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A protocol for a lung neovascularization model in rodents

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Abstract

By providing insight into the cellular events of vascular injury and repair, experimental model systems seek to promote timely therapeutic strategies for human disease. The goal of many current studies of neovascularization is to identify cells critical to the process and their role in vascular channel assembly. We propose here a protocol to analyze, in an *in vivo* rodent model, vessel and capillary remodeling (reorganization and growth) in the injured lung. Sequential analyses of stages in the assembly of vascular structures, and of relevant cell types, provide further opportunities to study the molecular and cellular determinants of lung neovascularization.

INTRODUCTION

A significant challenge to *in vivo* studies of neovascularization in adult tissues is the ability to trigger consistent changes within a model system. We have developed a protocol to study vascular remodeling in a rodent model of hyperoxic acute lung injury (HALI): vascular changes in the HALI model show a high degree of reproducibility[1-4] and so offer a basis for comprehensive studies of vascular precursor cells (see accompanying *Nature Protocol* by Jones *et al*.[5]) in addition to molecular and therapeutic studies.

In the human lung, both the pulmonary and bronchial circulations extensively remodel in response to injury and in disease[6]. On the vascular side, for example, in idiopathic (familial) and secondary forms of pulmonary hypertension, and in the severest form of acute lung injury, that is, the Adult Respiratory Distress Syndrome, the walls of pulmonary arteries remodel (thicken) throughout the lung, but it is in the smallest ones (<100 μm in diameter in the alveolar region) that this change imposes the greatest functional restriction and leads to increased pulmonary vascular resistance and pressure[7]. There are several well-established experimental models of vascular remodeling in different species, mostly focused on changes in the rodent and calf lung[7,8]. In the model described here, rodents breathe a high but sublethal oxygen tension (70-85%) at normobaric pressure for several weeks. This approach has the advantage of triggering significant vessel wall growth (thickening) in large arteries[9] and in the small pulmonary arteries and veins[1-4,10] similar to ones remodeling in human lung. However, note that, in the mouse, similar vessels do not remodel by the development of these cells (as robustly) as in the rat. The model permits analysis of the cellular basis of the response[1-4]. Furthermore, in a second phase, in which rodents are returned to breathing air after breathing high oxygen, the model provides additional information for the regenerative capacity of the lung's injured capillary bed[1]. These structural events are mirrored by changes in vascular endothelial growth factor—vascular restriction in the high oxygen-injured lung being associated with fall in lung

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expression levels of vascular endothelial growth factor[11], and vascular restoration on return to breathing air with increase[12].

Both by brightfield microscopy and high-resolution imaging, cells are readily characterized (in 1-μm-thick and 90-nm-thick resin sections, respectively) by morphology and by location —as intravascular (circulating through the lung) or as resident in vascular structures (endothelial cells, pericytes, smooth muscle cells or perivascular fibroblasts). The protocol we present here has the advantage that the quantitative analysis obtained by these combined approaches provide significant insight into the origin and role of cells assembling vessels and capillaries in the injured adult lung.

MATERIALS

REAGENTS

- Clidox disinfectant
- Nembutal sodium solution, 50 mg ml^{-1} (Abbott Laboratories)
- 10% (vol/vol) paraformaldehyde, methanol-free (PolySciences, cat. no. 04018-1)
- 25% (vol/vol) gluteraldehyde (PolySciences, cat. no. 01909)
- Araldite 502/Embed 812 kit (EMS, cat. no. 13940)
- 4% osmium tetroxide (EMS, cat. no. 19190)
- Propylene oxide (Fisher Scientific, cat. no. 04332-1)
- Sorensen's buffer solution A (13.8 g NaH_2PO_4 in 500 ml distilled/deionized water; Fisher Scientific, cat. no. S-369)
- Sorensen's buffer solution B (14.2 g Na_2HPO_4 in 500 ml distilled/deionized water; Eastman Kodak Co., cat. no. 19032)
- 10× Dulbecco's phosphate-buffered saline (PBS) (Gibco/Invitrogen, cat. no. 14200-075)
- Sterile saline
- Ethanol, 95% (AAPER Alcohol & Chemical Co., cat. no. 04 H12QB)
- Ethanol, 100% (AAPER Alcohol & Chemical Co., cat. no. 04 I13BA)
- Toluidine blue (Ernest Fullam, cat. no. 50180)
- Sodium borate (Fisher Scientific, cat. no. S-248)
- Permount mounting medium (Fisher Scientific, cat. no. SP15-500)
- Distilled/deionized water
- Uranyl magnesium acetate (Polysciences, cat. no. 01205)
- Lead citrate (Polysciences, cat.no.00378)

EQUIPMENT

- Adult male Sprague-Dawley rats or C57BL/6 or FVB strain mice (5-6 weeks old): examine the lungs of (a minimum of) 3-4 rodents at each time point **! CAUTION** Experiments using live rodents must conform to local and national regulations
- Irradiated food pellets (Prolab 5P75 Isopro RMH 3000, Scott's Distributing Inc., cat. no. 8670)

- Autoclaved bed-o-cobs (for rats 1/4 inch cat. no. 2670, for mice 1/8 inch cat. no. 2675, Scott's Distributing Inc.)
- High-pressure medical grade liquid oxygen (Airgas, cat. no. OXUSP240LT230)
- High-pressure medical grade liquid nitrogen (Airgas, cat. no. NINF230LT230)
- Drierite dessicant, 8 mesh (Fisher Scientific, cat. no. 07-578-3B)
- 2-0 silk ligature, black braided (Ethicon/Johnson & Johnson Health Care Systems, cat. no. SA-65)
- 4-0 silk ligature, black braided (Ethicon/Johnson & Johnson Health Care Systems, cat. no. A-303)
- Dissecting instruments
- Polyethylene (PE) catheters (Beckton-Dickinson) for rat trachea and pulmonary artery (cut with a beveled edge, cat. no. 427446 PE205, o.d. 2.08 mm, i.d. 1.57 mm; for mouse trachea—cat. no. 427411 PE50, o.d. 0.965 mm, i.d. 0.0.58 mm; and for pulmonary artery—cut at a sharp 45° angle, cat. no. 427401 PE10, o.d. 0.61 mm, i.d. 0.28 mm)
- Luer adapter needles (Beckton-Dickinson for rat trachea and pulmonary artery—16 gauge stub adapter, cat. no. 427561; for mouse trachea, 22G, 0.7 mm × 25.4 mm, cat. no. 305155; and for pulmonary artery, 27G, 0.4 mm × 12.7 mm, cat. no. 305109)
- 3-way stopcock with male luer-lock adapter (Mallinckrodt, cat. no. 91040)
- Sterile specimen containers, 4 oz (Fisher Scientific, cat. no. 14-375-147)
- Single-edged industrial blades (Ted Pella, cat. no. 121-72)
- Cotton gauze sponges, 2 inch × 2 inch (Fisher Scientific, cat. no. 22-362-178)
- Kendall Tendersorb underpads, 17 inch × 24 inch (Owens & Minor, cat. no. 3583 007105)
- Single-use disposable scalpels, no. 15 blade (Fisher Scientific, cat. no. 08-927-5C)
- Shell vials, 15 mm o.d. × 45 mm height (EMS, cat. no. 72631-10)
- Disposable transfer pipettes (Fisher Scientific, cat. no. 13-711-7)
- Wood applicator sticks, 6 inch × 1/2 inch diameter (Fisher Scientific, cat. no. 01-340)
- Silicone flat embedding molds (Polysciences Inc., cat. no. 02615-1)
- Precleaned superfrost glass slides (Fisher Scientific, cat. no. 12-550-15)
- Premium coverglass (Fisher Scientific, cat. nos. 12-548-5E and 12-548-B)
- Formvar film/copper grids 200 mesh (EMS, cat. no. FF200-CU)
- Dumont N-5 tweezers (EMS, cat. no. 72854-D)
- NUNC 245 mm square bioassay dish (Fisher Scientific, NNI no. 240835)
- Tissue-culture Petri dish, 150 mm × 25 mm (Beckton-Dickinson, cat. no.3025)
- Grid storage boxes (Leica Microsystems, cat. no. 705525)
- 5 cc Luer-Lok tip disposable syringe (Becton-Dickinson, cat. no. 309603)
- Millex GP 33 mm PES 0.22 mm Millipore syringe-driven filter unit (Fisher Scientific, cat. no. SLGP 033RS)

