intracellular calcium signaling events play an active role in airway epithelial morphogenesis.

TRPV4 Regulates Active Contractility of the Developing Lung

Building on the findings supporting the role of TRPV4 as a pressure sensitive Ca^{2+} channel, and our recent study demonstrating pressure-based regulation of airway contractility,[50] we hypothesized that TRPV4 mediates the peristaltic ASM contractility of the lung. High-frequency timelapse imaging (1 Hz) was used to observe lung explants following 48 h of culture (Supplementary Movie 1). As ASM contraction events are irregular and vary spatially throughout the organ, the analysis of contractions was performed in the left and right mainstem bronchi across multiple lung explants (Fig. 3a). ASM contraction events were quantified with kymographs (Fig. 3b). TRPV4 agonism (GSK101) produced an increase in ASM contraction frequency, and inhibition (GSK205) resulted in sharply reduced contraction frequencies (Fig. 3c). These studies are consistent with the well-established role for Ca^{2+} signaling in ASM contractility in the developing lung and lend strong support to the hypothesis that TRPV4 is an important mechanosensor that regulates several phenotypes associated with overall lung growth.

TRPV4 Regulates the Stability of the Pulmonary Vasculature

As multiple tissue compartments of the lung (epithelial, ASM, vascular) are known to extensively cross-regulate[65] and immunostaining demonstrated TRPV4 localization to each of these areas, we proceeded to investigate the impact of TRPV4 modulation on ASM and PV morphogenesis. *Ex vivo* explant culture was conducted over 24 h in the presence of TRPV4 modulators. After culture, samples were fixed and stained for whole-mount confocal imaging (Fig. 4a). To determine the impact of TRPV4 modulation on the ASM and PV tissue compartments, we stained for αSMA and PECAM, respectively. For ASM, we observed a minimal correlation between αSMA staining intensity and TRPV4 activation; however, it did not rise to significance (Fig. 4b). These data suggest that the change in ASM contraction frequency we observed is likely due to a modulation of ASM function, rather than an increase or decrease of ASM coverage.

Surprisingly, in the PV, TRPV4 activation (GSK101) led to a consistent increase in vascular coverage, whereas inhibition (GSK205) led to the near

