A Microfluidic System to Measure Neonatal Lung Compliance Over Late Stage Development as a Functional Measure of Lung Tissue Mechanics

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Premature birth interrupts the development of the lung, resulting in functional deficiencies and the onset of complex pathologies, like bronchopulmonary dysplasia (BPD), that further decrease the functional capabilities of the immature lung. The dysregulation of molecular targets has been implicated in the presentation of BPD, but there is currently no method to correlate resultant morphological changes observed in tissue histology with these perturbations to differences in function throughout saccular and alveolar lung development. Lung compliance is an aggregate measure of the lung's mechanical properties that is highly sensitive to a number of molecular, cellular, and architectural characteristics, but little is known about compliance in the neonatal mouse lung due to measurement challenges. We have developed a novel method to quantify changes in lung volume and pressure to determine inspiratory and expiratory compliance throughout neonatal mouse lung development. The compliance measurements obtained were validated against compliance values from published studies using mature lungs following enzymatic degradation of the extracellular matrix (ECM). The system was then used to quantify changes in compliance that occurred over the entire span of neonatal mouse lung development. These methods fill a critically important gap connecting powerful mouse models of development and disease to measures of functional lung mechanics critical to respiration and enable insights into the genetic, molecular, and cellular underpinnings of BPD pathology to improve lung function in premature infants. [DOI: 10.1115/1.4047133]

Introduction

Proper function of the lung is critical for survival and dependent on complex developmental programs that guide morphogenic processes to create an intricate tissue architecture. The main functional gas exchange units of the lung called alveoli are generated in the last phase of development in the womb (week 36 to term) and into adolescence [1,2]. Unsurprisingly, premature birth results in lungs that are not fully developed but are still required for respiration of the infant. Premature babies are born in the saccular stage of development prior to alveolarization wherein the organ is highly cellular with a primitive extracellular matrix (ECM) [3]. It is estimated that more than 40% of infants born prematurely are diagnosed with bronchopulmonary dysplasia (BPD) [4], which has an unknown cause and is correlated with necessary ventilator and supplemental oxygen treatments to overcome the functional developmental deficits in these infants. As such, there is a significant research focus and a critical need to understand the mechanisms of late-stage lung development and how genetic, environmental, and clinical treatment factors contribute to BPD pathology. Transgenic rodent models have been used to implicate overexpression of connective tissue growth factor, phosphodiesterase 4 (PDE4), and cyclooxygenase-2 (COX-2) in the impaired alveolarization associated with BPD based on histological quantification of tissue architecture [5–8]. However, these and other studies are only focused on alterations in lung morphology and do

not address the question of whether these structural abnormalities correlate to the lung dysfunction observed in BPD. Currently, there is no ability to connect observed structural changes in the lung to functional consequences of genetic, molecular, and cellular variations in normal development nor disease.

Lung compliance is a clinically relevant measure of the aggregate mechanical properties of the lungs over inspiration and expiration. Compliance provides a functional measure of the ability of the lungs to distend and is inclusive of and highly dependent on pulmonary cell-generated forces, ECM composition, tissue architecture and spatial heterogeneities, surface tension-mediated alveolar recruitment and derecruitment, and the volume of the breath, termed tidal volume [9–12]. In studies that measure lung compliance in the whole animal, compliance also incorporates the mechanics and properties of the pleural cavity, chest wall, diaphragm, and oropharynx. Compliance of the fully developed adult lung has been thoroughly characterized in a variety of animal models, but the changes in lung compliance that occur during late-stage development are still relatively unknown [13–16], particularly in neonatal animal models that serve as lung models of preterm birth [17].

