Matrix modulation of compensatory lung regrowth and progenitor cell proliferation in mice

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1Tufts University Cummings School of Veterinary Medicine, North Grafton, Massachusetts; 2Departments of Internal Medicine and Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri; 3Stem Cell Program, Children’s Hospital, Department of Genetics, Harvard Medical School, Boston, and Harvard Stem Cell Institute, Cambridge; and 4Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

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Hoffman AM, Shifren A, Mazan MR, Gruntman AM, Lascola KM, Nolen-Walston RD, Kim CF, Tsai L, Pierce RA, Mecham RP, Ingenito EP. Matrix modulation of compensatory lung regrowth and progenitor cell proliferation in mice. Am J Physiol Lung Cell Mol Physiol 298: L158–L168, 2010. First published November 13, 2009; doi:10.1152/ajplung.90594.2008.—Mechanical stress is an important modulator of lung morphogenesis, postnatal lung development, and compensatory lung regrowth. The effect of mechanical stress on stem or progenitor cells is unclear. We examined whether proliferative responses of epithelial progenitor cells, including dually immunoreactive (CCSP and proSP-C) progenitor cells (CCSP+/SP-C+) and type II alveolar epithelial cells (ATII), are affected by physical factors found in the lung of emphysematics, including loss of elastic recoil, reduced elastin content, and alveolar destruction. Mice underwent single lung pneumonectomy (PNY) to modulate transpulmonary pressure (mechanical stress) and to stimulate lung regeneration. Control mice underwent sham thoracotomy. Plombage of different levels was employed to partially or completely abolish this mechanical stress. Responses to graded changes in transpulmonary pressure were assessed in elastin-insufficient mice (elastin+/−, ELN+/−) and elastase-treated mice with elastase-induced emphysema. Physiological regrowth, morphometry (linear mean intercept; Lmi), and the proliferative responses of CCSP+/SP-C+, Clara cells, and ATII were evaluated. Plombage following PNY significantly reduced transpulmonary pressure, regrowth, and CCSP+/SP-C+, Clara cell, and ATII proliferation following PNY. In the ELN+/− group, CCSP+/SP-C+ and ATII proliferation responses were completely abolished, although compensatory lung regrowth was not significantly altered. In contrast, in elastase-injured mice, compensatory lung regrowth was significantly reduced, and ATII but not CCSP+/SP-C+ proliferation responses were impaired. Elastase injury also reduced the baseline abundance of CCSP+/SP-C+, and CCSP+/SP-C+ were found to be displaced from the bronchioalveolar duct junction. These data suggest that qualities of the extracellular matrix including elastin content, mechanical stress, and alveolar integrity strongly influence the regenerative capacity of the lung, and the patterns of cell proliferation in the lungs of adult mice.

pneumocyte; bronchioalveolar stem cell; elastase; elastin; pneumonectomy; lung regeneration

THE LUNG IS A UNIQUE ORGAN that conveys rhythmic and tonic mechanical stresses to resident cells through the extracellular matrix. These macroscalar forces are transmitted to integrins that span the cell membrane and communicate macroscalar forces to the cells interior, i.e., the cytoskeleton. The conversion of macroscalar to microscalar forces evokes intracellular processes (biochemical and transcriptional) that promote synthesis and growth and fend off apoptosis. A reduction in mechanical stress is deleterious, favoring apoptosis, for example, in anchorage-dependent cells. Complete detachment of these cells from the matrix (anoikis) results in cell death. Lung morphogenesis and postnatal lung development both depend on mechanical distortion of cells for proper growth. The extent to which adult lung tissues depend on mechanical stress for homeostasis in vivo is less clear. Important examples where mechanical forces in the lung are diminished in adults include emphysema, genetic diseases that impact lung elastin content or function, and aging. These conditions tend to propagate over time for reasons that are poorly understood.

Apoptotic and senescent cells are overabundant in the emphysematic lung (2, 29), and the capacity for lung regeneration (i.e., compensatory lung regrowth) is impaired in animal models of emphysema (27). One explanation for these observations is a defect in the transduction of mechanical signals that ordinarily stimulate the progression of the cell cycle. Should progenitor cells fail to respond to mechanical cues by proliferation, their role in lung repair or regeneration may be reduced. Alteration of matrix stiffness is known to alter lineage specification and the capacity for differentiation (5, 6), so it is plausible that the mechanical properties of the emphysematous lung is insufficient to support the functional pool of progenitor and stem cells in the distal lung. Study of this phenomenon has been hampered by the lack of techniques to identify progenitor or stem cells that may participate in lung repair or regeneration. However, progress has been made in characterizing several putative progenitor or stem cells from adult lung. For example, investigators have identified progenitor cells in the airway including basal cells (9), variant Clara cells (8), bronchiolar stem cells (28), and bronchioalveolar stem cells (BASC) (16). Bronchioalveolar stem cells express both airway (CCSP) and alveolar type II epithelial (proSP-C or SP-C) markers in vivo, and localized to a restricted zone, the bronchioalveolar duct junction (BADJ) in mice. The restricted site and dual immunoreactivity (CCSP+/SP-C+) have implied that these cells may repopulate the airway and alveolar compartments following injury. In support of this theory, CCSP+/SP-C+ cells proliferate in vivo in response to airway (naphthalene) and alveolar epithelial cell (bleomycin) toxins, or after pneumonectomy, suggesting that they respond to cues evoked by...
injury or mechanical stress, respectively. Moreover, dually
immunoreactive cells have been isolated by FACS (CD31-, CD45-, Sca-1+, CD34+) and induced in vitro to express aquaporin 5, proSP-C, or CCSP (15, 16), compatible with the property of multipotency. Whether CCSP+/SP-C+ exhibit multipotency, thus “stemness” in vivo, awaits lineage tracing experiments. Nevertheless, the properties of toxin resistance, mixed phenotype (CCSP+ and proSP-C+), restricted niche, and proliferative behavior have led us to ask whether CCSP+/SP-C+ and the more traditional progenitors of the alveolar space (alveolar epithelial type II cells; ATII) are responsive to perturbations in lung matrix biology. We hypothesized that lung regeneration (compensatory lung regrowth) and progenitor cell proliferation are activated by mechanical stress, and thus in animal models with reduced mechanical stress at the macroscarcular level (i.e., higher lung compliance), these responses would be significantly attenuated. Animal models that possess features of emphysema (alveolar destruction or reduced elastin content) or surgically reduced mechanical stress were employed to study these responses in vivo.

