Research Article

The critical role of collagen VI in lung development and chronic lung disease

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Abstract

Type VI collagen (collagen VI) is an obligate extracellular matrix component found mainly in the basement membrane region of many mammalian tissues and organs, including skeletal muscle and throughout the respiratory system. Collagen VI is probably most recognized in medicine as the genetic cause of a spectrum of muscular dystrophies, including Ullrich Congenital Myopathy and Bethlem Myopathy. Collagen VI is thought to contribute to myopathy, at least in part, by mediating muscle fiber integrity by anchoring myoblasts to the muscle basement membrane. Interestingly, collagen VI myopathies present with restrictive respiratory insufficiency, thought to be due primarily to thoracic muscular weakening. Although it was recently recognized as one of the (if not the) most abundant collagens in the mammalian lung, there is a substantive knowledge gap concerning its role in respiratory system development and function. A few studies have suggested that collagen VI insufficiency is associated with airway epithelial cell survival and altered lung function. Our recent work suggested collagen VI may be a genomic risk factor for chronic lung disease in premature infants. Using this as motivation, we thoroughly assessed the role of collagen VI in lung development and in lung epithelial cell biology. Here, we describe the state-of-the-art for collagen VI cell and developmental biology within the respiratory system, and reveal its essential roles in normal developmental processes and airway epithelial cell phenotype and intracellular signaling.

Introduction

Type VI collagen (collagen VI) is an extracellular matrix protein found mainly in the basement membrane region. It can be observed throughout much of the lung structure, including in the alveoli, airways, and vasculature. However, little is known about the role of collagen VI in lung development or disease. Recent work demonstrates that collagen VI genes are significantly differentially expressed in the chronic lung disease, bronchopulmonary dysplasia (BPD) and animal models of BPD. We also identified common and rare variants that are significantly associated with BPD in two of the collagen VI genes. The lack of knowledge on the function of collagen VI in the lung, despite its abundance and broad presence, makes it a compelling target for the study of disease and normal function. Collagen VI is required for proper lung structure and maintenance of proper pulmonary epithelial function. We examined the effects of collagen VI loss-of-function on the murine lung, as well as the influence of collagen VI on cell autonomous

functions that may have critical involvement in processes like lung development and injury repair.

Lung structure

The structure of the lung plays a critical role in its function as the respiratory organ. The vast majority of the lung volume and surface area is occupied by alveoli, thin-walled acinar structures that represent the base unit responsible for gas exchange. Adult human lungs contain an average of nearly 500 million alveoli, providing roughly 70 m^2 of surface area [1–3]. In contrast, there are roughly 2.3 million alveoli in the mouse lung, which yield around 80 cm^2 of surface area [4]. Each human alveolus has an average diameter of 200 μm (80 μm in mice) [5]. The alveoli, which are largely composed of epithelium, microvasculature and extracellular matrix, make up what is known as the lung parenchyma. The alveoli are highly elastic structures that expand to fill with air and recoil with each breath [6]. Much of this dynamic property, as well as the stiffness and resistance to stretch which protect the lung from mechanical stress due to overinflation, is imparted by the composition of the extracellular matrix. The majority of the extracellular matrix in the alveolus consists of basement membrane. Where the epithelium lies directly over a capillary, this basement membrane is very thin, and is shared between the alveolar epithelium and endothelium, creating the shortest possible distance for gasses to diffuse between blood and atmosphere. The remaining portion of the alveolus, where the epithelium is not in close proximity to the vasculature, has a slightly thicker wall, with separate epithelial and endothelial basement membranes. This thicker region also contains elastin and additional interstitial matrix components and fibroblasts [7,8].

The conducting airways of the lung function to allow the passage of gasses into and out of the lung parenchyma [9]. In humans, the bronchi, bronchioles, respiratory bronchioles and alveolar ducts, represent the branched structure connecting the alveoli with the trachea [10,11]. Mice do not have respiratory bronchioles, instead the bronchioles transition to the bronchoalveolar ducts and alveolar ducts [11]. In humans, the bronchi, the largest of the airways, are around 20 mm in diameter. The airways slowly decrease in diameter, down to around 0.5 mm at the respiratory bronchiole [11,12]. In mice, the bronchi are around 2 mm in diameter, decreasing to around 0.1 mm at the bronchoalveolar duct [13]. These bronchi are composed mainly of smooth muscle, cartilage and epithelial layers. The cartilage and muscle gradually diminish and become discontinuous as lower generations give way to bronchioles [10,11]. The cartilage forms continuous plates around the larger bronchi; moving distally, these become smaller and more discontinuous and are nonexistent at the point of the terminal bronchioles. The smooth muscle layer forms a continuous sheath around the bronchi, and gradually becomes thinner and less continuous, ending at the respiratory bronchiole [10,11]. In mice, the cartilage rings end at the main bronchus, while the smooth muscle layer extends to the terminal airway [11]. Unlike the alveoli, the airways are much more rigid, and retain their structure throughout the respiratory process [10,14].

In addition to these gas-handling airways and alveoli, the lung is highly vascularized. Larger vessels (arteries and veins) are typically found adjacent to airway structures (bronchi and bronchioles) [15]. These are the arteries and veins that serve to bring oxygen-depleted blood into the lung, specifically the alveoli, and oxygenated blood back to the heart. The alveoli contain a rich capillary bed, also known as the pulmonary microvasculature, with a large surface area to aid in efficient gas exchange [15,16]. These capillaries, like the alveoli, are somewhat dynamic structures that must handle the expansion and retraction associated with inspiration and expiration [17,18]. The matrix aids in contributing both elasticity and rigidity to these structures. The vasculature is completely lined by an endothelial layer and can be additionally supported by other cell types, including smooth muscle, fibroblasts, and pericytes [15]. The endothelium of the microvasculature found within the parenchyma serves two critical roles in the lung. It provides a barrier between the blood and the tissue environment, and allows for efficient exchange of molecules like oxygen and carbon dioxide across the alveolar epithelium between the blood and the atmosphere [19,20].

