Lung Mechanics in Mice:

Effect of Decorin Deficiency

By Anita Fust

Department of Physiology
McGill University
Montreal, Canada

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of the degree of MSc

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CONTRIBUTIONS OF AUTHORS

This thesis is presented in manuscript-based format. Each chapter represents a manuscript. In addition to the manuscripts, it conforms to all other requirements of the "Guidelines for Thesis Preparation" as outlined by the Faculty of Graduate Studies and research.

Chapter 2 is in the process of being submitted for publication with the following title and contributing authors: "Mechanical properties of mouse distal lung: in vivo vs in vitro comparison." Anita Fust, Jason H. T. Bates, Mara S. Ludwig. Dr. Jason Bates supplied the complex signal used to make in vitro measurements of complex impedance in the mice. I was responsible for the planning and executing of all the experiments. I was also responsible for the preparation of the manuscript, being guided by Dr. Ludwig. Both Dr. Bates and Dr. Ludwig aided significantly in the critical evaluation of the results and the preparation of the manuscript.

Chapter 3 is also in the process of being submitted for publication with the following title and contributing authors: "Alterations in lung mechanics in decorin deficient mice." Anita Fust, Frederique LeBellego, Renato V. Iozzo, Peter J. Roughley, and Mara S. Ludwig. Dr. Mara Ludwig had proposed the original hypothesis of the potential contribution of proteoglycans to lung tissue viscoelasticity and thus lung mechanics. As a part of my master's thesis, I, with the supervision of Dr. Ludwig, formed objectives, formulated experimental protocol, executed experiments to completion, as well as analyzed and interpreted
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ABSTRACT

Decorin is required for the normal fibrillogenesis and spatial arrangement of collagen. As collagen is important in determining the elastic behaviour of the lung, we hypothesized that lung tissue mechanics would be altered in decorin deficient (Den-/-) mice. Complex impedance, pressure-volume curves, and length-stress curves of lung parenchyma were measured in C57BL/6 mice, 6 Den-/­ and 6 wildtype (Den+/+), both in vivo and in vitro. Immunohistochemistry and Western blotting were performed to identify decorin and biglycan in the lung tissues. In vivo, airway resistance was decreased and lung compliance was increased in Den-/­ mice. In vitro, length-stress curves showed increased compliance in the Den-/­ mice. Immunohistochemistry showed decorin staining in the airway and vessel walls of Den+/+ but not Den-/­ mice; Western blots showed that biglycan levels were not different in the Den-/­ mice. These data support a critical role for decorin in the formation of the lung collagen network. Lack of decorin alters lung tissue mechanical behaviour. Additionally, the data from Den+/+ mice were compared to those from other species, and is consistent with the evidence in the literature that mouse lungs differ structurally from other species. Finally, differences observed in vivo vs. in vitro suggest that measurements made in the strip more accurately reflect lung tissue properties.

Key words: complex impedance, tissue mechanics, hysteresivity, lung compliance
RESUMÉ

La décorine (DCN) est nécessaire pour la fibrillogenèse et l'arrangement spatial normal du collagène, composé déterminant le comportement élastique du poumon. La mécanique des tissus du poumon pourrait donc être altérée chez les souris déficientes pour la DCN (Den-/-). L'impédance complexe, les courbes pression-volume, et les courbes longueur-contrainte ont été mesurées chez des souris C57BL/6, 6 souris Den-/- et 6 souris sauvages (Den+/+), in vivo et in vitro. In vivo, la résistance des voies respiratoires est diminuée et la compliance pulmonaire est augmentée chez les souris Den-/- . In vitro, les courbes longueur-contrainte ont montrées une augmentation de la compliance pulmonaire chez les souris Den-/- . Par immunohistochimie nous avons montré la présence de la DCN dans les parois des voies aériennes et des vaisseaux sanguins; par western-immunoblot nous n'avons montré aucune différence entre les niveaux de biglycan chez les souris Dcn-/- . Ces données suggèrent que la DCN ait un rôle critique dans la formation du réseau de collagène dans le poumon. L'absence de DCN modifie le comportement mécanique des tissus du poumon. Les données des souris Den+/+ ont été comparées à d'autres espèces et sont en accord avec les données de la littérature que les poumons de souris diffèrent structurellement des autres espèces. Aussi, les différences observées in vivo vs in vitro suggèrent que les mesures faites sur une petite bande de parenchyme reflètent plus précisément les propriétés du tissu pulmonaire.

Mots clés: impédance complexe, mécanique des tissus, hysteresivity, compliance pulmonaire
CHAPTER 1: INTRODUCTION
1.1 Lung physiology

1.1.1 Anatomy

The major function of the respiratory system is to bring oxygen into the bloodstream and remove carbon dioxide. Gas exchange occurs at the alveolar or parenchymal level, the site of the blood-gas barrier. This barrier is comprised of layers – the alveolar type I and II cells, basement membrane, capillary endothelial cells, and pulmonary interstitium. The thinness of the alveolar capillary membrane allows for the ready diffusion of gases, while still acting as a barrier against the leakage of water and solutes from the capillaries into the alveolar spaces. The alveoli are lined with surfactant – a substance secreted by the alveolar type II cells that controls surface tension. The interstitium of the parenchyma is made up of extracellular matrix, which consists of collagen and elastin fibers, glycoproteins, and proteoglycans, including decorin. These components determine to a large extent the mechanical properties of the lung tissue. Abnormalities in the anatomic constituents that make up the blood-gas barrier may compromise ideal gas exchange.

1.1.2 Physiological parameters

In order for gas exchange to occur, air must move into and out of the lungs. How readily ventilation occurs will depend on the mechanical properties of the system. In disease conditions, the mechanical properties of the lungs change. For example, in emphysema, a lung disease characterized by destruction of alveolar walls, compliance of the lung is increased when compared to the
healthy lung. It is therefore extremely valuable to be able to measure the mechanical properties of the lungs and respiratory system. Pertinent variables include:

- airway resistance
- tissue damping or tissue resistance
- tissue elastance or compliance
- tissue hysteresivity

Airway resistance is a measure of the resistance to airflow in the airways. Flow through airways is essentially flow through a tube and the principles of Poiseuille apply. The Poiseuille equation describes the dynamics of laminar flow through a tube.

\[ R = \frac{\Delta P}{V} = \frac{8\eta l}{\pi r^4} \]  

(1)

where \( R \) = resistance to flow, \( \Delta P \) = pressure difference from one end of the tube to the other, \( V \) is flow through the tube, \( \eta \) is viscosity of the flowing medium, \( l \) is the length of the tube, and \( r \) is the radius of the tube. As portrayed in the equation, the resistance of the tube is inversely proportional to its radius.

\[ R \propto \frac{1}{r^4} \]  

(2)

This means that the narrower, more distal, airways will have higher resistances, while the larger airways, such as the trachea and main bronchi, will have relatively lower resistances. However, because of the branching of the airways, in
the distal lung there are many small airways in parallel. The addition of parallel airways will decrease the resistance of the system according to the equation:

\[ \frac{1}{R} = \frac{1}{R_1} + \frac{1}{R_2} + \frac{1}{R_3} + \cdots \quad (3) \]

This is in contrast to airways in series, where the resistances are additive:

\[ R = R_1 + R_2 + R_3 + \cdots \quad (4) \]

The resistance of an airway is not fixed, and can change in response to a number of factors. There are, for example, alveolar attachments around the airways that result in an interdependence between airways and parenchyma. Therefore, the stiffness of the parenchyma can influence the diameter of the airways by exerting a pull on the airways via the alveolar attachments.

*Tissue damping or tissue resistance* are terms used to reflect the resistive properties of the parenchymal tissue. The materials that comprise the tissue, such as collagen and elastin fibers and other small molecules, have intrinsic elastic and resistive properties. Tissue damping reflects how much energy is dissipated in overcoming the intrinsic resistive properties of the tissue when it is stretched. This is a dynamic property.

*Tissue compliance* is a measure of the distensibility of the lung or the ease with which the tissue can be stretched. Compliance is expressed as the change in volume for a given change in pressure. Another term used is *tissue elastance*, which is simply the inverse of compliance. Elastance, or compliance, also reflects the ability of the lung to recoil after a volume change.
Lung tissues are viscoelastic insofar as they display both elastic and resistive properties, such as hysteresis, stress-relaxation, and creep. Fredberg et al. (15) hypothesized that the dissipative and elastic properties of lung tissue are intrinsically coupled at the level of the stress-bearing element. This hypothesis led to the development of a parameter that would readily measure dissipative behaviour in relation to elastic behaviour. The parameter was called tissue hysteresivity, and was defined as the ratio of dissipated energy to the stored potential energy at maximal stretch (15). This parameter is frequency independent, decreases only slightly with operating stress, and is thought to be relatively conserved across species. Hysteresivity is altered in pathological conditions where the fibers of the extracellular matrix are affected (10).

1.2 Methods of measuring mechanics

1.2.1 Dynamic measurements

The forced oscillation technique is used to measure dynamic properties of lung (30). Dolhnikoff et al. (10) studied rat lungs using this method both in the whole lung and in the lung tissue strip. In the intact lung in vivo, this technique involves applying a sinusoidal volume to the lungs and simultaneously measuring the flow and the pressure generated in the trachea. Total lung resistance \( R_L \) and lung elastance \( E_L \) can then be calculated by fitting the equation of motion to the tracheal pressure \( P_t \):

\[
P_t = R_L \cdot V + E_L \cdot V + K \quad (5)
\]
where \( \dot{V} \) = flow in the trachea, and \( K \) is a constant reflecting positive end expiratory pressure (PEEP). As these parameters change with frequency, measurements are generally made at numerous frequencies, and the results (values of \( R_L \) and \( E_L \)) displayed as functions of frequency. The forced oscillation technique can also be used in the organ bath with a tissue strip. The tissue strip is stretched sinusoidally while the tension and length of the tissue are measured. Tissue resistance (\( R_t \)) and tissue elastance (\( E_t \)) are then determined by fitting another form of the equation of motion to the tension (\( T \)):

\[
T = E_t \Delta l + R_t \left( \frac{\Delta l}{\Delta t} \right) + K
\]  

(6)

where \( l = \) length, \( \frac{\Delta l}{\Delta t} \) is the length change per unit time, and \( K \) is a constant reflecting the operating stress. Similar to the \textit{in vivo} situation, measurements must be made at many frequencies, since both \( R_t \) and \( E_t \) vary with frequency. In the whole lung and the tissue strip, hysteresivity (\( \eta \)) can be calculated at each frequency from the values of \( R \) and \( E \):

\[
\eta = 2\pi f \left( \frac{R}{E} \right)
\]  

(7)

More recently, a method of measuring complex impedance has been used to study lung mechanics. This is also a forced oscillation technique, except that instead of applying a single frequency oscillation to the lung, a signal comprised of multiple mutually primed sinusoids ranging from about 0 to 20 Hz is applied to the airway opening. The amplitudes of the sinusoids need to decrease hyperbolically with
frequency so that each frequency component has equal power. During the application of the volume signal, pressure ($P$) and flow ($\dot{V}$) are measured at the airway opening. Complex impedance is calculated from the Fourier transforms of $P$ and $\dot{V}$:

$$Z(f) = \frac{P(f)}{\dot{V}(f)}$$  \hspace{1cm} (8)

When a complex signal is applied to the tissue strip, measurements of tension ($T$) and length ($l$) are made. In this case, complex impedance is calculated from the Fourier transforms of $T$ and $\frac{\Delta l}{\Delta t}$:

$$Z(f) = \frac{T(f)}{\frac{\Delta l}{\Delta t}(f)}$$  \hspace{1cm} (9)