MATERIALS AND METHODS

Animals and study design. All protocols were approved by the Institutional Animal Care and Use Committee at Tufts University. Animal strains that were employed included C57BL6 (WT, Jackson Laboratories, 3-mo-old females) and an elastin-insufficient construct (mouse elastin hemizygote: ELN+/−, 3-mo-old females) previously described. For ELN+/−, C57BL/6 mice bearing a deletion of exon 1 in the elastin gene were backcrossed for more than five generations, resulting in mice possessing 55% of wild-type lung elastin content (17) (homoyzogotes are lethal). ELN+/− litter mates were used as controls. Mutant (ELN+/−) mice have significantly higher lung compliance (in vivo) and lower mechanical stiffness (tissue strips) than ELN+/+, but chord lengths and gross morphology of the lung are indistinguishable from each other (26). Experimental emphysema (alveolar destruction, increased lung volumes and compliance) was produced by the injection of porcine pancreatic elastase (1.5 IU porcine pancreatic elastase; Elastin Products, St. Louis, MO) intratracheally delivered in a volume of 40 μl of PBS via the orotracheal cannula (20 g Abbocath) in anesthetized mice (female C57BL6/J) at 6 wk of age. Following instillation, mice received 0.3-mI inflations two to three times, and the endotracheal tube was removed. A control group received an equal volume of PBS. Six weeks after injection of elastase or PBS, mice underwent surgery, either pneumonectomy (PNY) or sham. The PBS-treated mice underwent PNY only. Hence, the elastase-injured mice were age matched to the other groups immediately before surgery.

Study design. Mice underwent PNY and physiological measurements (pleural-esophageal pressure during spontaneous breathing and static lung mechanics) on day 0 before and after PNY (+ = plombage procedure) and on days 7 and 14 after PNY. Hence, we measured mechanical stress (pleural pressure) in each mouse immediately before and after surgical treatment, and on days 7 and 14 for correlation with the extent of compensatory lung regrowth at those time points. Mice in all groups (PNY, PNY+plombage, elastin haploinsufficient, elastase injured) were killed after physiological assessment on day 14 or 28, and the lung was processed for histomorphometry and immuno-fluorescence (IF) for CCSP+/SP-C+ and ATII (precursor cell) enumeration. The CCSP+/SP-C+ counts were expressed as CCSP+/SP-C+ per BADI. The BADI was defined as the juncture between the most distal low-lying terminal bronchiolar epithelium and adjacent fibers of the proximal alveolar duct(s). Counts were derived from examination of ≥30 BADI, whereas the ATII were quantified as the number of ATII per nucleated cells, derived from counting all nucleated cells within 10 high-power fields (×400 magnification).

Physiology. Mechanical stress on lung was characterized by measurement of pleural pressure (Ppl) before, immediately after, and on day 7 after surgery in wild-type mice with or without plombage. The extent of compensatory lung regrowth was measured physiologically in terms of lung volumes. Mice were anesthetized (IP) with ketamine (50–75 mg/kg) and xylazine (3.8–5 mg/kg). Once anesthetized, mice were intubated orotracheally using a 20-gauge IV catheter. A calibrated fluid-filled catheter (18-gauge plastic feeding tube; Instech Laboratories, Plymouth Meeting, PA)-pressure transducer (TRD 0110; Buxco Electronics, Wilmington, NC)-strain gauge amplifier (MAX 2270, Buxco) system was calibrated with a water manometer. The amplitude of the pressure was frequency independent up to 10 Hz. The Ppl catheter was inserted into the distal esophagus where maximal shifts in pleural pressure were evident during spontaneous breathing in the sedated mouse. Data were recorded for 5 min using a commercial data acquisition system (XA Biosystem v. 2.9, Buxco). During spontaneous breathing, the mean Ppl at end-expiration (Ppl-EEP), the mean Ppl at end-inspiration (Ppl-EEP), and the maximum shift in Ppl (DPpmax) were measured. The pressure-time integral [∫Pp[pl]dt] for Ppl-EEP, dpPmax, and the total pressure-time integral was computed post hoc using Acknowledge (v. 3.7.3; BioPac, Goleta, CA). Following measurement of pleural pressure, mice were placed on a digitally controlled mechanical ventilator (AUT6110, Buxco) in supine position within a flow-type whole body plethysmograph (PLY3111, Buxco) that controlled forced and passive inflation/deflation maneuvers. Ventilator settings were f = 200/min, Fio2 = 0.21, tidal volume = 0.3 ml, and PEEP = 0. The lung was inflated to total lung capacity (TLC; 25 cmH2O) at least three times before measurements to normalize volume history, and total lung capacity (25 cmH2O), residual volume (~25 cmH2O), and vital capacity were measured using commercial software (XA Biosystem v. 2.9, Buxco). Finally, PEEP was removed, three inflations (25 cmH2O) were provided, followed by airway occlusion (8 s) at end-expiration for measurement of Boyle’s law FRC [([V/6][SP] × (P(atm) − P(H2O))] (18). Measurement of FRC was used to compute the volume of plombage to introduce into the chest of each mouse, assuming removal of 32% of the lung by pneumonectomy (31). On the day of death (day 14), mice were given an additional dose of ketamine/xylazine, and the chest was opened for a second measurement of vital capacity (VC) unencumbered by the plombage material and chest wall. Elastase mice with TLC >1.5 ml were excluded from PNY or sham procedures since in preliminary studies these mice exhibited a lower survival rate (50–75% vs. >90% in mice with lower TLC). Elastin mutant mice were measured by one of the authors (A. Shiren) in a separate laboratory using a modification of these methods. Mice were anesthetized with ketamine/xylazine cocktail, orally intubated with an 18-gauge cannula, and ventilated (tidal volume = 10 ml/kg, rate = 240 bpm, PEEP = 3 cmH2O) using a Flexivent small-animal ventilator (SCI REQ, Montreal, Canada). TLC was determined as the volume of inflation to 30 cmH2O plateau airway pressure averaged over three such maneuvers. Quasistatic PV loops were obtained by stepwise inflation and deflation of the lungs as described (3).