Lung epithelium

This alveolar surface is covered by specialized epithelium comprised of alveolar epithelial type I (ATI) and type II (ATII) cells. Type I cells are very flat, thin cells that cover over 95% of the alveolar surface area. Each ATI cell covers an average of 5400μm^2 [21]. These membranous cells are found covering the microvascular bed of the alveoli, and along with the vascular endothelial cells, form a boundary between the blood and atmosphere known as the air-blood-barrier. The primary responsibilities of ATI cells are creating a thin barrier that allows gasses to diffuse as efficiently as possible and controlling the movement of water between the external and internal environments [22,23]. The movement of water across the pulmonary epithelium is critical to the transition from the aqueous intrauterine environment to the external environment at the time of birth, maintaining water homeostasis, and in forming and attenuating pulmonary edema during injury response [22]. In addition, ATI cell spreading and folding appear to be crucial properties in the development and maturation of alveoli [24].

Though ATII cells cover roughly 5% of the alveolar surface area, these cuboidal cells make up as much as 60% of the alveolar epithelial cell

population [25]. These cells are commonly found at the "corners" of alveoli, where the septa of two alveoli meet. ATII cells contain microvilli on the apical cell surface, and lamellar bodies, which are specialized organelles involved in pulmonary surfactant secretion [26]. The ATII cells provide support to the alveolar epithelium by acting as a progenitor to ATI cells during development and repair. In addition, they produce and recycle pulmonary surfactant, a lipid-rich fluid that helps to regulate surface tension in the lung and promote dissolution of gasses [25,27,28].

The airway epithelium runs continuously from the trachea to the alveoli, but varies in composition from the larger upper airways to the lower respiratory bronchioles. The upper airways (trachea, bronchi and large bronchioles) are lined with pseudostratified, ciliated, columnar epithelium which transitions to ciliated columnar epithelium in small airways and terminal bronchioles, and finally to cuboidal epithelium in respiratory bronchioles and alveolar ducts [29,30]. The epithelium of the upper airways is referred to as pseudostratified because it is a single layer in which all cells are in contact with the basement membrane, but not all cells reach the airway lumen. Histologically, this gives the impression of multiple layers of epithelial cells [31]. In mice, the pseudostratified epithelium is more proximal in location, found mainly in the trachea, transitioning to columnar epithelium through the majority of the bronchi and intrapulmonary airways [11,32].

There are multiple cell types in the pseudostratified airway epithelium, including ciliated columnar epithelial cells, which constitute roughly 50% of the cells in this layer, basal cells, goblet cells and club cells [10,29,33]. Ciliated columnar epithelial cells function in the movement of mucous from the lung to the trachea [29]. Goblet cells produce mucus, which is an acidic, glycoprotein-rich fluid that covers the apical surface of the airway epithelium. The main function of mucus is to protect the lung from foreign particles and chemicals [34]. Goblet cells and ciliated cells work in concert in a continuous process of clearance and renewal of the mucus layer. By clearing inhaled contaminants and pathogens, as well as providing a warm, humid environment, the airway also functions to condition the air as it enters the lung parenchyma [10,30].

Club cells are also secretory, producing numerous glycoproteins, lipids and proteins. The functions of these secretions include maintaining chemical and physical properties of the lung, aiding in the prevention of lung collapse, for example [29,35]. Basal cells function as a progenitor in the airway epithelium and are able to proliferate and differentiate into ciliated and goblet cells. In addition to their role as progenitor cells, basal cells are firmly attached to the basement membrane and support the attachment of other airway epithelial cell types [29,36]. The more distal, columnar airway epithelium consists mainly of ciliated and goblet cells [11]. The cuboidal epithelium represents the transition between airway and alveolar epithelium, and is made up of nonciliated cells that closely resemble ATII cells [37,38].

Lung extracellular matrix

The extracellular matrix, comprised of noncellular structural and signaling components of the lung, plays critical roles in normal function and homeostasis, as well as development and injury repair. The main components of the matrix are fibrous proteins (collagens, elastin, fibronectin, *etc.*), proteoglycans and glycoproteins [39,40]. Structurally, the matrix is responsible for imparting tensile strength and elasticity to the lung tissue, allowing for resistance to expansion and recoil [39,41]. In addition to structural properties, the matrix also provides a niche for adjacent cells, ensuring proper cell function, and regulating processes like differentiation and proliferation [42–44]. In the lung, the matrix is often divided into two categories, defined by location: the interstitial matrix and the basement membrane [42]. The interstitial matrix, composed mainly of collagens I and III, and elastin, is found in the lung parenchyma, the region of the lung responsible for gas exchange. It serves to connect the major structures of the lung and imparts most of the mechanical properties attributed to the lung [45]. The basement membrane is a thin, specialized sheet of matrix that can be found at the basal side of epithelial and endothelial cells, as well as surrounding muscle and nerve cells [40,42]. The basement membrane is thought to serve as an interface between the cellular and matrix components of the lung epithelium, endothelium and musculature, and acts as a barrier between the epithelium and external environment, and the internal environment (Fig. 1) [48].