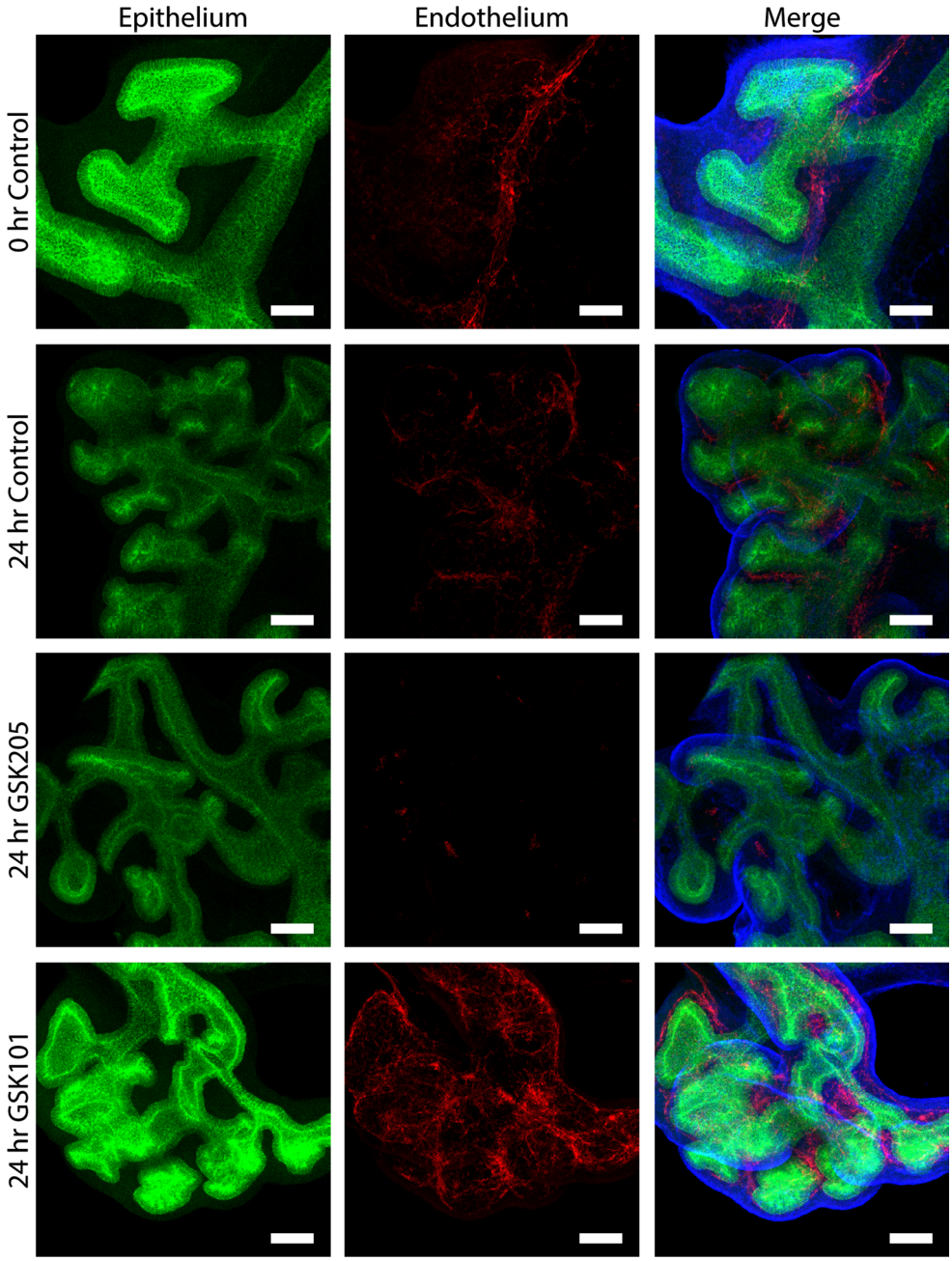

FIGURE 5. TRPV4 activity regulates the stability of PV during lung morphogenesis. Embryonic mouse lungs were isolated at E12.5 and cultured for ~ 24 h before fixation, staining, and whole mount imaging. At isolation (0 h Control), lungs demonstrate an intricate vascular network (Endothelium; PECAM; red), and this is maintained during routine culture (24 h Control). However, TRPV4 inhibition (24 h GSK205; 10 μM) nearly eliminates PECAM positive cells, while TRPV4 activation (24 h GSK101; 100 nM GSK1016790A) results in a more robust capillary network. Scale bars 50 μm.

complete ablation of the PV (Figs. 4a and 4c). An intricate, hierarchical vascular network can be observed in freshly explanted mouse lungs (Fig. 5) with an organization that is maintained over culture. TRPV4 agonism results in extensive new vascular formation and a similar hierarchical organization. Conversely, TRPV4 antagonism appears to destabilize the existing PV resulting in a loss of hierarchical organization and the widespread resorption of de novo angiogenesis in the lung. These data demonstrate that TRPV4 activity is an essential regulator of PV stability during early embryonic lung development. Further, given the known reciprocal signaling between the airway and PV during lung development,[65] loss of TRPV4-mediated PV stability would likely impair overall lung growth.