Pneumonectomy. The technique of left unilateral pneumonectomy was modified from the methods of Sakurai et al. (24) and Voswinckel et al. (31). Mice acclimated to their housing for at least 72 h, were anesthetized (IP) with ketamine (75 mg/kg) and xylazine (5 mg/kg), and received buprenorphine (0.1 mg/kg sc) before and twice per day for 48 h after PNY. A single dose of amoxicillin (100 mg/kg sc) and 2 ml of lactated Ringer solution (sc) were given immediately before surgery as prophylaxis. Once anesthetized, mice were intubated orotracheally using a 20-gauge angiocatheter and placed on a mechanical ventilator (AUT6110; Buxco Electronics, Wilmington, NC) using the following settings: Fio2 = 0.21, f = 200, tidal volume = 0.3 ml, PEEP = 5 cmH2O. The left side of the chest was clipped and a sterile field created using chlorhexidine antiseptic solution and alcohol. A 15
blade was used to make a small (7-mm) incision in the left fifth intercostal space, and the lung was exteriorized carefully using blunt-ended forceps. Once lifted such that the left (L) lung hilum was visualized, the mainstem bronchus was ligated using silk (4-0), and the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised. Two sustained inflations (25 cmH2O) were used to fully inflate the right (R) lung to the level of the lung distal to this ligature excised.

Plombage. Plombage involved the instillation of sterile dental wax into the pleural cavity to prevent the expansion of the remaining lung past midline and therefore blunt changes in pleural pressure provoked by PNY (21). The volume of plombage was therefore the independent experimental variable for modulating regrowth. Dental wax was formed into balls (~0.02–0.03 g each) for easy insertion through the chest incision by sterile technique. Groups of balls were weighed and sterilized, and their total volume was established on the basis of known density (0.85 g wax/cm³). Following excision of the L lung, sterile wax of varying volumes (0% of measured FRC) was carefully fed into the L hemithorax using blunt forceps, and the chest was closed as for pneumonectomy. This method was intended to induce three levels of mechanical load in wild-type mice, ranging from increased stress to subphysiological levels as observed in the diseased (emphysematous) lung.

Histomorphometry. Immediately following pulmonary function tests and while still under anesthesia with ketamine and xylazine, the degassed lung was immediately fixed at total lung capacity (equivalent to 25 cmH2O pressure) in neutral buffered formalin (10%) for 12–24 h. Once fixed, the right lungs were sectioned longitudinally (cranial to caudal) into 10 equidistant slices for examination of medial vs. lateral stem cell proliferation. The right lung samples were sectioned (5 μm) apart. Slides were stained with hematoxylin and eosin, and 10 randomly oriented nonoverlapping fields (×200 magnification) were photographed (Zeiss M1 AxioImager, Axiocam 1.0× digital camera). For random orientation of fields, a spaced noncontinuous grid (of total grid length 1,000 μm) was placed on each field at an angle of [Rand(n) × 90°] where Rand(n) is a random number between 0 and 1. Processing was performed using a custom program in commercial imaging software (SigmaScanPro, v.5.0, San Jose, CA) that permitted input of an appropriate threshold, and automatically identified points of grid-image overlap, counting the number of overlaps. Lmi was defined as the total grid length (1,000 μm) divided by the number of overlaps.