One of the benefits of applying a complex signal is that the data can be gathered in a much shorter time instead of applying each frequency individually. Additionally, the constant phase model can be fit to the complex impedance data:

$$Z(f) = R_N + j(2\pi f)I + \frac{(G - jH)}{(2\pi f)^\alpha}$$  \hspace{1cm} (10)

where $\alpha = \frac{2}{\pi} \tan^{-1} \left( \frac{H}{G} \right)$  \hspace{1cm} (11)

The constant phase model, developed by Hantos et al. (19), describes the real and imaginary components of impedance in terms of parameters representing Newtonian or airway resistance ($R_N$), inertance ($I$), tissue damping ($G$) and tissue
elastance \((H)\). In this model of respiratory mechanics, \(I\) is most often negligible, as it is a parameter that reflects the viscosity of the air moving through the airways. This model allows for the partitioning of airway from tissue properties. The model is intended to fit impedance data obtained \(in vivo\), and is perhaps not the most appropriate model to describe \(in vitro\) impedance, since there is no flow \(per se\) occurring in the tissue strip. However, the model is still a valuable tool for estimating tissue mechanical parameters, and calculating hysteresivity \((\eta)\), which can easily be obtained from the values of \(G\) and \(H\):

\[
\eta = \frac{G}{H} \quad (12)
\]

### 1.2.2 Static measurements

The quasistatic pressure-volume curve is a way of measuring the static properties of the lung. A pressure-volume curve is made by slowly inflating the lungs from zero pressure to total lung capacity and then slowly deflating the lungs. During the inflation and deflation, pressure \((P)\) and volume \((V)\) in the lung are recorded. In order to get an accurate reflection of the static properties (i.e. compliance) of the lung, the inflation and/or deflation must be done slowly enough to allow for stress-adaptation. This can be done by increasing or decreasing the volume in a step-wise fashion. The Salazar-Knowles equation can be fit to the recorded values of pressure and volume \((45)\):

\[
V = A - Be^{-kp} \quad (13)
\]
where $A$ and $B$ are constants, and $\kappa$ is a constant describing the shape of the curve. We can determine the static compliance ($C_L$) of the lungs from the relationship between $P$ and $V$:

$$C_L = \frac{\Delta V}{\Delta P} \quad (14)$$

The shape of the curve, as described by the parameter $\kappa$ in the equation, can change depending on the properties of the lung. For example, emphysematous lungs have been shown to have different $\kappa$ values from normal lungs (11, 12, 31). Also, the inflation curve is generally different from the deflation curve, and the difference between these curves is referred to as hysteresis. Hysteresis can be explained by the surface tension at the air-liquid interface of the alveoli, by airway and alveolar recruitment, and by volume-induced changes in the mechanical behaviour of the tissues themselves (plasticity).

In order to assess the static properties of the tissue strip, the relationship between tension ($T$) and length ($l$) is measured. As in the whole lung, the strip is slowly stretched from zero to maximal strain, and then slowly allowed to relax in a step-wise fashion so as to allow stress-adaptation at each point along the curve. The $T$ and $l$ data can be fit by a five-parameter exponential equation:

$$l = l_o + a\left(1 - e^{-bT}\right) + c\left(1 - e^{-dT}\right) \quad (15)$$

where $l_o$ is the length of the strip at $T=0$, and $a, b, c,$ and $d$ are constants describing the shape of the curve. The compliance of the strip ($C_S$) can be calculated as follows:
1.3 Structural determinants

1.3.1 Airways

Airways contribute to the overall mechanical properties of the lung by providing a resistance to airflow. The airways are made up of an inner wall, a smooth muscle layer, and an outer wall. The inner wall consists of the surface lining liquid, the epithelial cells, basement membrane, lamina propria, and the submucosa, which consists of vessels, loose connective tissue, and mucous glands. The smooth muscle layer is interspersed with fibrous connective tissue bands. The outer wall is the adventitia, consisting of loose connective tissue and vessels. Narrowing of the airways increases the resistance of the respiratory system. There are many factors involved in determining the luminal diameter of the airways. Thickening of the inner or outer wall or the smooth muscle layer will increase the resistance of the airways. Remodelling of the airways, which may include hypertrophy and hyperplasia of the smooth muscle cells, increased deposition of extracellular matrix, alterations in the mucous secreting glands, and other changes, can all cause narrowing of the lumen and thus increase resistance. Additionally, because of airway-parenchymal interdependence, the mechanical state of the parenchyma can influence the diameter of the airways. The effect of this interdependence may be decreased when the adventitia, or outer wall, is thickened. Remodelling occurs in several pathological conditions involving the airways, including asthma and chronic obstructive pulmonary disease (COPD).
asthmatics, airways are hyperresponsive, the smooth muscle layer thickens, and there are also changes in proteoglycan deposition (21). The remodelling seen in these pathological conditions naturally leads to changes in lung mechanics, and many studies have observed and recorded the changes and their effects (39).

1.3.2 Air-liquid interface

The alveoli of the lung are lined with surfactant, a phospholipid substance that reduces the surface tension of the alveolus. Surfactant has the characteristic quality of altering surface tension with changes in surface area. This permits the air space to open more readily during inspiration, and prevents alveolar collapse at end expiration. Surfactant, however, does not account completely for pressure-volume hysteresis. Parenchymal tissues also contribute. When the air-liquid interface is abolished, such as occurs when the lungs are fluid-filled, hysteresis is substantially reduced (40).

1.3.3 Extracellular matrix

The extracellular matrix is the key determinant of the biomechanical properties of most tissues, including the lung. The extracellular matrix is made up of collagen and elastin fibres, as well as proteoglycans and glycoproteins, which represent the ‘ground substance’ between the fibres. The matrix is a viscoelastic material that displays both dissipative and elastic properties. Several explanations exist for the dissipative behaviour of the lung tissue. Collagen and elastin are fibers that are intrinsically elastic. Isolated elastin and collagen fibers display
negligible hysteresivity, but when these fibers are arranged in a network as they are in the lung parenchyma, hysteresivity may be increased (7). A further hypothesis is that energy loss occurs when friction is generated at the surfaces of fibers sliding past each other (36). Ground substance also contributes to the viscoelastic nature of lung tissue. Mijailovich et al. (35) compared the hysteresivity of pigeon ligamentum propatagiale, a material consisting primarily of elastin fibers, to that of rabbit lung parenchymal tissue, which, in addition to elastin and collagen fibers, also contains ground substance. They found 10-20 fold higher hysteresivity in the lung parenchyma as compared to the ligamentum propatagiale. Suki et al. (51) have proposed that the matrix can be modelled as a polymer-like material in which molecules deform and thereby generate a strain. This strain causes conformational changes that result in energy dissipation or viscoelastic behaviour. Another study showed that, although the connective tissue network dominates parenchymal mechanics in the intact lung, mechanics can be influenced by the tone or contraction of interstitial cells (58). Navajas et al. (38) suggested that one third of the dissipative behaviour observed during the cycling of dog parenchymal lung strips is attributable to plasticity while the other two thirds can be accounted for by tissue viscoelasticity.

The proteoglycans found in the matrix may also contribute to the viscoelastic nature of the tissue. There are four different classes of proteoglycans (PGs): the large aggregating PGs, basement membrane PGs, cell associated PGs, and small leucine rich PGs (SLRPs). Proteoglycans are a family of molecules consisting of a protein core and glycosaminoglycan (GAG) sidechains. The GAG
sidechains have high ionic charges and are therefore hydrophilic. Their ability to attract water molecules into the matrix can alter tissue turgor and viscoelasticity. Removal of these sidechains with chondroitinase and heparitinase results in altered tissue resistance and hysteresivity (3). SLRPs are a class of PGs that are characterized by leucine-rich repeat regions in the core protein. Generally multifunctional, many of these SLRPs interact with fibrillar collagen, growth factors, and may be involved in regulatory pathways of cell growth and proliferation. Decorin and biglycan belong to the Class I subfamily of SLRPs, grouped in this way because of the similarity in the sequence of their core protein and the type of sidechains (22). They both have dermatan or chondroitin sulfate sidechains; while decorin only has one sidechain, biglycan has two. Other SLRPs include fibromodulin and lumican of Class II, and epiphycan of Class III.

1.3.4 Decorin

Decorin is a SLRP that can bind collagen and regulate collagen fibrillogenesis and assembly in the matrix (22). The protein core of decorin binds collagen, while the GAG sidechains maintain inter-fibrillar distance. This interaction of decorin with collagen has the potential to affect tissue resistance and hysteresivity, either because of the intrinsic mechanical properties of the molecule, and/or its effect on the collagen fiber network. Decorin also has the ability to bind TGF-β (23, 57), a cytokine that has been implicated in pathologic processes characterized by remodelling (37, 56). Decorin is also involved in the signalling pathways regulating cell growth and proliferation (48, 53). Through
these latter interactions, decorin may also be involved in determining tissue viscoelasticity in a more indirect way. In lung fibrosis, a pathological condition involving increased amounts of TGF-β, the deposition of extracellular matrix is also increased. There are marked differences in the proteoglycan composition of these tissues, resulting in altered lung mechanical properties (10, 55).

Decorin has been shown to be present in tissues with fibrillar collagen, such as the lung (6, 41, 42). Small quantities of decorin have been found in the submucosal regions of the airway and vessel walls of the lung (41, 42). Decorin may also play an important role in the remodelling associated with such respiratory diseases as pulmonary fibrosis and asthma (6, 41). The process of remodelling involves the deposition of new matrix, including decorin regulated collagen fibrillogenesis.

Danielson et al. (9) created a genetically modified mouse in which the decorin gene is disrupted, resulting in decorin deficiency. Electron microscopic images of cross-sections from the skin and tendon of decorin deficient mice show differences in the uniformity and arrangement of collagen fibrils in the matrix. Decorin deficient mice have collagen fibres that vary greatly in terms of shape and size. In addition, fibres are arranged relatively randomly in the matrix, whereas wildtype mice have uniform collagen fibers that are packed in an orderly fashion. In addition to the morphological findings, these authors showed increased skin fragility and markedly reduced tensile strength in the decorin deficient mice. Bone tissue is also affected by decorin deficiency; the collagen
fibrils of the matrix are smaller compared to wildtype (8). To date, no studies have examined the effect of decorin deficiency on the lungs.

In addition to studies in decorin deficient animals, several groups have studied the effects of decorin overexpression, both in vivo and in cell culture. Overexpression of decorin differentially affects matrix metalloproteinases and tissue inhibitors of metalloproteinases (2). This suggests that decorin might play an important role in balancing the synthesis and degradation of the extracellular matrix. A study in which the decorin gene was overexpressed in airway epithelium showed that exogenous TGF-β-induced inhibition of lung growth was abolished after epithelial transfer of the decorin gene (60). This finding suggests that decorin can antagonize bioactive TGF-β during lung growth and differentiation. TGF-β is also a key cytokine in the pathogenesis of pulmonary fibrosis. Mice in which TGF-β was overexpressed developed marked lung fibrosis. However, when decorin was concomitantly overexpressed, fibrosis was significantly reduced (23). Decorin overexpression has been shown to increase the protein levels as well as expression of p21 and p27, inhibitors of cyclin-dependent kinases (48). Finally, overexpression of decorin has also been shown to inhibit tumor growth via the p21 pathway (53). Hence, not only is decorin able to bind and regulate extracellular matrix molecules such as collagen, it is also in the signalling pathways involved in cell growth and proliferation.
CHAPTER 2: MANUSCRIPT No. 1 –

“MECHANICAL PROPERTIES OF MOUSE DISTAL LUNG: IN VIVO VS IN VITRO COMPARISON”
2.1 Introduction to Manuscript No. 1