CONCLUSION

In these studies, we have shown that the putative mechanosensor, TRPV4, is present in the embryonic murine lung and positively regulates its development. With pharmacological perturbation of TRPV4 we demonstrate that its regulation directly correlates with murine lung contractions and growth, providing further support for the hypothesis of contraction mediated growth.[32,33,35] Importantly, we observed a marked decrease in PECAM expression after TRPV4 inhibition, when compared to both culture controls and freshly isolated lungs prior to culture. This demonstrates that TRPV4 activity is essential for stabilization of the PV during lung development. Whereas this is a completely novel finding in lung development, a similar role of TRPV4 has been shown in stabilizing vessels in tumors and after stroke,[2,13,60] suggesting that TRVP4 may be a broad regulator of vessel behavior in tissues undergoing rapid angiogenesis. Importantly, through use of an *ex vivo* model, these studies have demonstrated that TRPV4 regulates multiple tissue compartments in an intact and growing organ. Future work will build on these whole organ studies by investigating the molecular mechanisms downstream of TRPV4-mediated Ca^{2+} signaling, for example, HIF-1α and its transcriptional target VEGF. Recent work identifying calcium signaling as an upstream regulator of HIF-1α,[27,46] makes it a likely downstream target of TRPV4 activation. VEGF, a protein downstream of HIF-1α activation has been shown to be important in lung growth[1,14,42,55] and is a known mediator of endothelial to epithelial intracellular growth signaling in lung. By identification of a novel mechanosensitive signaling modality in lung growth, this work provides a new avenue for the development of future therapies for BPD and PH, as well as providing novel directions for research into disease etiology.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1007/s12195-018-0538-7) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

Joshua T. Morgan, Wade G. Stewart, Robert A. McKee and Jason P. Gleghorn report no conflicts of interest.

HUMAN AND ANIMAL STUDIES

No human studies were carried out by the authors for this article. All institutional and national guidelines for the care and use of laboratory animals were followed and approved by the appropriate institutional committees at the University of Delaware.

REFERENCES

[1]Acarregui, M. J., S. T. Penisten, K. L. Goss, K. Ramirez, and J. M. Snyder. Vascular endothelial growth factor gene expression in human fetal lung in vitro. *Am. J. Respir. Cell Mol. Biol.* 20:14–23, 1999.

[2]Adapala, R. K., R. J. Thoppil, K. Ghosh, H. C. Cappelli, A. C. Dudley, S. Paruchuri, V. Keshamouni, M. Klagsbrun, J. G. Meszaros, W. M. Chilian, D. E. Ingber, and C. K. Thodeti. Activation of mechanosensitive ion channel TRPV4 normalizes tumor vasculature and improves cancer therapy. *Oncogene* 35:314–322, 2016.

[3]Alcorn, D., T. M. Adamson, T. F. Lambert, J. E. Maloney, B. C. Ritchie, and P. M. Robinson. Morphological effects of chronic tracheal ligation and drainage in the fetal lamb lung. *J. Anat.* 123:649–660, 1977.

[4]Ali, Z., P. Schmidt, J. Dodd, and D. L. Jeppesen. Predictors of bronchopulmonary dysplasia and pulmonary hypertension in newborn children. *Dan. Med. J.* 60:A4688, 2013.

[5]Alvira, C. M. Aberrant pulmonary vascular growth and remodeling in bronchopulmonary dysplasia. *Front. Med.* 3:21, 2016.

[6]An, H. S., E. J. Bae, G. B. Kim, B. S. Kwon, J. S. Beak, E. K. Kim, H. S. Kim, J.-H. Choi, C. I. Noh, and Y. S. Yun. Pulmonary hypertension in preterm infants with bronchopulmonary dysplasia. *Korean Circ. J.* 40:131–136, 2010.

[7]Benza, R. L., D. P. Miller, R. J. Barst, D. B. Badesch, A. E. Frost, and M. D. McGoon. An evaluation of long-term survival from time of diagnosis in pulmonary arterial hypertension from the REVEAL Registry. *Chest* 142:448–456, 2012.

[8]Berkelhamer, S. K., K. K. Mestan, and R. H. Steinhorn. Pulmonary hypertension in bronchopulmonary dysplasia. *Semin. Perinatol.* 37:124–131, 2013.