Immunostaining. Immunofluorescence staining (IF) was performed on formalin-fixed (10%, 12–24 h) paraflin-embedded sections (5 μm). Tissue sections were deparaffinized and hydrated using standard methods, and antigen retrieval was performed using a citrate buffer (pH 6.0) and microwave heating, followed by slow cooling to room temperature. Buffers included PBS with 0.1% Triton X-100 (Sigma) (immunofluorescence). Tissues were washed with buffer three times after antigen retrieval. Nonspecific binding was blocked using 10% serum from an appropriate species and then diluted in wash buffer (2 h, 23°C). The tissues were subsequently incubated with primary antibodies diluted in wash buffer at 4°C overnight, washed three more times, and then incubated with secondary antibodies (37°C, half an hour). As primary antibodies, the polyclonal goat antibody anti-CCSP (Santa Cruz, dilution 1:200), the polyclonal rabbit antibody anti-proSP-C (Chemicon AB3786, dilution 1:1,000), and the monoclonal mouse antibody anti-BrdU (Santa Cruz, dilution 1:200) were used. Detection was performed with the following antibodies: when triple staining for colocalization of pro-SPC, CCSP, and BrdU, donkey anti-rabbit Alexa Fluor 488 (green), donkey anti-goat Alexa Fluor 350 (blue), and donkey anti-mouse Alexa Fluor 594 (red); when staining for CCSP and pro-SPC alone, donkey anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 594; for noncolocalizing studies, donkey anti-rabbit Alexa Fluor 594 for IF, secondary antibodies were diluted 1:200 in PBS, except Alexa Fluor 350, which was diluted 1:100. After washing three times in PBS, tissues were coverslipped with Vectashield mounting medium without DAPI when triple staining was performed, and with DAPI when single or double staining was performed. For determination of CCSP+/SP-C+, tissues were double-stained with CCSP and proSP-C. For immunohistochemistry, the Vectastain ABC system (Vector Labs) was used according to the manufacturer’s instructions. Antigen retrieval was performed as described above for IF. Briefly, after application of primary antibodies and blocking, biotinylated secondary antibodies were added at a 1:100 dilution, followed by Vectastain ABC solution 1:100. Slides were then incubated in DAB solution (0.06% 3,3′-diaminobenzidine in PBS, 0.003% H2O2) for 2–5 min, dehydrated, and counterstained with hematoxylin. Controls, employed in all experiments in triplicate, consisted of tissue specimens from each animal that were incubated each with 1 conjugated secondaries (no primary immunostain control) or 2 single antibodies (single primary + secondary combination).

Fig. 1. A and B: pleural pressure (Ppl) was measured immediately before (No surg) and after pneumonectomy (PNY) on day 0 and on postoperative days 7 and 14 in PNY (i.e., control), PNY-low, and PNY-high groups. Mechanical stress was expressed as the pressure-time integral (jPpl) over 1 s for Ppl at FRC, over the maximum shift in Ppl, and their sum (total jPpl). There was a significant (*P < 0.05) difference in jPpl at FRC between PNY-low and no surgery and both PNY (control) and PNY-high. Thus, PNY-low most closely mimicked the sham group. The total jPpl was different between PNY and the other groups (*P < 0.05). Cyclic jPpl was greater in PNY, but this did not reach significance.
Hence, the plombage material did not introduce a restrictive component in the significantly lower values in plombage groups vs. PNY for both techniques. 

**Fig. 2.** A: vital capacity (VC) of mice before, immediately after, and 7 and 14 days after surgery showing a marked diminution of regrowth as a function of plombage. Compared with baseline, VC measured in vivo (closed chest) was significantly lower on days 7 and 14 postoperatively in the plombage groups, on day 14 significantly lower in the PNY-low vs. PNY (†), and PNY-high vs. PNY (‡). B: VC measured in closed- and open-chest conditions showing significantly lower values in plombage groups vs. PNY for both techniques. Hence, the plombage material did not introduce a restrictive component in the closed-chest measurements of VC.

Examples of immunostaining with CCSP, proSP-C, or serum alone (control) are shown in Supplemental Fig. S1. (Supplemental data for this article are available online at the *AJP-Lung* web site.)

**Enumeration of CCSP+/SP-C+ and ATII in situ.** The lung was evaluated first on low power (×100 magnification) to locate and enumerate BADJ in the sections. At high power (×400), we counted the number of CCSP+/SP-C+ (dual expressing CCSP and proSP-C + a single nucleus) at each BADJ. Deconvolution microscopy was used selectively to confirm that these antigens were present within the cytoplasm of each CCSP+/SP-C+ cells. A minimum of 30 BADJ was evaluated in each mouse spanning at least three sections. The number of CCSP+/SP-C+ per BADJ was used as an index of abundance in each mouse, and BrdU uptake was evaluated in three randomly selected mice per group as a measure of active DNA synthesis. In addition, we counted the number of proSP-C-positive and CCSP-negative cells (ATII) per total nucleated cells from at least 10 high-power fields (×400). The percentage of nucleated cells that were ATII was used as an index of cell density.

**Statistical analyses.** ANOVA was employed to compare data between three groups of mice and the Student’s *t*-test for post hoc testing of differences between any two groups, or paired *t*-test when appropriate. Two-way ANOVA was employed to examine the effect of independent groups (PNY vs. sham) and time (2 or 4 wk after surgery) and their interactions in the elastase-injured mice. Correlations were tested using Spearman’s correlation coefficient (*ρ*). A *P* value less than or equal to 0.05 was designated as significant. Data are expressed as means ± SE.

**RESULTS**

**Mechanical stress and compensatory lung regrowth is reduced after plombage.** There was a significant increase in the ∫Pppl-EEP in mice that received PNY, which was reversed to control values (obtained from unoperated mice) by plombage in the PNY-low group. However, the ∫Pppl-EEP was significantly lower in the PNY-high vs. all the other groups, suggesting that PNY-high reduced mechanical stress to below physiological levels (Fig. 1A). These statistical differences were abolished by day 7 (Fig. 1B). Compensatory lung regrowth was significantly impaired in the plombage groups. There was significantly lower VC on days 7 (ante-mortem) and 14 (day of death) in the plombage groups (Fig. 2A). At the end of the experiment, VC was lower in the PNY-low and PNY-high groups compared with the PNY alone whether it was measured in the open- or close-chested preparations in vivo (Fig. 2B).