- Parafilm, 4 inch × 250 ft (Fisher Scientific, cat. no. 13-374-12)
- Staining box with rubber plate (Ted Pella, knife station no. 8092)
- Thermometer/hygrometer (Durotherm, cat. no. 39408)
- Plexiglas canister for dessicant (Fisher Scientific, cat. no. 09-204)
- Tank wrench (1 1/8 inch)
- Log sheets
- Teflon tape (1/4 inch)
- Dissecting board, 5/8 inch × 23 inch × 16 inch (Fisher Scientific, cat. no. 09-002-16)
- Plexiglas exposure box (40 inch × 30 inch × 34 inch) consisting of a main chamber (~27 cu. ft) with an air-lock and gloved portholes (four small holes positioned at the top, side and back of the main chamber allow some of the gas to escape and dissipate)
- Autoclaved polysulfone cage with micro-filter top (Ancare, for rats cat. no. R20PLF, for mice cat. no. N10PLF)
- Autoclaved 500 ml water bottle (Ancare, cat. no. N40HTSEC) with filtered water
- Oxygen analyzer (Servomex, cat. no. 572/A1310 with internal pump 00570983) 2-stage gas tank regulator (to register gas flow and amount of gas in the tank): max inlet pressure 3,000 psi; delivery pressure 200 psi (Airgas, cat. no. 12Y12-2150)
- Dual gas flowmeter (Cole Parmer, cat. no. GMR2-01001): for rats, O_2 and N_2 capillary tubes cat. no. N092-04 with stainless steel float balls; for mice, O_2 capillary tube cat. no. 112-02-N with stainless float ball and N_2 capillary tube cat. no. 092-04-N with carboloy float ball
- PE tubing i.d. 0.156 inch, o.d. 1/4 inch, wall thickness 0.47 inch (Cambridge Valve and Fitting, Swage-Lok-Solon cat. no. PFA-T4-047-100)
- Tubing adapters 1/4 inch × 1/8 inch NPT (Cole Palmer)
- Vacuum pump, 1/3 hp (Emerson, cat. no. SA55NXGTC-4143; VWR Scientific, cat. no. G180DX/mfg no. J85-7)
- Fume hood
- Stereomicroscope (Wild Heerbrugg M5A) with Intralux 6000 fiber-optic light source
- 4 °C refrigerator and -20 °C freezer
- Vacuum oven (Fisher Scientific, cat. no. 280)
- Leica-Jung Supercut Rotary Microtome 2065 (Leica Instruments GmbH)
- Reichert-Jung tungsten carbide knife (16 cm D profile, code B 32720, Leica instruments GmbH)
- Reichert-Jung Ultracut E Microtome Type 701704/FABR (C. Reichert Optische Werke AG, cat. no. 396385: preferably on a `floating' table, Kinetics System Inc. Vibraplane Model 1201)
- Diatome knives, 1 rough/1 fine cut (Diatome Histoknife 8 mm, 45° angle; Diatome ultraknife 3.5 mm, 45° angle, Diatome)
- Corning hotplate/stirrer (Corning, cat. no. PC351)

- Zeiss Axioplan brightfield microscope with ×10 eyepieces and ×10, ×25, ×40 objectives (system equipped with a Sony CCD-Iris color video DXC-107A and SPOT camera options, MVI Inc.)
- Rotomix (Thermolyne, cat. no. 48200)
- Transmission electron microscope (EM300, Philips/FEI Co.)
- CCD-300-RC high-sensitivity camera (Dage/MTI, Advanced Microscopy Techniques)

REAGENT SETUP

Liquid gas—Obtain liquid oxygen (3) and liquid nitrogen (2) tanks before the start of the exposure schedule. ▲ **CRITICAL** It is recommended to store a spare liquid O_2 and liquid N_2 tank to prevent interruption to the exposure schedule once started.

24 h before the end of the high oxygen/air-breathing schedule—Prepare the following reagents: 1× PBS from 10× PBS using distilled/deionized water; 0.2 M Sorensen's buffer with 20 ml solution A and 80 ml solution B (pH 7.2) and then a 0.1 M solution using distilled/deionized water; 3% (vol/vol) paraformaldehyde/0.1% gluteraldehyde (vol/vol) in 1× PBS and 3% paraformaldehyde/2% gluteraldehyde in 0.1 M Sorensen's buffer, store at 4 °C and bring to room temperature (RT; 18-25 °C) for use; 50%, 70% ethanol from 100% ethanol with distilled/deionized water; store 95% and 100% ethanol at RT; for Epon/araldite resin mixture follow manufacturer's instructions; 2% (vol/vol) solution of osmium tetroxide in 0.1 M Sorensen's buffer; 0.1% toluidine blue (vol/vol) in distilled/deionized water from a stock solution of 1% toluidine blue (wt/vol) in 1% sodium borate (wt/vol) and filter immediately before use through a PES 0.22-μm-filter unit attached to a 5 ml disposable syringe.

