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RESEARCH ARTICLE

Integrative Cardiovascular Physiology and Pathophysiology

Hypoxia-induced inhibition of mTORC1 activity in the developing lung: a possible mechanism for the developmental programming of pulmonary hypertension

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Abstract

Perinatal hypoxia induces permanent structural and functional changes in the lung and its pulmonary circulation that are associated with the development of pulmonary hypertension (PH) in later life. The mechanistic target of the rapamycin (mTOR) pathway is vital for fetal lung development and is implicated in hypoxia-associated PH, yet its involvement in the developmental programming of PH remains unclear. Pregnant C57/BL6 dams were placed in hyperbaric (760 mmHg) or hypobaric chambers during gestation (505 mmHg, *day 15* through *postnatal day 4*) or from weaning through adulthood (420 mmHg, *postnatal day 21* through 8 wk). Pulmonary hemodynamics and right ventricular systolic pressure (RVSP) were measured at 8 wk. mTOR pathway proteins were assessed in fetal (*day 18.5*) and adult lung (8 wk). Perinatal hypoxia induced PH during adulthood, even in the absence of a sustained secondary hypoxic exposure, as indicated by reduced pulmonary artery acceleration time (PAAT) and peak flow velocity through the pulmonary valve, as well as greater RVSP, right ventricular (RV) wall thickness, and RV/left ventricular (LV) weight. Such effects were independent of increased blood viscosity. In fetal lung homogenates, hypoxia reduced the expression of critical downstream mTOR targets, most prominently total and phosphorylated translation repressor protein (4EBP1), as well as vascular endothelial growth factor, a central regulator of angiogenesis in the fetal lung. In contrast, adult offspring of hypoxic dams tended to have elevated p4EBP1 compared with controls. Our data suggest that inhibition of mTORC1 activity in the fetal lung as a result of gestational hypoxia may interrupt pulmonary vascular development and thereby contribute to the developmental programming of PH.

NEW & NOTEWORTHY We describe the first study to evaluate a role for the mTOR pathway in the developmental programming of pulmonary hypertension. Our findings suggest that gestational hypoxia impairs mTORC1 activation in the fetal lung and may impede pulmonary vascular development, setting the stage for pulmonary vascular disease in later life.

HIF1α; mTOR; perinatal hypoxia; pulmonary vascular disease; VEGF

INTRODUCTION

Developmental programming centers on the concept that exposures during critical developmental periods, such as perinatal life, affect physiological function and disease susceptibility throughout the life span by altering organ system development (1). The lung and its pulmonary circulation are particularly vulnerable to perinatal exposures, given that developmental processes vital for efficient pulmonary gas transfer are incomplete at birth. Fetal growth restriction and insufficient oxygenation in early life, for instance, induce durable structural and functional changes in the pulmonary vascular bed and peripheral airspaces that are, in turn, predictive of abnormal pulmonary vascular function or pulmonary hypertension (PH) in later life (2–9).

PH is a progressive, life-threatening condition that often develops secondary to the chronic hypoxia of cardiopulmonary disease of high-altitude (≥2,500 m) residence. Hypoxia-associated PH is a leading cause of morbidity and mortality in Bolivia, where nearly two-thirds of the population lives above 3,000 m (10). Our and others' retrospective studies have shown that young adult Andean highland males have elevated pulmonary artery pressures and are more likely to have experienced hypoxic complications in perinatal life or to have been born to a preeclamptic mother compared with their healthy counterparts (5, 11). However, retrospective

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human studies are limited by the inability to assess the level of hypoxic exposure directly or to account for other environmental exposures across the life span fully. In the present study, we used an experimental murine model that allowed for precise control of the duration, timing, and severity of hypoxic exposure to determine whether perinatal hypoxia was associated with PH in later life and, if so, to explore potential mechanisms responsible for observed associations.

Although the mechanistic target of the rapamycin (mTOR) pathway appears vital for fetal lung development (12) and has been implicated in hypoxia-associated PH (13–17), it is unclear whether mTOR contributes to the effect of perinatal hypoxia to trigger PH in later life.

mTOR, a well-conserved serine/threonine kinase, is a hypoxia-responsive regulator of cellular metabolism and proliferation. mTOR forms the catalytic core of two distinct multiprotein complexes, mTORC1 and mTORC2, which are distinguished by complex-specific proteins (i.e., raptor for mTORC1 and rictor for mTORC2). In the developing fetus, mTORC1 influences the elongation and branching of conducting airways and, through its interaction with the hypoxia-inducible factor (HIF1α) signaling pathway, the expression of vascular endothelial growth factor (VEGF) and, subsequently, pulmonary vascular development (12). Inhibition of mTORC1 activity in the fetal lung, therefore, would be expected to interrupt healthy pulmonary vascular and airway development. Here we tested the hypothesis that perinatal hypoxia and hypoxic exposure during gestation induce the development of pulmonary hypertension in later life in association with the suppression of mTORC1 activity in the fetal lung.

MATERIALS AND METHODS

Ethical Approval

All protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado Denver [Protocol No. B-95911(06)1E] and comply with the U.S. National Research Council's Guide for the Care and Use of Laboratory Animals as well as with the ethical principles for animal research outlined by the *American Journal of Physiology*.

Experimental Animals

Nulliparous C57/BL6 female and male mice aged 10–14 wk (Jackson Laboratories) were paired under standard conditions (21°C, 12 h dark-light cycles: lights on at 0600 and off at 1800, 60% humidity) and ambient oxygen tensions in the animal care facility at the University of Colorado Denver Anschutz Medical Campus (P_B ~ 640 mmHg). Animals were supplied with food and water ad libitum at all times. Confirmation of mating was determined by the presence of a copulatory plug, which was considered to be *gestational day* (*GD*) *0.5*. In the current study, only male offspring were included. We recognize the importance of sex differences and, in particular, their relevance for prenatal programming studies. In this study, we elected to study male offspring only, given that our foundational work in humans focused on the association between perinatal hypoxia and the development of pulmonary vascular dysfunction associated with excessive erythrocytosis in Andean male highland residents.