The basement membrane contains many components, including fibronectin, collagens IV, VI, and XVII, laminins and multiple other proteoglycans and glycoproteins [49,50]. The basement membrane regulates pulmonary epithelial and endothelial cell function and plays multiple roles in lung development. For example, during development, basement membrane is thought to be involved in directing branching morphogenesis by patterned localization of certain components, including fibronectin, and collagens I, and III [51–54]. Under homeostatic conditions, the basement membrane provides an anchoring point for epithelial cells, and supports survival and differentiation [55]. Alterations in the composition of the basement membrane are also known to impact pulmonary epithelial function and can lead to reduced adhesion and apoptosis of the epithelial cell layer [50,56]. Dysregulation of basement membrane components is known to accompany multiple diseases that affect the structure and function of the lung, including

chronic obstructive pulmonary disease (COPD), asthma, idiopathic pulmonary fibrosis (IPF), and bronchopulmonary dysplasia (BPD) [57–61].

Fibrillar collagens, particularly collagens I and III, represent major constituents of the interstitial matrix of the lung. Major functions of fibrillar collagen include forming three-dimensional frameworks and providing mechanical strength to a tissue [62]. Concordant with these functions, collagens I and III are critical in regulating stiffness of the lung structure [63]. These collagens undergo cross-linking *via* lysyl oxidase; the extent of this cross-linking is a factor that controls the stiffness of the matrix [63]. These collagens are also involved in regulating developmental processes, including branching morphogenesis and alveolarization [64]. Dysregulation of the deposition, cross-linking of these collagens and other interstitial matrix components is involved in the pathogenesis of multiple lung pathologies, including IPF, and cancer [63,65]. In IPF, aberrant cross-linking of fibrillar collagens can cause an increase in stiffness in the lung, resulting in increased proliferation, contraction, and activity of lung fibroblasts, which contributes to fibrosis of the lung tissue [63,66].

Type IV collagen is a critical component of lung basement membrane. Unlike fibrillar collagens, collagen IV is known as a network-forming collagen, which generates a mesh-like structure [67]. It is thought that collagen IV is necessary for proper assembly of the basement membrane. Studies of collagen IV have shown that it is necessary for proper development of the alveoli, patterning of lung epithelium and endothelium, and is involved in regulating the deposition of other critical basement membrane components, including elastin [68,69]. Collagen IV mutants display abnormal angiogenesis and severely reduced development of the terminal air spaces [70,71]. Thickening of the basement membrane and accumulation of collagen IV has been shown in asthma [72]. Multiple studies have provided evidence for increased degradation of collagen IV in IPF, COPD, and BPD [73,74].

Elastin is a unique extracellular matrix component that is essential to proper lung function. The primary function of elastin is to provide elastic recoil to the tissue, that is, the force returning the lung to its original state after it is expanded upon inhalation [75]. Elastin forms a complex and highly crosslinked network in the extracellular matrix. Similar to fibrillar collagen, lysyl oxidase is responsible for cross-linking elastin proteins, which form a mature fibrillar network when associated with fibrillin-containing microfibrils [75]. The cross-linked form of elastin functions as an elastomer, allowing for expansion of the lung and providing recoil during exhalation [76]. The deposition of elastin contributes to lung development, as a director of alveolar development, and may play a role in vascular growth [64,75,77]. Elastin can be found throughout much of the lung structure, including the airways and larger vessels, though it is most abundant in the lung parenchyma, where it has been shown to be critical for function and development of the tissue [75,77–79]. Elastin has been shown to interact with many other extracellular matrix proteins, several of which are also known to interact with collagen VI, including microfibril-associated glycoprotein 1 (MAGP1), perlecan, and biglycan [80,81]. Alterations in elastin have been shown in multiple lung diseases. Destruction of elastin is a key factor in the development of emphysema [82,83]. Patients with COPD show a reduced ability to repair elastin [84]. Lung tissue from patients with BPD shows disorganization of elastic fiber structure [57,85,86]. The alteration of the ratio of collagen to elastin in these lungs is thought to affect both alveolarization, as well as pulmonary function [57].