EQUIPMENT SETUP

24 h before the start of the exposure schedule—Determine the set O_2 tension to be used to induce vascular remodeling in rodent lung, that is, 85% O_2 for rats and 75% O_2 for mice. Determine the O_2 and N_2 capillary tubes of correct bore diameter, and `float' balls of the appropriate type/material (e.g., carboloy or stainless steel, as described under EQUIPMENT), to deliver a gas flow sufficient to maintain the set oxygen tension, and appropriate temperature and humidity levels, in the main chamber (see Fig. 1a, and vendor `flowmeter calibration sheets,' for additional details). Optimal gas flow is determined by the size and number of rodents housed in the main chamber: aim to cycle one total gas exchange (atmosphere) through the main chamber (~27 cu. ft) every 10 min (at ~2 cu.ft per min). Connect the following components of the exposure system with PE tubing: 2-stage gas regulators to two liquid O_2 tanks and a single liquid N_2 tank using Teflon tape and a wrench to obtain tight seals; 2-stage regulators on one O_2 tank and the N_2 tank to the two flowmeter inlet valves (these tanks provide a continuous gas flow to the main chamber and are replaced by fresh tanks as needed); single outlet valve of the flowmeter to the main supply valve of the main chamber (Fig. 1b); 2-stage gas regulator of the second liquid O_2 (flush) tank to an inlet at the top of the air-lock (this flush tank is used to rapidly return the air-lock to the set O_2 tension); sensor supply valve from the main chamber to the inlet of a container of Drierite and the outlet of the Drierite container to the inlet of the analyzer (Fig. 1b); analyzer to the sensor return valve of the main chamber (Fig. 1b). Also, check that the battery of the analyzer is charged and connect the analyzer to an (emergency) electrical outlet. Clean the chamber inside with 70% ETOH and outside with Clidox. Place the combined thermometer/hygrometer inside the main chamber. Calibrate the O_2 and N_2 settings for gas flow through the flowmeter to deliver the set O_2 tension to the main chamber: first turn on the analyzer (to activate its pump); next open the 2-stage regulator valves of the main O_2 and N_2 tanks; and finally use the flowmeter needle valves to adjust the amount

of O_2 and N_2 gas delivered into the flowmeter. When adjusting the flowmeter needle valves, monitor the O_2 tension of the (mixed) gas leaving the flowmeter outlet valve using the analyzer. This test is quickly completed by feeding the mixed gas from the flowmeter (via the calibration outlet of the main supply valve) and from the sensor supply valve line to the analyzer, into a rubber glove: reading and adjusting the O_2 tension of this small gas volume (versus the large volume of gas in the chamber) significantly reduces the time needed to calibrate the correct O_2 and N_2 flowmeter settings. To equilibrate the set O_2 tension in the main chamber, divert the set O_2 gas mixture from the flowmeter to the main chamber via the main supply valve. Typically, it will take 2-3 d for the main chamber (~27 cu. ft) to equilibrate to the set O_2 tension. To accelerate the process, flood the main chamber and air-lock with 100% O_2 from the flush tank until the analyzer reads the set O_2 tension minus 10%. Close the regulator valve of the flush tank and close the connecting door between the main chamber and air-lock. Gas still being delivered from the main tanks via the flowmeter and the main supply valve will continuously feed the system to equilibrate at the set O_2 tension, and then maintain it. Monitor the system for 1-3 h to establish that the set O_2 tension in the main chamber is stable (±2%). See TROUBLESHOOTING for further guidance.

PROCEDURE

Induction of vascular remodeling in rat lungs by exposure to high oxygen

1| Place 6-8 standard cages with rodents in the air-lock of the exposure chamber.

2| Open the regulator valve of the O_2 flush tank to (gently) flood the air-lock until the analyzer reads the set O_2 tension minus 10% and then close the regulator valve of the tank.

3| Open the connecting door between the main chamber and air-lock, move the cages with rats from the air-lock into the main chamber and close the connecting door.

4| In preparation for the next change-out of (used) cages, place an appropriate number of clean cages with bedding, food (in a Ziploc bag to keep it dry) and water bottles in the air-lock and close the exterior door. Use the flush tank to return the air-lock to the set O_2 tension minus 10%, to minimize gas leak/drift through the connecting door to the main chamber.

▲ **CRITICAL STEP** Once the exposure schedule has started, the O_2 tension in the main chamber must be maintained (optimally ±2%) at the recommended level to induce the desired vascular changes. Depending on the flow rate, at this stage of the schedule, a full liquid O_2 tank typically lasts ~5-7 d and a liquid N_2 tank typically lasts ~2weeks.

5| Maintain daily logs throughout the exposure schedule: record the health status of the animals, monitor gas consumption (by the level of gas within the O_2 and N_2 tanks), the oxygen tension, and temperature and humidity in the main chamber, twice a day. The set O_2 tension may alter slightly with the addition or removal of animals to the chamber or when a new O_2 or N_2 tank is connected to the system.

6| Change the cages in the main chamber three times each week. First check that there are sufficient clean cages with bedding, food and fresh water bottles available in the air-lock and that the O_2 tension in the air-lock matches the set tension in the main chamber. As needed, adjust the gas tension in the air-lock using the O_2 flush tank. When equilibrated to the set O_2 tension (±2%), open the connecting door between the air-lock and the main chamber; move the clean cages with food and water bottles into the main chamber. Transfer the rodents into clean cages (one at a time), move the used cages into the air-lock and close the connecting door to the main chamber. Open the exterior door to the air-lock, remove the used materials and replace with clean cages, fresh food (in a Ziploc bag to keep dry) and water bottles, in

preparation for the next change of cages. Close the exterior door and flood the air-lock with O_2 from the flush tank to return to the set O_2 tension minus 10%.

7| Run the system continuously for up to 4 weeks: plan to examine the lungs of rats after breathing high O_2 for 1-4 weeks.

Induction of vascular remodeling in rodent (rat and mouse) lungs by breathing high oxygen followed by breathing air

8| If desired, gradually reduce the set O_2 tension in the main chamber over a further ~1 week until equivalent to air (21%), and then remove the cages from the chamber onto standard racks in a controlled environment room: plan to examine the lungs of rodents returned to air breathing for 2 or 4 weeks. This variation exposes rats or mice to high oxygen followed by return to breathing air to induce vascular remodeling, that is, the restoration of capillary networks injured by breathing high oxygen. Note that in both the rat and mouse, the lower oxygen tension of air (sensed as 'relative hypoxia' in the high oxygen-adapted lung) triggers this response.

▲ **CRITICAL STEP** As the O_2/N_2 gas balance is gradually reversed during the weaning period, more liquid N_2 tanks than O_2 tanks are needed. Careful weaning (to prevent dyspnea, cyanosis and asphyxia) is essential for rodents to breathe air after adapting, over the course of several weeks, to breathe the high oxygen tension(s) used in the protocol.

? TROUBLESHOOTING

Lung tissue preparation for high-resolution microscopy studies

9| Administer 200 mg per kg body weight i.p. sodium pentobarbitol, perform a midsternal thoracotomy, and keep the thorax open using hemostat clamps (for rat) or taped suture loops (for mouse).

▲ **CRITICAL STEP** Rodents breathing high oxygen at the end of the exposure schedule must be anesthetized immediately after they are removed from the exposure chamber (to prevent dyspnea, cyanosis and asphyxia); rodents that are weaned from high oxygen and that re-adapt to breathe the lower oxygen tension in air can be anesthetized after 1-4 weeks in air.

10| Fill the tracheal and vascular reservoirs of the fixation apparatus (see Fig. 2) with 3% paraformaldehyde/0.1% gluteraldehyde. Bleed each fixative delivery line of air bubbles to ensure that fixative flows freely.

! CAUTION Reagents used to fix and process tissue are toxic. It is recommended that all steps of tissue fixation, and processing into resin, be completed under a chemical fume hood. Use a mask and double nitrile gloves. Dispose of hazardous waste in accordance with local regulations.

11| Place a silk ligature under the trachea, insert a PE catheter into the trachea via a small incision in the front midline, and tie the ligature tight around the trachea and catheter.

12| Place two silk ligatures behind the pulmonary artery (for rat) or three silk ligatures behind the pulmonary artery and ascending aorta (for mouse, use a stereoscopic microscope).