Previous studies that have investigated neonatal lung compliance typically used esophageal balloons, manometers, or pressure transducers to measure changes in lung pressure in response to stepwise volume addition to generate discrete points on a pressure–volume (P–V) curve to calculate compliance [18–20]. More recently, mechanical ventilators have been used for the quantification of lung mechanics in some rodent models of BPD assessing drug efficacy on structural and functional properties of the lung in late-stage alveolarization [21,22]. Mechanical ventilators typically used for quantification of mechanical properties

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Fig. 1 A microfluidic system for the measurement of neonatal lung compliance made with 3D printed and off-the-shelf components. (a) Exploded 3D computer-aided design rendering of device components. (b) Cartoon schematic of system setup including position of the heart (dotted line)–lung explant (in chamber) with the central chamber and intubated on the micro-needle. Placement and connections of pressure transducers, fluid inlet port and valve, and connection to syringe pump. (c) Photograph of a P7 lung explant intubated via a pulled glass micropipette and secured with sutures in a partially assembled chamber for clarity.

have transitioned to using the forced oscillation technique to determine total respiratory system resistance, elastance, and compliance [23], but suffer from the inability to deviate from the nonphysiological 1:1 inspiratory to expiratory time ratio (physiological – 1:2 [24]) and are unable to test whole explanted lungs. Considering the numerous physiological roles of many proteins implicated in abnormal lung development, it becomes difficult to decouple lung specific structural changes from thoracic and skeletal alterations based on whole animal compliance measurements [25,26] creating the need to test lung explants in isolation. Further, all of the methods generated to date have been unable to assess lung compliance at early neonatal ages in rodents due to their small size. As such, progress has been slowed in understanding the functional outcomes of cellular signaling and structural organization in a genetically tractable animal model at critical developmental transitions between terminal sacculization and early alveolarization relevant for preterm infants.

Therefore, we have developed a microfluidic system to measure pulmonary compliance in the early neonatal mouse lung to provide functional assessments of molecular and genetic perturbations in BPD mouse models. We developed a novel method to calculate changes in lung volume to generate continuous P–V curves for the calculation of inspiratory and expiratory compliance. To validate the compliance measurements, lungs were treated with elastase or collagenase to mimic pathologic ECM degradation and compliance was subsequently measured and compared to that of age-matched healthy lung explants and compliance measurements from similarly treated adult mouse lung explants found in the literature. This novel microfluidic system allowed, for the first time, the quantification of normal mouse lung compliance over the complete range of postnatal lung development from saccular (postnatal day zero (P0)) through early alveolarization (P7), through completion of alveolarization and lung maturation (P21). This system quantifies a functionally relevant parameter needed for respiration and enables the ability to decipher functional outcomes for genetic, molecular, cellular, and ECM signaling and organization in late-stage lung developmental processes relevant for the treatment of preterm infants.

Materials and Methods

Animals. For all studies, CD-1 mice were used and all procedures and protocols were approved by the University of Delaware Institutional Animal Care and Use Committee. Postnatal pups (P0, P7, P21) were euthanized, and the chest cavity was opened for

surgical removal of heart–lung en bloc. Care was taken to not puncture the lungs and to maintain tracheal length by dissection proximal to the oropharynx.

Microfluidic Compliance Chamber Design and Pressure Sensor Calibration. A microfluidic chamber was designed in SolidWorks and 3D printed from a photocurable polymer (FormLabs, Form 2, clear resin, Somerville, MA). The device consisted of three stacked layers each containing a single port and a central cavity to contain the lung explant which were fastened together along with rubber gaskets to form an airtight chamber (Fig. 1(a)). A fabricated glass microneedle used to intubate the trachea was inserted into the lowermost chamber inlet. The end of the microneedle was attached to a syringe pump (Chemxyx, Fusion 200, Stafford, TX) for infusion and withdrawal of air from the lung explant. A 0–1 psi differential pressure transducer (Honeywell, 24PCAF6D, Charlotte, NC) was used at a T-junction proximal to the microneedle to continuously measure lung pressure (Fig. 1(b)). The inlet in the middle layer contained a fluid-filled port that connected to the fluid filled chamber containing the lung explant. This port was used for volume calibration but closed for compliance measurements. In the uppermost layer, or the “roof” of the chamber, the port was connected to a 0–4 inH₂O differential pressure transducer (Honeywell, CPCL04DFC). This transducer is for the continuous measurement of pressure within an air bubble that resides in the sealed fluid filled chamber and is used for measurement of lung volume. For all external connections, noncompliant polyethylene tubing was used (Becton Dickinson & Co, 427440, Franklin Lakes, NJ). Pressure transducers were connected to signal conditioners (Omega Engineering, DMD4059-DC, Norwalk, CT) and DAQs (Vernier, SDAQ, Beaverton, OR) for signal amplification and noise reduction and subsequently recorded with a custom LabVIEW VI. Pressure transducers were calibrated using a water manometer at varying, randomized hydrostatic pressure heads to determine calibration constants for voltage to pressure conversions. All analyses were done using MATLAB (Mathworks, Natick, MA). After device setup, lung–heart explants were intubated with the pulled glass micropipette and secured via 9-0 suture as described previously [16,27] (Fig. 1(c)). For all experiments, explant medium was used as a fluid bath that consisted of Dulbecco’s modified Eagle medium with 1% penicillin/streptomycin and 5% fetal bovine serum.