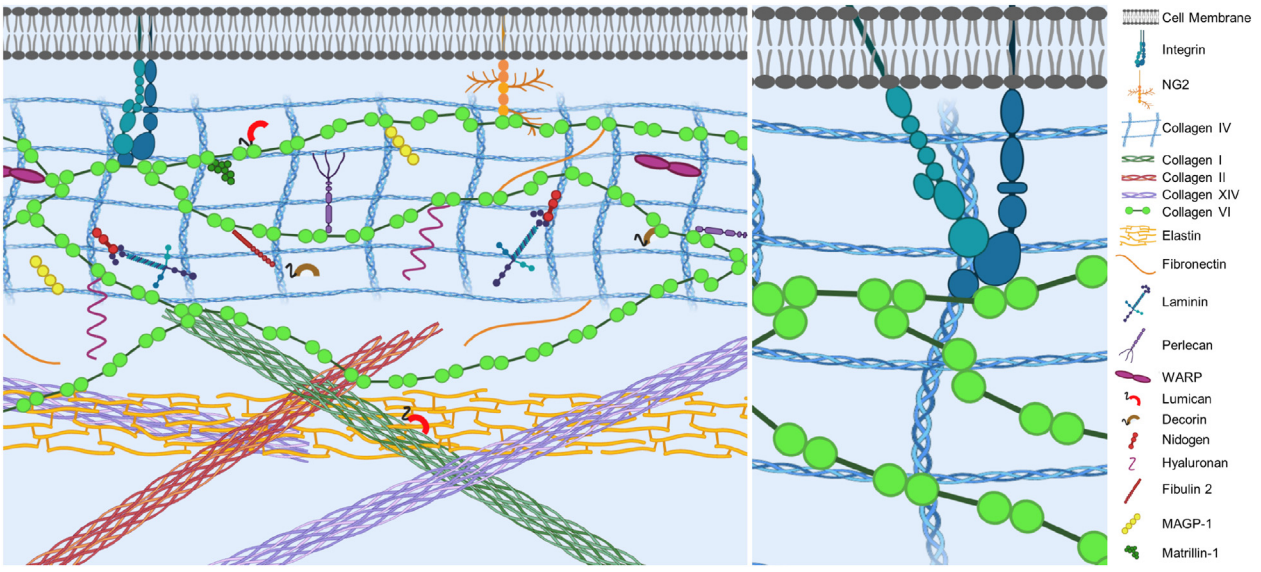

Fig. 1. Schematic representation of extracellular matrix underlying epithelial cells. Image adapted from [46,47].

Collagen VI

Collagen VI is primarily localized to the basement membrane region and can be found in association with epithelial and endothelial cells, as well as muscle and adipose tissue (Fig. 1). There are six collagen VI genes: *COL6A1*, *COL6A2*, *COL6A3*, *COL6A4*, *COL6A5* and *COL6A6*, which encode six separate peptide chains, annotated as α1(VI), α2(VI), α3(VI), α4(VI), α5(VI), and α6(VI) [87,88]. The *COL6A1* and *COL6A2* genes are located on chromosome 21q22.3, while *COL6A3* is found on chromosome 2q37 and *COL6A4*, *COL6A5* and *COL6A6* are located in tandem on chromosome 3q21 [47]. The *COL6A4* gene is not expressed in humans, due to a large chromosomal inversion separating the gene into two segments [88]. Each collagen VI peptide chain shares a similar general structure, though they vary in size. The majority of the chains are comprised of a triple-helix forming domain that is 335–336 amino acids in length, flanked by N-terminal and C-terminal Von Willebrand Factor A (VWF-A) domains [89]. The α1(VI) and α2(VI) chains are between 130 and 150 kDa in size and contain 3 VWF-A domains, while the α3(VI), α4(VI), α5(VI), and α6(VI) are between 220 and 300 kDa and contain between 7 and 10 VWF-A domains [47,90]. In addition, the α3(VI), α4(VI), α5(VI), and α6(VI) chains contain short, unique domains at the C-terminal end, and the α3(VI) and α4(VI) chains contain Kunitz-like domains, which are known to inhibit degradation by certain proteases [47,90,91]. In addition, there are known splice variants of *COL6A2* and *COL6A3*, which produce distinct isoforms of these chains that also appear to be differentially expressed across tissues, indicating additional variation and complexity in collagen VI structure, and potentially function, in different tissues [92–94].

Collagen VI chains form extensive quaternary structures intracellularly to produce functional collagen VI filaments. First, a triple helix containing an α1(VI), α2(VI) and an α3(VI), α5(VI), or α6(VI) chain form, referred to as the collagen VI monomer [95–99]. The α4(VI), α5(VI), and α6(VI) chains are structurally similar to the α3(VI) chain, with which they are thought to be interchangeable in the monomer [47,88]. Next, two of these monomers will associate to form antiparallel dimers stabilized by disulfide bonds, which then align to produce large disulfide-bond stabilized tetramers. These ~2000 kDa tetramers are secreted into the extracellular space, where they non-covalently interact end-to-end with other collagen VI tetramers to form the unique beaded-filament structure of collagen VI [47,100–105].

Once in the extracellular space, collagen VI is known to bind and interact with multiple other matrix constituents, and is thought to be involved in maintaining tissue organization and structure by connecting cells and matrix components with one another [47,106]. Known binding partners of collagen VI include Collagens I, II, IV and XIV, as well as MAGP1, perlecan, biglycan, and hyaluronan [81,90,106–110]. In addition to structural properties, collagen VI is also involved in signaling to cells it is in contact with. It is known to bind α1β1, α2β1, α3β1, α10β1 and αvβ3 integrins, as well as chondroitin sulfate proteoglycan 4 (CSPG4/NG2) [111–115]. The absence of collagen VI is linked with reduced survival in muscle cells, which is attributed to dysregulated autophagy and polarization. Collagen VI deficiency is also associated with an increased rate of apoptosis in pulmonary epithelial cells [116–118]. It has been shown that collagen VI promotes spreading and wound closure of lung epithelial cells *via* phosphoinositide 3-kinase (PI3k) and cell division control 42 homolog (CDC42) downstream of interaction with β1 integrins [56].

Collagen VI in disease

The importance of collagen VI in the basement membrane is emphasized by its central role in the spectrum of muscular dystrophies broadly referred to as the collagen VI muscular dystrophies. These include the better known forms of the diseases, Bethlem myopathy and Ullrich congenital muscular dystrophy (UCMD). These disorders are known to be caused by mutations in the *COL6A1*, *COL6A2* and *COL6A3* genes, which can affect collagen VI structure, or cause complete loss of collagen VI [119–121]. Collagen VI muscular dystrophies occur in around 1 in 100,000 individuals, and can range from mild, to severe and debilitating in presentation. The most severe form of the disease, UCMD, causes laxity of distal joins, and severe contractures of the hips, knees, elbows and spine, as well as muscular weakness [122]. If untreated, UCMD can lead to death due to respiratory insufficiency within the first few years of life [123]. Bethlem myopathy, commonly the more mild form of the disease, can cause joint laxity early in life, with development of joint contractures over time, and progressive increases in muscular weakness beginning later in life [124]. Patients with Bethlem myopathy can have a normal lifespan, though the myopathy can be fatal if respiratory insufficiencies are not treated [120]. In addition, there are intermediate phenotypes that cannot be classified as either Bethlem myopathy or UCMD, leading to the classification of the collagen VI muscular dystrophies as a broader spectrum [125–127].