13| Lift the heart and tie the pulmonary veins with a single ligature (to prevent pulmonary venous outflow and establish a closed pulmonary vascular system): in preparation for securing the pulmonary artery catheter, make snares with the remaining ligature(s). Carefully cut the trachea close to the larynx.

14| Use blunt-ended scissors to excise the thoracic block: gently pull the trachea (with the PE catheter in place) away from the spine, and dissect away connective tissue (and adjacent subclavian vessels) in the thoracic cavity.

15| Cut the esophagus above the diaphragm to release the thoracic block, and place it on a damp gauze pad on a dissecting board.

▲ **CRITICAL STEP** Avoid nicking or cutting the lung surface when excising the thoracic block, as fixative will leak and prevent good lung distension.

16| Insert a pulmonary artery catheter (use a stereomicroscope for the mouse). At this step, the protocol for tissue fixation differs slightly for rats (option A) or mice (option B).

(A) For rat experiments

- **i.** Use small sharp scissors to make an incision in the bottom of the right ventricle, insert a PE catheter through the incision (3-4 mm) upward into the opening of the main pulmonary artery (it will be visible through the artery wall).
- **ii.** Secure the catheter (1-2 mm) into the main pulmonary artery by tightening the snare around the catheter, the artery wall and some heart muscle.

(B) For mouse experiments

- **i.** Connect the PE catheter to a needle (which subsequently will connect to the fixative delivery tube of the vascular reservoir).
- **ii.** Under a stereomicroscope, puncture the right ventricle with the PE catheter (about 1 mm below the main pulmonary artery), slide the PE catheter into the main pulmonary artery (it will be visible through the artery wall) and secure it by tightening the snare around the pulmonary artery and the aorta.
- **iii.** To facilitate insertion of the mouse pulmonary artery catheter, and ensure that it remains stable after insertion, carefully position the thoracic block and secure the catheter horizontally, and at the right height, to align with the pulmonary artery.

▲ **CRITICAL STEP** Place the catheter far enough into the trachea to be held securely but not so far as to jam against the bronchial bifurcation or pass into the right or left main bronchus. Similarly, the tied vascular catheter should float free in the lumen of the main pulmonary artery and not jam against the wall: avoid cutting the wall of the pulmonary artery with the ligature but tie it tight enough to ensure that it is secure, and that the catheter will not be displaced by the pressure of inflowing fixative.

? TROUBLESHOOTING

17| Turn on the pump and use the gate-clip to set the pressure in the vascular reservoir system to 100 cm H_2O to obtain maximum vascular distension (this pressure is essential to fully distend vessels in the hypertensive rat lung, that is, in rats breathing 85% ± 2% O_2 for 4 weeks, which have pulmonary artery pressures of ~60-90 mm Hg, and is the standard pressure of choice in all our studies).

18| Connect the tracheal catheter with a luer-adapted needle (via a stopcock) to the tubing connecting to the tracheal reservoir of fixative (Fig. 2).

19| Connect the pulmonary artery catheter to a luer-adapter (via a stopcock) and to tubing leading to the vascular reservoir (Fig. 2).

20| Open the hemostat clamping the tubing to the pulmonary artery catheter, and as the vascular bed begins to fill (and the lungs begin to inflate), open the other hemostat to allow fixative to flow into the tracheal catheter.

21| Distend the lungs with fixative via both catheters (for ~1-2 min) until the peripheral margins are sharp (Fig. 3a).

▲ **CRITICAL STEP** Fill the vascular bed with fixative ahead of the airways, as fluid pressure in alveoli can prevent (collapsed) capillaries from opening and filling with fixative.

22| Clamp the vascular catheter, and then the tracheal catheter, with mosquito hemostats to maintain distension of the vascular bed and airway.

23| Clamp the delivery tubes to each reservoir with hemostats; shut off the pump and open the gate-clip to release the pressure in the vascular reservoir system.

24| Continue to maintain a closed lung vascular and airway system as the fixation process starts: leave the rat and mouse tracheal catheter, and rat pulmonary artery catheter, still clamped with a mosquito hemostats; remove the mouse pulmonary artery catheter—simultaneously tightening the third snare around the pulmonary artery to prevent fluid leak. Submerge the thoracic block in a container of fixative, and float in fixative for 2 h.

25| Separate the left and right lungs with a single-edge blade, and cut tissue blocks 3 mm^3 from each lung: collect ~6-12 blocks from each rodent, taking the blocks from comparable peripheral regions of each lung to avoid including the large airways and vessels present in the central zone (Fig. 3b).

? TROUBLESHOOTING

26| Transfer the 3 mm^3 tissue pieces into labeled 'shell vials' with fresh 3% paraformaldehyde / 2% gluteraldehyde and leave for a further 2 h at RT.

■ **PAUSE POINT** These blocks can be stored overnight in fixative at 4 °C.

27| Rinse the tissue for 2 × 20 min in 0.1 M Sorensen's buffer; leave overnight at 4 °C.

28| Pipette off 0.1 M Sorensen's buffer, and immerse tissue in 2% osmium tetroxide in 0.1 M Sorensen's buffer for 2 h at 4 °C.

▲ **CRITICAL STEP** Use a rotator for Steps 29-31.

29| Pipette off osmium tetroxide, rinse tissue in 0.1 M Sorensen's buffer at RT for 3 × 15 min and proceed to dehydrate in ethanol at RT: 50% for 30 min; 70% for 30 min (under vacuum); 95% for 15 min (use capped vials); 100% for 2 × 15 min; clear with propylene oxide 2 × 15 min.

30| Infiltrate tissue with a 1:1 mixture of Epon/araldite and propylene oxide at RT: cap vials and leave overnight.

31| Remove vial caps; leave 3-4 h until propylene oxide evaporates, leaving volume of vial approximately halved. Pour off remaining Epon/araldite; add fresh 100% Epon/araldite and infiltrate for 3-4 h at RT.

32| Embed tissue with identifying label in fresh Epon/araldite in flat embedding molds, and polymerize at 60 °C for 24-48 h.

■ **PAUSE POINT** The polymerized blocks are stable and can be stored at RT indefinitely.

? TROUBLESHOOTING

Tissue block sectioning and staining for high-resolution microscopy

33| Under a stereomicroscope, use a single-edge industrial blade to remove excess resin from the surface of the block and expose the tissue face; proceed to cut 1-μm-thick and 90-nm-thick sections for analysis.

34| Collect several 1-μm-thick toluidine blue-stained sections, each prepared from 3-4 randomly selected tissue blocks (from ones available from each rodent), to quantify vessel populations (see below) and collect representative digital images. Collect the sections on glass slides, gently dry on a hotplate, stain with 0.1% toluidine blue, rinse with distilled/deionized water and mount under a coverslip using Permount.

35| Collect a series of 90-nm-thick sections onto formvar-coated copper grids with a (locking) Dumont tweezer, and place the tweezers with the grids in a closed Petri dish to air-dry.

■ **PAUSE POINT** Store and catalog these toluidine blue-stained sections at RT and grids (with attached unstained tissue sections) in calibrated storage boxes at RT until proceeding with high-resolution imaging. Plan on using these within weeks.