Calibration of System to Measure Instantaneous Lung Volume. For each gestational age tested, we determined a system of equations and calibration protocol such that the changes in the

Fig. 2 Methods to measure small volume changes in neonatal lung explants. (a) Cartoon demonstrating how changes in lung volume result in changes to reference air bubble volume. Lung inflation decreases air bubble volume and increases P_B , whereas expiration results in an increase in bubble volume and decrease in P_B . (b) System calibration is performed by stepwise fluid addition to the chamber to measure corresponding P_L and P_B to calculate K_L and K_B using Boyle's law. (c) Time-dependent changes in lung volume were calculated using K_B and a pressure-dependent correction factor, δ .

volume of the lung could be measured. A lung was intubated and affixed as described above and the chamber was assembled. The chamber that contained the lung explant was completely filled with explant media via syringe pump from the center layer chamber port. This resulted in a small air bubble within the chamber that also filled the connection tube to the top pressure transducer. As such, this bubble deforms from an increase in lung volume due to the closed system and the incompressible liquid phase that couples the air-filled lung and the bubble. The pressure inside this bubble, P_B , was continuously recorded by the pressure transducer (Fig. 2(a)). Similarly, once the chamber was setup, the tubing to the lung was closed resulting in a second effective air bubble within the lung with a pressure sensor continuously measuring pressure within the lung, P_L . To determine calibration coefficients and relationships between pressure and volume changes in the lung and bubble, a syringe pump applied defined volumes of fluid in a stepwise manner to the central well. The system was allowed to equilibrate and the mean pressure at equilibrium was recorded for the respective chamber fluid addition step (Fig. 2(b)). Fluid volume that was added to the chamber for each step ($P_0 = 5 \mu\text{L}/\text{step}$, $P_7 = P_{21} = 10 \mu\text{L}/\text{step}$) was related to the gestational ages investigated and based on approximate tidal volumes from previous experiments to minimize injurious compression of the organ with fluid addition. Following stepwise fluid addition for calibration, an equivalent volume was withdrawn from the chamber.

Compliance Measurements. For compliance measurements, the chamber inlet for calibration fluid addition was sealed and a syringe pump added air to the lung through the intubation micro-needle. Lung tidal volume is typically defined as the volume per

breath that is achieved for 25 cmH₂O transmural pressure applied to the lung [28]. For older rodents, tidal volume is related to animal body weight and is approximately 6–10 $\mu\text{L}/\text{g}$ body weight [29]. We verified that this approximate relationship holds true for neonatal animals as well. The lung was inflated with air via syringe pump at a flowrate of 50 $\mu\text{L}/\text{min}$, until the calculated tidal volume was achieved. Once the measured change in lung volume was equal to the calculated tidal volume for the given explant, the syringe pump was set to withdraw air from the lung at a rate of 25 $\mu\text{L}/\text{min}$. Inflation and withdrawal rates were set to match the physiological 1:2 inspiratory to expiratory time ratio of normal breathing [24] and air withdrawal continued until the lung pressure returned to the baseline prior to the test. Forced inspiration–expiration loops were performed on each lung explant at least three times and pressure baselines for the bubble and lung were collected following each loop. P–V curves were plotted and the isovolumetric pressure increase, inspiratory, and expiratory regions of individual P–V loops were identified. Inspiratory and expiratory compliance was defined as the absolute value of the slope of a linear regression fit to data for the inspiratory and expiratory phases of the P–V curve, respectively. To calculate hysteresis, MATLAB was used to fit linear regressions of the isovolumetric pressure increase and inspiratory phases and an exponential curve was fit to the expiratory phase. Using these envelope curves, hysteresis was calculated as:

$$H = \int_0^{P_{\text{tidal vol}}} (\text{Isovol}(x) - \text{Exp}(x)) dx + \int_{P_{\text{tidal vol}}}^{P_{\text{isovol=insp}}} (\text{Isovol}(x) - \text{Insp}(x)) dx \quad (1)$$

Enzymatic Treatments for Selective Extracellular Matrix Depletion. Intubated lung explants were submerged in 1.5 mg/mL type I collagenase (Worthington Biochemical, LS004194, Lakewood, NJ) or 7.5 mg/mL porcine pancreatic elastase (Worthington Biochemical, LS006363) in 0.2 M tris(hydroxymethyl) amino-methane (Tris) buffer pH 8.8 for 15 min at room temperature. The enzyme solution was then aspirated, and lungs rinsed with phosphate buffered saline prior to compliance testing in explant medium. Compliance testing was conducted on enzyme treated lungs using the same procedure and determination of tidal volume as untreated controls.

Quantitative assessment of enzyme function was confirmed with the hydroxyproline assay (Cell Biolabs, STA-675, San Diego, CA) [30,31] for collagen content and elastin immunofluorescent staining and western blotting was used to assess elastase treatment [32]. For immunostaining, following enzymatic treatment, the pulmonary vasculature was flushed with 4% wt/vol sodium citrate solution. Lung explants were pressure fixed using standard protocols [33]. They were intubated and inflated with 4% paraformaldehyde (PFA) to 20 cmH₂O, tied off and fixed in a PFA bath overnight at 4 °C prior to paraffin embedding. Paraffin sections (5 μm) were rehydrated and heated to 65 °C for 3 h in 4% sodium citrate for antigen retrieval. Sections were incubated in blocking buffer overnight at 4 °C, followed by incubation with rabbit polyclonal elastin antibody (Abcam, ab21610, Cambridge, MA) and goat antirabbit secondary antibody (Invitrogen, 84541, Waltham, MA) sequentially for 1 h each at room temperature followed by fluorescent imaging using a Zeiss epifluorescent microscope. For western blotting, following enzymatic treatment, lung explants were flash frozen in liquid nitrogen, homogenized, and protein was extracted in lysis buffer (10 μL/mg of tissue). Western blotting was performed using standard methods [34,35] and probed with rabbit elastin antibody (Abcam, ab217356). Bands were normalized to beta actin and intensity was quantified using IMAGEJ.

Hematoxylin and Eosin Staining. Lung explants (P0, P7, and P21) were dissected, flushed with 4% wt/vol sodium citrate, perfusion fixed with 4% PFA, and paraffin embedded using the same protocols as described above. Lungs sections (5 μm) were stained using hematoxylin and eosin and imaged to observe morphological differences between lung tissue architecture over lung development.

Statistical Analysis. Statistical analysis, students *t* test, was completed for all measures of compliance, tidal volume, and hysteresis using JMP Pro predictive analytics software. Statistical significance was determined as *p* < 0.05.

Results

We successfully developed a microfluidic chamber and method to measure whole explant lung compliance from neonatal mice (Fig. 1). Times to measure an individual heart–lung explant are of the order of 90 min including setup of a harvested heart–lung block (device assembly, intubation and securing), bubble volume–pressure calibration, and at least four repeated P–V loops performed.