This study was done in order to further characterize mouse respiratory system mechanics, which has become especially pertinent of late as mice are a valuable tool for genetic studies. Gene overexpression or knockout is easily done in the mouse genome. Therefore, it is important to have a good understanding of the mechanical behaviour of the mouse respiratory system and the parenchymal tissues. Much of the literature concerning lung mechanics reports studies done in dogs, rats or guinea pigs. There is evidence to suggest that there are differences between the species – both anatomical and mechanical. Therefore, we studied mouse lungs in order to better characterize the mechanical properties of their lungs. We studied the dynamic and static properties of the respiratory system using a small animal ventilator. By measuring complex impedance and the pressure-volume curve, we were able to calculate dynamic tissue resistance and elastance, airway resistance and hysteresivity, as well as static compliance. We also studied the dynamic and static properties of the mouse parenchymal strip by measuring complex impedance and stress-length curves. To date, there is no report in the literature of such measurements being made in mice. With this study we show that it is possible to make measurements of complex impedance in mouse lung strips. Also, we show the importance of describing the lung mechanical behaviour of species separately, since there are differences in the mechanical properties of the lungs across species. Finally, we show the value of making measurements in strips, in addition to making measurements in the \textit{in vivo} lung, since the data obtained from the strips is different from that obtained \textit{in vivo}.
and is a more direct reflection of the viscoelastic properties of the parenchymal tissue itself.
2.2 Abstract

While measurements of tissue mechanics have been made in several species, relatively little has been reported in the mouse. Therefore, we measured complex impedance in C57BL/6 mice. Mice were anesthetized, intubated and connected to a small animal ventilator and impedance was measured by applying a volume signal with multiple frequencies to the airway opening. A constant phase model was applied to changes in volume ($V$) and pressure ($P$) to calculate Newtonian resistance ($R$), tissue damping ($G$) and tissue elastance ($H$). Hysteresivity ($\eta$) was calculated as $G/H$. Quasistatic $PV$ curves were also obtained. Lungs were then excised and tissue strips mounted in the organ bath for in vitro measurements of complex impedance and quasistatic stress-strain curves. Values of $\eta$ were significantly higher in vivo than in vitro ($0.115 \pm 0.017$ vs. $0.043 \pm 0.008$). The higher values of $\eta$ in vivo may represent the effects of airway heterogeneities, the air-liquid interface, or changes in alveolar geometry. Measurement of mechanics in the tissue strip offers a better assessment of pure tissue properties.

Key words: complex impedance, tissue mechanics, hysteresivity, lung compliance
2.3 Introduction

Lung tissue mechanical behaviour has been characterized in several different species, both in vivo and in vitro (5, 25). However, relatively little is known about the mechanical properties of mouse lungs. Mice represent a valuable tool for genetic studies as their genome is well described. Therefore, establishing appropriate methodologies for measuring mechanical behaviour in mice is especially pertinent. Mouse lung architecture is known to be different from that of other species (13, 34, 54). Proportional to body weight, the airways of mice have larger diameters and end rather abruptly as they reach the terminal alveoli (54). In addition, there is evidence in the literature that the components of the elastic fiber system are different in mice as compared to other rodents (13). Therefore, one would expect mouse lungs to display different mechanical properties compared to other species. Recently, a comparative study was done in small rodents in which the complex mechanical input impedance of the lung was measured in vivo (17). These data showed that mice have relatively lower airway resistance ($R$), higher tissue elastance ($H$), and lower hysteresivity ($\eta$) than rats, guinea pigs and rabbits.

The measurement of complex input impedance represents a significant improvement over resistance and elastance at a single frequency for the characterization and partitioning of lung mechanics. However, impedance measurements cannot entirely distinguish between effects due to airway heterogeneities and changes in intrinsic tissue viscoelasticity (29). Therefore, in order to better describe peripheral lung mechanics, we measured complex
impedance and quasi-static pressure-volume curves in vivo, and complex impedance and quasistatic stress-strain curves in tissue strips in vitro. In the in vitro experimental condition, the air-liquid interface is removed so the measured stress-strain relationships reflect only the intrinsic tissue properties.

The parenchymal strip consists primarily of lung alveolar tissue with a small proportion of airways and vessels (46). The parenchyma demonstrates viscoelastic behaviour, which contributes significantly to the overall mechanical properties of the lung (15, 19, 27). Lung parenchymal strips have been used to study peripheral lung mechanical and pharmacological behaviour (47, 59). While parenchymal strips have been studied in a number of species, to our knowledge there are no data currently available in strips from mice. We hypothesized that mouse lung mechanical properties would be different from those reported in other species.

The mechanical properties of parenchymal strips have generally been assessed in terms of resistance and elastance at a single frequency of oscillation (47). In order to accurately describe the mechanical behaviour of the lung tissue using this approach, measurements must be repeated at multiple frequencies since $R$ and $E$ have been shown to depend markedly on frequency (3, 15, 19, 28, 51). However, this assumes that the mechanical properties of the tissues do not change over the entire course of the experiment, which may be extensive. By applying many oscillation frequencies at the same time and using Fourier transform methods to reconstruct impedance at each frequency separately (19), the time of
experimentation may be greatly reduced, thereby minimizing the likelihood of
plastic changes taking place within the tissue.

Therefore, we measured the complex impedance of mouse lungs in vivo, and of mouse parenchymal strips in vitro. In addition, we characterized the quasi-static pressure-volume relationship of the lungs and the stress-strain curve of the strips. Our goal was to establish how the intrinsic mechanical properties of the lung parenchyma are reflected in the overall properties of the lung in a species that is currently of central importance in respiratory research.

2.4 Methods

2.4.1 In vivo

Animal preparation

Male and Female C57BL/6 mice (aged 7 weeks old) were obtained from Charles River (St. Constant, PQ) and housed in a regular animal facility at McGill University (Montreal, PQ). Experiments were performed when animals were 8-10 weeks old. Each mouse was anesthetized with an injection of xylazine (12 mg/kg i.p.) followed five minutes later by an injection of sodium pentobarbital (40 mg/kg i.p.). After tracheostomy, an 18-gauge metal cannula was inserted into the trachea and tightly bound. The mouse was connected via the tracheal cannula to a computer-controlled small-animal ventilator (flexiVent, Montreal, Canada; (49)). Mice were mechanically ventilated at 150 breaths/min with a tidal volume (Vt) of 6ml/kg at a positive end expiratory pressure (PEEP) of 1.5 cm H₂O. The animals were paralyzed with an injection of pancuronium bromide (1.2 mg/kg
Heart rate was monitored by a 3 lead EKG. All animals received humane care in compliance with the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council of Animal Care, and an institutional animal ethics committee approved the protocol.

**Measurement of complex impedance**

A computer generated volume signal comprised of 19 mutually primed sinusoids ranging from 0.25 to 19.625 Hz was applied to the airway opening. The amplitudes of the sinusoids decreased hyperbolically with frequency such that each frequency component had equal power. The signal had a peak-to-peak volume of 0.17ml and lasted 16 seconds. The piston displacement (ml) and cylinder pressure (cm H\textsubscript{2}O) of the ventilator were measured during the application of the signal. A total lung capacity (TLC) manoeuvre was performed to standardize volume history. One minute later, the complex input impedance of the respiratory system (Z) was measured. Measurements were repeated three or four times for each mouse and the results averaged.

**Calculation of parameters**

Z is defined by the equation:

$$Z(\omega) = \frac{P(\omega)}{dV(\omega)/dt}$$  \hspace{1cm} (1)

where $P$ is the Fourier transform of pressure (cm H\textsubscript{2}O) at the airway opening and $V$ is the transform of volume (ml) applied to the lungs. Both are functions of angular frequency ($\omega$). Z was determined using cross- and auto-power spectra of
$P$ and $V$, together with a correction for the physical properties of the mechanical ventilator, as described previously (16, 17, 20). $Z$ was fit with the constant-phase model (19):

$$Z(\omega) = R + j\omega I + (G - jH)/\omega^\alpha$$

(2)

where $R$ approximates airway resistance, $I$ is inertance of the gas in the central airways, $G$ is tissue damping, $H$ is tissue elastance, $j$ is the imaginary unit, and $\alpha = (2/\pi)\tan^{-1}(H/G)$. Hysteresivity ($\eta$), previously defined by Fredberg et al. (15) as a dimensionless variable coupling dissipative and elastic behaviour, was calculated as:

$$\eta = G/H$$

(3)

**Measurement of Pressure-Volume curve**

Following the complex impedance measurements, $P$ and $V$ measurements were made during quasistatic deflation of the lung. The lungs were slowly inflated to total lung capacity (TLC), and then deflated step-wise over 18 seconds. This procedure was applied three times and the data averaged to make a single $P$-$V$ curve.

The $P$-$V$ curve was characterized by the equation (45):

$$V = A - Be^{-\kappa P}$$

(4)

The value of $\kappa$ was determined for each animal. Static compliance of the lung ($C_L$) was calculated between 5 and 10 cm H$_2$O as $\Delta V/\Delta P$. 

33
2.4.2 In vitro

Tissue preparation

Mice were disconnected from the small animal ventilator after the in vivo experiments were completed. In vitro methodology is similar to that used previously by Al Jamal et al. (3). Through an abdominal incision, the diaphragm was cut and a bilateral pneumothorax was induced. The thorax was opened, the animal exsanguinated, and the heart, lungs and trachea were carefully resected en bloc ensuring that the lungs were not punctured. The lungs were rinsed with a modified Krebs solution (mM: NaCl 118, KCl 4.5, NaHCO₃ 25.5, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 10; Sigma, St. Louis, MO; pH=7.40, 6°C) by filling the lungs three times to TLC. A strip (1 x 1 x 6-7 mm) was cut from the periphery of the left lobe. The pleura was removed and the unloaded length \( l_o \) and wet weight \( W_o \) were recorded. The strips were kept in a bath of iced Krebs solution which was continuously bubbled with 95% O₂ / 5% CO₂.

Experimental Setup

Metal clips were glued to either end of the tissue strip with cyanoacrylate. Steel music wires (0.5 mm diam) were attached to the clips and the strip suspended vertically in an organ bath. A mercury bead was placed in the bottom of the organ bath, allowing the wire to pass through the bath, but preventing the Krebs solution from leaking out. The bath was filled with 20 ml of Krebs solution, maintained at 37°C and continuously bubbled with 95%O₂/5%CO₂. One end of
the strip was attached to a force transducer (model 400A, Cambridge Technologies, Watertown, MA, USA) which had an operating range of ±10 g, resolution of ±200 μg and compliance of 1 μm/g, while the other end was connected to a servo-controlled lever arm (model 300B, Cambridge Technologies, Watertown, MA, USA), which delivered length perturbations to the mounted strip. The lever arm was capable of peak to peak length excursions of 8 mm and length resolution of 1 μm and was in turn connected to a computer, which controlled the frequency, amplitude and waveform of the oscillation. Movement of a screw thumb wheel system, which effected slow vertical displacements of the force transducer, set the resting tension (T). Length and force signals, as obtained by the lever arm and force transducer respectively, were converted from analog to digital with an analog to digital converter (DT2801-A, Data Translation Inc., Marlborough, MA) low pass filtered and recorded on an A/T compatible computer at a sampling frequency of 256 Hz.

Measurement of complex impedance

To measure the complex impedance (Zs) of the tissue strips, an 8 second broad-band pseudorandom displacement input signal composed of 17 mutually primed frequencies ranging from 0.5 to 19.75 Hz was generated by a computer and delivered to the lever arm. The linearity and hysteresis of the system were tested by measuring the moduli of a steel spring of stiffness comparable with that of the tissue strip. The spring was suspended in the bath by music wire in the same manner as the strip. The frequency and amplitude dependence of the system
were assessed over a range of frequencies (0.1-10 Hz). The spring stiffness did not show any dependence upon oscillatory frequency below 5 Hz. The hysteresivity of the system was independent of frequency and had a value <0.003.

Lung parenchymal strips were preconditioned by slowly cycling tension from 0 to 2 grams three times; on the third cycle, the strip was unloaded to a stress of approximately 500 mg/mm² and allowed to stabilize for 45 minutes at the end of which time the stress was approximately 450 mg/mm². Zs was then measured. Measurements consisted of eight consecutive eight-second recordings; the average of these eight recordings was calculated.