Pregnant dams were randomly assigned to one of four experimental groups, distinguished by the timing of hypoxic exposure (Fig. 1). Hypobaric chambers were used to simulate altitudes of 3,500 m (P_B ~505 mmHg) and 5,000 m (P_B ~420 mmHg) to provide hypoxic (Hx) exposures during perinatal life and early adulthood, respectively. A more modest (3,600 m) hypoxic exposure was chosen for the perinatal period, given that the higher (5,000 m) altitude exposure caused high fetal and neonatal mortality and thereby prohibited prospective studies. Hyperbaric chambers were also used to simulate sea level (P_B ~760 mmHg), given the laboratory altitude of 1,600 m, and to achieve normoxic (Norm) exposures. The study groups were as follows: *1*) normoxic controls (control), *2*) perinatal normoxia followed by hypoxia from 21 days to 8 wk of age (Norm-Hx), *3*) perinatal followed by hypoxia from 21 days to 8 wk of age (Hx-Hx), and *4*) perinatal hypoxia followed by normoxia from 21 days to 8 wk of age (Hx-Norm).

For the perinatal exposures, separate pregnant dams were placed in either normoxic or hypoxic chambers from *GD 14* through *postnatal day 4*. From *postnatal day 5* through *postnatal day 20*, all offspring were housed under normoxic conditions to ensure that we were isolating the influence of perinatal exposures on pulmonary vascular function. Secondary normoxic or hypoxic exposures occurred from *postnatal day 21* to 8 wk of age. Our study design was such that pups and dams could be housed together until weaning on *postnatal day 21*.

To evaluate the effect of gestational hypoxia on mTOR pathway protein expression in fetal lung, separate sets of pregnant dams were randomly assigned to one of two experimental groups: *1*) normoxic controls (control, 760 mmHg from *GD 14* to *GD 18.5*) and *2*) perinatal hypoxia (Hx, 505 mmHg from *GD 14* to *GD 18.5*). Fetal lung tissue was harvested from animals in both groups at *GD 18.5*.

Hemodynamic Analyses

Echocardiograms were performed in offspring at 8 wk of age to assess PH, pulmonary artery blood flow, and right ventricular hypertrophy (RVH). Echocardiography was performed using a Vevo-770 high-resolution imaging system from Visual Sonics (VisualSonics, Ontario, Canada) and a 30-MHz mechanical transducer. For this procedure, mice were anesthetized with 2% inhaled isoflurane, breathing room air [FiO_2 = 0.21, Denver, Colorado (P_B 630 mmHg)], and maintained at a body temperature of 37°C for the duration of the noninvasive imaging. Heart rate and blood pressure were continuously monitored throughout the procedure. Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal short-axis view at the level of the aortic valve. Pulse-wave Doppler of the pulmonary artery was used to measure pulmonary artery acceleration time (PAAT) and the pulmonary artery flow velocity-time integral. Mouse hearts were imaged in a right parasternal long axis on the right side of the mouse. M-mode images were recorded to measure the right ventricle anterior wall (RVAW) thickness as an index of RVH.

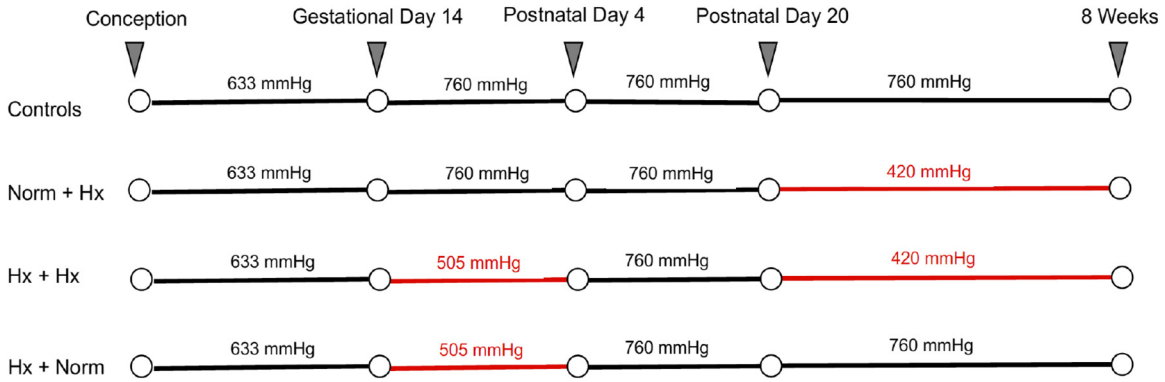

Figure 1. Experimental protocol. Pregnant C57/BL6 dams were housed under ambient oxygen tensions (Denver, CO, 1,609 m, $P_B \sim 640$ mmHg) from *gestational day 0.5* through *day 14*. During the perinatal period, defined as *gestational day 14* through *postnatal day 4*, mice were either exposed to hypobaric hypoxia ($P_B \sim 505$ mmHg) or hyperbaric normoxia ($P_B \sim 760$ mmHg). From *postnatal day 5* through *day 21*, all of the offspring were housed under normoxic conditions. For the hypoxic exposure in early adulthood, defined as *postnatal day 20* to 8 wk, offspring were again either housed under hypobaric hypoxia ($P_B \sim 420$ mmHg) or hyperbaric normoxia ($P_B \sim 760$ mmHg). As shown, the study groups included *1*) controls ($n = 15$), *2*) perinatal normoxia and early adulthood hypoxia (Norm-Hx; $n = 15$), *3*) perinatal and early adulthood hypoxia (Hx-Hx; $n = 13$), and *4*) perinatal hypoxia followed by early adulthood normoxia (Hx-Norm; $n = 8$); n = number of mice.

Direct Measurement of Right Ventricular Systolic Pressure

One day after echocardiography, mice were anesthetized for the duration of the procedure with 2% inhaled isoflurane, placed on a regulated heating pad to maintain body temperature at 37°C, intubated with a 20 G angiocath, and immediately connected to a positive pressure ventilator (Microvent). Throughout the procedure, body temperature, heart rate, and blood pressure were continuously monitored. Respiratory frequency was set at 120 breaths per min, and peak inspiratory pressure was set at 10 cm H_2O. An incision was made on the left side of the chest at the level of the fifth or sixth thoracic vertebra. The intercostal muscles were then separated using a hemostat to expose the lungs and heart. An incision was made in the pericardial sac surrounding the heart by pulling the sac away from the heart using forceps. The right ventricular (RV) septum was visualized, and a small Millar catheter (SPR-839) was immediately inserted into the RV apex, making sure that the pressure sensor was entirely inside the RV. Only cases with no or minimal bleeding were included for analysis. After steady-state hemodynamics were obtained, the mice were euthanized by cervical dislocation and vital tissue harvested for biological sample collection. Since RVSP closely parallels sP_{PA} (18), an elevation of right ventricular systolic pressure (RVSP) was used as an index of PH.