Measuring Small Changes in Lung Volume in an Irregularly Shaped Lung. As neonatal lung explants are small and air is a compressible fluid, we needed to develop a new method to measure microliter scale changes in lung volume to generate P–V curves for the determination of lung compliance. Average weight of these neonatal mice ranged from 1.5 g at birth (P0) to 15 g at the completion of lung development and maturation (P21), resulting in tidal volumes of 17 μL–123 μL, respectively. To measure such small changes in volume, we took advantage of the sealed microfluidic chamber, the pressure in a reference air bubble, and

Boyle’s Law. Within the closed system, the lung and air bubble are the only deformable bodies (Fig. 2(a)). Changes in the volume/pressure of one are communicated through the incompressible liquid resulting in concomitant changes in volume/pressure in the other. As such, as the lung volume, V_L , increases, the volume of the air bubble, V_B , will decrease, resulting in an increase in the bubble pressure, P_B . Likewise, upon expiration and withdrawal of air from the lung, V_B increases resulting in P_B decreasing.

To calibrate this system and determine a relationship to couple changes in pressure and volume in the lung to changes in pressure and volume in the air bubble, we serially added defined volumes of media, V_f , to the chamber and monitored resultant changes in P for the isolated lung and air bubble (Fig. 2(b)). Given the short testing times and assuming that the chamber is sealed, and that temperature is constant over the testing window, the ideal gas law simplifies to Boyle’s law at each equilibration “state” of the system after fluid addition. For each compressible fluid-filled space, the air bubble and the lung, this results in P_1V_1 after fluid addition step 1 must be equal to P_2V_2 for step 2, etc., which must be equivalent to a constant K . Because the volume of each lung tested and the volume of the air bubble is variable and dependent on the exact setup of the system, we developed the calibration protocol to be independent of volume. To do this, we use conservation of volume

$$V_{L1} + V_{B1} = V_{L2} + V_{B2} + V_f \quad (2)$$

and a system of equations coupled to Boyle’s law to derive

$$V_{f1} = \left(\frac{1}{P_{L1}} - \frac{1}{P_{L2}} \right) K_L + \left(\frac{1}{P_{B1}} - \frac{1}{P_{B2}} \right) K_B \quad (3)$$

$$V_{f2} = \left(\frac{1}{P_{L2}} - \frac{1}{P_{L3}} \right) K_L + \left(\frac{1}{P_{B2}} - \frac{1}{P_{B3}} \right) K_B \quad (4)$$

Solving the system of equations generates values for K_L and K_B , which are dependent on gestational age in the specific experimental setup (Table 1). We also derive a proportionality constant, δ from the pressure measurements collected during calibration. This coefficient is the ratio of the pressure changes between the bubble and lung that result from the addition of given liquid volumes added to the chamber. This relationship takes the form

$$\delta = \frac{P_{L2} - P_{L1}}{P_{B2} - P_{B1}} \quad (5)$$

For experiments, to calculate the changes in lung volume, V_L , as a function of time (Fig. 2(c)), we couple V_L to the volume of the bubble, V_B , multiplied by the proportionality constant

$$V_L(t) = \frac{K_B}{P_B(t)} \times \delta \quad (6)$$

The piecewise form of δ is needed due to the variability in deformation of the lung explants at various gestational ages. In this system, we are not controlling for the size of the air bubble; however, in all cases the volume of the bubble is larger than the volume of the lung. Thus, $\delta < 1$. In most cases, and all lung explants from age P7 and older, the addition of fluid resulted in a larger deformation to the bubble than the lung causing ΔP_B to be

Table 1 Calibration constants K_B and K_L for each gestational age determined for these studies

Gestational age	K_B	K_L
P0	-2.47×10^7	1.33×10^4
P7	-4.50×10^7	2.79×10^5
P21	-7.93×10^8	1.92×10^7

Fig. 3 Generation of P–V curves and measurement of compliance. (a) A measured P–V loop with labeled isovolumetric pressure increase, inspiratory, and expiratory phases with model fits for calculation of inspiratory and expiratory compliance. (b) P–V curves for repeated compliance testing of explants to validate noninjurious testing parameters. (c) Inspiratory and expiratory compliance and (d) hysteresis values.

larger than ΔP_L . However, in P0 lung explants, the deformation from external fluid addition was much greater in the lung than the bubble resulting in ΔP_L to be greater than ΔP_B . In these cases, δ must be replaced with $(1/\delta)$ to maintain a ratio <1 .