**Calculation of parameters**

Zs is defined as:

\[
Zs(\omega) = \frac{T(\omega)}{dL(\omega)/dt}
\]  

(5)

where \( T = \) tension in mg, and \( L = \) length in mm. \( T \) and \( L \) signals were recorded as functions of time, and the mean cross-power (\( T-L \)) and auto-power (\( L-L \)) spectra calculated by Fourier transformation of 4 s data windows overlapping by 50%. Zs was calculated as the ratio of the averaged spectra. Mechanical parameters were estimated by fitting the constant phase model (19) to Zs according to equation 2. In this instance, \( R \) reflects the Newtonian resistance of the tissues. Results were standardized for strip size by multiplying the values of \( G \) and \( H \) by \( l_o/A_o \). \( A_o \) is the unstressed cross sectional area in mm² of the lung parenchymal strip obtained from the formula:

\[
A_o = W_o/(\rho \times l_o)
\]  

(6)
where $W_0$ is the weight of the strip in mg, $\rho$ is the mass density of the tissue taken as 1.06 mg/mm$^3$, and $l_o$ is the resting length of the strip in mm. Hysteresivity, $\eta$, defined above, was calculated using equation 3.

**Measurement of Stress-Strain curves**

When the complex impedance measurements were completed, the strip was manually stretched to approximately 3g tension and then relaxed in a stepwise fashion, slowly returning to zero stress. This generated a length-tension curve, which was then converted to a stress-strain curve, with stress ($\sigma$) calculated as $T/A_o$ and strain ($\varepsilon$) calculated as $(l-l_o)/l_o$. In order to obtain a mean value for compliance, the data points were fit with a five-parameter exponential curve:

$$l = l_o + a\left(1 - e^{-bT}\right) + c\left(1 - e^{-dT}\right)$$

This model was chosen to fit the data because the Salazar-Knowles model (equation 4) yielded a poor fit, whereas the above equation minimized the residual sum of the squares. Static compliance of the strip ($C_s$) was calculated as $\Delta l/\Delta \sigma$ between 500 and 1000 mg/mm$^2$.

All *in vivo* data manipulations were performed with flexiVent Software (Scireq, Montreal, Quebec), while all *in vitro* data manipulations were performed with the ANADAT software package (RHT-InfoDat, Montreal, Quebec). T-tests were used to assess whether $\eta$ was different *in vivo* than *in vitro*. Results were considered statistically significant at a probability level of 5%. Values are reported as mean ± standard error (SE).
2.5 Results

Data of length and force as recorded during the application of the complex signal are shown in Figure 1. Representative plots of Z and Zs are shown in Figure 2, together with their fits provided by Eq. 2. A good fit was considered to have a standard deviation of the model fit of less than 3. Complex impedance is plotted as a function of frequency, where the real part represents resistance and the imaginary part represents reactance. Both Z and Zs are dependent on frequency. The values of the parameters R, G, H, and η, in vivo and in vitro, are shown in Table 1. In vivo, G is approximately 10% of the value of H while in vitro, G is approximately 5% of H. As a consequence, η is greater in vivo than in vitro (p<0.01).

$P-V$ curves obtained in vivo, and $σ-ε$ curves obtained in vitro from all animals are shown in Figure 3. Both in vivo and in vitro curves show the typical curvilinear pattern. The average values for $k$ and $C$ are shown in Table 2.

2.6 Discussion

This study shows that the dynamic mechanical behaviour of mouse lungs is different in vivo compared to in vitro. This difference is reflected in the values of η, a dimensionless index that affords a particularly robust way to describe lung tissue behaviour because it varies minimally with oscillation amplitude, frequency, volume history, or across species (15). A second finding of our study is that mouse lung in vitro mechanical behaviour is different than that described in
other rodents (Table 3), as has been previously shown for in vivo behaviour (17). This may be accounted for by structural differences between species (13, 34).

Certain technical issues warrant discussion. In vivo experiments were performed on closed-chested animals. Therefore, the mechanical properties that were measured (R, G and H) reflect not only that of the lung, but the entire respiratory system. G and H have been shown to be affected by the presence of a chest wall; both G and H were reported to be higher in the closed-chested than in the open-chested animal (20, 44). Sly et al. (50) also report an effect of the chest wall on R_w, G, and η, but not on H. However, η remains relatively unchanged between the open and closed chest conditions at low PEEP, a condition of the current experiment. Since the value of η is not affected by inclusion of the chest wall at low PEEP, we can consider it to reflect the properties of the lung. The chest wall has been shown to contribute to the shape of the pressure-volume curve at lower lung volumes (1). In order to analyze the pressure-volume curves, we fit the Salazar-Knowles equation (45) to the data points above FRC, thereby minimizing the contribution of the chest wall to the shape of the PV curve.

The parenchymal strip includes some proportion of airways. Faffe et al. (13) characterized the proportion of anatomic constituents of the mouse parenchymal strip. They reported that the strip consisted of 2.5% bronchial wall, 2.3% blood vessel wall, and 95% alveolar wall. Previous data in rats from our laboratory, in which the anatomic constituents of peripheral and more proximal parenchymal strips were sampled, showed that baseline mechanics were unchanged despite a variation in the proportion of small airways from 6-20% of
the sample (46). Hence, in a strip with only 2.5% bronchial wall, such as has
been measured in the mouse, the presence of airways are unlikely to unduly
influence the assessment of tissue mechanics. Therefore, we felt we could use the
mouse lung tissue strips as a fair representation of the parenchymal tissues.

In preliminary experiments, we determined that an operating stress of 500
mg/mm² was required in order to record an adequate impedance measurement for
each frequency component. This *in vitro* stress is likely higher than the
respective *in vivo* operating pressure of 1.5 cmH₂O. As has been shown by
Navajas et al. (38), η decreases slightly as operating stress increases. These
authors made measurements at three different operating stresses, 0.60, 1.05 and
2.00 kPa. The value of η decreased by approximately 10 % over this range of
stress. We measured almost a three-fold variation in η in the *in vivo* vs. *in vitro*
situation. Therefore, while some of the decrease in η might be due to the
comparatively higher operating stress *in vitro*, the magnitude of change cannot be
accounted for by this mechanism alone.

One major finding of our experiment is that η measured *in vitro* was less
than that measured *in vivo*. There are several possible explanations for this
observation. The absence of surface tension in the *in vitro* condition may
contribute to η, as surfactant displays hysteretic properties. Fluid filling removes
the air-liquid interface normally found at the alveolar level. Studies in isolated
saline-filled lungs demonstrate that the hysteresis of the quasistatic PV loop
decreased dramatically compared to that of air-filled lungs (40). However, a
study comparing isolated lung with an intact air-liquid interface with that of fluid
filled lung tissue strips showed nearly identical values of $\eta$ (44). Therefore, surfactant is unlikely to account for all of the difference between the *in vivo* and *in vitro* states.

Another factor that may contribute to $\eta$ *in vivo* is the heterogeneity of the airway and respiratory system. There is some debate as to the degree of influence that airway heterogeneities have on the measured values of lung tissue properties. Lutchen *et al.* (28) initially suggested that in a moderately constricted, inhomogeneous lung, an input impedance from 0.1 to 5 Hz could be used to provide a reasonable separation of airway and tissue properties. After methacholine-induced constriction, increased airway heterogeneities largely accounted for the observed increase in tissue resistance, and the altered frequency dependence of lung elastance (29). A more recent study by the same group (44) proposed that heterogeneities of the lung even at baseline, might be important in determining tissue damping. Lung mechanics were measured in heterogeneous and homogeneous conditions. Higher values for $\eta$ were found in the more heterogeneous conditions. Additionally, a model that incorporated branching asymmetry best fit the data in the heterogeneous conditions. Hence, the authors suggested that compartment-like heterogeneities of the lung significantly increased tissue damping and $\eta$. The extent to which branching asymmetry contributes to respiratory mechanics has been studied in detail by Gomes *et al.* (16). They found a higher value of $\eta$ in the giant pouched rat, which displayed a greater degree of asymmetry, than in the harvest mouse. In our study, we compared closed chested lung mechanics with the mechanics of the tissue strip.
The strip, being primarily composed of parenchyma, has few of the heterogeneities that are present in the closed-chested system. Our observation of higher $\eta$ values in vivo is in accordance with this analysis.

Hysteresivity has been defined as the ratio of the energy dissipated per cycle to the stored potential energy at maximum stretch (15). The anatomic constituents that contribute to $\eta$ are not well defined. One theory to account for this energy dissipation is that of fiber interaction kinetics (36). Mijailovich et al. (35) showed that $\eta$ of the parenchymal strip was 20-fold greater than that of isolated collagen and elastin fibers. They suggested that the frictional forces that acted at the surface of the fibers of the parenchymal network as they slid past each other accounted for the increased $\eta$ in the intact strip. Another explanation has been proposed by Suki et al. (51) who suggested that the matrix can be modelled as a polymer-like material in which the molecules generate a strain as they deform. The conformational changes that occur result in energy dissipation or viscoelastic behaviour.

Mechanics in the mouse compared with that in other species is shown in Table 3. In vivo, $H$ is higher in mice than in other species; $\eta$ is lower. Our data are similar to what was reported by Gomes et al. (17) who measured $Z$ in vivo in mice, rats, guinea pigs, and rabbits. In mice $R$ was lower, $H$ was higher, and $G$ was relatively similar, resulting in lower $\eta$ as compared to other species. In the current experiment, in vitro measurements showed that $G$ and $H$ were higher in mice than values reported in other species in the literature; $\eta$ was again lower. A similar observation was made by Faffe et al. (13) who measured resistance and
elastance at a single frequency in rat and mouse lung tissue strips. These authors reported higher $\eta$ in rats compared to mice. Our data confirm that mouse lung properties are substantially different from those reported in other species, both in vivo and in vitro.

One potential explanation to account for the species dependant differences in mechanical properties of lung tissue relates to differences in lung architecture (13, 16, 34, 54). Mercer and colleagues (34) compared the relative amounts of collagen and elastin fibers in the alveolar entrance ring, as well as other components of the alveolar interstitial spaces in a number of species, including mice, rats, and humans. They found substantial variations across species. They also measured thickness of the alveolar septum and again found species related variations. Faffe et al. compared the composition of lung tissue strips in rats and mice (13). In contrast to the findings of Mercer et al. (34), these authors found that the collagen and total elastic fiber content was not different in mice compared to rats. However, the specific components of the elastic system varied. They found that mouse lung strips had fewer fully developed elastin fibers and more oxytalan, a component of the elastic fiber system, than rat lung strips (13). These differences may play a role in the observed differences in $G$, $H$ and $\eta$ of mice compared to other species. Further, mice have proportionately larger airways that end abruptly, instead of tapering off as in other species (54). Mice also display more symmetry in the branching of their airway tree (16). As discussed above, these differences in airway geometry could affect the measurement of in vivo tissue properties.
In conclusion, we have shown that complex impedance is readily obtainable in mice. Parenchymal strips provide a truer measure of actual tissue mechanical properties. The different mechanical behaviour of lung tissue of mice compared with that of other species may be due to differences in branching patterns, differences in the relative sizes of airways and alveoli, as well as differences in the protein and cellular composition of the parenchymal tissue.

2.7 Acknowledgements

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2.8 Figure legends

Figure 1. Representative tracings of the length and force signals vs. time during the application of the complex signal to the tissue strip in vitro.

Figure 2. Representative plots of complex impedance (Z) vs. frequency (f), in vivo (A) and in vitro (B). The real component of Z represents resistance (positive values of Z) and the imaginary component represents reactance (negative values of Z).