The dysregulation or complete loss of functional collagen VI in these individuals significantly impacts muscle satellite cells, reducing their ability to self-renew [128]. Previous work has identified a potentially protective role of collagen VI against apoptosis in fibroblasts [129]. In a *Col6a1*$^{-/-}$ mouse that is unable to produce functional collagen VI, spontaneous apoptosis of muscle fibers due to mitochondrial dysfunction have been observed [117]. It has been shown that this mitochondrial dysfunction is due to inappropriate opening of the mito-

chondrial permeability transition pore due to aberrant cyclophilin D activity [130]. The nature of the effects of collagen VI loss on cyclophilin D activity is not yet known. This work implies a degenerative phenotype in collagen VI-related myopathies, consistent with the progressive nature of the diseases [131].

There is a high degree of respiratory involvement in UCMD and intermediate collagen VI muscular dystrophies, presenting as a progressive, restrictive phenotype [123,132]. Respiratory insufficiency is not common at birth, and commonly develops following the loss of ambulation, appearing first at night as nocturnal hypoxemia [133]. However, in contrast to other forms of muscular dystrophy, like Duchenne muscular dystrophy (DMD), the development of respiratory insufficiency in collagen VI muscular dystrophies can occur before the loss of ambulation, indicating that respiratory decline may occur more rapidly in collagen VI-related muscular dystrophies than other non-collagen VI related muscular dystrophies [123]. This is often attributed to the increased stiffness of the chest wall and diaphragmatic weakness caused by the loss of collagen VI [134]. Individuals with respiratory insufficiency often only need ventilatory support at night. However, left untreated, this ventilatory defect is often fatal in the late teenage years [132,133].

Studies directly examining the role of collagen VI in the lung are limited. The presence of collagen VI in the lung in close proximity to airway, alveolar epithelium, and vascular endothelium, has been established [50,56,135]. Recent mass-spectrometry based proteomic analysis identified COL6A1, COL6A2, and COL6A3 as the most abundant collagen proteins in adult mouse lung tissue [42]. Case studies of both neonates and adults with collagen VI muscular dystrophies have reported spontaneous pneumothoraces, subpleural blebbing, and fragile lung parenchyma in patients requiring surgery [136]. A murine model of Annexin A2 (ANXA2) loss-of-function, which is unable to secrete collagen VI, has been developed. These mice exhibit impaired adhesion and increased apoptosis of airway epithelial cells. In addition, *AnxA2*$^{-/-}$ and *Col6a1*$^{-/-}$ mice have altered lung tissue elasticity and reduced exercise tolerance [50,137].

Lung development

Lung development is divided into five stages, each resulting in increasing structural complexity of the lung. This leads to a fully functional respiratory organ with proper airway branching and distribution, and enough surface area and vasculature for efficient gas exchange at birth. The first stage of lung development is the embryonic stage, which begins around week 4 and lasts until week 7, corresponding with embryonic day (e) 9.5-e12 in mice. The initial formation of the trachea and lung buds from the ventral wall of the primitive foregut occurs early in this stage [64,138]. The process of branching morphogenesis begins during this time, forming bronchi and the basis of the lobular structure of the lung [64,139]. Precursors of the airway cartilage localize to the trachea and differentiate during the embryonic phase [64]. In addition to airway formation, the early pulmonary arteries and veins form in parallel to the branching airways [64].

Lasting from week 5 to week 17 (e12–e16.5 in mice), the second, or pseudoglandular stage, marks the formation of the bronchial tree and early parenchyma [64]. During this stage, extensive branching morphogenesis forms the first 20 generations of the airway, including some of the first alveolar ducts [140]. The early acinar structures of the respiratory airway are present at the terminal ends of the conducting airways after this phase [140]. Columnar and cuboidal epithelial cells are found lining the airways. By the end of the pseudoglandular stage, some ciliated, goblet and basal cells have begun to differentiate in the proximal airways [141]. The first Surfactant protein C (SFTPC) expressing precursors to ATII cells are also present during this phase [140]. Cells positive for α-smooth muscle actin begin to form around the proximal airways, and begin to contract, moving fluid through the lung and rhythmically expanding the airway structure [142]. Similar to the embryonic stage, the vasculature continues to grow in parallel to the airways, but more slowly [139,143].

The third stage of lung development, known as the canalicular stage, lasts from week 16 to 26 (e16.5-e17.5 in mice). In this phase, the distal airways form, and the lung nears the end of the process of branching morphogenesis. The air-blood barrier, thin regions of parenchymal tissue consisting of alveolar epithelium, endothelial cells, and a shared basement membrane that will be the future sites of gas exchange, also forms during the canalicular stage [143]. The airway epithelium differentiates during this phase, and the cuboidal cells lining the acinus differentiate to ATI and ATII cells. This allows for the distinction between the conducting airway and the respiratory acinus, which will eventually form the lung parenchyma [64,138,144]. The growth and elongation of the alveolar ducts results in a structure resembling “canaliculi,” giving this stage its name [64,145]. The capillary network surrounding the acinus undergoes extensive angiogenesis, resulting in a dense capillary bed around these structures [64]. The differentiated alveolar epithelial cells and the capillaries come into closer proximity, due in part, to remodeling and condensation of the extracellular matrix [145]. The differentiation of ATII cells is also accompanied by the production of pulmonary surfactant [146].