The extent of compensatory lung regrowth was measured as the difference between the final and initial pre-PNY VC (delta VC) and the absolute final VC. The delta VC was inversely correlated with indices of mechanical stress, including ∫Pppl-EEP (ρ = −0.7, *P < 0.001*) and ∫Pppl-total (ρ = −0.54, *P = 0.007*), and the mean value for Ppl-mean (ρ = −0.59, *P = 0.003*), but not the dPPlmax (Fig. 3). Absolute final VC was significantly correlated with ∫Pppl-EEP (ρ = 0.52, *P = 0.01*) and ∫Pppl-total (ρ = 0.43, *P = 0.028*), but not for dPPlmax or Pmean. Final (day 14) lung compliance was significantly (*P < 0.01*) lower in the PNY-low (0.040 ± 0.001 ml/cmH₂O) and PNY-high (0.036 ± 0.002) groups vs. PNY alone (0.047 ± 0.002). There was a significant increase (*P = 0.025*) in Lmi in the PNY-high (36 ± 1.5 μM) vs. PNY-low groups (32 ± 0.39 μM), but there was no difference between PNY (33.3 ± 1.4 μM) and either plombage group. Plombage had no effect, however, on the SD or...
CV (heterogeneity) of Lmi. These data show that lowering mechanical stress below physiological levels by plombage altered lung architecture following PNY, whereas morphometry is normally unaffected by PNY without plombage.

Cell proliferation following PNY as modulated by plombage. PNY induced marked cell proliferation (BrdU uptake) in CCSP+/SPC+ (BASC), ATII, and Clara cells (Fig. 4, A–C). However, only CCSP+/SPC+ cell BrdU uptake was sensitive to low levels of plombage (PNY-low), i.e., CCSP+/SPC+ cell proliferation was more sensitive to mechanical modulation of PNY with plombage than the other two cell types. Also, CCSP+/SPC+ cells showed a greater fraction of BrdU+ cells after PNY than the other cell types. In this regard, CCSP+/SPC+ proliferation mirrors more closely the effects of mechanical stress modulation on compensatory lung regrowth than does the BrdU uptake data in ATII or Clara cells.

We further examined the regional effects of PNY on CCSP+/SP-C+ density since PNY may cause greater mechanical stress in the axial/medial lobes (e.g., cardiac + accessory lobes). It was hypothesized that plombage would ameliorate mechanical stress (which is greater medially after PNY) and thus cause expansion of CCSP+/SP-C+ to occur nonuni-

Fig. 4. A–C: BrdU uptake (14 days) in CCSP+/SPC+ cells, ATII, or Clara cells following PNY or PNY + either low levels of plombage (PNY-low PLB) or high levels of plombage (PNY-high PLB) to modulate mechanical stress caused by PNY. There was a significant proliferative response in all 3 progenitor cell types, but CCSP+/SPC+ cells showed greater responses to PNY and a greater reduction due to PNY-low PLB. D: abundance of CCSP+/SP-C+ in lung tissue sections on day 14 partitioned into medial and lateral lung regions. There was a significant reduction in CCSP+/SP-C+ per bronchioalveolar duct junction (BADJ) in the lateral regions in the plombaged groups compared with PNY, whereas the medial region had lower CCSP+/SP-C+ per BADJ only after PNY-high PLB. *P < 0.01 vs. PNY and PNY-low PLB; †P < 0.01 vs. PNY only. E: example of CCSP, proSP-C, and BrdU triple immunostaining (see MATERIALS AND METHODS) of a single BADJ (X400 magnification, AxioImager, Zeiss). The arrows point to 2 CCSP+/proSP-C+/BrdU+ cells at the BADJ.
formly in the lung. Indeed, the increase in CCSP+/SP-C+ per BADJ was completely abolished in the medial sections in the PNY-high group, but not in the PNY-low group (Fig. 4D). Thus more of the mechanical effects on CCSP+/SP-C+ are concentrated in the medial sections.

Since CCSP+/SPC+ cells were most sensitive to the effects of both PNY and plombage, we examined the relationship between mechanical stress imposed after surgery or the extent of regrowth on day 14 with the abundance of CCSP+/SPC+ cells at BADJ on day 14. The number of CCSP+/SP-C+ per BADJ on day 14 correlated significantly with the imposed mechanical stress on day 0 (i.e., ∫Ppl-EEP; ρ = 0.51, P = 0.02). The CCSP+/SP-C+ per BADJ also correlated with the extent of compensatory lung regrowth as measured by VC (ρ = 0.59, P = 0.007) (Fig. 5). Thus the full spectrum of mechanical stress influenced both the extent of regrowth and CCSP+/SP-C+ abundance concomitantly in the PNY model in wild-type mice.

Reduced lung elastin content in mutants caused failure of PNY to increase CCSP+/SP-C+. There was no significant effect of lowering elastin content by altering gene dose on physiological indices of compensatory lung regrowth. Using water displacement for measurement in this group, right (remaining) lung volumes on day 14 after PNY vs. sham was 0.46 ± 0.04 vs. 0.36 ± 0.03 ml for ELN+/+, and 0.50 ± 0.02 vs. 0.41 ± 0.04 ELN+/- [not significant (N/S)]. However, the response of CCSP+/SP-C+ to PNY was significantly different between groups, whereby PNY failed to evoke any proliferative response of CCSP+/SP-C+ in ELN+/- in contrast to the brisk response of CCSP+/SP-C+ wild-type littermates (ELN+/+) (P < 0.01) (Fig. 6A). A similar pattern was observed for ATII cells, which proliferated significantly in wild-type (ELN+/+) littermates but not in hemizygous (ELN+/-) mice. BrdU uptake was not assessed to measure cell proliferation in these mutant mice.