Tissue Sampling

In adult offspring, intracardiac blood samples were collected following the physiological measurements to assess hematocrit levels in triplicate by microcentrifugation. Hearts were dissected to obtain the ratio of right ventricular to left ventricular (LV) and septal (S) weight [RV/(LV + S)] as a measure of RVH. Lung tissue was also obtained following physiological measurements and immediately snap-frozen for protein measurements. To obtain fetal lung tissue for protein measurements, pregnant dams were euthanized at *GD 18.5*; fetuses were immediately removed, euthanized, and weighed; and fetal lung tissue was isolated in ice-cold PBS supplemented with 1× Halt Protease and Phosphatase Inhibitor (Thermo Fisher Scientific, Cat. No. 1861281) to protect against protein degradation by endogenous proteases and phosphatases released during protein extraction and purification.

Semiquantitative Protein Analysis

We sought to determine whether hypoxia suppressed mTORC1 activity in the fetal lung and, if so, whether such changes were sustained into adulthood. Using the Wes System (ProteinSimple, CA) with a 12-230kDa Separation Module, we performed semiquantitative analysis of mTOR pathway proteins in lung homogenates of fetuses (*GD 18.5*) and adult offspring (8 wk) that had gestated under normoxic or hypoxic conditions. Protein targets included several upstream regulators of mTORC1 regulators [adenosine monophosphate kinase α (AMPKα), protein kinase B (Akt), and phosphoinositide 3-kinase (PI3K)]. AMPKα inhibits mTOR through the direct phosphorylation of tuberin (TSC2) and raptor (19, 20). PI3K, a vital component of the insulin signaling pathway, can phosphorylate and activate Akt, which, in turn, inactivates TSC2, a negative regulator of mTOR within the mTOR-raptor complex (21, 22). Proteins downstream of mTORC1 were also measured and included translation repressor protein (4EBP1), S6 ribosomal protein (S6), peroxisome proliferator-activated receptor γ (PPARγ), and vascular endothelial growth factor A (VEGFA). Activated mTOR promotes mRNA translation through the phosphorylation of 4EBP1 and S6 ribosomal protein.

Fetal lung tissue (~10 mg/snap-frozen sample) was added to 1× PBS with 1× Halt Protease and Phosphatase Inhibitor (Thermo Fisher Scientific, Cat. No. 1861281) and lysed using MP bio Lysing Matrix D beads with the Fast Prep-24 homogenizer (MP Biomedicals, Irvine, CA). Adult mouse lung tissue (~10 mg/snap-frozen sample) was added to radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and

5 mmol/L sodium vanadate] supplemented with 2× Halt Protease and Phosphatase Inhibitor cocktail. Adult lung tissue was minced on ice using dissecting scissors and lysed using a TT-30K digital handheld homogenizer (Hercuvan Lab Systems, Cambridge, UK).

Total protein concentration was determined by bicinchoninic acid analysis (Pierce BCA Protein Assay; Thermo Fisher Scientific, Cat. No. 232277) using a 1:10 dilution of each sample. To ensure even loading, lysates were normalized to a 1 mg/mL concentration in 0.1× sample buffer (ProteinSimple, Cat. No. 042–195). All primary antibodies, purchased from Cell Signaling unless otherwise noted, were prepared using ProteinSimple antibody diluent to reach the following concentrations: AMPK (Cat. No. 4811, 1:25), pAMPKα Thr172 (Cat. No. 2535, 1:25), 4E-BP1 (Cat. No. 9452, 1:10), p4EBP1 Thr37/46 (Cat. No. 9459, 1:10), S6 ribosomal protein (Cat. No. 2217, 1:100), pS6 ribosomal protein Ser235/236 (Cat. No. 2211, 1:50), AKT (Cat. No. 9272, 1:10), pAKT Ser473 (Cat. No. 4060, 1:10), PI3K (Cat. No. 3811, 1:10), PPARγ (Cat. No. 2446, 1:25), and VEGFA (Novus Biologicals, 1:10). Proteins were normalized to vinculin (Sigma Aldrich, Cat. No. V9131, 1:120,000) using a 90% rabbit/10% mouse secondary antibody mix (ProteinSimple Cat. No. 042–206 and 042–205, respectively). Exceptions to the above normalization include *1*) PPARγ, which was normalized to vinculin (1:960,000) and diluted in 10% BSA (Sigma Aldrich, Cat No. A2153) with the above secondaries; *2*) PI3K, which was normalized to β-actin (Cat. No. 4970, 1:800) using rabbit secondary only; and *3*) VEGF, which was normalized to β-actin (Cat. No. 4970, 1:6,400) using rabbit secondary only. Data were extracted using Compass software (ProteinSimple, v. 4.1.0).

Statistics and Analytical Strategy

GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) was used to generate aligned or scattered dot plots, and SPSS v. 26 (IBM, Armonk, NY) was used for data analysis. Analysis of variance (ANOVA) with Bonferroni post hoc testing was used to determine whether echocardiographic measures of pulmonary hemodynamics, RVSP, or hemoglobin concentration differed between exposure groups (controls, Norm-Hx, Hx-Norm, and Hx-Hx). Pearson's correlations were used to evaluate whether hemodynamic indices of elevated pulmonary artery pressure were positively associated with hematocrit levels, as such associations would suggest that pulmonary circulation abnormalities may be due to increased blood viscosity. Normalized protein levels were compared between exposure groups using independent Student's *t* tests. The numbers of animals for each comparison are noted in the figures or figure legends. For all graphs, values represent means ± SD. A two-sided $P < 0.05$ was taken as evidence of significant association or difference, and trends were considered when $0.05 < P < 0.10$.

RESULTS

Our late-gestation, hypoxia-exposure protocol produced a significant reduction of fetal weight at *GD 18.5* (0.90 ± 0.16 g vs. 0.81 ± 0.13 g, $P < 0.05$) of similar magnitude to our and others' previous rodent models (23–25).

Pulmonary Hemodynamics and PH in Control versus Norm-Hx

To determine whether exposure to hypobaric hypoxia from 3 to 8 wk of age was sufficient to induce PH and pulmonary vascular dysfunction, we first contrasted key outcome variables between controls and mice exposed to hypoxia during later life only (Norm-Hx). Compared with controls, Norm-Hx reduced PAAT by 15% ($P < 0.001$) and increased RVSP by 79% ($P < 0.0001$), hematocrit by 34% ($P < 0.0001$), and the RV:LV + S weight ratio by 15% ($P < 0.05$; Fig. 2).