36| Double-stain the grids/sections at RT with uranyl magnesium acetate and lead citrate.

37| Place each grid/section into a drop of 7.5% aqueous uranyl magnesium acetate for 20 min.

38| Rinse in distilled/deionized water and rotate for 10 min. Repeat twice.

39| Place each grid/section into a drop of 0.2% aqueous lead citrate for 5 min.

40| Rinse in distilled/deionized water and rotate for 10 min. Repeat twice.

41| Collect each grid/section with a (locking) Dumont tweezer and place in a closed Petri dish to air-dry.

? TROUBLESHOOTING

High-resolution imaging of lung vessels in tissue sections

42| Quantify cell populations in vessels <50 μm in diameter by brightfield microscopy (using ×10 eyepiece and ×40 objective) in 1-μm-thick toluidine blue-stained sections. Count 20 vessels per lung.

43| Use an eyepiece reticule (graticule) to measure vessel diameter. Class intra-acinar vessels by location (associated with a respiratory bronchiolus or alveolar duct or present in the alveolar wall): quantify at least ≤ 100 alveolar duct and alveolar wall vessels per rodent group[1,4]. Record the wall structure of each vessel including cells and elastic laminae, that is, no lamina, a single lamina, double laminae. Class cells as follows: ones forming the luminal surface as endothelial, ones between double lamina as smooth muscle cells (as these are the only cells identified at this location in high-resolution images, see below), and intimal cells beneath endothelium as `subendothelial' cells. The phenotype of `subendothelial' cells is not identified in 1-μm-thick toluidine blue-stained sections, but high-resolution imaging confirms that a variety of cell types (intermediate cells, fibroblasts and smooth muscle cells, as well as those cells in transition) form this layer at different time points in the hyperoxia-injured lung. Count only nucleated cell processes.

44| View stained grids/sections by transmission electron microscopy (TEM). This can be done repeatedly if carefully handled and stored in calibrated grid-boxes to ensure reliable retrieval. Typically, use a magnification of ×6,689-9,818 to identify cell morphology/organelles and position in a vessel or capillary wall.

45| Collect a library of high-resolution images of cells forming vessel or capillary walls. Images collected digitally or scanned from standard (3 inch × 4 inch) negatives are each suitable for analysis. For each time point, collect 20-30 vessels per rodent group: analyze all cells in each vessel. Typically, a wall of a small alveolar vessel may include four cells (in normal lung) or as many as 10 cells (in the hyperoxic lung).

● **TIMING**

EQUIPMENT SETUP: it requires 6-8 h to set up, calibrate and equilibrate the high oxygen exposure system

Steps 1-8: over the course of 4-6 weeks, 90 minutes will be required 3 times per week

Steps 9-23: it requires 30-40 min (per rodent) to excise the thoracic block, place tracheal and pulmonary artery catheters, and inflate the lungs with fixative

Steps 24-26: to fix the lungs and cut 6 × 3 mm^3 tissue blocks each from the right and left lung requires 5-6 h (i.e., to prepare 12 × 3 mm^3 tissue blocks each from 3-4 rodent lungs)

Steps 27-31: it requires 8 h to dehydrate and pre-infiltrate the 36-48 × 3 mm^3 tissue blocks with resin and another 10-16 h to complete the resin infiltration

Step 32: it requires 48 h to polymerize the labeled tissue blocks in the embedding molds at 60 °C

Steps 33-35: it will take 2-2.5 h to rough-cut excess resin from the surface of one tissue block, and prepare 1-μm-thick toluidine blue-stained sections and approximately fifteen to twenty 90-nm-thick sections on formvar-coated grids (plan to cut a total of 3-4 tissue blocks in 8 h)

Steps 36-41: to double-stain 15-20 grids/sections, to enhance electron-density of cell membranes and organelles requires 4 h

Steps 42 and 43: it requires 6 h to quantify vessels in 4 × 1-μm-thick toluidine blue-stained sections

Steps 44 and 45: to view multiple grids/sections by transmission electron microscopy, allow sufficient time to collect overviews and determine the appropriate analysis (~2-3 d per lung); proceed to collect images for analysis (it takes ~8 h for ~100-150 digital images per rodent group)

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

After breathing a high but sublethal oxygen tension for 4 weeks, most small alveolar vessels in the rat lung will have developed numerous perivascular cells[1] (Fig. 4). In 1-μm-thick toluidine blue-stained sections, for example, ~5-6 alveolar vessels are present per section, and quantitative data (Fig. 4a-d) demonstrate a shift in the size, wall thickness and distribution of

cells within this vessel population and in associated cell types (endothelial, subendothelial, smooth muscle, fibroblasts) in the hyperoxia-injured lung[1,4]. Few, if any, small alveolar vessels have subendothelial or medial smooth muscle cells in normal lung and many alveolar vessels of similar size have these cells after hyperoxia[1,4]. Thickening of vessel walls by these perivascular cells results in significant lumen restriction, that is, narrowing (Fig. 4c,d). The advantage of this type of analysis of 1-μm-thick resin sections is that the resulting data set will be larger than that can be acquired in the same time frame by high-resolution imaging.

Depending on how a section aligns on the grid, alveolar vessels are usually visible by TEM in ~10% of grid openings (Fig. 4e-h). Endothelial cells, pericytes, fibroblasts and smooth muscle cells are identified by their distribution of organelles (endoplasmic reticulum, mitochondria, Golgi complex and micropinocytotic vesicles), presence of a basal lamina and location in relation to other cells and wall components[1,4]. Detailed analyses of sequential (time-related) images show that fibroblasts in the interstitium that lie close to small alveolar vessels are recruited to align as new perivascular cells in the hyperoxia-injured lung and develop filament networks characteristic of smooth muscle cells[2,3]. Use of immunogold labels further identifies expression of α-smooth muscle actin and smooth muscle myosin heavy chain as these precursor cells a cquire a smooth muscle phenotype[2,3]. Vessel wall remodeling by this precursor cell population is progressive, that is, greater after 4 weeks breathing high oxygen than after 1 week[4]: other studies in a rat and calf model of pulmonary hypertension induced by hypoxia report that (GFP^+) bone marrow-derived fibrocytes (expressing collagen) remodel vessel walls[8].

Lung capillaries (injured by high oxygen) undergo repair in both rats and mice that breathe high oxygen for 4 weeks, followed by weaning to air for 1 week, and then breathing air for 1-4 weeks (Fig. 5). As for vessel wall remodeling, capillary repair is progressive, that is, greater after 4 weeks of air breathing than after 1 week. By TEM, capillaries are usually visible in >50% of grid openings. As a result of injury or of a program of vascular loss (triggered by the persistently high oxygen tension), capillary shutdown and loss in HALI leaves nonfunctional (unperfused) residual capillary segments and avascular zones[1]: on return to air breathing, however, the spontaneous repair of these disrupted capillary networks returns their pattern and density close to that in normal lung[1]. Vascular precursor cells can be detected, quantified and immunophenotyped during the process of capillary repair[2,3] (see accompanying *Nature Protocol* by Jones *et al*.[5]).