The fourth stage, called the saccular stage, begins at week 26 and lasts until week 36, or e17.5 through postnatal day (p) 4 in mice. During

this stage, the branching morphogenesis program ends, and the acini expand prior to the process of alveolarization. It is thought that the processes of branching morphogenesis and alveolarization cannot occur at the same time [138,147]. During the saccular stage, the acini grow in length and width, and form clusters of airspaces, which are referred to as sacculi. Due to the expansion of the airspaces, the mesenchyme condenses as sacculi come in contact with one another. These contact points are referred to as primary septa. The capillary network surrounding each saccule remains in close proximity to the alveolar epithelium, resulting in two layers of microvasculature in the primary septa [145]. It is important to note that while this stage occurs entirely *in utero* in humans, mice are born near the midpoint of the saccular stage, and the remainder of the stage occurs during the neonatal period.

The final, alveolar stage, begins shortly before birth in humans at week 36 and can last for up to 20 years after birth (in mice, this occurs from P4 to P36), though the majority of alveolar development occurs before age 3 (mouse P21). During this time, the thick saccular wall thins and secondary septa rise from the luminal surface [148]. Secondary septa are thin sections of alveolar wall that form out of the existing primary septa to increase surface area and separate individual alveoli [64]. This occurs by folding of the alveolar epithelium, which forces this wall of tissue upward from the existing surface of the septal wall (Fig. 2) [145]. The initiation of the secondary septal fold is assisted by the underlying vasculature. During the alveolar stage, the capillaries of the primary and secondary septa, which consist of two layers, fuse to form a single-layered network [64,139]. Connective tissue of the septa thins, and the airspaces grow in volume, forming alveoli that are able to efficiently exchange gasses between the blood and atmosphere [145].

ECM during lung development

The extracellular matrix is constantly evolving during lung development and is essential to multiple developmental processes. There are components of the lung extracellular matrix that are present and potentially involved in guiding airway branching in the embryonic and pseudoglandular stages. As early as 8 weeks into embryonic development, collagens I, III and VI are found surrounding the airways, but not the buds. It is postulated that these may stabilize the airways, while promoting growth and branching from the terminal lung buds [51]. Interaction between the airway and additional matrix components, including fibronectin and laminin, has been shown to drive airway branching [86]. Deposition of the collagenous extracellular matrix, which is very primitive until the saccular stage, increases, and it influences further growth and development of the pulmonary epithelium [140]. The α-smooth muscle actin positive myocyte precursors surrounding the airway deposit both fibrillar collagens and elastin around the saccule. Specific locations around the saccule, where elastic fibers and collagen fibrils have been deposited, mark the sites of future secondary septa formation [77,79]. Elastin deposition causes the secondary crest, the ridge that elongates to form the secondary septum, to elevate from the saccular wall. It is hypothesized that the elastin and fibrillar collagens prevent movement of the saccular wall, while the secondary crest elongates to form the alveolus [78].

Bronchopulmonary dysplasia (BPD)

Bronchopulmonary dysplasia is a chronic lung disease that is the most common morbidity associated with premature birth and very low birth weight (<1000 g) [149,150]. Clinical definitions of BPD typically include the need for supplemental oxygen at 28 days of life, or 36 weeks corrected gestational age [150–152]. Common characteristics of BPD include simplified alveoli, reduced pulmonary vasculature, and increased inflammation in the lung [16,85,151,153]. In addition to complications in the neonatal period, there are significant long-term sequelae associated with a BPD diagnosis. Individuals with BPD often suffer delays in growth and development, as well as cognitive impairments [154,155]. There are often long-term respiratory complications due to structural alterations of the lung tissue, including pulmonary hypertension and sleep hypoxia [156,157]. Finally, there is significantly increased risk of recurrent respiratory infections in individuals with BPD [158].

The risk for developing BPD significantly increases with birth prior to 30 weeks of gestational age and increases further with birth at earlier times [150,159]. Birth at these early gestational ages can occur in the saccular stage, or even late canalicular stage, long before the lung is prepared to supply the body with oxygen, or even to be exposed to the atmosphere [160]. This is prior to key developmental events, including alveolar formation, considerable deposition and remodeling of the extracellular matrix, sufficient surfactant production, and potentially prior to the completion of branching morphogenesis [139,140,160]. In order to ensure proper oxygenation of the blood in premature infants, one of the most crucial forms of treatment is supplemental oxygen (>21%) [161]. Though it is necessary, it has been shown in animal models that this treatment can cause damage and structural changes to the lung [162,163]. It is thought that immaturity of the lung, coupled with exposure to high oxygen levels and influence from additional risk factors, is responsible for the development of BPD [150,152].