Figure 3. A; pressure-volume curves in vivo. The model $V = A - Be^{kP}$ was fit to the data to generate the individual pressure-volume curves. B; stress-strain curves in vitro. A five-parameter exponential curve ($l = l_o + a(1 - e^{-bT}) + c(1 - e^{-dT})$) was fit to the data to generate individual stress-strain curves.
<table>
<thead>
<tr>
<th></th>
<th>$R$</th>
<th>$G$</th>
<th>$H$</th>
<th>$\eta$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo</strong></td>
<td>0.425 ± 0.038 cm H$_2$O·s/ml</td>
<td>3.51 ± 0.25 cm H$_2$O/ml</td>
<td>31.61 ± 2.02 cm H$_2$O/ml</td>
<td>0.111 ± 0.004</td>
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<tr>
<td><strong>In vitro</strong></td>
<td>12.76 ± 0.82 mg·s/mm²</td>
<td>187.8 ± 15.5 mg/mm²</td>
<td>4496 ± 226 mg/mm²</td>
<td>0.042 ± 0.003</td>
</tr>
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$R$, Newtonian resistance; $G$, tissue damping; $H$, tissue elastance; $\eta$, hysteresivity
### Table 2 Quasi-static mechanics *in vivo* and *in vitro* (Mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>$\kappa$</th>
<th>$C$</th>
</tr>
</thead>
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<tr>
<td><em>In vivo</em></td>
<td>0.118 ± 0.004</td>
<td>0.057 ± 0.002 ml/cm H$_2$O</td>
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<tr>
<td><em>In vitro</em></td>
<td>0.0009 ± 0.001 mm/(mg/mm$^2$)</td>
<td>0.0001</td>
</tr>
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</table>

$k$, exponential constant describing the shape of the curve: $V = A - Be^{-kp}$; $C$, compliance.
Table 3  Lung mechanics in rodents *in vivo* and *in vitro*

*In vivo*

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>$G$ (cmH$_2$O/ml)</th>
<th>$H$ (cmH$_2$O/ml)</th>
<th>$\eta$</th>
<th>PEEP (cmH$_2$O)</th>
<th>$\kappa$</th>
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<td>2.0</td>
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<td>0.23</td>
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<td>3.6</td>
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<td>0.15</td>
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<td>32</td>
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*In vitro*

<table>
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<th>Reference</th>
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<th>$G$ (mg/mm$^2$)</th>
<th>$H$ (mg/mm$^2$)</th>
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<th>Operating stress</th>
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<td>50 mg/mm$^2$</td>
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<td>Guinea Pig</td>
<td>72</td>
<td>680</td>
<td>0.11</td>
<td>50 mg/mm$^2$</td>
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<tr>
<td>Sakai <em>et al.</em> (44)</td>
<td>Rat</td>
<td>0.3</td>
<td>3.0</td>
<td>0.09</td>
<td>100 mg/mm$^2$</td>
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<tr>
<td>Faffe <em>et al.</em> (13)</td>
<td>Rat</td>
<td></td>
<td></td>
<td>0.08</td>
<td>100 mg/mm$^2$</td>
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<tr>
<td>Faffe <em>et al.</em> (13)</td>
<td>Mouse</td>
<td></td>
<td></td>
<td>0.06</td>
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</tr>
<tr>
<td>Fust <em>et al.</em></td>
<td>Mouse</td>
<td>190</td>
<td>4500</td>
<td>0.04</td>
<td>500 mg/mm$^2$</td>
</tr>
</tbody>
</table>

$G$, tissue damping; $H$, tissue elastance; $\eta$, hysteresivity; $\kappa$, exponential constant
Figure 1

Length (mm)

Force (mg)

Time (s)
Figure 2

A

$Z$ (cm H$_2$O·s/ml)

B

$Z$ (mg·s/mm)

$f$ (Hz)
Figure 3

A

Volume (ml) vs. Pressure (cm H_2O)

B

Strain vs. Stress (mg/mm^2)
CHAPTER 3: MANUSCRIPT No. 2 –

“ALTERATIONS IN LUNG MECHANICS IN DECORIN DEFICIENT MICE”
3.1 Introduction to Manuscript No. 2

The purpose of the following study was to determine the effects of decorin deficiency on mouse lung mechanics. The results of this study will increase our understanding of the determinants of lung tissue viscoelasticity and lung mechanics in general. Decorin may affect lung mechanics via different mechanisms: directly through the intrinsic properties of the molecule itself, or indirectly through its interactions with collagen and TGF-β. Previous work has shown that decorin deficiency significantly affects collagen fibers in the skin, and consequently, the skin mechanical properties of decorin deficient mice are altered. It is likely then, that lung mechanics would also be altered in decorin deficient mice. In order to test this hypothesis, both dynamic and static properties of the respiratory system and the isolated parenchymal tissue strip were measured in wildtype and decorin deficient mice. A better understanding of the roles and functions of decorin, especially as they lead to mechanical changes, may aid in understanding pathological processes relevant to the lung.
3.2 Abstract

Decorin is required for the normal fibrillogenesis and spatial arrangement of collagen. As collagen is important in determining the elastic behaviour of the lung, we hypothesized that lung tissue mechanics would be altered in decorin deficient (Dcn-/−) mice. C57BL/6 mice, (Dcn-/- and Dcn+/+) were anesthetized, intubated and connected to a small animal ventilator. Complex impedance was measured by applying a volume (V) signal with multiple frequencies to the trachea at a PEEP of 1.5 cmH2O. Changes in V and pressure (P) were fit to a constant phase model to calculate airway resistance (Raw), tissue damping (G) and tissue elastance (H). A PV curve was obtained by inflating the lungs to 25 cmH2O and measuring P during stepwise deflations. Lungs were excised and parenchymal tissue strips mounted in the organ bath for in vitro measurement of complex impedance and quasistatic length-stress curves. In addition, one lung was fixed with OCT for immunohistochemistry, and a portion of the remaining lung was reserved for protein extraction. In the Dcn-/- mice, Raw was decreased in vivo when compared to Dcn+/+ mice. Lung Compliance (Ct) was increased in Dcn-/- mice (0.0175±0.000 vs. 0.0203±0.001 ml/cmH2O, p<0.02). Similarly, length-stress curves showed increased compliance in the Dcn-/− mice. These data, showing an alteration in compliance, support a critical role for decorin in the proper formation of the lung collagen network; lack of decorin alters lung tissue mechanical behaviour.

Key words: complex impedance, pressure-volume curves, length-stress curves.
3.3 Introduction

Viscoelastic behaviour of the lung parenchymal tissues is determined primarily by the extracellular matrix and its components. The tissue matrix is comprised of collagen and elastin fibers, glycoproteins, and proteoglycans. Collagen and elastin fibers are essentially elastic, but when arranged in a network display prominent hysteretic properties (35). Decorin, a small leucine-rich proteoglycan (SLRP), regulates collagen fibril formation and the spatial arrangement of collagen fibers in the matrix (22). Via this mechanism, decorin may play an important role in normal lung tissue mechanics.

Decorin has been shown to be present in tissues with fibrillar collagen, such as the lung (6, 41, 42). Decorin has been described in the submucosal region of the airway wall, as well as in the lung vasculature (41, 42). Decorin may also play a role in the remodelling associated with such respiratory diseases as pulmonary fibrosis and asthma (6, 41). The process of remodelling involves the deposition of new matrix, including decorin-regulated collagen fibrillogenesis. Decorin has the ability to bind TGF-β (57), a cytokine that has been implicated in pathologic processes characterized by remodelling (37, 55).

Danielson et al. (9) have recently published studies in mice in which the decorin gene has been disrupted, such that decorin mRNA is not transcribed and the decorin protein is not produced. They described that the collagen fibers in the skin of the Dcn−/− mice were more heterogeneous, both in terms of size and structure, than those in Dcn+/+ animals. In studies characterizing the mechanical behaviour of the skin, they documented increased skin fragility, altered
compliance, and reduced tensile strength in the *Dcn*-/− mice as compared to *Dcn*+/+ controls.

We questioned whether lung mechanical properties would also be altered in *Dcn*-/− mice. We were particularly interested in the behaviour at the top portion of the quasistatic pressure vs volume (*PV*) curve, as it is thought to be governed more by the mechanical behaviour of collagen at these higher lung volumes (33). Therefore, we studied the dynamic and quasistatic mechanical lung tissue properties, both *in vivo* and *in vitro* of *Dcn*-/− and *Dcn* +/+ mice. We measured complex impedance of the respiratory system as well as quasistatic *PV* curves *in vivo*. Additionally, we measured the complex impedance and the length-stress relationship of parenchymal tissue strips *in vitro*. We also performed Western blotting and immunohistochemistry to quantify and localize both decorin and biglycan, another SLRP. Biglycan, like decorin, belongs to the Class 1 subfamily of SLRPs (22). Of the SLRPs, biglycan and decorin show the highest internal identity in amino acid sequence (~57%) (22). They have the same types of glycosaminoglycan (GAG) sidechains, i.e., dermatan and/or chondroitin sulfate. Finally, and perhaps most relevant to this study, biglycan and decorin deficiencies seem to have similar effects on collagen fibril structure, at least in the skin of mice (8). There is evidence for upregulation of one member of a SLRP family in response to a deficiency in another family member. For example, in the case of the fibromodulin null mouse, lumican protein increases 4-fold in the tail tendon as compared to the tail tendon of wildtype mice (52). We
reasoned, therefore, that biglycan might be upregulated in the \textit{Dcn}^{-/-} mice, to compensate for the lack of decorin, and subserve its usual physiologic function.

3.4 Methods

3.4.1 Determination of genotype

Breeding was initiated with a male C57BL/6 mouse heterozygous for decorin \textit{Dcn}^{+/-} (9) and \textit{Dcn}^{+/+} C57BL/6 females. Mice were housed in a regular animal facility at McGill University (Montreal, PQ). The mutation of the decorin gene was created by targeted disruption of exon 2 of murine decorin by the insertion of the Pgk-neo cassette (9). The genotype of the offspring was determined by extracting DNA from the tail tissue using a Genomic DNA Purification Kit (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Real-Time Polymerase Chain Reaction (PCR) (Roche, Basel, Switzerland) was used to analyse DNA. Sense and antisense primers corresponding to exon 2 of murine decorin, and an additional primer corresponding to the PGK promoter of the PGK-neo cassette, were generated (9). The reaction mixture consisted of 1 ul DNA, 1ul sense primer, 1 ul antisense primer or 1ul PGK primer, 7ul H2O, and 10 ul SBr-green mix (Qiagen, Hilden, Germany). Reaction conditions were as follows. Melting: 95°C, 900s. PCR (45 cycles): segment 1: 94°C, 15s. segment 2: 57°C, 20s. segment 3: 72°C, 25s. Melting curve: 57°C, 45s. Cooling: 30°C, 30s.

To verify that the products of the RT-PCR reaction were of the appropriate size, PCR products were run on 1.8% agarose gel for 1.5 hrs at 60 V.
3.4.2 In vivo measurements

Animal preparation

Ten Dcn/- and seven Dcn+/+ C57BL/6 mice (8-12 weeks old) were studied. Selected measurements were carried out in different animals. Mice were anesthetized with an injection of xylazine (12 mg/kg i.p.) followed five minutes later by an injection of sodium pentobarbital (40 mg/kg i.p.). After tracheostomy, an 18-gauge metal cannula was inserted into the trachea and tightly bound. The mouse was connected via the tracheal cannula to a computer-controlled small-animal ventilator (flexiVent, Montreal, Quebec; (49)). Mice were mechanically ventilated at 150 breaths/min with a tidal volume (\(V_t\)) of 6ml/kg at a positive end expiratory pressure (PEEP) of 1.5 cmH\(_2\)O. The animals were paralyzed with an injection of pancuronium bromide (1.2 mg/kg i.p.). Heart rate was monitored by a 3 lead EKG. All animals received humane care in compliance with the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council of Animal Care, and an institutional animal ethics committee approved the protocol.