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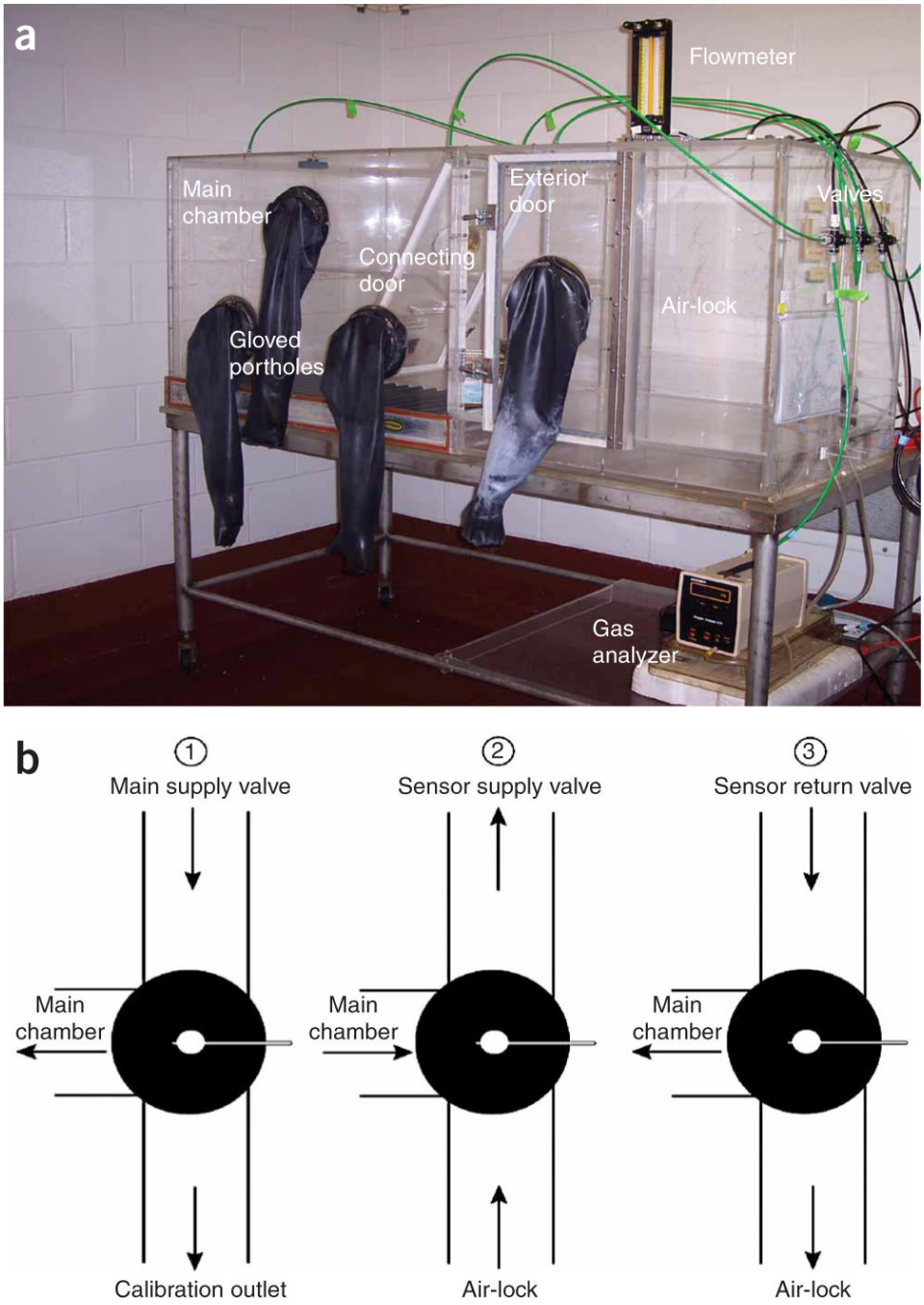

Figure 1.

Equipment setup. (**a**) Overview of Plexiglas chamber in which rodents breathe a high oxygen tension. Animals are housed in standard cages in the main chamber. An air-lock with a connecting door to the main chamber and an exterior door facilitate transfer of cages and food and water during the course of the exposure schedule. Gloved portholes assist in the transfer of these materials in and out of the main chamber. Note the location of the flowmeter, the oxygen analyzer and the valves that direct the flow of gas from adjacent liquid oxygen/nitrogen tanks. (**b**) Illustration of gas supply valves connecting the flowmeter and oxygen analyzer to the main chamber and airlock. A tube carries mixed gas from the flowmeter to the main supply valve (1) where it is directed either to the main chamber or to a calibration outlet for sampling

the oxygen tension (by a glove test). Gas drawn from the main chamber, or from the airlock, is directed via a sensor supply valve (2) through a container of Drierite dessicant (not shown) to the analyzer. Gas sampled by the analyzer is returned to the main chamber, or air-lock, via a sensor return valve (3).