[9]Berridge, M. J., M. D. Bootman, and H. L. Roderick. Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* 4:517–529, 2003.

[10]Berridge, M. J., P. Lipp, and M. D. Bootman. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1:11–21, 2000.
[11]Bhat, R., A. A. Salas, C. Foster, W. A. Carlo, and N. Ambalavanan. Prospective analysis of pulmonary hypertension in extremely low birth weight infants. *Pediatrics* 129:e682–e689, 2012.
[12]Brennan, S. C., B. A. Finney, M. Lazarou, A. E. Rosser, C. Scherf, D. Adriaensen, P. J. Kemp, and D. Riccardi. Fetal calcium regulates branching morphogenesis in the developing human and mouse lung: involvement of voltage-gated calcium channels. *PLoS ONE* 8(11):e80294, 2013.
[13]Chen, C.-K., P.-Y. Hsu, T.-M. Wang, Z.-F. Miao, R.-T. Lin, and S.-H. H. Juo. TRPV4 activation contributes functional recovery from ischemic stroke via angiogenesis and neurogenesis. *Mol. Neurobiol.* 2017. https://doi.org/10.1007/s12035-017-0625-0.
[14]Compernolle, V., K. Brusselmans, T. Acker, P. Hoet, M. Tjwa, H. Beck, S. Plaisance, Y. Dor, E. Keshet, F. Lupu, B. Nemery, M. Dewerchin, P. Van Veldhoven, K. Plate, L. Moons, D. Collen, and P. Carmeliet. Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat. Med.* 8:702–710, 2002.
[15]Evans, N. J., and L. N. Archer. Doppler assessment of pulmonary artery pressure during recovery from hyaline membrane disease. *Arch. Dis. Child.* 66:802–804, 1991.
[16]Featherstone, N. C., M. G. Connell, D. G. Fernig, S. Wray, T. V. Burdyga, P. D. Losty, and E. C. Jesudason. Airway smooth muscle dysfunction precedes teratogenic congenital diaphragmatic hernia and may contribute to hypoplastic lung morphogenesis. *Am. J. Respir. Cell Mol. Biol.* 35:571–578, 2006.
[17]Featherstone, N. C., E. C. Jesudason, M. G. Connell, D. G. Fernig, S. Wray, P. D. Losty, and T. V. Burdyga. Spontaneous propagating calcium waves underpin airway peristalsis in embryonic rat lung. *Am. J. Respir. Cell Mol. Biol.* 33:153–160, 2005.
[18]Fewell, J. E., A. A. Hislop, J. A. Kitterman, and P. Johnson. Effect of tracheostomy on lung development in fetal lambs. *J. Appl. Physiol.* 55:1103–1108, 1983.
[19]Fouron, J. C., J. C. Le Guennec, D. Villemant, G. Perreault, and A. Davignon. Value of echocardiography in assessing the outcome of bronchopulmonary dysplasia of the newborn. *Pediatrics* 65:529–535, 1980.
[20]Galambos, C., Y.-S. Ng, A. Ali, A. Noguchi, S. Lovejoy, P. A. D'Amore, and D. E. DeMello. Defective pulmonary development in the absence of heparin-binding vascular endothelial growth factor isoforms. *Am. J. Respir. Cell Mol. Biol.* 27:194–203, 2002.
[21]Galiè, N., H. A. Ghofrani, A. Torbicki, R. J. Barst, L. J. Rubin, D. Badesch, T. Fleming, T. Parpia, G. Burgess, A. Branzi, F. Grimminger, M. Kurzyna, and G. Simonneau. Sildenafil use in pulmonary arterial hypertension (SUPER) study group. Sildenafil citrate therapy for pulmonary arterial hypertension. *N. Engl. J. Med.* 353:2148–2157, 2005.
[22]Hamanaka, K., M.-Y. Jian, M. I. Townsley, J. A. King, W. Liedtke, D. S. Weber, F. G. Eyal, M. M. Clapp, and J. C. Parker. TRPV4 channels augment macrophage activation and ventilator-induced lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 299:L353–L362, 2010.
[23]Hamanaka, K., M.-Y. Jian, D. S. Weber, D. F. Alvarez, M. I. Townsley, A. B. Al-Mehdi, J. A. King, W. Liedtke, and J. C. Parker. TRPV4 initiates the acute calcium-dependent permeability increase during ventilator-induced lung injury in isolated mouse lungs. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 293:L923–L932, 2007.