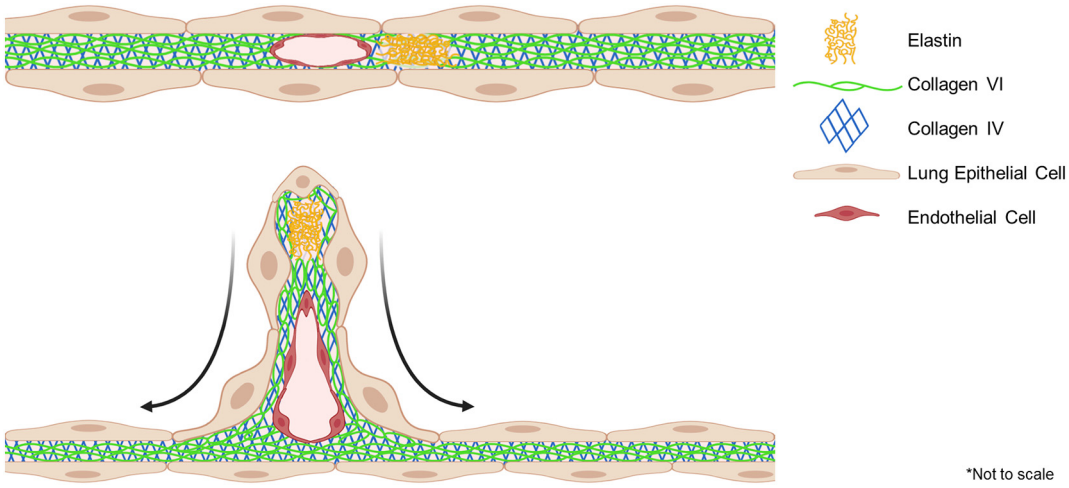

Fig. 2. Schematic representation of secondary septa formation.

Identification of genetic influence in BPD susceptibility

Multiple studies have shown that genetic influences are a major risk factor for the development of BPD. However, the specific genes, and their roles, have not yet been fully characterized [150,151]. Using twin-pair studies, the concordance of BPD among monozygotic twins is consistently higher than would be expected, and much higher than is observed in dizygotic twins [164–166]. These studies found that genetic factors account for as much as 53–82% of the risk for developing BPD. Many additional genome and transcriptome-wide studies have been conducted to identify candidate genes that may convey susceptibility and participate in BPD pathogenesis. Genome-wide association studies (GWAS) and copy-number variation (CNV) studies have been conducted to identify genes that may have sequence variants associated with BPD [167–169]. Of the few key findings from genetic studies of BPD are variants in sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2 (*SPOCK2*) that are associated with BPD at genome-wide significance. SPOCK2 is a proteoglycan component of the extracellular matrix known to increase in expression through the alveolar phase of lung development [170]. Exome sequencing studies have identified both rare and common variants that are associated with BPD. One such study identified 258 variants in genes that were largely associated with lung structure and function, particularly with the formation and organization of collagen fibrils, and embryonic airway development [171].

Some of the challenges in analyses of GWAS and CNV studies are thought to be due to small contributions from many genes [172–175]. The potential for multigenic influence can create an issue of power and disparate results across studies [176,177]. Each patient may not have the same subset of genetic influences, though the end result on lung development, injury repair and response to environmental influences may be similar [175]. The clinical definition of BPD, based on oxygen requirement, also allows for heterogeneity in the factors leading to the diagnosis [150,178]. Determination of the need for supplemental oxygen is somewhat subjective, and may lead to variation in the diagnosis of individuals, particularly those with more mild forms of the disease [179].

Studies examining the extracellular matrix have identified aberrant expression, organization and deposition of additional extracellular matrix components in BPD. Elastin, a critical matrix component in lung function and development, shows significant alterations in deposition and turnover in BPD lung tissue [85,160]. The deposition and organization of collagen is also aberrantly regulated in BPD, with thicker, disorganized collagen fibers in the lungs of BPD patients [180,181]. Lysyl oxidase enzymes, which function to crosslink collagens and elastin in the matrix, as well as lysyl hydroxylases, which catalyze the generation of hydroxylysine residues that serve as the substrate for lysyl oxidase show abnormal expression patterns in BPD and animal models of BPD [182–184]. In addition to structural constituents of the matrix, dysregulated expression of the matrix metalloproteinases MMP2 and MMP9 has been reported in BPD [185,186]. Matrix metalloproteinases are enzymes that degrade ECM and are necessary for matrix remodeling, development, and homeostasis. MMP2 and MMP9 are known as gelatinases, which degrade collagens IV and VI, and fibronectin, among other targets [187].

Perspectives

Prior to recent studies, there was little knowledge of the function of collagen VI in the lung. The presence of collagen VI had previously been shown in the basement membrane region of the airway, subjacent to the epithelium [135]. Individu-

als with intermediate and severe (UCMD) forms of collagen VI muscular dystrophy nearly always have significant respiratory morbidity. One study has provided evidence for structural abnormalities of the lung parenchyma in these patients [136]. Work examining a mouse model lacking the ability to deposit Collagen VI has identified altered pulmonary function, reduced exercise tolerance, and altered airway epithelial cell morphology. Integrin β1 and the proteoglycan NG2 have been identified as cell surface molecules that may interact with collagen VI [188]. In addition, collagen VI is thought to anchor cells, including muscle and epithelial cells, with other matrix constituents and cells within the mesenchyme. These studies have begun to identify collagen VI as an important component of lung ECM, and potential regulator of pulmonary epithelial cell function. However, additional studies examining the effects of collagen VI on lung structure and function are called for [136,189].

Collagen VI in lung development

Studies in the mouse lung have identified potentially significant role for collagen VI in the development of three major components of lung tissue: the airways, alveoli, and vasculature. Examination of collagen VI in mouse and human lung tissue revealed broad distribution of collagen VI protein throughout the lung. There was strong staining of collagen VI in the basement membrane regions of the alveoli, airways, and large vessels. Studies of a *Col6a1*$^{-/-}$ mouse that does not produce any detectable collagen VI protein identified simplified alveolar structure, similar to what is observed in BPD and animal models of BPD [137,190]. These mice also display alterations in respiratory system resistance and compliance consistent with this emphysematous phenotype. Within this simplified parenchyma, there was significantly reduced microvasculature and alveolar epithelial type II cells [137].