Elastase injury impaired lung regeneration, but CCSP+/SP-C+ and ATII cell densities increased after PNY. Elastase injury significantly impaired compensatory lung regrowth in mice (Fig. 7). In the group that was killed 2 wk after surgery, VC was 85 ± 3.3% presurgery in the elastase-PNY group compared with 107 ± 1.5% in the elastase-sham-operated group.

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**Fig. 5.** Correlations between indices of mechanical stress measured immediately after PNY, and final index of CCSP+/SP-C+ abundance (CCSP+/SP-C+ per BADJ) (A–D) and correlations between CCSP+/SP-C+ abundance and final VC (E and F) (closed- and open-chest measurements shown).
DISCUSSION

This study demonstrates that modulation of mechanical stress measured at the macroscale level significantly alters the proliferative responses of lung progenitor cells and the magnitude of compensatory lung regrowth. In wild-type mice, progenitor cell proliferation and lung regrowth in response to PNY were reduced in tandem by plombage. Lowering of the gene dose of elastin completely abolished CCSP+/SP-C+ and ATII proliferation, but moderate loss of elastin content in ELN+/− did not interfere with lung regrowth, suggesting discordance between the progenitor cell response and the potential for lung regrowth in this model. Elastase injury resulted in yet another unique pattern, whereby compensatory lung regrowth was significantly impaired yet ATII and CCSP+/SP-C+ proliferation were unaffected (opposite to elastin insufficiency). Together, these data show that alterations to the extracellular matrix (ECM) via mechanical, biochemical, or structural alterations can have profound yet distinct effects on the capacity for lung regeneration and progenitor cell function. While these data clearly demonstrate some of the influences of ECM on lung regeneration in mice, it does not elucidate whether the population of cells that synthesize ECM was perturbed in the mutant or injured mice in such a way that growth was limited by their paracrine or precursor functions beyond the mechanical-matrix disorders that were generated.

A cautionary statement concerning our use of the term “proliferation” when referring to changes in progenitor cell abundance in elastin mutants and elastase-injured animals is essential. Our previous data concerning CCSP+/SP-C+ cell kinetics showed that in wild-type mice, CCSP+/SP-C+ cells do not have detectable BrdU uptake in shams (over 7 days of BrdU feeding after surgery), such that all of the increase in abundance of this cell type after PNY could be explained by cell division (20). We assume that this phenomenon similarly governs the cell kinetics of CCSP+/SP-C+ in elastin mutants and 99 ± 2.8% baseline VC in the PBS-PNY group (P < 0.005, elastase-PNY vs. either group). At 4 wk, the gap in VC between PNY and sham remained statistically significant (P < 0.01). Elastase markedly increased mean Lmi, e.g., 59 ± 9.9 μM in elastase-sham vs. 33 ± 1.8 μM in non-elastase PNY group (P < 0.001) derived from the plombage portion of this investigation. The effect of PNY (vs. sham) in elastase-injured mice was to reduce Lmi at both time points after surgery (2 wk postsurgery: PNY = 47 ± 5.3 μM vs. sham = 59 ± 9.9 μM; 4 wk postsurgery PNY = 42 ± 2.4 μM vs. sham = 50 ± 4.9 μM), but the model (group, time, group × time) was not statistically significant (P = 0.09). PNY also failed to reduce heterogeneity of alveolar dimensions as represented by the SD and CV of Lmi.

Prior to PNY, the abundance of CCSP+/SP-C+ (per BADJ) was ~50% lower in elastase-injured sham-operated mice (Fig. 6B) than uninjured sham-operated mice (Fig. 6A). Furthermore, in 9 out of 12 (75%) elastase-injured vs. 1 out of 6 of the PBS-treated mice (P < 0.05, Fisher Exact test), CCSP+/SP-C+ were found outside the BADJ (Fig. 8). This occurred at a frequency of 14.4 ± 0.05% CCSP+/SP-C+ observed for the sham and 14.5 ± 0.05% CCSP+/SP-C+ for the PNY-operated mice (N/S). In comparison, CCSP+/SP-C+ were rarely (<2%) found outside the BADJ in elastin mutants or nonelastase-treated mice. Thus elastase injury rather than PNY induced a “migration” of CCSP+/SP-C+ from its niche.

PNY in elastase-injured mice caused a marked increase in CCSP+/SP-C+ (P < 0.001) to >3-fold sham values. PNY elicited a significant increase in ATII cells (per total nucleated cells in parenchyma) in PBS-treated mice only, i.e., there was no significant change in elastase-injured mice (vs. sham) at 2 or 4 wk after PNY. BrdU uptake was not assessed as a measure of cell proliferation in these mice. There was no correlation between the CCSP+/SP-C+ counts and final VC in elastase-injured groups of mice as seen in the noninjured wild-type mice.