[24]Hara, A., C. J. Chapin, R. Ertsey, and J. A. Kitterman. Changes in fetal lung distension alter expression of vascular endothelial growth factor and its isoforms in developing rat lung. *Pediatr. Res.* 58:30–37, 2005.
[25]Harding, R., and S. B. Hooper. Regulation of lung expansion and lung growth before birth. *J. Appl. Physiol. Bethesda Md* 1985(81):209–224, 1996.
[26]Hislop, A. A. Airway and blood vessel interaction during lung development. *J. Anat.* 201:325–334, 2002.
[27]Hui, A. S., A. L. Bauer, J. B. Striet, P. O. Schnell, and M. F. Czyzyk-Krzeska. Calcium signaling stimulates translation of HIF-alpha during hypoxia. *FASEB J.* 20:466–475, 2006.
[28]Isakson, B. E., W. H. Evans, and S. Boitano. Intercellular Ca^{2+} signaling in alveolar epithelial cells through gap junctions and by extracellular ATP. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 280:L221–L228, 2001.
[29]Isakson, B. E., G. J. Seedorf, R. L. Lubman, W. H. Evans, and S. Boitano. Cell-cell communication in heterocellular cultures of alveolar epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 29:552–561, 2003.
[30]Jakkula, M., T. D. Le Cras, S. Gebb, K. P. Hirth, R. M. Tuder, N. F. Voelkel, and S. H. Abman. Inhibition of angiogenesis decreases alveolarization in the developing rat lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 279:L600–L607, 2000.
[31]Jesudason, E. C. Small lungs and suspect smooth muscle: congenital diaphragmatic hernia and the smooth muscle hypothesis. *J. Pediatr. Surg.* 41:431–435, 2006.
[32]Jesudason, E. C. Exploiting mechanical stimuli to rescue growth of the hypoplastic lung. *Pediatr. Surg. Int.* 23:827–836, 2007.
[33]Jesudason, E. C. Airway smooth muscle: an architect of the lung? *Thorax* 64:541–545, 2009.
[34]Jesudason, E. C., N. P. Smith, M. G. Connell, D. G. Spiller, M. R. H. White, D. G. Fernig, and P. D. Losty. Developing rat lung has a sided pacemaker region for morphogenesis-related airway peristalsis. *Am. J. Respir. Cell Mol. Biol.* 32:118–127, 2005.
[35]Jesudason, E. C., N. P. Smith, M. G. Connell, D. G. Spiller, M. R. H. White, D. G. Fernig, and P. D. Losty. Peristalsis of airway smooth muscle is developmentally regulated and uncoupled from hypoplastic lung growth. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 291:L559–L565, 2006.
[36]Khemani, E., D. B. McElhinney, L. Rhein, O. Andrade, R. V. Lacro, K. C. Thomas, and M. P. Mullen. Pulmonary artery hypertension in formerly premature infants with bronchopulmonary dysplasia: clinical features and outcomes in the surfactant era. *Pediatrics* 120:1260–1269, 2007.
[37]Kim, H. Y., M.-F. Pang, V. D. Varner, L. Kojima, E. Miller, D. C. Radisky, and C. M. Nelson. Localized smooth muscle differentiation is essential for epithelial bifurcation during branching morphogenesis of the mammalian lung. *Dev. Cell* 34:719–726, 2015.
[38]Kim, H. Y., V. D. Varner, and C. M. Nelson. Apical constriction initiates new bud formation during monopodial branching of the embryonic chicken lung. *Dev. Camb. Engl.* 140:3146–3155, 2013.
[39]Lazarus, A., P. M. Del-Moral, O. Ilovich, E. Mishani, D. Warburton, and E. Keshet. A perfusion-independent role of blood vessels in determining branching stereotypy of lung airways. *Dev. Camb. Engl.* 138:2359–2368, 2011.