Measurement of complex impedance

A computer generated volume signal comprised of 19 mutually primed sinusoids ranging from 0.25 to 19.625 Hz was applied to the airway opening. The amplitudes of the sinusoids decreased hyperbolically with frequency such that each frequency component had equal power. The signal had a peak-to-peak
volume of 0.17ml and lasted 16 seconds. Piston displacement (ml) and cylinder pressure (cmH\textsubscript{2}O) were measured during the application of the signal. A total lung capacity (TLC) manoeuvre was performed to standardize volume history. One minute later complex impedance was measured at a PEEP of 1.5 cmH\textsubscript{2}O. Measurements were repeated three or four times for each mouse and the parameter estimates from each of the signal applications were averaged.

**Calculation of parameters**

Respiratory impedance ($Z$) was determined using the equation:

$$Z(f) = \frac{P(f)}{V(f)}$$  \hspace{1cm} (1)

where $P$ is cylinder pressure (cmH\textsubscript{2}O) and $V$ is flow through the piston (ml/s). Both are functions of frequency ($f$). The respiratory system impedance ($Z(f)$) was fit with the constant-phase model (19):

$$Z(f) = R_N + j(2\pi f)I + \frac{(G - jH)}{(2\pi f)^\alpha}$$  \hspace{1cm} (2)

where $R_N$ is airway (flow dependent) resistance, $I$ is airway inertance, $G$ is tissue damping, $H$ is tissue elastance, $j$ is the imaginary unit, and $\alpha = (2/\pi)\tan^{-1}(H/G)$.

**Measurement of Pressure-Volume curve**

Following the complex impedance measurements, quasistatic measurements of the pressure-volume relationship of the respiratory system were performed. Lungs were slowly inflated to TLC and then step wise deflated over
18 seconds. The signal was applied three times and the data were summed to make a single pressure-volume curve.

The pressure-volume curve was characterized by the equation (45):

\[ V = A - Be^{-\kappa P} \quad (3) \]

where \( V \) is volume, \( P \) is pressure, and \( \kappa \) is an exponential constant. The value of \( \kappa \) was determined for each animal. Compliance of the lung \( (C_L) \) was calculated between 15 and 20 cm H\(_2\)O as:

\[ C_L = \frac{\Delta V}{\Delta P} \quad (4) \]

where \( \Delta V \) is change in volume and \( \Delta P \) is change in pressure over the given range.

3.4.3 In vitro measurements

Tissue preparation

Mice were disconnected from the small animal ventilator after the \textit{in vivo} experiments were completed. Through an abdominal incision, the diaphragm was cut and a bilateral pneumothorax was induced. The thorax was opened, the animal exsanguinated, and the heart, lungs and trachea were carefully resected \textit{en bloc} ensuring that the lungs were not punctured. The lungs were rinsed with a modified Krebs solution (mM: NaCl 118, KCl 4.5, NaHCO\(_3\) 25.5, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, glucose 10: Sigma, St. Louis, MO; pH=7.40, 6°C) by filling the lungs three times to TLC. The right lung was reserved for pathologic study and a strip \((1 \times 1 \times 6-7 \text{ mm})\) was cut from the periphery of the left lobe. The remainder of the left lung was set aside for protein extraction. The pleura
was removed and the unloaded length ($l_0$) and wet weight ($W_o$) were recorded. The strips were kept in a bath of iced Krebs solution which was continuously bubbled with 95% $O_2$ / 5% $CO_2$.

*Experimental Setup*

Metal clips were glued to either end of the tissue strip with cyanoacrylate. Steel music wires (0.5 mm diam) were attached to the clips and the strip suspended vertically in an organ bath. A mercury bead was placed in the bottom of the organ bath, allowing the wire to pass through the bath, but preventing the Krebs solution from leaking out. The bath was filled with 20 ml of Krebs solution, maintained at 37°C and continuously bubbled with the 95%O2/5%CO2. One end of the strip was attached to a force transducer (model 400A, Cambridge Technologies, Watertown, MA, USA) which had an operating range of ±10 g, resolution of ±200 $\mu$g and compliance of 1 $\mu$m/g, while the other end was connected to a servo-controlled lever arm (model 300B, Cambridge Technologies, Watertown, MA, USA), which delivered length perturbations to the mounted strip. The lever arm was capable of peak to peak length excursions of 8 mm and length resolution of 1 $\mu$m and was in turn connected to a computer, which controlled the frequency, amplitude ($\epsilon$) and wave form of the oscillation. Movement of a screw thumb wheel system, which effected slow vertical displacements of the force transducer, set the resting tension ($T$). Length and force signals, as obtained by the lever arm and force transducer respectively, were converted from analog to digital with an analog to digital converter (DT2801-A,
Data Translation Inc., Marlborough, MA) low pass filtered and recorded on an A/T compatible computer at a sampling frequency of 256 Hz.

**Measurement of complex impedance**

For measurements of complex impedance, an 8 second broad-band pseudorandom displacement input signal composed of 17 mutually primed frequencies ranging from 0.5 to 19.75 Hz with a maximum amplitude of 0.18 mm was generated by a computer and delivered to the lever arm. The linearity and hysteresis of the system were tested by measuring the moduli of a steel spring of stiffness comparable with that of the tissue strip. The spring was suspended in the bath by music wire in the same manner as the strip. The frequency and amplitude dependence of the system were assessed over a range of frequencies (0.1-10 Hz). The spring stiffness did not show any dependence upon oscillatory frequency below 5 Hz. The hysteresivity of the system was independent of frequency and had a value <0.003.

Lung parenchymal strips were preconditioned by slowly cycling tension from 0 to 2 grams three times; on the third cycle, the strip was unloaded to a stress of approximately 500 mg/mm² and allowed to stabilize for 45 minutes, at the end of which time the stress was approximately 450 mg/mm². Complex impedance was then measured. Measurements consisted of eight consecutive eight-second recordings; the average of these eight recordings was calculated.
Calculation of parameters

Tissue impedance was calculated as:

\[ Z(f) = \frac{T(f)}{\frac{\Delta l}{\Delta t}(f)} \]  \hspace{1cm} (5)

where \( T \) = tension in mg, \( l \) = length in mm and \( t \) = seconds.

Force and length signals were obtained and recorded as a function of time, converted to functions of frequency \((f)\) using Fourier transforms, and then complex impedance was calculated. The mechanical parameters were estimated by fitting the constant phase model (19) to the impedance data according to equation 2. In this instance the parameter \( R_N \) reflects the Newtonian (flow dependent) resistance of the tissues. Results were standardized for strip size by multiplying the values of \( G \) and \( H \) by \( l_0/A_o \). \( A_o \) is the unstressed cross sectional area (in mm\(^2\)) of the lung parenchymal strip obtained from the formula:

\[ A_o = \frac{W_o}{\rho \times l_o} \hspace{1cm} (6) \]

where \( W_o \) is the weight of the strip in mg, \( \rho \) is the mass density of the tissue taken as 1.06 mg/mm\(^3\), and \( l_o \) is the length of the strip in mm.

Measurement of Length-Stress curves

When the complex impedance measurements were completed, the strip was manually stretched to approximately 3g tension and then relaxed in a stepwise fashion, allowing 5 seconds between steps. This generated a length-tension curve, which was then converted to a length-stress curve, with stress \((9)\)
calculated as $T/A_0$. The data points were fit with a five-parameter exponential curve:

$$l = l_o + a(1 - e^{-bT}) + c(1 - e^{-dT}) \quad (7)$$

where $l_o$ is the length of the strip at $T=0$, and $a, b, c,$ and $d$ are constants describing the shape of the curve. The compliance of the strip ($C_s$) was calculated between 300 and 500 mg/mm$^2$ as follows:

$$C_s = \frac{\Delta l}{\Delta \mathcal{G}} \quad (8)$$

**Tissue Fixation and Immunocytochemistry**

The right lung was filled with histocon, submerged in OCT, and immediately frozen in isopentane cooled in liquid nitrogen. Sagittal sections (7 mm thickness) were cut from medial and lateral aspects of the lobe. Sections incubated overnight at 4°C with the primary antibody, (either a 1:200 dilution of rabbit anti-mouse decorin antiserum (LF-113), or a 1:100 dilution of rabbit anti-mouse biglycan antiserum (LF-159) (14)). The slides were then washed and subsequently incubated for 30 minutes in a 1:100 dilution of biotinylated swine antibody against rabbit immunoglobulin (DAKO, E 0353, Glostrup, Denmark). Finally, the slides were incubated for 30 minutes in a 1:100 dilution of alkaline phosphatase-conjugated avidin (DAKO, D 0365, Glostrup, Denmark). The slides were developed with Fast Red TR (Sigma, F-2768, Oakville, Ontario), counterstained with Gill II Haematoxylin, and fixed in lithium carbonate. After allowing the slides to dry, they were covered with a thin layer of crystal mount.
Protein extraction and Western blotting

Proteoglycans were extracted from the left lung in 4 M guanidine HCl. The extract was dialyzed extensively and the pellet resuspended. The amount of protein was determined with the Bio-Rad Protein Assay (BioRad, Mississauga, Ontario). An aliquot of 10 µg of protein from each sample was incubated with protease-free chondroitinase ABC (0.1 U/ml) (Sigma, Oakville, Ontario). Proteins were separated on 10% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Baie d’Urfé, Quebec). Membranes were incubated in a 1:1000 dilution of a rabbit anti-mouse decorin antiserum (LF-113) or a rabbit anti-mouse biglycan antiserum (LF-159), which were generated against synthetic decorin and biglycan peptides of the mouse protein (14). Membranes were then incubated with biotin-labeled swine anti-rabbit secondary antibody (Dako, Mississauga, Ontario) and finally with streptavidin-biotinylated HRP complex (Amersham Biosciences, Baie d’Urfé, Quebec). The antigen/antibody complexes were detected with ECL detection reagents (Amersham Biosciences, Baie d’Urfé, Quebec) according to the manufacturer's instructions. Membranes were then stripped, reblocked and probed with polyclonal antibody against actin. Densitometric analysis of biglycan and actin was accomplished with image analyzer software (Fluorchem; Alpha Innotech, San Leandro, CA), which measures the sum of all the pixel values after background correction.
3.4.4 Data analysis

All in vivo data manipulations were performed with flexiVent software (Scireq, Montreal, Quebec), while all in vitro data manipulations were performed with the ANADAT software package (RHT-InfoDat, Montreal, Quebec, Canada). T tests were used to assess whether mechanical parameters were different in Dcn-/- vs Dcn +/+ mice. Results were considered statistically significant at a probability level of 5%. Values are reported as mean ± standard error (SE).

3.5 Results

Sample results of the genotyping of the mice are shown in Figure 1. Primers for the wildtype allele produced a product of 161 bp. Primers for the disrupted allele were chosen so that the 3’ end began in the region of the inserted Pgk-neo cassette. The product for the disrupted allele was 250 bp. The melting points of the PCR products of the wildtype and disrupted alleles were ~79 °C and ~84 °C respectively. As shown in Figure 1A, Dcn +/+ mice had only one product that melted at ~79 °C. Heterozygous mice had two products, one melting at ~79 °C and the other at ~84 °C. Dcn-/- mice had one product, which melted at ~84 °C. In Figure 1B, agarose gels of PCR products from the respective animals are shown.

Qualitatively, we observed that the skin, cartilage and lung tissue of the Dcn-/- mice were more fragile and tore more readily than those of the Dcn +/+ mice.
The results for the dynamic measurements of complex impedance for both the in vivo and in vitro preparations are shown in Table 1. In vivo, $R_{aw}$ was greater in the $Dcn^{-/-}$ mice than in the $Dcn^{+/+}$ mice. However, tissue damping and elastance were not significantly different. In vitro, no differences were observed in the complex impedance of the tissue strips. Again, tissue damping and elastance were equivalent in the two groups.