Effect of Perinatal Hypoxia on Pulmonary Hemodynamics and PH in Adulthood

Perinatal hypoxia augmented hemodynamic indices of PH and polycythemia in response to a secondary hypoxic exposure during adulthood. Specifically, comparing Hx-Hx with Norm-Hx, we found that perinatal hypoxia exaggerated the hypoxia-associated reduction in PAAT ($P < 0.0001$) and PV peak flow velocity ($P < 0.001$) and enhanced the hypoxia-associated increase of RVAW thickness ($P < 0.0001$) and hematocrit ($P < 0.001$) (Fig. 2).

Perinatal hypoxia alone (Hx-Norm) had lasting effects on pulmonary vascular function even in the absence of a secondary hypoxic exposure during adulthood (Fig. 2). Specifically, compared with controls, mice exposed to perinatal hypoxia had reduced PAAT and PV peak flow velocity ($P < 0.05$, both) and greater RVSP ($P < 0.01$), RVAW thickness ($P < 0.001$), and RV:LV + S weight ($P < 0.001$) at 8 wk of age under normoxic conditions. Similar elevations in RVSP, PAAT, PV peak velocity, and RV:LV + S weight values at 8 wk of age were seen in mice exposed to perinatal hypoxia as well as a secondary hypoxic exposure during adulthood.

Relationship between Hematocrit and Pulmonary Hemodynamics

Hematocrit levels were not associated with hemodynamic indices, RVSP, or RVH in any of the four study groups (data not shown), indicating that the observed effects of hypoxia were not due to accompanying polycythemia or hence, likely, increased blood viscosity.

Effect of Hypoxic Gestation on mTOR Pathway Protein Expression in Fetal and Adult Lung

To test the hypothesis that gestational hypoxia suppressed mTORC1 activation in the fetal lung and, if so, whether such changes were sustained into adulthood, we first compared hypoxia-sensitive, upstream regulators of mTOR. In fetal lung homogenates, total AMPK, pAMPK, and pAMPK:AMPK were similar between normoxic and hypoxic groups (Fig. 3*A*). Compared with controls, total AKT and PIK3 protein expression were lower in hypoxic fetal lung, but neither pAKT nor pAKT:AKT differed (Fig. 3, *B* and *C*). Several downstream mTOR targets were lower in hypoxic compared with normoxic fetal lung. In particular, total 4EBP1, p4EBP1, and p4EBP1:4EBP1 were reduced in hypoxic relative to normoxic lung (Fig. 3*D*). Total S6 (but not pS6 or pS6:S6; Fig. 3*E*) and PPARγ [Norm: 0.29 ± 0.06 (expressed as the ratio between PPARγ and vinculin) and Hx: 0.22 ± 0.03, $P = 0.051$] followed

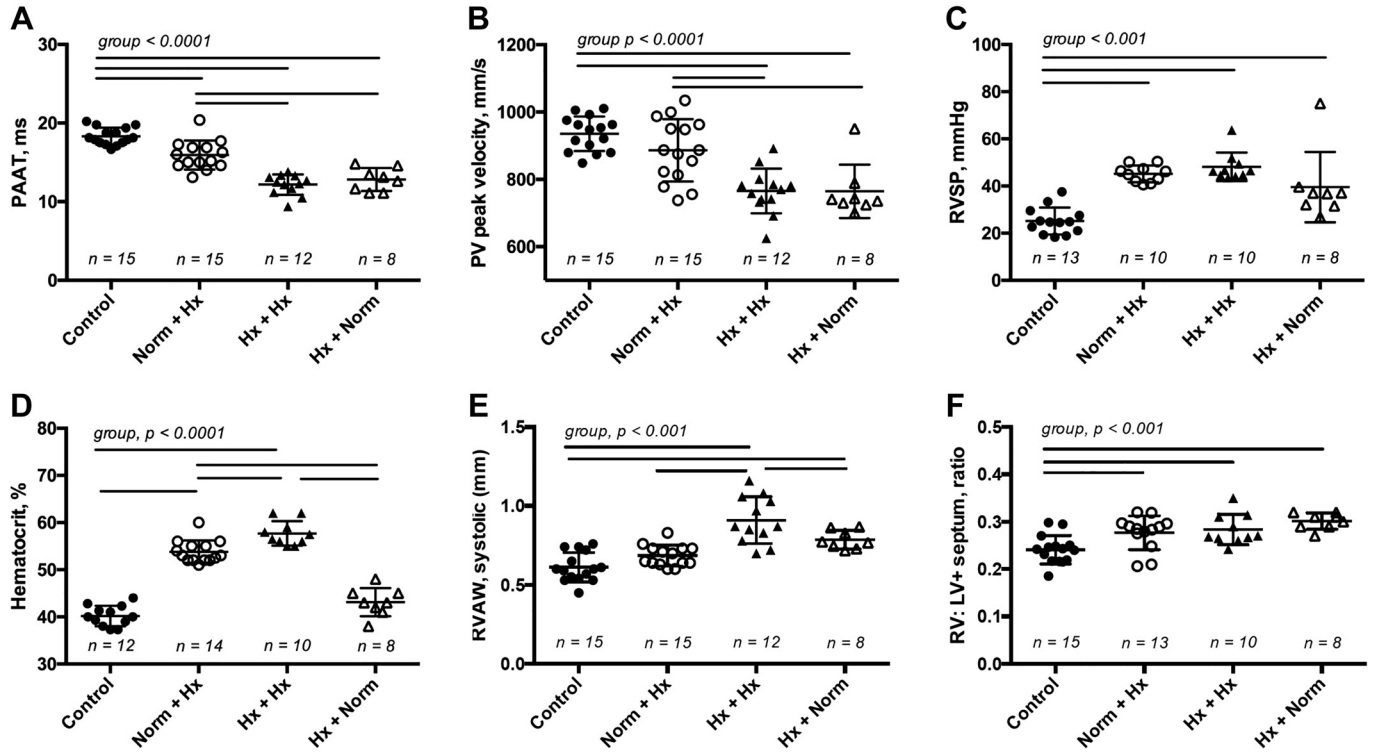

Figure 2. Effect of hypoxic (Hx) exposure on pulmonary hemodynamics and indices of pulmonary hypertension (PH) in mice. Pulmonary hemodynamics and indices of pulmonary hypertension are contrasted between four groups of 8-wk-old mice. The four study groups were *1*) normoxic controls, *2*) perinatal normoxia and early adulthood hypoxia (Norm-Hx), *3*) perinatal and early adulthood hypoxia (Hx-Hx), and *4*) perinatal hypoxia followed by early adulthood normoxia (Hx-Norm). Shown are pulmonary artery acceleration time (PAAT) (*A*), peak flow velocity through the pulmonary valve (PV, peak velocity) (*B*), right ventricular systolic pressure (RVSP) (*C*), hematocrit (Hct) levels (*D*), right ventricular anterior wall (RVAW) thickness (*E*), and right ventricular to left ventricular plus septal weight ratio (RV:LV + S) (*F*). Exposure groups were compared using one-way ANOVA and Tukey's multiple-comparisons tests to identify sources of significant between-group differences. One-way ANOVA *P* values are shown above each graph, and significant between-group multiple comparisons denoted by a solid black bar; specific *P* values for the latter are provided within the manuscript text. *n*, sample sizes.

a similar trend. VEGF protein expression was also lower in hypoxic fetal lung homogenates ($P < 0.05$, Fig. 3*F*).