Generation of Lung P–V Curves and Validation. P–V curves were generated during compliance testing. For compliance tests, the syringe pump connected to the lung intubation microneedle was programmed such that the inspiratory flowrate was twice the expiratory flowrate to replicate the I:E ratio observed during spontaneous breathing [24]. P_L and P_B were monitored (8 Hz) and these data were used to plot V_L as a function of P_L to create a P–V loop (Fig. 3(a)). The P–V loop consists of three distinct regions, an isovolumetric pressure increase phase, inspiratory phase, and an expiratory phase. Lungs were inflated to tidal volume (6–10 $\mu\text{L/g}$ body weight) with care taken to ensure inspiratory and expiratory volumes were tracked so as to not over inflate and induce ventilation injury, nor over withdraw air and induce atelectasis [29,36,37]. To verify P–V loops were not a result of injury, a few P7 lung explants were subjected to repeated forced inspiration and expiration cycles (Fig. 3(b)). P–V loops for each mouse were generated in replicate and the shape of the isovolumetric pressure increase, inspiratory, and expiratory curves was maintained across replicates and explants of the same gestational age. Marginal variation in P–V loops was observed in repeated measures and values for inspiratory and expiratory compliance (Fig. 3(c)) and hysteresis (Fig. 3(d)) were similar across explants.

To validate our compliance testing method, we treated P7 lung explants with elastase or collagenase to confirm that mechanical property changes to these perturbations in our system match similar treatments to adult rodent lung explants found in the literature [38–40]. Elastin and collagen of various isoforms are ubiquitous structural proteins throughout the lung ECM and provide different functional characteristics to the ECM mechanical properties. Given that these proteins are critical for lung function and differentially contribute to inspiratory and expiratory tissue properties, specific alterations to the P–V curve should be captured in our testing method. Elastin immunostaining (Fig. 4(a)) validated widespread decrease in elastin signal uniformly throughout treated explants and the decrease in elastin protein content was confirmed with western blotting with treatment of 7.5 mg/mL elastase (Fig. 4(b)). Degradation of collagen via collagenase exposure was confirmed based on hydroxyproline as says, where doses of 1.0 and 1.5 mg/mL decreased the overall hydroxyproline content of the tissue when normalized to age-matched controls (Fig. 4(c)). Elastin western blotting and hydroxyproline assays on collagenase and elastase treated lung explants, respectively, demonstrated that

enzymatic treatment did not have off target effects for these key ECM proteins. Functionally, elastase treatment increased values for both inspiratory and expiratory compliance, whereas collagenase treatment only had an effect on inspiratory compliance relative to untreated controls (Fig. 4(d)), consistent with studies with similar treatment in adult lung explants ($n=2$ for all conditions) [38–40]. Resultant tidal volume was increased in both enzymatic treatments in comparison to age-matched normal lungs (Fig. 4(e)); however, P–V curve hysteresis was markedly decreased in collagenase-treated explants (Fig. 4(f)).

Changes in Compliance Over Alveolar Development. Lung explant tissue sections were stained with hematoxylin and eosin to compare tissue architecture at P0 (saccular developmental stage), P7 (early alveolarization) and P21 (late alveolarization/maturation). At birth, P0, lung tissue was characterized by irregular air spaces that were separated by thick, highly cellular septa (Fig. 5(a)). Over gestation, the lung transitions from the saccular stage to the alveolar stage. By P7, the lung septal walls decreased in thickness and discrete alveoli could be observed (Fig. 5(b)). By P21, corresponding to lung maturation in the mouse, the final tissue organization could be observed consisting of numerous open airspaces and alveoli having thin septal walls, composed of connected cells and ECM (Fig. 5(c)). Compliance testing for animals at these ages produced characteristic P–V curves (Fig. 5(d)) and compliance measurements revealed an expected increase in both inspiratory and expiratory lung compliance over gestation ($n=4$ for all gestational ages, Fig. 5(e)). Tidal volume also increased with gestational age, as was expected based on the significant size differences in lung explants, increasing dramatically from P0 to maturation at P21 (Fig. 5(f)). Interestingly, differences in hysteresis of the P–V loop are observed at P0 relative to reasonably constant values at P7 and P21 (Fig. 5(g)).