The average pressure-volume curves obtained in vivo from all animals are shown in Figure 2. The average values for $\kappa$, the slope of the tangent between 15 and 20 cmH\(_2\)O ($C_L$), and the volume of the lungs at 18 cm H\(_2\)O ($V_{18cmH2O}$) are shown. The $\kappa$ values were not different in the two groups of mice. However, $C_L$ was higher in the $Dcn^{-/-}$ mice compared to the $Dcn^{+/+}$ mice ($C_L = 2.03 \pm 0.09$ vs $1.75 \pm 0.04$ ml/cm H\(_2\)O, respectively, $p<0.02$). $V_{18cmH2O}$ was also greater in the $Dcn^{-/-}$ mice than in the $Dcn^{+/+}$ mice ($0.865 \pm 0.026$ vs $0.781 \pm 0.026$ ml, respectively, $p<0.05$).

The length-stress curves obtained in vitro are shown in Figure 3. The average values for the slope of the tangent between 300 and 500 mg/mm\(^2\) ($C_S$) for the $Dcn^{-/-}$ strips compared to the $Dcn^{+/+}$ strips were $3.2 \pm 0.2 \times 10^{-3}$ mm\(^3\)/mg vs. $2.2 \pm 0.3 \times 10^{-3}$ mm\(^3\)/mg, respectively ($p < 0.01$). The length of the strip at 1000 mg/mm\(^2\) ($L_{1000mg/mm2}$) for the $Dcn^{-/-}$ strips compared to the $Dcn^{+/+}$ strips was $1.32 \pm 0.09$ mm vs. $1.09 \pm 0.07$ mm, respectively ($p < 0.05$).

Results of the western blots done for decorin and biglycan are shown in Figure 4. As expected, no decorin was detected in the $Dcn^{-/-}$ mice. Biglycan,
when controlled for protein loading, was not different in the *Den-/-* mice when compared to the *Den+/* mice.

We identified the presence of decorin by immunohistochemical staining of the lung tissue. In the *Den+/* mice, decorin was seen in the airways and around the blood vessels, as is depicted in panel A of Figure 5. An airway from a *Den-/-* mouse lung is shown in panel B; no decorin was detectable.

3.6 Discussion

The main finding of this study is that the lung tissue compliance of *Den-/-* mice is significantly altered as compared to *Den+/* mice. This is likely due to the interaction of decorin with collagen, a structural protein that is recruited at higher stresses.

Certain technical issues warrant discussion. As described in the results section, the lung tissue of the *Den-/-* mice was very fragile. This made measurement of *in vitro* mechanics difficult, especially measurement of the length-stress curve, as the tissues were stretched over a wide range of stresses. Stress failure (tearing of the tissue) often occurred at relatively low tensions in the *Den-/-* parenchymal strips. This reduced the range over which the stress-length curve could be obtained.

Traditionally, a stress-strain curve (where strain = \((l - l_o)/l_o\)) is obtained to gather information about *in vitro* tissue mechanics. However, because of the different compliance of the *Den-/-* vs *Den+/* tissue strips, the length \((l_o)\) of the tissue strips once mounted in the fluid-filled organ bath and the operating stress
applied, was not consistent. Therefore, we measured the stress-length relationship, where length was the absolute change in length from the original length in the organ bath. Had we measured actual strain, the differential between the two curves would have been even more substantial, i.e., the strain necessary to generate a given tension in the $Dcn^{-/-}$ strips would have been much higher than in the $Dcn^{+/+}$ strips, and the $Dcn^{-/-}$ stress-strain curve would have been shifted even further away from the $Dcn^{+/+}$ curve. In other words, the difference between the two curves was minimized by plotting length instead of strain. Moreover, the parenchymal strips that tore most readily, were likely the strips in which the compliance was most altered.

As shown in figure 2, the difference between the two groups of mice occurred in the values of the slope at higher pressures. The compliance of the $Dcn^{-/-}$ mice seemed to be greater only in the higher pressure range. It has been suggested that elastic fibers account for lung compliance in the lower pressure range, i.e., around functional residual capacity, whereas collagen fibrils become more important as lung volume becomes limited. Mercer and Crapo (33) have conducted elegant morphometric studies examining the configuration of collagen and elastic fibers in the alveolar duct and wall. They concluded that at low levels of strain, collagen fibers have a “wave-like” configuration and are readily extensible; stress is borne by adjacent elastic fibres. At higher levels of strain, collagen fibers act to limit further distension. Recently, Sly et al. (50) reported a decrease in the hysteresivity of mouse lungs as lung volume increased. These authors suggest that their findings support the idea that at high lung volumes, the
tissue matrix contributes less to the mechanics of the lung while the individual collagen fibres become more important. Decorin binds collagen and is involved in both collagen fibrillogenesis and the spatial alignment of the fibres in the matrix. It seems reasonable, therefore, to expect that the absence of decorin would affect lung mechanics at a lung volume where the mechanical behaviour of collagen becomes limiting.

In vivo measurements include the behaviour of many different elements, in addition to the collagen-elastin-proteoglycan matrix. As the pressure-volume curve was measured in closed chested animals, we were also sampling the mechanical behaviour of the chest wall. In addition, the air liquid interface, or surfactant, contributes significantly to the compliance of the lungs (40). However, studies by Bachofen and colleagues (4) measuring the relations among surface tension, surface area and alveolar geometry showed that, at higher lung volumes, tissue forces become predominant in determining pressure volume behaviour.

The differences in the stress vs length curves measured in vitro address the issue of which elements are contributing to the difference in in vivo compliance observed in the Dcn -/- vs Dcn +/+ animals. The parenchymal strip is comprised primarily of alveolar walls (26); hence the behaviour of the fibrous matrix is mainly sampled. Moreover, the preparation is fluid-filled, thereby eliminating the contribution of surface forces. The consistent in vivo and in vitro results implicate the tissue matrix as the source of the different mechanical properties in the two populations of mice.
One issue that remains, however, is the question of why there were no observable differences in the values for $G$ and $H$ in the dynamic measurements of complex impedance. *In vivo* measurements were performed at 1.5 cmH$_2$O PEEP — a relatively low transpulmonary pressure, where collagen was perhaps not yet recruited. *In vitro* measurements were performed at an operating stress of 500 mg/mm$^2$. This represents a relatively high stress, where collagen fibers should contribute to mechanical behaviour. One possibility to explain the lack of differences in dynamic measurements between the two sets of tissues, is that *in vitro* measurements are made in a system where the tissue is strained in a biaxial fashion, rather than in three dimensions, as is the case *in vivo*, and the relative effects of increasing strain and volume may, therefore, differ. Another possible explanation is that the amplitude of the length signal during impedance measurements was relatively modest (the largest amplitude component was 0.18mm or approximately 3% $l_0$) as compared to the change in length during the quasistatic measurement. A large length perturbation may be required for the compliance characteristics of collagen to be revealed, even at higher stresses.

One dynamic parameter which was different between the $Dcn-/-$ and $Dcn+/+$ mice was airway resistance ($R_{aw}$) (Table 1). Immunohistochemical staining demonstrated the presence of decorin in the wall of airways and blood vessels in the $Dcn+/+$ mice. This distribution of decorin has also been reported by Redington and co-workers in the lungs of normal and asthmatic humans (41). Mice deficient in decorin may have decreased $R_{aw}$ because of the impact of decorin on the compliance of the airway wall. A more compliant airway wall
could be relatively dilated because of the mechanical interdependence between airways and the surrounding parenchymal attachments (32). The parenchyma would more effectively tether open the more compliant airway wall, resulting in an airway with a larger luminal diameter, and a lower airway resistance. It would be of interest to determine whether decorin deficient mice respond differently to contractile challenge.

In addition to changes in the mechanical properties of the lung, we questioned whether, in the absence of decorin, other proteoglycans might be upregulated. Specifically, we hypothesized that biglycan, a SLRP structurally similar to decorin, would be increased. Decorin and biglycan have core proteins of approximately the same length and composition (22). Moreover, they both have chondroitin sulfate side chains; decorin has one and biglycan has two. A study recently reported by Svensson et al. (52), showed that lumican in the tail tendon of fibromodulin-null mouse increased 4-fold as compared to the tail tendon of wildtype mice. However, we observed no measurable differences in the amount of biglycan in the lung tissues of Dcn-/- and the Dcn+/+ mice.

In conclusion, we have shown that lung tissue mechanics are altered in the Dcn-/- mice. Compliance was increased at higher pressures and stresses, and $R_{aw}$ was decreased. In Dcn+/+ mice, decorin was present in the vessel and airway walls. Biglycan was not upregulated to compensate for the absence of decorin. The alteration in tissue properties likely occurred as a result of the abnormal collagen fibril formation that occurs in Dcn-/- mice (9). Further imaging studies
to document the precise abnormalities in the formation of the fibrils comprising the lung matrix would be of interest.

3.7 Acknowledgements

Supported by the JT Costello Memorial Research Fund and the Canadian Institutes of Health Research. A.F. is a recipient of a studentship award from the Montreal Chest Institute Research Center.
3.8 Figure legends

Figure 1. A) Melting point derivatives for the real-time-PCR product of genomic DNA from wildtype (+/+, heterozygous (+/-), and decorin deficient (-/-) mice. B) PCR product of genomic DNA from wildtype (+/+), heterozygous (+/-), and decorin deficient (-/-) mice on a 1.8% agarose gel.

Figure 2. Pressure-volume curves during quasistatic deflation *in vivo*. The model \( V = A - Be^{-kp} \) was fit to the data to generate the curves. Compliance \((C_L)\) was measured from the slope of the tangent to the curve between 15 and 20 cmH\(_2\)O. \(Dcn^{+/+}\), wildtype; \(Dcn^{-/-}\), decorin deficient. *, \( p < 0.05 \) vs \(Dcn^{+/+}\); †, \( p < 0.02 \) vs \(Dcn^{+/+}\).

Figure 3. Stress-length curves during step-wise relaxation of parenchymal strips *in vitro*. A five-parameter exponential curve \((l = l_o + a(1 - e^{-bt}) + c(1 - e^{-dt}))\) was fit to the data to generate the curves. Compliance \((C_s)\) was measured from the slope of the tangent to the curve between 300 and 500 mg/mm\(^2\). \(Dcn^{+/+}\), wildtype; \(Dcn^{-/-}\), decorin deficient. *, \( p < 0.05 \) vs \(Dcn^{+/+}\). **, \( p < 0.01 \) vs \(Dcn^{+/+}\).
Figure 4. A) Expression of decorin and biglycan in decorin deficient ($Dcn^{-/-}$) and wild type ($Dcn^{+/+}$) mice. Cartilage (Ct) was used as a positive control, and actin as a control for protein loading. B) Amount of biglycan corrected for protein loading in $Dcn^{+/+}$ and $Dcn^{-/-}$ lungs. Results are expressed as means ± S.E.M.