Adult offspring of hypoxic dams tended to have elevated p4EBP1 protein expression as well as p4EBP1:4EBP1 ratios compared with normoxic controls (Fig. 4), which was the opposite of observations made in fetal lung. No other proteins assessed differed between adult offspring gestated under normoxic versus hypoxic conditions.

DISCUSSION

With precise control of hypoxic exposure, our present experimental animal study showed that insufficient oxygenation in early life not only exacerbated PH in response to a secondary hypoxic exposure in later life but also impaired pulmonary vascular function under normoxic conditions. Furthermore, in this study, exploring the role of the mTOR pathway for the developmental programming of pulmonary hypertension, our protein expression studies suggest that gestational hypoxia impairs mTORC1 activation in the fetal lung but that such effects normalize by 8 wk of age, even though perinatal hypoxia induces pulmonary circulation abnormalities that persist in later life. Based on these findings, we propose that inhibition of the mTORC1 activity in early life as a result of antenatal hypoxia may impede pulmonary vascular and airway development, setting the stage for pulmonary vascular disease in later life.

Our perinatal hypoxia model effectively produced PH, as indicated by the hemodynamic indices of reduced PAAT and PV flow velocity, elevated RVSP, and RVH. Our findings were consistent with previous experimental animal studies demonstrating that oxygen deficit in early life adversely affects the function and structure of the pulmonary circulation. Neonatal mice, for instance, develop RVH following exposure to hypobaric hypoxia (380 mmHg) for 7 days within 48 h after delivery (26). Neonatal rats exhibit a more pronounced response soon after birth, with severe RVH accompanied by elevated pulmonary vascular resistance and remodeling after 7 to 14 days of hypoxic exposure (27, 28). Experimental sheep models of uteroplacental insufficiency or fetal growth restriction also show that impaired fetal oxygenation induces pulmonary vascular remodeling and alveolar simplification (29, 30).

In support of our observation that perinatal hypoxia alone induces persistent pulmonary circulation abnormalities even in the absence of sustained hypoxia, others have shown that rat pups exposed to hypoxia in early life showed increased medial thickness of the small pulmonary arteries, RVH, reduced PAAT, and an exaggerated hypoxic pulmonary vasoconstrictor response during adulthood (8, 31, 32).