[40]Liedtke, W. TRPV channels' role in osmotransduction and mechanotransduction. *Handb. Exp. Pharmacol.* 2007. https://doi.org/10.1007/978-3-540-34891-7_28.
[41]Means, A. R. Calcium, calmodulin and cell cycle regulation. *FEBS Lett.* 347:1–4, 1994.
[42]Miquerol, L., M. Gertsenstein, K. Harpal, J. Rossant, and A. Nagy. Multiple developmental roles of VEGF suggested by a LacZ-tagged allele. *Dev. Biol.* 212:307–322, 1999.
[43]Moore, K. A., S. Huang, Y. Kong, M. E. Sunday, and D. E. Ingber. Control of embryonic lung branching morphogenesis by the Rho activator, cytotoxic necrotizing factor 1. *J. Surg. Res.* 104:95–100, 2002.
[44]Moore, K. A., T. Polte, S. Huang, B. Shi, E. Alsberg, M. E. Sunday, and D. E. Ingber. Control of basement membrane remodeling and epithelial branching morphogenesis in embryonic lung by Rho and cytoskeletal tension. *Dev. Dyn.* 232:268–281, 2005.
[45]Morrisey, E. E., and B. L. M. Hogan. Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev. Cell* 18:8–23, 2010.
[46]Mottet, D., G. Michel, P. Renard, N. Ninane, M. Raes, and C. Michiels. Role of ERK and calcium in the hypoxia-induced activation of HIF-1. *J. Cell. Physiol.* 194:30–44, 2003.
[47]Mourani, P. M., and S. H. Abman. Pulmonary vascular disease in bronchopulmonary dysplasia: pulmonary hypertension and beyond. *Curr. Opin. Pediatr.* 25:329–337, 2013.
[48]Muratore, C. S., H. T. Nguyen, M. M. Ziegler, and J. M. Wilson. Stretch-induced upregulation of VEGF gene expression in murine pulmonary culture: a role for angiogenesis in lung development. *J. Pediatr. Surg.* 35:906–912, 2000; (discussion 912–913).
[49]Nayak, P. S., Y. Wang, T. Najrana, L. M. Priolo, M. Rios, S. K. Shaw, and J. Sanchez-Esteban. Mechanotransduction via TRPV4 regulates inflammation and differentiation in fetal mouse distal lung epithelial cells. *Respir. Res.* 16:60, 2015.
[50]Nelson, C. M., J. P. Gleghorn, M.-F. Pang, J. M. Jaslove, K. Goodwin, V. D. Varner, E. Miller, D. C. Radisky, and H. A. Stone. Microfluidic chest cavities reveal that transmural pressure controls the rate of lung development. *Development* 144:4328–4335, 2017.
[51]Olver, R. E., D. V. Walters, and S. M. Wilson. Developmental regulation of lung liquid transport. *Annu. Rev. Physiol.* 66:77–101, 2004.
[52]Phan, M. N., H. A. Leddy, B. J. Votta, S. Kumar, D. S. Levy, D. B. Lipshutz, S. H. Lee, W. Liedtke, and F. Guilak. Functional characterization of TRPV4 as an osmotically sensitive ion channel in porcine articular chondrocytes. *Arthritis Rheum.* 60:3028–3037, 2009.
[53]Rubin, L. J., D. B. Badesch, R. J. Barst, N. Galie, C. M. Black, A. Keogh, T. Pulido, A. Frost, S. Roux, I. Leconte, M. Landzberg, and G. Simonneau. Bosentan therapy for pulmonary arterial hypertension. *N. Engl. J. Med.* 346:896–903, 2002.