Discussion

We have developed a novel microfluidic system to measure neonatal mouse lung compliance by applying forced inspiration and expiration cycles in lung explants to generate functional measures of lung structure. This system uses 3D printed parts and standard off-the-shelf components including syringe pumps and pressure transducers. Our approach overcomes several challenges with current methods to fixture and apply controlled mechanical loading to early neonatal lung explants including the ability to measure microliter scale changes in lung volume by measuring pressure in a reference air bubble. Further, as this system is based on measuring compliance in an isolated lung explant, it is

Fig. 4 Validation of compliance testing methods with enzymatic treatment. (a) Immunofluorescent staining for elastin demonstrated a global decrease in intensity following elastase treatment. (b) Western blotting and (c) hydroxyproline content was measured in explants to confirm changes in elastin content from elastin treatment and collagen concentration changes from collagenase treatment. Quantification of (d) inspiratory and expiratory compliance, (e) tidal volume, and (f) hysteresis in response to enzymatic treatment.

compatible with other methods that have been developed for *ex vivo* lung culture to leverage investigations using pharmacological and enzymatic studies as illustrated herein with a genetically accessible animal model [16,27]. Such studies are difficult to perform in whole animals due to heterogeneous administration, they have possibility to induce inflammatory responses, and they are typically performed in larger animals [41,42]. Our system and methods enable the measurement of compliance in mouse lung explants from birth through animal maturity and demonstrates, for the first time, the successful measurement of terminal sacular and alveolarization stages of neonatal mouse lung development.

Our testing methods allowed for the calculation of lung compliance from a measurement of instantaneous P-V loops, rather than from P-V loops from a series of discrete quasi-static equilibrium points. A continual challenge with interpreting lung compliance measurements is the fact that compliance is an extensive property that aggregates cell, architectural, material, and contextual forces, properties, and geometries to define a measure of the lung's resistance to inflation. As such, direct comparisons between methods for generation of P-V loops are difficult and differ between species, age, and study. However, the P-V curves generated with our microfluidic system consist of key phases observed in forced inspiratory-expiratory experiments, including an isovolumetric pressure increase, and inspiratory and expiratory phases. These phases are observed with other testing methods including mechanical ventilators [43,44] in adult rodents and are observed clinically. The isovolumetric pressure increase region of the P-V curve is a hallmark of lung P-V curves, during which air compresses and the increase in lung pressure initiates alveolar recruitment and airspace opening, but do not yet cause large changes in lung volume [45,46]. The large region of isovolumetric pressure

increase in our P-V curves is reflective of the fact that we are performing measurements on isolated lung explants in a fixed closed fluid-filled cavity. As such, changes in lung volume occur once the combination of lung pressure and tissue resistance is larger than the pressure in the reference air bubble. Once this point is achieved, rapid lung inflation occurs with extensive alveolar recruitment and avalanching [46,47]. Due to the closed chamber, this point is effectively an instability, which once overcome, rapidly inflates the lung with a corresponding decrease in pressure. As such, the P-V curve has an inspiratory curve that has a negative slope, and the expiratory phase of the P-V curve has an exponential decrease. The airtight chamber mimics a mechanical chest wall to act as the an equivalent of whole body plethysmograph chambers previously used to determine lung volumes [48,49] and deviates from the shape of other P-V curves due to the lack of the chest wall [50,51].

Given that these compliance measurements require the measurement of small fluid volumes and pressures, it is important to understand various sources of error that can be introduced into the system. The pressure measurements of the lung and reference bubble are highly sensitive. Physical contact between the lung explant and walls of the chamber could alter lung deformation and generate inaccurate measurements of lung pressure over time. Additionally, the constants derived from calibration runs K_B and K_L are a function of the experimental system. These constants would be expected to be different for different chamber sizes, different reference bubble volumes, and different lung sizes and properties. Changes to any of these parameters requires a calibration run prior to an experiment to determine the K_B and K_L of that particular experimental configuration to calculate correct P-V curves. In our experiments, we used the same microfluidic system