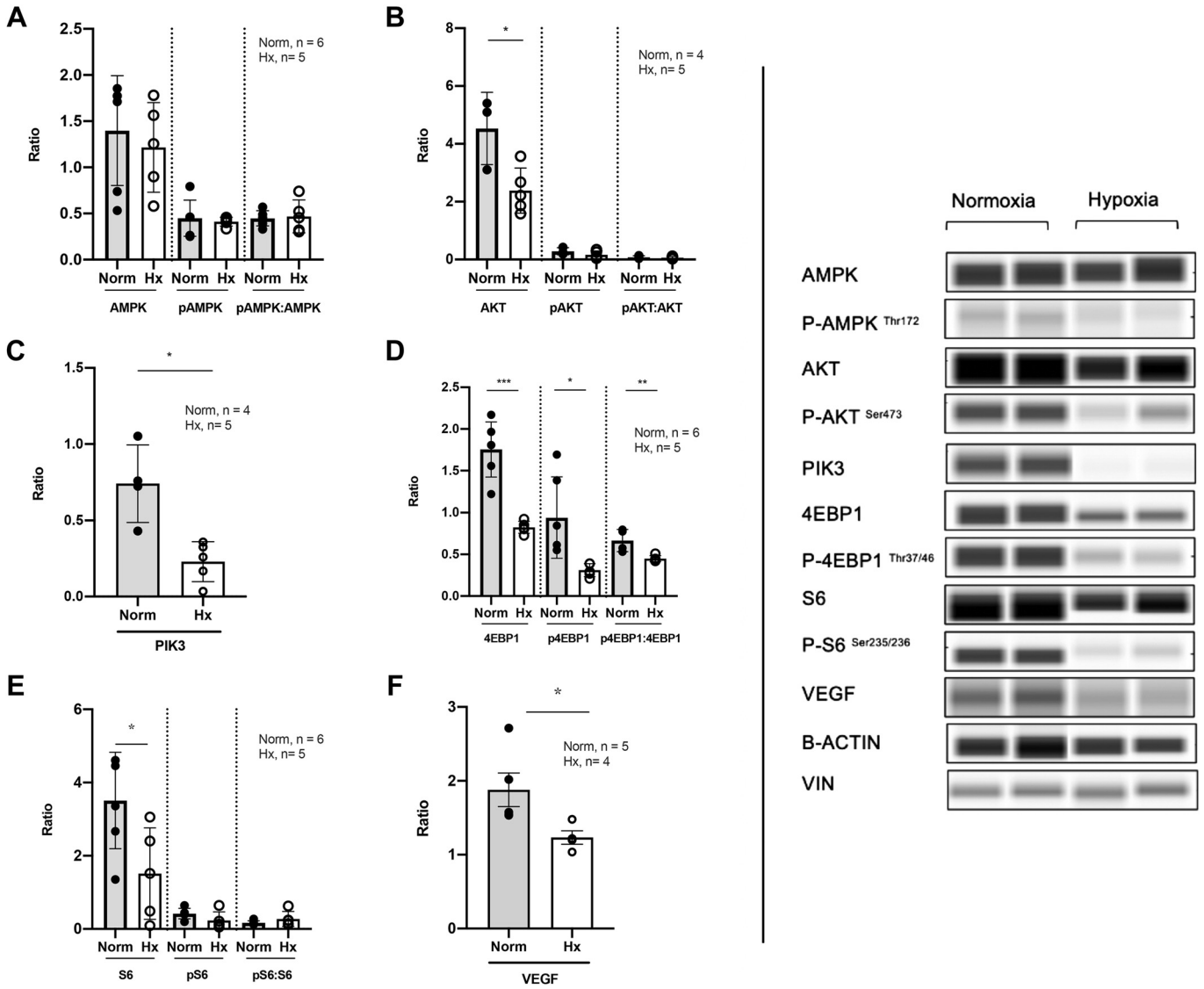

Figure 3. Effect of gestational hypoxia on mechanistic target of the rapamycin (mTOR) pathway protein expression in fetal mouse lung. To evaluate the effect of gestational hypoxia on mTOR pathway protein expression in fetal lung, pregnant dams were randomly assigned to one of two experimental groups: *1*) normoxic controls (control, 760 mmHg from *GD 14* to *GD 18.5*) and *2*) perinatal hypoxia (Hx, 505 mmHg from *GD 14* to *GD 18.5*). Fetal lung tissue was harvested from animals in both groups at *GD 18.5*. Total protein and, where applicable, phosphorylated protein expression are shown for AMPK (*A*), AKT (*B*), PIK3 (*C*), 4EBP1 (*D*), S6 (*E*), and VEGFA (*F*). AMPK, adenosine monophosphate kinase alpha; Akt, protein kinase B; 4EBP1, translation repressor protein; PIK3, phosphoinositide 3-kinase; S6, S6 ribosomal protein; VEGFA, vascular endothelial growth factor A. Unpaired *t* tests were used to compare protein levels between normoxic and hypoxic conditions. Asterisks are used to show significance values are shown in each graph: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. *n*, sample sizes. Representative images for each protein are shown on the *right*.

Insufficient oxygenation during perinatal life also results in irreversible alveolar simplification and slows down the postnatal increase in lung volume in rats (33, 34), which likely contributes to persistent hypoxia and, in turn, adverse pulmonary vascular outcomes. Similarly, highland-gestated lambs develop elevated basal pulmonary vascular resistance and sP_{PA}, exaggerated postnatal pulmonary vascular contractile response to hypoxia and pharmacological agonists, and increased muscularity of the small pulmonary arteries; several of these features remain under normoxic, low-altitude conditions (35, 36).

In our model, pulmonary circulation abnormalities associated with perinatal hypoxia or secondary hypoxic exposure during adulthood were not associated with hematocrit levels, suggesting that neither polycythemia nor increased blood viscosity is likely to be a primary determinant of the PH observed. Consistent with this finding, we have previously shown that, among highlanders residing in La Paz-El Alto, Bolivia, adverse perinatal oxygenation raises the risk of excessive erythrocytosis accompanied by a modest elevation of systolic pulmonary artery pressure during early adulthood and that this association is independent of increased blood viscosity (11).

One mechanism potentially influencing an association between perinatal hypoxia and the development of PH is the mTOR pathway. In the developing fetus, mTORC1 influences elongation and branching of the conducting airways and, through its interaction with the HIF1α signaling pathway,