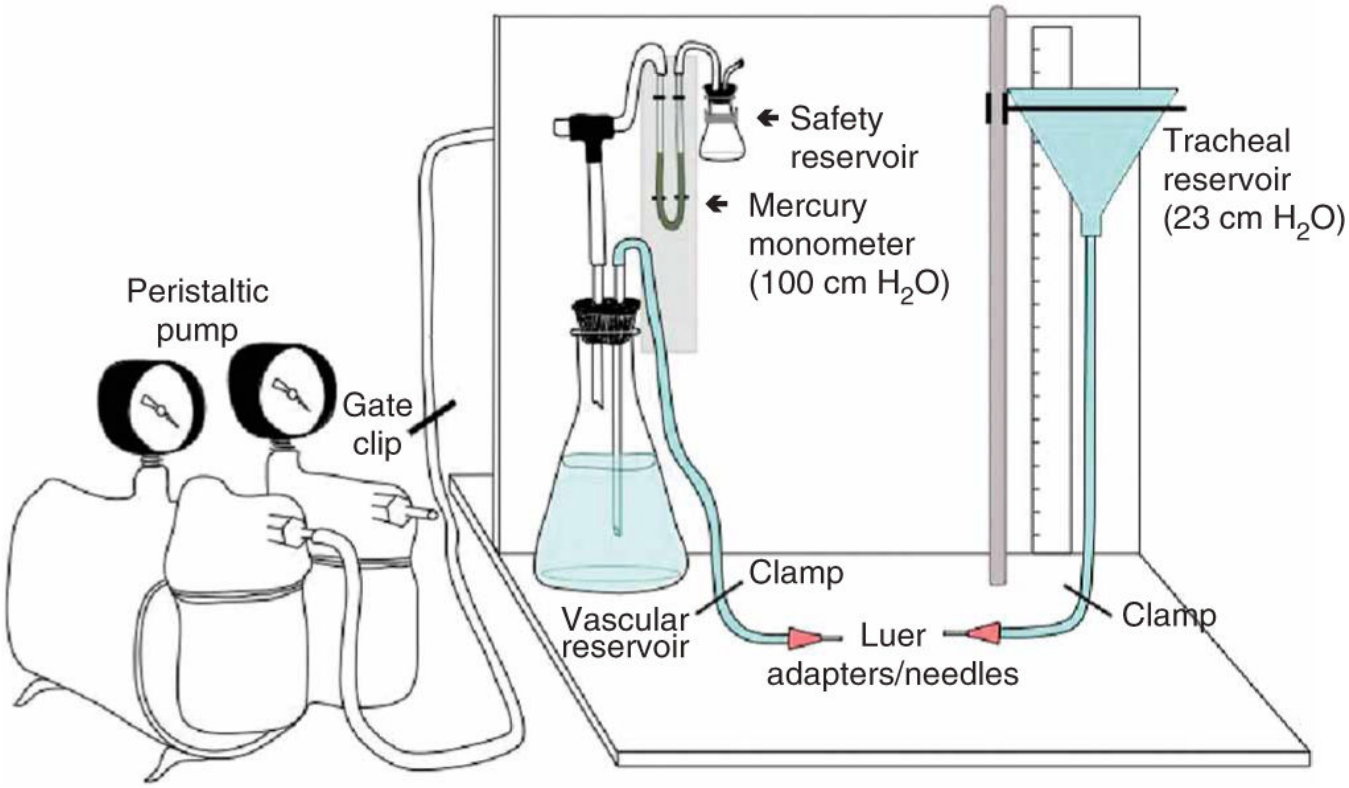

Figure 2.

Drawing illustrating the inflation apparatus to distend lungs initially with a 3% paraformaldehyde/0.1% gluteraldehyde solution (shown in blue). Fixative is delivered to the tracheal catheter at 23 cm H_2Opressure (determined by gravity) and to the vascular catheter at 100 cm H_2O (determined by positive pressure from a peristaltic pump). *Note*: use a standard pressure monometer to predetermine the height of the mercury column needed for the pump to generate this pressure. The catheters connect first to a luer adapter, or a luer-adapted needle, and then, via a 4-way stopcock, to the tubing delivering fixative from each reservoir. Hemostat clamps (indicated by black bars) regulate the flow of fixative through the delivery tubes from each reservoir. A gate clip (heavy black bar) positioned on the tubing from the peristaltic pump is used to obtain/regulate pressure in the vascular reservoir.

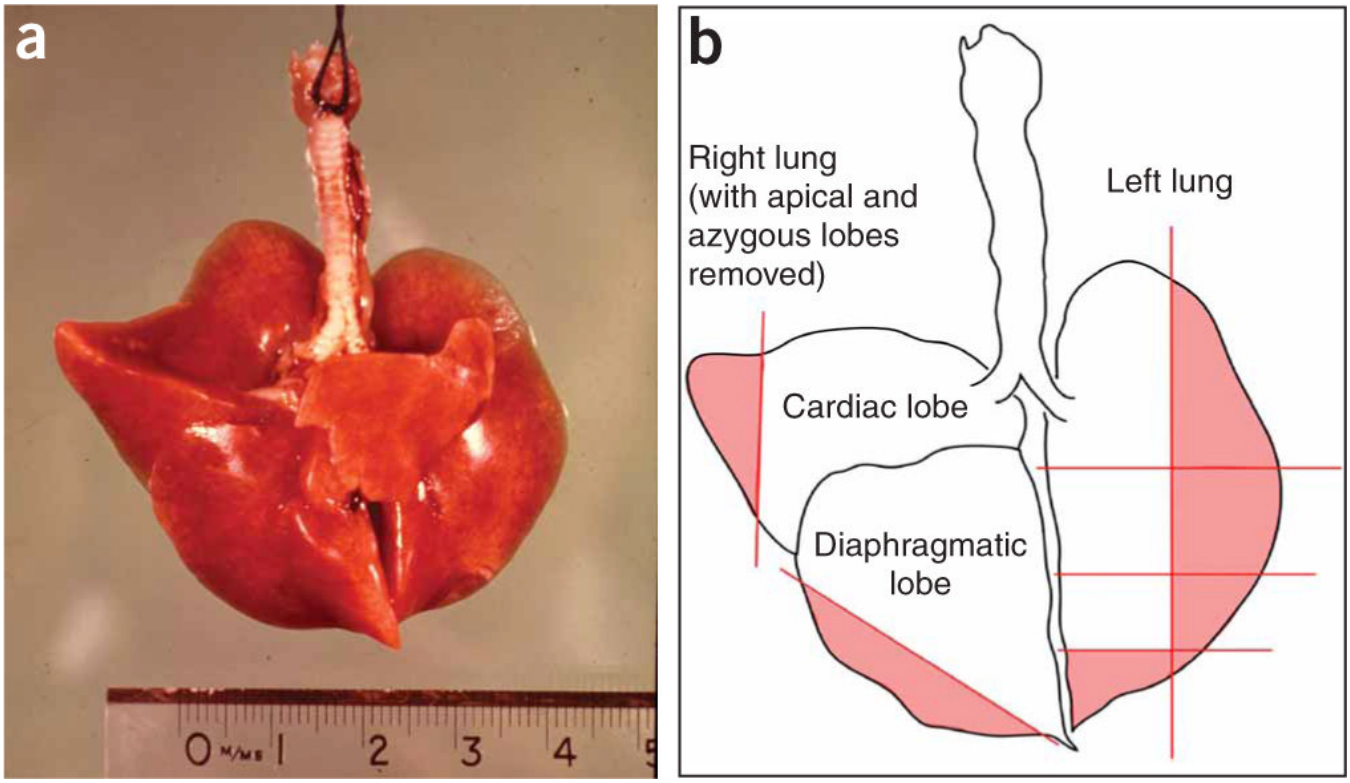

Figure 3.
Image and cartoon of fixative-distended rodent (rat) lung. (**a**) Note the sharply distended peripheral lung margins denoting well-inflated lungs. (**b**) Optimal tissue blocks are collected from peripheral zones (red) of the right and left lung. These zones provide the maximum number of small lung vessels (<100 μm in diameter) for analysis, while avoiding the central large airway and vascular structures that enter at the hilum and subsequently branch throughout the lung.