Fig. 6. A: effect of modification of elastin gene dose on the CCSP+/SP-C+ and ATII response to PNY or sham procedure at 2 wk postsurgery in wild-type littermates (ELN+/+) vs. elastin haploinsufficient (ELN+/-) animals only. *P < 0.01 vs. sham. PNY caused a significant increase in both CCSP+/SP-C+ and ATII in ELN+/- animals only. B: effect of elastase (EL) on the CCSP+/SP-C+ and ATII responses to PNY or sham procedure (SH) at 2 or 4 wk after surgery. PBS noninjured mice 2 wk after PNY or sham are shown for comparison; *P < 0.05 PNY vs. sham groups; †P < 0.05 PBS sham 2 wk vs. EL SHAM 2 or 4 wk. There was no significant effect of PNY vs. sham on ATII cell densities in elastase-injured mice.
and elastase-injured animals, although this was not validated with specific markers of cell division. For example, the contribution of cell migration to these areas in these models of elastin disturbances cannot be excluded.

Compensatory lung regrowth following PNY is thought to be initiated by mechanical stress, as shown by the profound reduction in regrowth caused by plombage in rodents and immature dogs (1, 33). Indeed, plombage was shown to have a dose-dependent effect on mechanical stress and compensatory lung regrowth in our model, using pleural pressure in spontaneous breathers or lung compliance in nonspontaneous breathers as proxies for mechanical stress. In contrast, Olson and Wardle (23) measured no significant effect of unilateral PNY (with or without plombage) on transpulmonary pressure in rabbits. The difference may relate to a unique shift in macro- or microscalar forces created by PNY in the mouse (vs. the rabbit) or specific effects of body position that have obscured the response to PNY in the rabbit. The authors are unaware of past studies that have documented mechanical stress by direct means, following PNY or plombage. In a previous study in dogs, plombage at a volume equivalent to 10% below FRC (in prone position) was shown to block mediastinal shift and inhibit the bulk of compensatory lung regrowth (10). In that study, mechanical stress per se was not measured directly, rather it was inferred by CT. Hence, it is plausible that the blockage of mechanical stress was insufficient to arrest lung regrowth, similar to the PNY-low group. Our data suggest that replacement of volume equivalent to PNY in the mouse (PNY-low), which presumably should stabilize the mediastinum, was insufficient to completely block regrowth (based on closed-chest measurements), a process that has previously been shown to continue in the cranial-caudal direction (22). This would explain why ATII and Clara cell proliferation was not significantly reduced in the PNY-low group compared with PNY alone. PNY also increased BNP uptake to a greater extent in CCSP+/SPC+ than ATII or Clara cells, and lowering of mechanical stress in the PNY-low group reduced CCSP+/SPC+ proliferation to a greater degree than in the other cell types, which implies that CCSP+/SPC+ may be more sensitive to changes in mechanical forces, either intrinsically or due to greater effects of PNY and plombage on mechanical stress at the BADJ. Further study would require isolation of the CCSP+/SPC+ and studies that involve mechanical stretching.

The effect of plombage on pleural pressures ($\Delta$EEPpl, $\Delta$dPPlmax) dissipated over the first 7 days after surgery, thus stimulus for regrowth is transient, coinciding with the period of maximal regrowth. It is this early time period (days 1–7) where the cellular and molecular mechanisms of compensatory lung regrowth in the mouse are most likely discernible through future investigations. The effect of plombage on mechanical stress can also be viewed qualitatively, permitting insight into the nature of stress-elicited regrowth. The effect of plombage was to reduce the end-expiratory pressure ($\Delta$EEPpl) following PNY, i.e., shift the pressure to a less negative value in a plombage-volume-related fashion. Plombage did not produce a similar dose-dependent effect on the maximum shift in pleural pressure (i.e., $\Delta$dPPlmax or “cyclic” pressure). These data suggest that prestress represented by $\Delta$EEPpl might be more important to compensatory lung regrowth and suggest that failure of regrowth in the mutant or elastase injury models may relate to reduced prestress (as supported by the higher total lung compliance in these models).

One critique of the plombage model might be the potential for the inserted material to directly interfere with the antemortem measurement of VC within the closed chest. In past studies, the prosthesis used in dogs for plombage caused mild pleural inflammation (10), which might also interfere with proper measurement, although we did not observe inflammation or adhesions in this study. Measurements in the open-chest animals were performed, with results that mirrored closed-chest recordings. Thus plombage specifically impaired lung tissue regeneration independently of affects on the chest wall.

The failure to show a progressive increase in Lmi or deterioration in pulmonary function (between 2 and 4 wk post-PNY or post-sham) in elastase-injured mice supports the concept that emphysema was not propagated acutely by forces imposed by PNY or tidal breathing forces in the sham-operated animals. Since mice generate new alveoli following PNY (7), they may be “protected” to some extent from apparent degradation of the fiber network after initial injury. Indeed, the elastase-injured

Fig. 7. A: compensatory lung regrowth was delayed in elastase-injured (EL) mice compared with PBS-treated (uninjured) mice. B: elastase caused marked loss of lung architecture. C: Linear mean intercept (Lmi) was markedly increased due to elastase injury but declined significantly by 4 wk postoperatively. *$P < 0.05$ vs. EL-SHAM 4 wk.
PNY-treated mice were found to exhibit a trend toward lower Lmi values than sham counterparts, inconsistent with the notion that PNY caused emphysema to be propagated by increased mechanical forces in this model. Interestingly, it was the loss of mechanical forces caused by plombage that appeared to exert more deleterious effects on regrowth. For example, in the PNY-high group, there was not only failure of regrowth, but Lmi increased significantly over 2 wk, albeit at levels that were difficult to distinguish upon inspection with light microscopy. In the elastin mutants where the loss of microscalar mechanical stress would be predicted due to the uniformity of the lesion, regrowth was compromised and CCSP+/SP-C+ and ATII proliferations were completely abolished. Hence, we propose that “force-failure” should be considered as an important contribution to the progressive nature of emphysema, which is characterized by progressive loss of elastic recoil and progenitor cell abundance.