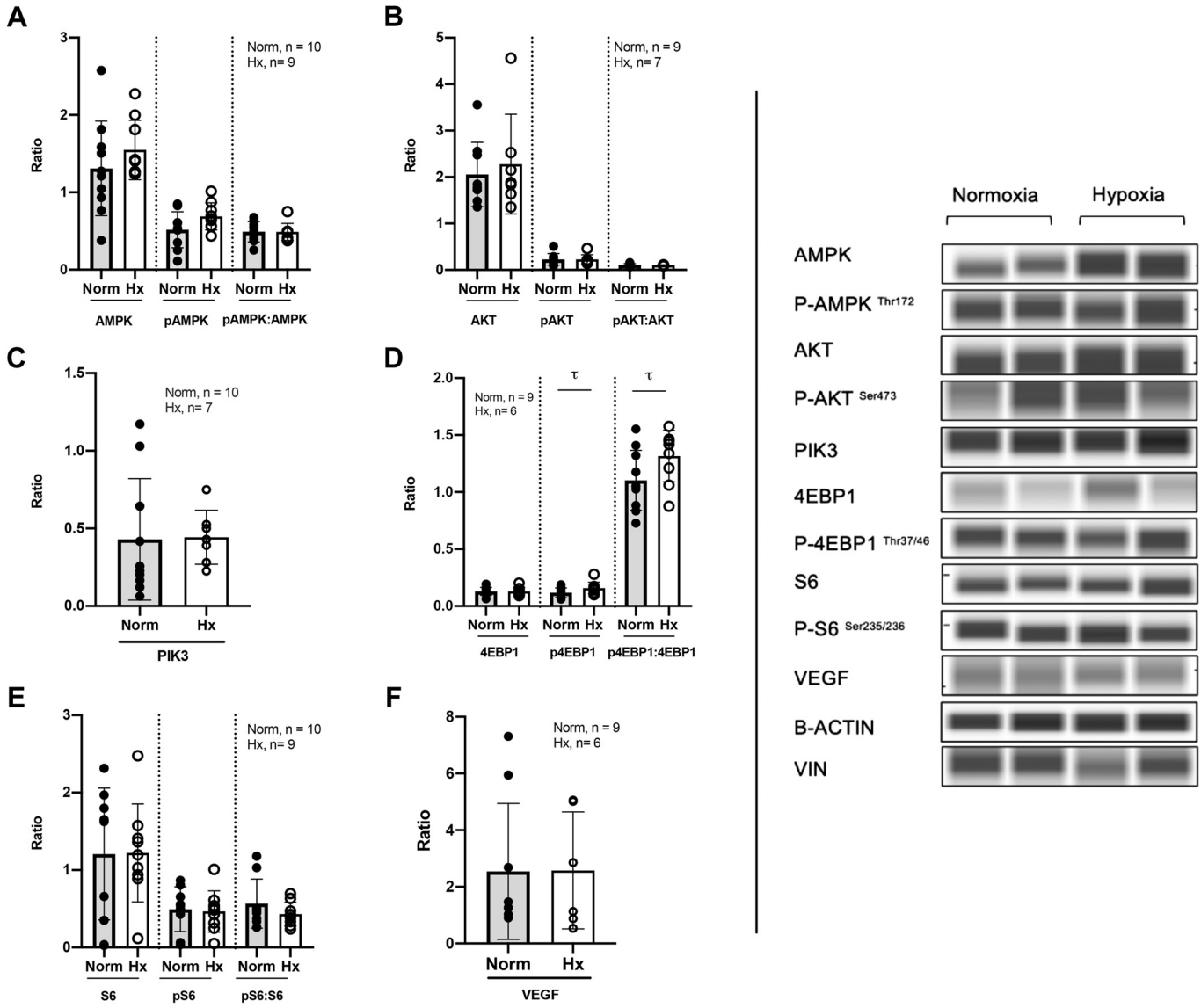

Figure 4. Effect of gestational hypoxia on mechanistic target of the rapamycin (mTOR) pathway protein expression in adult mouse lung. To evaluate whether gestational hypoxia modified mTOR pathway protein expression during adulthood, protein levels were assessed in lung tissue obtained from 8-wk-old mice gestated under normoxic (760 mmHg from *GD 14* to *GD 18.5*) or hypoxic (Hx, 505 mmHg from *GD 14* to *GD 18.5*). Total protein and, where applicable, phosphorylated protein expression are shown for AMPK (*A*), AKT (*B*), PIK3 (*C*), 4EBP1 (*D*), S6 (*E*), and VEGFA (*F*). AMPK, adenosine monophosphate kinase alpha; Akt, protein kinase B; 4EBP1, translation repressor protein; PIK3, phosphoinositide 3-kinase; S6, S6 ribosomal protein; VEGFA, vascular endothelial growth factor A. Unpaired *t* tests were used to compare protein levels between normoxic and hypoxic conditions. Trends ($0.05 < P < 0.10$) are shown using the tau symbol (τ). *n*, sample sizes. Representative images for each protein are shown on the *right*.

temporally and spatially unifies the airway branching morphogenesis program with pulmonary vascular development (Fig. 5). Specifically, in the developing lung, angiogenesis is initiated through the activation of a mTORC1-HIF1α complex and, in turn, increased VEGFA transcription and secretion from advancing airway epithelial cells (Fig. 5) (37, 38). HIF1α has two transactivation domains, an oxygen-sensitive C-terminus transcriptional activation domain and a more centrally located N-terminal transactivation domain. The transcription of HIF1α-target genes is initiated when the HIF1α C-terminus transcriptional activation domain binds to the co-activator CBP/p300. The functional interaction of the mTORC1 component raptor and HIF1α increases HIF1α-CBP/p300 binding and, subsequently, amplifies HIF1α activity (39). Evidence further suggests that raptor recruits mTOR to HIF1α through the presence of an mTOR signaling (TOS) motif located within the N-terminus of HIF1α (40). Although our study assessed VEGF and protein targets indicative of mTORC1 activation, functional studies are needed to determine the specific mechanisms by which mTORC1 may be involved in the regulation (or dysregulation) of angiogenesis and airway development in the context of developmental programming of pulmonary hypertension (Fig. 5) as well as histological evaluation of pulmonary vascular remodeling that has been observed other animal models of neonatal hypoxia (41).

In summary, existing literature supports our observation that hypoxia reduces mTORC1 signaling and VEGFA