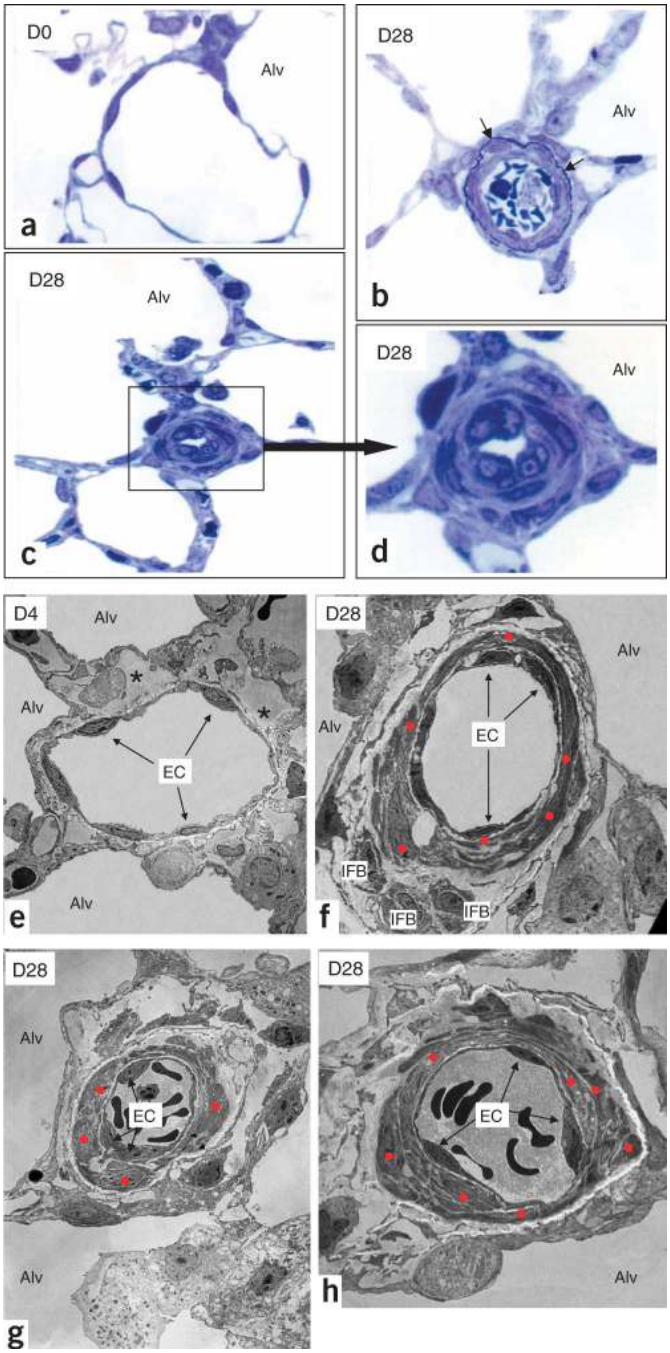

Figure 4.

Illustration of vessel wall thickening by brightfield microscopy and by TEM in normal rat lung and in the rat lung after breathing high oxygen. (**a-d**) Brightfield microscopy; (**e-h**) TEM. Representative examples of wall thickening by perivascular cells in alveolar wall vessels present in 1-μm-thick Epon/araldite sections stained with toluidine blue: (**a**)D0, 45-μm-diameter vessel; (**b**)D28,31-μm-diameter vessel (note the development of a single elastic lamina, see arrows); (**c** and **d**)D28, 21-μm-diameter vessel (note lumen encroachment by cells). Original magnification: ×630. (Reproduced with permission from [ref. 4].) Representative images of wall thickening by perivascular cells in alveolar wall vessels obtained by TEM: (**e**) 23-μm-diameter vessel, (**f-h**) 17-, 15-, 18-μm-diameter vessels, respectively. Typically, in the

early stage of breathing high oxygen, vessel wall thickness is close to normal and consists mainly of endothelial cells (**e**, D4). Atthis time, cell injury by high oxygen causes edema to widen the interstitium surrounding the vessel (*). Later (**f-h**, D28), wall thickness increases as endothelial cells are surrounded by interstitial fibroblasts aligning as perivascular cells (indicated by red dots). Alv=alveolus, EC=endothelial cell, IFB=interstitial fibroblast. Original magnification: (e) ×1,416, (**f**) ×1,662, (**g**) ×1,416,(**h**) ×1,662.

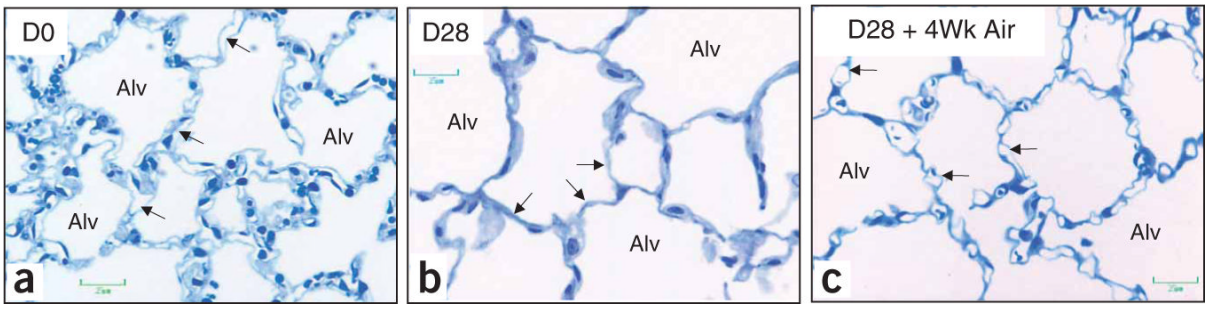

Figure 5.

Illustration of alveolar-capillary membrane structure in the normal rat lung and in the rat lung after breathing high oxygen. The alveolar capillary membrane of normal lung is characterized by its complex, open (lace-like) structure (**a**, arrows). After 4 weeks in high oxygen, regions of the membrane now lack patent capillary segments (**b**, arrows). These are demonstrated by TEM to consist of avascular zone and residual capillary structures. After 4 weeks in high oxygen, 1 week of weaning and then 4 weeks of breathing air, patent capillary segments are restored— resulting in an alveolar capillary membrane structurally close to normal (**c**, arrows). Alv=alveolus. Original magnification: ×400.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason(s)	Solution
EQUIPMENT SETUP	Inconsistent or unstable 0_2 tensions in the exposure chamber	Failure to deliver or maintain the set oxygen tension can result from faulty tanks and regulators, a leaking flowmeter, leaking connectors to tubing or mechanical failure of the analyzer pump	Check all connections in the system and tank regulators Replace faulty tanks and test the analyzer (pump) for performance
			Frequent monitoring (twice daily) will minimize such failures
			If a leak persists, check the gloved portholes and integrity of the sealing gaskets around the connecting door between the main chamber and the air-lock, and around the air-lock's exterior door
EQUIPMENT SETUP and Step 8	Failure to induce vascular remodeling	Rodents were incorrectly exposed to the set 0_2 tension	Keep strictly to the schedule described for the 0_2 tension and length of exposure for the high 0_2/air cycle
			If rat or mouse strains different from the ones described are used, check for reported differences in the 'angiogenic' response of different strains
10-16	Incomplete lung inflation with fixative	Cut in lung surface or pulmonary artery	(Typically) Discard the lung
		Poor placement and/or ligation of catheters, or bubbles or kinks in the fixative delivery line	If the lungs do not (visibly) begin to fill within the first 30 seconds, check the placement of each catheter and check if there are bubbles or kinks in the delivery lines. If nonfilled regions appear as fixative distends the lungs, gently move each lobe until all regions become well distended
25	Insufficient vessels or cells available for analysis	Blocks taken from regions too central and contain larger vascular/airway structures	Choose additional blocks or recut original blocks to attempt to expose other vessels deeper in the lung tissue
26-32	Lung tissue blocks embedded in Epon-araldite resin do not cut well	Air trapping in the lung or poor tissue dehydration, infiltration and polymerization steps	Poorly prepared blocks cannot be retrieved. Review all steps of the procedure and extend times in reagents. Blocks larger than the size (3 mm^3) recommended will not process adequately for the procedure times listed. Check quality of the Histoknife or diamond knife
33-41	High levels of extraneous 'dirt' in the resolution images	Dirty knife boat, tools or reagents used for double-staining steps	Change water in knife boat between blocks Ensure tweezers and cutting tools used are clean Use freshly prepared reagents. Filter all reagents well and extend rinse times