[54]Schittny, J. C., G. Miserocchi, and M. P. Sparrow. Spontaneous peristaltic airway contractions propel lung liquid through the bronchial tree of intact and fetal lung explants. *Am. J. Respir. Cell Mol. Biol.* 23:11–18, 2000.
[55]Shinkai, M., T. Shinkai, S. Montedonico, and P. Puri. Effect of VEGF on the branching morphogenesis of normal and nitrofen-induced hypoplastic fetal rat lung explants. *J. Pediatr. Surg.* 41:781–786, 2006.
[56]Steinhorn, R. H. Neonatal pulmonary hypertension. *Pediatr. Crit. Care Med.* 11:S79–S84, 2010.
[57]Subhedar, N. V., and N. J. Shaw. Changes in pulmonary arterial pressure in preterm infants with chronic lung disease. *Arch. Dis. Child. Fetal Neonatal Ed.* 82:F243–F247, 2000.
[58]Taghizadeh, A., and E. O. Reynolds. Pathogenesis of bronchopulmonary dysplasia following hyaline membrane disease. *Am. J. Pathol.* 82:241–264, 1976.
[59]Tang, N., W. F. Marshall, M. McMahon, R. J. Metzger, and G. R. Martin. Control of mitotic spindle angle by the RAS-regulated ERK1/2 pathway determines lung tube shape. *Science* 333:342–345, 2011.
[60]Thoppil, R. J., H. C. Cappelli, R. K. Adapala, A. K. Kanugula, S. Paruchuri, and C. K. Thodeti. TRPV4 channels regulate tumor angiogenesis via modulation of Rho/Rho kinase pathway. *Oncotarget* 7:25849–25861, 2016.
[61]Thorneloe, K. S., A. C. Sulpizio, Z. Lin, D. J. Figueroa, A. K. Clouse, G. P. McCafferty, T. P. Chendrimada, E. S. R. Lashinger, E. Gordon, L. Evans, B. A. Misajet, D. J. Demarini, J. H. Nation, L. N. Casillas, R. W. Marquis, B. J. Votta, S. A. Sheardown, X. Xu, D. P. Brooks, N. J. Laping, and T. D. Westfall. N-((1S)-1-{[4-((2S)-2-{[(2,4-dichlorophenyl)sulfonyl]amino}-3-hydroxypropanoyl)-1-piperazinyl]carbonyl}-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part I. *J. Pharmacol. Exp. Ther.* 326:432–442, 2008.
[62]Tomashefski, J. F., H. C. Oppermann, G. F. Vawter, and L. M. Reid. Bronchopulmonary dysplasia: a morphometric study with emphasis on the pulmonary vasculature. *Pediatr. Pathol.* 2:469–487, 1984.
[63]Unbekandt, M., P.-M. del Moral, F. G. Sala, S. Bellusci, D. Warburton, and V. Fleury. Tracheal occlusion increases the rate of epithelial branching of embryonic mouse lung via the FGF10-FGFR2b-Sprouty2 pathway. *Mech. Dev.* 125:314–324, 2008.
[64]Walther, F. J., M. J. Benders, and J. O. Leighton. Persistent pulmonary hypertension in premature neonates with severe respiratory distress syndrome. *Pediatrics* 90:899–904, 1992.
[65]Warburton, D., A. El-Hashash, G. Carraro, C. Tiozzo, F. Sala, O. Rogers, S. De Langhe, P. J. Kemp, D. Riccardi, J. Torday, S. Bellusci, W. Shi, S. R. Lubkin, and E. Jesudason. Lung organogenesis. *Curr. Top. Dev. Biol.* 90:73–158, 2010.
[66]Yin, J., and W. M. Kuebler. Mechanotransduction by TRP channels: general concepts and specific role in the vasculature. *Cell Biochem. Biophys.* 56:1–18, 2010.