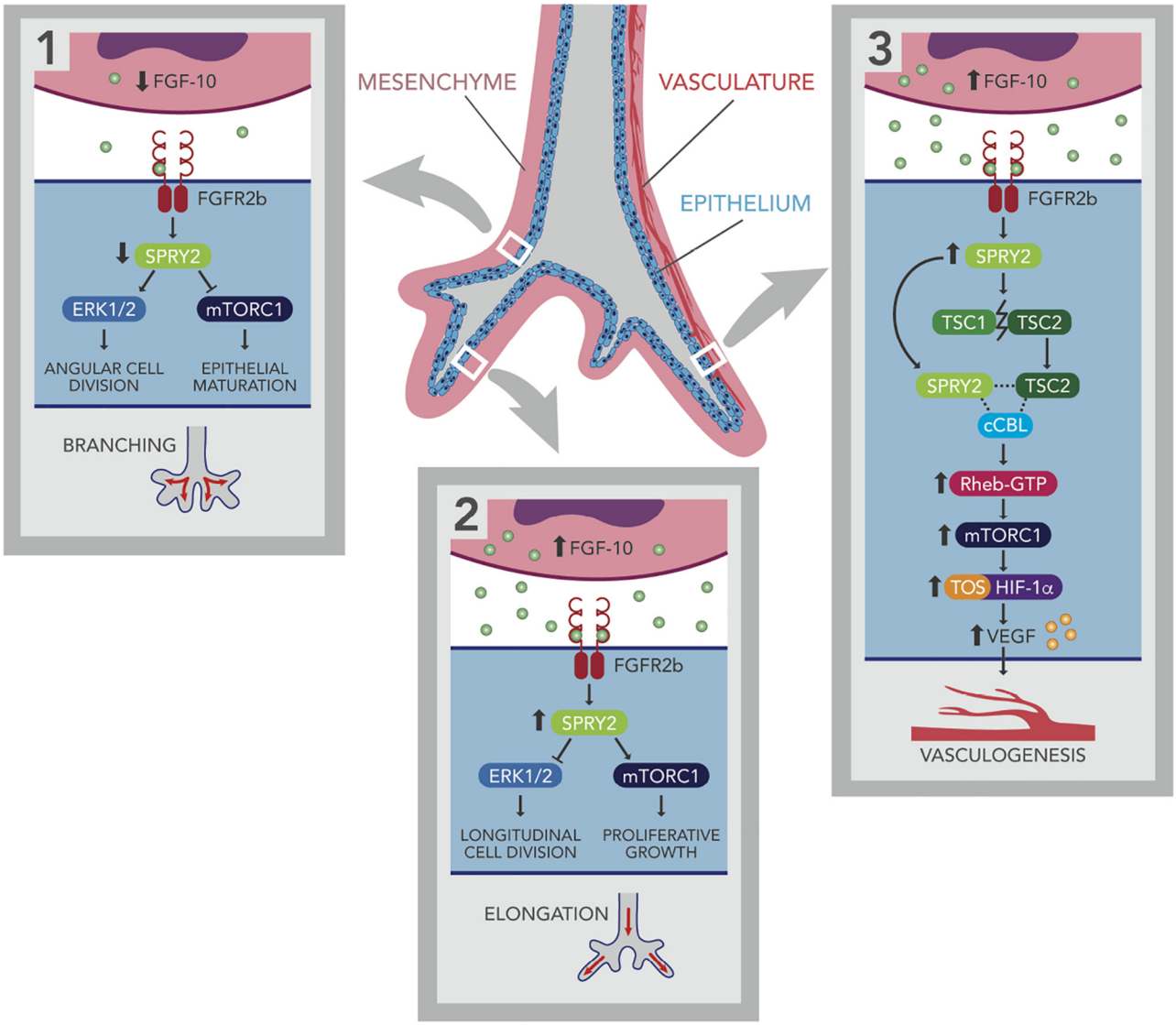

Figure 5. Targets for future study: putative mechanisms by which mechanistic target of the rapamycin (mTOR) influences the development of the pulmonary vasculature and peripheral airways. mTORC1 influences elongation and branching of the conducting airways in the fetal lung and, through its interaction with the hypoxia-inducible factor (HIF1α) signaling pathway, temporally and spatially unifies the airway branching morphogenesis program to pulmonary vascular development. *1*) airway branching is induced by the mesoderm-derived fibroblast growth factor-10 (FGF-10) binding to a tyrosine kinase receptor, FGFR2b, expressed in cells at the leading end of endodermal airway tube and ahead of prospective airway branches. Reduced FGFR2b activity stabilizes and inactivates sprouty 2 (Spry2). Spry2 inactivation, in turn, suppresses airway elongation and stimulates branching epithelial growth through the repression of mTORC1 activity and enhanced ERK1/2 activity, the latter of which enhances angular cell division. *2*) conversely, enhanced FGFR2b activity within airway epithelial cells promotes tubular outgrowth toward the FGF-10 signal by increasing epithelial mTORC1 activity and, therefore, proliferative growth. *3*) vasculogenesis in the fetal lung is initiated through the activation of a mTORC1-HIF1α complex and, in turn, increased vascular endothelial factor A (VEGFA) transcription and secretion from advancing airway epithelial cells (37, 38). FGF-10 is expressed in the mesenchyme ahead of nascent airway buds and diffuses towards the airway epithelium. FGF-10 induces autophosphorylation and signaling to Spry2 whose activation induces tuberin (TSC2) clearance enabling Rheb to bind GTP and activate mTORC1. HIF1α is regulated through the FGF-10-Spry2-mTORC1 signaling axis by a direct interaction involving the putative mTOR signaling (TOS) motif located within the N-terminus of HIF1α (40).

expression in the fetal lung and is consistent with our hypothesis that such effects may impair pulmonary vascular development. mTORC1 activation promotes mRNA translation (protein synthesis) by phosphorylating its downstream effectors, with 4EBP1 being a central target. Our findings show that, compared with normoxic controls, p4EBP1 and p4EBP1:4EBP1 ratios were reduced in lung homogenates from hypoxic versus normoxic fetuses, suggesting that gestational hypoxia inhibited mTORC1 signaling. Our observation that VEGFA expression was reduced in hypoxic fetal lung is supported by evidence that the inhibition of mTORC1 by rapamycin impairs angiogenesis by reducing the VEGFA expression (42). It is well established that hypoxia downregulates mTORC1 activity via AMPK-dependent and AMPK-independent pathways. Specifically, hypoxia impairs mitochondrial respiration and, therefore, reduces ATP levels, leading to AMPK activation and, in turn, mTORC1 inhibition (19). Given that we did not observe differences in pAMPKα expression between normoxic and hypoxic fetal lung, we consider it likely that inhibition of mTORC1

activity operated through AMPK-independent mechanisms (43).

In contrast to our observations in the fetal lung, adult offspring of dams exposed to hypoxia during pregnancy tended to have elevated p4EBP1 protein expression as well as p4EBP1:4EBP1 compared with normoxic controls, suggesting increased mTORC1 activation. Hypoxia-induced mTORC1 activation in developed pulmonary vasculature has been linked to a detrimental propagation of apoptosis-resistant vascular smooth muscle cell proliferation, whereas inhibition of mTORC1 reverses pulmonary artery vascular smooth muscle cell proliferation in monocrotaline-induced PH (44) and protects against hypoxia-induced PH in adult animals (45). Given evidence of reduced mTORC1 activity in human fetal growth restriction and evidence that growth-restricted fetuses are often hypoxemic, we consider the mTOR pathway may also be central to well-established links between impaired fetal growth and pulmonary vascular dysfunction.

The interpretation of our findings should take into consideration our study strengths but also its limitations. Our experimental animal model faced several constraints, predominantly related to differences between the pathophysiology of hypoxia-associated PH in humans compared with mice. For instance, although mice reliably develop increased RVSP and RVH in response to chronic hypoxia, pulmonary vascular remodeling is minimal compared with that observed in humans (46). Temporal variation concerning the developmental stages of the lung should also be taken into consideration. Neonatal mouse lung development at birth until approximately 2 wk of age, for example, parallels human lung development from week 24 of gestation through the first 2 years of age (47). More specifically, in the mouse, alveolar development occurs postnatally, whereas in humans, alveolarization occurs during gestation and continues well into postnatal life. In addition, whole lung homogenates were used for semiquantitative protein measurements; thus, we were not able to identify the specific cell types responsible for differences observed. Our experimental murine model, however, offers several strengths, including the ability to precisely control exposures encountered across the life span (including the duration and severity of hypoxic exposure), access to biological specimens from an organ of direct relevance in a timely manner, and the ability to eliminate concerns regarding variable genetic background or unknown exposures across the life span. Of equal importance, the influence of interoperator effects on pulmonary vascular outcomes was eliminated by having a single operator perform the Doppler ultrasound studies to assess pulmonary hemodynamics and only one operator conduct the direct RVSP measurements.

In summary, our findings indicate that insufficient oxygenation in early life impairs pulmonary vascular function under normoxic conditions and augments PH induced by hypoxic exposure in later life. Our data further suggest that inhibition of the mTORC1 activity in early life as a result of gestational hypoxia may interrupt pulmonary vascular development and thereby contribute to the developmental programming of PH. We consider such studies to be of importance, given that understanding links between early-life exposures and pulmonary vascular health outcomes across the life span has emerged as a critical step in the prevention of pulmonary vascular disease and the timely identification of at-risk individuals (48).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.G.J. conceived and designed research; G.W., J.A.H., D.P., and C.G.J. performed experiments; J.A.H. and C.G.J. analyzed data; C.G.J. interpreted results of experiments; C.G.J. prepared figures; C.G.J. drafted manuscript; W.M., G.W., L.G.M., J.A.H., D.P., and C.G.J. edited and revised manuscript; W.M., G.W., L.G.M., J.A.H., D.P., and C.G.J. approved final version of manuscript.

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