Figure 5. Photomicrographs of immunohistochemical staining for decorin in A) wild type and B) decorin deficient animals. In wild type mice, decorin is prominent around airways (large arrow) and blood vessels (small arrow). Magnification: x200.
Table 1
Dynamic mechanics in wildtype (Dcn+/+) and decorin deficient (Dcn-/-) mice.
(Mean ± SE)

<table>
<thead>
<tr>
<th>In vivo</th>
<th>Raw (cmH₂O·s/ml)</th>
<th>G (cmH₂O/ml)</th>
<th>H (cmH₂O/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dcn+/+</strong></td>
<td>0.42 ± 0.04</td>
<td>3.51 ± 0.25</td>
<td>31.6 ± 2.0</td>
</tr>
<tr>
<td><strong>Dcn-/-</strong></td>
<td>0.30 ± 0.01 *</td>
<td>3.72 ± 0.45</td>
<td>32.6 ± 4.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>In vitro</th>
<th>RN (N·s/m²)</th>
<th>G (N/m²)</th>
<th>H (N/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dcn+/+</strong></td>
<td>125 ± 8</td>
<td>1840 ± 150</td>
<td>44090 ± 2220</td>
</tr>
<tr>
<td><strong>Dcn-/-</strong></td>
<td>118 ± 5</td>
<td>1880 ± 90</td>
<td>41020 ± 1810</td>
</tr>
</tbody>
</table>

Raw, airway resistance; G, tissue damping; H, tissue elastance; RN, Newtonian resistance of the tissues. N=6 in each group. *, p < 0.05 vs Dcn+/+
Figure 1

A

![Graph A](image)

B

![Graph B](image)
Figure 2

\[ k = 0.114 \]
\[ C_L = 2.03 \pm 0.09 \times 10^{-2} \text{ ml/cmH}_2\text{O} \quad *(p<0.02) \]
\[ V_{18\text{cmH}_2\text{O}} = 0.855 \pm 0.026 \text{ ml} \quad *(p<0.05) \]
\[ n = 5 \]

\[ k = 0.118 \]
\[ C_L = 1.75 \pm 0.04 \times 10^{-2} \text{ ml/cmH}_2\text{O} \]
\[ V_{18\text{cmH}_2\text{O}} = 0.781 \pm 0.026 \text{ ml} \]
\[ n = 6 \]

- **Dcn +/+**
- **Dcn -/-**
- Tangent
- \( V_{18\text{cmH}_2\text{O}} \) (Dcn +/+)
- \( V_{18\text{cmH}_2\text{O}} \) (Dcn -/-)
Figure 3

\[ C_S = 3.2 \pm 0.2 \times 10^{-3} \text{ mm}^3/\text{mg} \quad \text{**(p<0.01)**} \]
\[ L_{1000\text{mg/mm}^2} = 1.32 \pm 0.09 \text{ mm} \quad \text{* (p<0.05)} \]
\[ n = 5 \]

\[ C_S = 2.2 \pm 0.3 \times 10^{-3} \text{ mm}^3/\text{mg} \]
\[ L_{1000\text{mg/mm}^2} = 1.09 \pm 0.07 \text{ mm} \]
\[ n = 6 \]
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>Dcn -/-</th>
<th>Dcn +/-</th>
<th>Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decorin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biglycan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

46 kD

B

<table>
<thead>
<tr>
<th></th>
<th>Dcn -/-</th>
<th>Dcn +/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biglycan/Actin</td>
<td></td>
<td></td>
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</table>

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CHAPTER 4: DISCUSSION
4.1 Summary of Findings

The main finding of this work is that the lung parenchymal tissue of Den-/- mice is significantly affected by the absence of decorin. The tissue is significantly more compliant in the higher pressure range. This is likely due to the absence of the interaction of decorin with the structural protein collagen that is recruited at higher stresses. Another finding is that \( \eta \) is different in vivo compared to in vitro. \( \eta \) is a dimensionless index that affords a particularly robust way to describe lung tissue behaviour because it varies minimally with oscillation amplitude, frequency, and volume history. Finally, this work demonstrates that mouse lung in vivo and in vitro mechanical behaviour is different than that described in other rodents. This may be accounted for by structural differences between species.

4.2 Technical Issues

Certain technical issues warrant discussion. The lung tissue of the Den-/- mice was very fragile, which made measurement of in vitro mechanics difficult, particularly the length-stress curve. In the parenchymal strips from the Den-/- mice, stress failure occurred at relatively low tensions. This reduced the range over which the stress-length curve could be obtained. Also, because of the variable compliance of the Den -/- vs Den +/- tissue strips, we did not measure the traditional stress-strain curve, but instead made measurements of length vs stress. If we had measured strain, we would have seen a more pronounced difference between the curve of the Den-/- tissue compared to the Den +/- tissue.
In other studies, $G$ and $H$ were affected by the presence of a chest wall (20, 44). The in vivo experiments reported here were performed on closed-chested animals and therefore the measurements made reflect not only the lung, but the entire respiratory system. However, $\eta$ is not affected by inclusion of the chest wall at low positive end expiratory pressure (PEEP), so we can consider it to reflect the properties of the lung. Additionally, the chest wall has been shown to contribute to the shape of the pressure-volume curve at lower lung volumes (1), but by fitting the Salazar-Knowles equation (45) to the data points above functional residual capacity (FRC) only, the contribution of the chest wall to the shape of the $PV$ curve is minimized.

The parenchymal strip includes some proportion of airways, which may contribute to the mechanics. Data from this lab showed that baseline mechanics in rat lung strips were unchanged despite a variation in the proportion of small airways from 6-20% of the sample (46). Hence, in a strip with only 2.5% bronchial wall, such as has been measured in the mouse (13), the airways are unlikely to influence the assessment of tissue mechanics significantly. Therefore, we felt we could use the mouse lung tissue strips as a fair representation of the lung parenchymal tissues.

In order to record an adequate in vitro complex impedance measurement, we determined that an operating stress of 500 mg/mm$^2$ was required. This in vitro stress is likely higher than the corresponding in vivo operating pressure of 1.5 cmH$_2$O. As has been shown by Navajas et al. (38), $\eta$ decreases slightly as operating stress increases. However, we measured almost a three-fold variation in
in the *in vivo* vs. *in vitro* situation. Therefore, while some of the decrease in $\eta$ might be due to the comparatively higher operating stress *in vitro*, the magnitude of change cannot be accounted for by this mechanism alone.

### 4.3 Discussion of Results

The pressure-volume curves obtained from the *Dcn-/−* and *Dcn+/+* mice show that the difference between the two groups occurs in the values of the slope at higher pressures. The compliance of the *Dcn-/−* mice seems to be higher only in the higher pressure range. We know collagen to be recruited at higher pressure ranges while elastin is responsible for most of the elastic behaviour of the lung tissue at lower pressures (33). Therefore, since decorin can bind collagen and is involved in collagen fibrillogenesis and the spatial alignment of the fibres in the matrix, it is reasonable to expect that the absence of decorin would affect mechanics where collagen is most important; that is, the higher pressure range. This may also be the reason why values for $G$ and $H$ are not different. *In vivo* measurements were performed at 1.5 cmH2O — a relatively low pressure, where collagen was perhaps not yet recruited. *In vitro* measurements were performed at a relatively higher stress, but other factors of the experimental setup, such as the amplitude of the signal, may have contributed to $G$ and $H$ values remaining unchanged in the two groups.

We also observed a change in the Newtonian, or flow-related mechanics of the lungs. Raw in the *Dcn-/−* mice was decreased. Using ICC to stain for decorin, we saw that decorin was present in the vessel and airway walls. Other
studies have also reported decorin to be present in airway wall (41). If there is more decorin present in the airway wall than in the parenchymal tissues, then a parameter reflecting airway properties ($R_{aw}$) may be more significantly affected by the absence of decorin than parameters reflecting tissue properties ($G$ and $H$). The lack of positive decorin staining in the parenchymal tissues may reflect either a lesser amount of decorin in these structures or a technical problem related to the immunohistochemical stain. A more sensitive imaging technique may be required to adequately visualize decorin in the alveolar wall.

We had hypothesized that in the absence of decorin, other proteoglycans might be affected. Specifically we questioned whether biglycan might be upregulated because of its structural similarity to decorin. Decorin and biglycan have core proteins of approximately the same length and composition (22). As well, they both have chondroitin sulfate side chains. Biglycan seemed the most likely candidate to be upregulated to perform the functions of decorin. A study done by Svensson et al. (52) showed that lumican protein increased 4-fold in fibromodulin-null mouse tail tendon tissue when compared to wildtype tail tendon tissue. If other small leucine rich proteoglycans behave in this manner, it seemed possible that biglycan could function similarly. However, the results of our western blots showed no differences in the amount of biglycan in the tissues from $Dcn^{-/-}$ and $Dcn^{+/+}$ mice.

Another finding of our experiment is that $\eta$ measured in vitro was less than that measured in vivo. There are several possible explanations for this observation. The lack of air-liquid interface and surfactant in the in vitro
condition would decrease $\eta$, as surfactant displays hysteretic properties and the air-liquid interface generates surface tension. Quasistatic pressure-volume curves of isolated saline-filled lungs, where the air-liquid interface is abolished, demonstrate that hysteresis is markedly reduced compared to that of air-filled lungs (40). However, a study comparing isolated lung with an intact air-liquid interface with that of lung tissue strips in an organ bath showed nearly identical values of $\eta$ (44). Therefore, the air-liquid interface and surfactant are unlikely to account for all of the difference between the \textit{in vivo} and \textit{in vitro} states.

Another factor that may contribute to $\eta$ \textit{in vivo} is the heterogeneity of the airway and respiratory system. Sakai \textit{et al.} (44) reported that compartment-like heterogeneities of the lung significantly increased tissue damping and $\eta$. The extent to which branching asymmetry contributes to respiratory mechanics has been studied in detail by Gomes \textit{et al.} (16) who found a higher value of $\eta$ in the giant pouched rat, which displayed a greater degree of asymmetry, than in the harvest mouse. In our study, we compared closed chested lung mechanics with the mechanics of the tissue strip. The strip has few of the heterogeneities that are present in the closed-chested system. Our observance of higher $\eta$ values \textit{in vivo} is in accordance with the reported theories of the effects of heterogeneities and branching asymmetry on lung mechanics.

Finally, we reported that the mechanics in the mouse, when compared to other species, are different. \textit{In vivo}, $H$ is higher in mice than in other species; $\eta$ is lower. Our data are similar to what was reported by Gomes \textit{et al.} (17) who measured impedance ($Z$) \textit{in vivo} in mice, rats, guinea pigs, and rabbits. In our
experiment, in vitro measurements showed that $G$ and $H$ were higher in mice than values reported in other species in the literature; $\eta$ was again lower. Faffe et al. (13) measured resistance and elastance at a single frequency in rat and mouse lung tissue strips and reported higher $\eta$ in rats compared to mice. Our data confirm that mouse lung mechanics are different from those reported in other species, both in vivo and in vitro.

One potential explanation to account for the species dependant differences in mechanical properties of lung tissue relates to differences in lung architecture (13, 16, 34, 54). Mercer and colleagues (34) found variations in the relative amounts of collagen and elastin fibers in the lung tissues across a number of species, including mice, rats, and humans. They also measured thickness of the alveolar septum and again found species-related variations. Faffe et al. (13) compared the composition of lung tissue strips in rats and mice and found that the specific components of the elastic system varied. Mouse lung strips had fewer fully developed elastin fibers and more oxytalan, a component of the elastic fiber system, than rat lung strips. Further, mice have proportionately larger airways that end abruptly, instead of tapering off as in other species (54). Mice also display more symmetry in the branching of their airway tree (16). These differences in anatomy and airway geometry may account for the lung mechanical properties of mouse lungs being different compared to other species.
4.4 Conclusion

In conclusion, we have shown that lung tissue mechanics are altered in the
*Den-/-* mice. Compliance is increased in the *Den-/-* mice at higher pressures and
stresses. *R*<sub>aw</sub> is decreased in the *Den-/-* mice. In *Den+/* mice, decorin was
detected in the vessel and airway walls. Biglycan protein in the lung tissue was
not different in the two groups; thus biglycan was not upregulated to compensate
for the absence of decorin. The absence of decorin seems to cause a change in the
mechanical properties of the tissues of the lung in such a way as to increase the
overall respiratory system compliance, increase the tissue compliance specifically,
and, via airway-parenchymal interdependence, reduce the *R*<sub>aw</sub>. This alteration in
tissue properties likely results as a consequence of the abnormal collagen fibril
formation that occurs in *Den-/-* mice (9). Additionally, we have added to the
characterization of mouse lung mechanical properties and have shown that
complex impedance is readily measured in mice, both *in vivo* and *in vitro*. 
REFERENCES


56. Venkatesan, N., P. J. Roughley, and M. S. Ludwig. Proteoglycan expression in bleomycin lung fibroblasts: role of transforming growth factor-