The potent effect of elastin haploinsufficiency (ELN+/−) to abolish CCSP+/SP-C+ and ATII proliferation suggests that elastin possesses a critical property for sensing or responding to mechanical stress or proliferation, or that reduced synthesis of elastin perturbs the proliferative stimulus of these cells. An alternative hypothesis is that elastin insufficiency results in a lowering of abundance of a specific subset of proliferative CCSP+/SP-C+ cells.

The effect of elastin insufficiency on the vasculature cannot be ignored, although elastin content of capillary walls (where neovascularization associated with neoalveolarization is greatest) is minimal, so the role of elastin as a supportive structure in the matrix may be more important. It is less likely that elastin has trophic effects, since elastin is a poor mitogen. We speculate that the diffuse loss of elastic recoil in elastin mutants, resembling the reduced mechanical stress caused by plombage, is the reason why CCSP+/SP-C+ and ATII did not

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Fig. 8. Immunohistochemistry of CCSP+/SP-C+ showing displacement from the BADJ. Stains include CCSP (rhodamine, red), proSP-C (FITC, green), and DAPI (blue) for nuclear staining. A: CCSP+/SP-C+ (arrows) at the BADJ and positioned outside the BADJ (B and C) in elastase-injured mice. Fourteen percent of CCSP+/SP-C+ were found detached from the BADJ in elastase-treated mice vs. <2% of CCSP+/SP-C+ in noninjured animals.
expansion. Although lung compliance is also increased in elastase-injured mice at the macroscalar level, elastase creates a heterogeneous injury in this model whereby many, if not most, alveoli are unperturbed, and thus integrity of microscalar forces on cells is maintained on most alveoli. In contrast, in elastin mutants, microscalar forces would be predicted to falter uniformly throughout the lung. To establish whether mechanical forces are responsible for elastin mutant failure to stimulate progenitor cells will require isolation of the cells to study intrinsic capacity to proliferate in the face of various mitogens and mechanical stretch, for example.

It is noteworthy that compensatory lung regrowth was markedly reduced in elastase-treated mice, yet CCSP+/SP-C+ proliferation was similar to uninjured mice at 2 wk after PNY. In contrast, the response of ATII was significantly lower than observed in noninjured mice after PNY, thus corresponding better with physiological evidence of regrowth. These data support the notion that ATII rather than CCSP+/SPC+ cells are responsible for regrowth, an observation that is consistent between animal models in this study. This further underscores our past study using cell kinetic modeling that showed that ATII are critical for restoration of lung surface area during post-PNY lung regeneration (20). Indeed, our data are consistent with predictions by Shapiro (25) who hypothesized that “BASC” or other “stem cells” in emphysema would respond to injuries (e.g., cigarette smoke, elastase) by proliferation, yet the repair of alveoli would fail due to insufficient matrices. An alternative explanation for reduced physiological regrowth after elastase injury is the lower abundance of CCSP+/SP-C+ before PNY, and thus before the onset of lung regrowth. Alternatively, the loss of CCSP+/SP-C+ before PNY may simply reflect damage to the niche (BADJ), which is rich in elastin. Overall, the relevance of CCSP+/SP-C+ cell proliferation and abundance to the physiological outcome cannot be determined by this study.

An unexpected finding in this study was the high prevalence of CCSP+/SP-C+ displaced from the niche, specifically in elastase-injured mice (~14% CCSP+/SP-C+ displaced). Ventura et al. (30) observed increased CCSP+/SP-C+ away from the niche in mice with deletion of MAPK14 (e.g., p38α) that showed marked CCSP+/SP-C+ proliferation. Our data show that elastase independent of the proliferative effects of PNY caused CCSP+/SP-C+ to appear some distance from the niche, but the mechanism was not defined. The long-term affects of elastase injury to displace CCSP+/SP-C+ warrants further study.

The molecular mechanisms underlying CCSP+/SP-C+, ATII, and Clara cell proliferation were not addressed by this study. It is noteworthy that expansion of CCSP+/SP-C+ has been observed in mice with reduced expression of proteins that suppress cell proliferation such as PTEN (4, 34), MapK14 (i.e., p38α) (30), and GATA-6 (36). These mechanisms may play a role in transduction of mechanical stress to CCSP+/SP-C+ proliferation.

In conclusion, we have observed the pattern of CCSP+/SP-C+ and ATII proliferation during compensatory lung regrowth in three animal models, each with attributes found in emphysema, namely loss of mechanical forces, reduced elastin content, and alveolar destruction. It appears that each model introduced a unique alteration to the response to PNY either by reducing CCSP+/SP-C+ or ATII proliferation (elastin mutant, plombage) or reducing CCSP+/SP-C+ abundance (elastase), mobilization of CCSP+/SP-C+ from the BADJ (elastase), or by delaying or abolishing compensatory lung regrowth (elastase injury). It is unclear what role the CCSP+/SP-C+ plays in compensatory lung regrowth, but these animal models demonstrate that the compensatory lung regrowth and CCSP+/SP-C+ proliferation, although well correlated in uninjured mice, may have distinct determinants in more complex animal models involving abnormalities of the extracellular matrix.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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