Fig. 5 Compliance increases with neonatal lung development. Representative H&E stained tissue sections for (a) P0, (b) P7, and (c) P21 lung explants illustrate the formation of alveoli and tissue architectural changes over saccular and alveolar stages of murine lung development. (d) Representative P–V loops, (e) inspiratory and expiratory compliance, (f) tidal volume, and (g) hysteresis measurements for corresponding age lung explants.

for all explants tested, ensured that the chamber was large enough to create a free lung boundary when at max inspiration, and controlled for the volume of the reference bubble with careful fluid addition between samples. The physical controls we employed meant that K_L and K_B were only functions of the architecture and composition of the lung and thus K constants were calculated from calibrations and found to only vary between gestational ages. As such, we used the same K values for all lungs of a given gestational age.

To validate our methods, we tested lung explants over a range of developmental stages, including full maturity. Repeated compliance tests were performed on the same lung explant to ensure testing protocols did not result in over inflation injury nor atelectasis due to tissue structural collapse. Enzymatic treatments that have been performed in adult mouse lung explants from literature were compared to changes in lung compliance observed in our system. Similarly, we observed an increase in inspiratory and expiratory compliance with elastase treatment [38–40], and only an increase in inspiratory compliance with collagenase treatment. These data are consistent with the role of collagen playing a structural role and resisting deformation, with less of an impact in lung recoil.

As expected from the changes in tissue architecture, inspiratory and expiratory compliance increased with gestational age. The lung architecture changes dramatically in the last part of development moving from a dense highly cellular organization to extensive extracellular matrix deposition and organization to create alveoli. These architectural features are observed in histological staining of lung sections from P0 (saccular stage of development) through P21 (maturation) and are directly relevant as a model for both human preterm and adult lungs. Importantly, we are the first to report a functional measure of lung mechanics for this early developmental window, measurements of which are critical to understand the impact of biological mechanisms and efficacy of therapeutic interventions. Currently, precise architectural measurements [52–55], called airway stereology, are a central comparative feature of most studies to account for differences in molecular, cellular, or pharmacological treatment. Whereas these

are important architectural characteristics, it is unclear exactly how these geometrical properties correspond to functional outcomes. Our method enables the quantification of functional lung mechanical properties that, with further work, may be able to be coupled to morphological tissue features over development. Interestingly, in testing neonatal lung explants at various ages, measurements revealed differences in hysteresis in P0 explants relative to P7 and P21. This difference is likely due to the fact that we are measuring compliance at birth and fluid within the airspaces is still being resorbed from the transition of a fluid-filled lung in utero [16]. It is well appreciated that hysteresis in the lung P–V curve is significantly diminished due to less surface tension forces in a lung that is liquid filled compared to air filled [16,56].

These data validate a new system and methods to quantify the changes in pulmonary compliance at early stages of neonatal development in the mouse. Dysregulation of the developmental programs, as in the case of premature birth often results in acute and long-term respiratory complications. This work fills a critically important gap connecting powerful mouse models of development and disease to measures of functional lung mechanics critical to respiration. These compliance measurements on isolated lung explants allow for the direct quantification of lung mechanics as a function of lung tissue architecture and composition independent of changes in other tissues (e.g., diaphragm, chest wall, etc.) involved in respiration. As such, these methods can advance the mechanistic understanding of lung function over normal development, enable insights into the genetic, molecular, and cellular underpinnings of BPD pathology, and help assess potential therapeutic targets to improve lung function in premature infants.

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Nomenclature

Exp	=	expiratory trendline
H	=	hysteresis of pressure-volume curve
Insp	=	inspiratory trendline
Isovol	=	isovolumetric trendline
K_B	=	bubble pressure-volume constant
K_L	=	lung pressure-volume constant
P_B	=	bubble pressure
$P_{\text{isovol=insp}}$	=	pressure where isovolumetric pressure equals inspiratory pressure
P_L	=	lung pressure
$P_{\text{tidal vol}}$	=	pressure at tidal volume
V_B	=	change in bubble volume
V_f	=	volume of fluid addition
V_L	=	change in lung volume
δ	=	proportionality coefficient between bubble and lung

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