The ontogeny of the structural alterations observed in the *Col6a1*$^{-/-}$ mouse lung have yet to be described. This appears to be a developmental defect, rather than a degenerative effect, for several reasons. The alveolar simplification phenotype is observed relatively early in life, soon after the completion of lung development. An increased number of airways suggest an overgrowth rather than a destructive process. In addition, acute inflammation was not detected in *Col6a1*$^{-/-}$ mouse lungs, and their lifespan appears to be unaffected. This contrasts with the collagen VI muscular dystrophies. There are some developmental aspects, especially in the more severe forms of collagen VI muscular dystrophies, like UCMD, that can lead to obvious muscular weakness and contractures at the time of birth [191]. However, there is significant destruction of muscle tissue that leads to the progressive nature of these muscular dystrophies [116,120,192,193]. Examination of defects in the lung may provide additional insight into developmental and destructive pathophysiology associated with these muscular dystrophies.

Development and maturation of the alveoli requires extensive participation of the matrix. Studies have shown that matrix deposition is a required event for alveolar septation [77]. In addition, spreading and migration of both epithelial cells and fibroblasts are necessary for the coordination of alveolar septation [194]. It is thought that elastin and collagen stabilize the newly formed ridge in the saccule that elongates to become the septa. This stabilization may prevent movement while the saccular wall expands, extending the secondary septum, and forming the alveolus [43]. Due to the localization of collagen VI to the basement membrane, and its interactions with biglycan, perlecan, and MAGP1, all of which are also known to bind elastin, it may be participatory in this event. The loss of collagen VI may lead to destabilization of the developing septa or the interactions between epithelium and matrix, hindering the formation of the alveoli. During the alveolar stage, ATI cells spread rapidly, sometimes spanning across multiple alveoli. This spreading event is concurrent with alveolar septation [24]. It is possible that alveolar septation is enhanced by the expanding surface area of the epithelium, causing epithelial buckling, or forcing elongation of the septa. Reduced spreading capacity in ATI cells may prevent this from occurring, which could slow alveolarization, or result in the formation of fewer alveoli. This hypothesis is supported by the observation of reduced total surface area of the lung epithelium in *Col6a1*$^{-/-}$ mice.

In addition to altered alveolar structure, we identified an apparent increase in airway number that would indicate a defect causing excessive airway growth or branching morphogenesis [137]. Previous work has shown that the presence or absence of extracellular matrix components can influence branching morphogenesis. During branching, collagen I accumulates at the end bud and constricts branching. Conversely, fibronectin, which accumulates in the clefts, has been shown to promote the propagation of epithelial buds from the globular primitive airway structure [54]. Early in the branching process, collagen III also accumulates at the cleft, and is thought to provide a more rigid structure to stabilize this newly formed cleft [195]. Collagen III has also been shown to be necessary for the formation of collagen I into fibers. The localization of collagen VI with collagens I and III in the branching airway suggests that it may share a similar role, or aid in the function of other ECM components during these critical events [51]. The presence of collagen VI may be required to direct either bud formation or branch elongation. The loss of collagen VI may result in destabilization of the early buds, or clefts, resulting in multiple branches forming from what should be a single

bud prior to elongation. Also, collagen VI, along with collagen I, collagen III, and multiple other ECM components, is found surrounding the elongating branches, but not in the terminal surface of the elongating bud. This suggests that these components may also stabilize the elongating airway branch. The absence of these components at the end bud may promote the formation of new branches at the terminal surface, while preventing new branches from forming along the airway. However, the loss of collagen VI may result in disorganization of the ECM and destabilization of this matrix sheath surrounding the airway, allowing for the aberrant formation of additional airways during branching morphogenesis.

The finding that airway epithelial cells spread, and perhaps migrate more rapidly in the presence of collagen VI, presents an additional mechanism for its involvement in branching morphogenesis [56]. One method of branch formation thought to be observed in lung airway branching is by epithelial folding, typically induced by differential growth of the epithelium or epithelial buckling. This can occur by greater proliferation of the epithelium relative to nearby tissue, or constriction of the apical surface of the epithelial cells, causing a bulge that becomes the new branch. Compressive forces on the epithelial layer can also cause folds to form, which then propagate into additional branches [196].

In addition to the reduced spreading efficiency of cells plated on matrices not containing collagen VI, we observe an increase in cell density and reduction in overall cell size of confluent airway epithelial cells in the absence of collagen VI [56]. The increased density and altered morphology of airway epithelial cells in the absence of collagen VI may result in aberrant bulging or compression of the epithelial layer, resulting in the formation of additional airway branches. Further, the lack of collagen VI may reduce the structural integrity or rigidity of the matrix, allowing for these events to occur more readily.

Because development of the microvasculature is closely associated with the development of the alveoli, it is likely that reduced alveolar development would contribute to a reduction in microvasculature, as well. However, we find a disproportionately large reduction in vasculature relative to the extent of alveolar simplification, which is potentially indicative of a direct effect of collagen VI loss on vascular development [137]. The lung vasculature develops through a branching process by invasion into the surrounding tissue, which requires spreading and morphogenesis of the endothelial cells [196]. Numerous studies have determined that matrix composition, and the presence of particular matrix components is critical for proper cell spreading and shape [197]. If endothelial cells respond to collagen VI similarly to airway epithelial cells, the loss of collagen VI could suppress this process, causing a reduction in overall vascularization of the lung.

Modifications of the extracellular matrix are known to be important steps in the development of the pulmonary epithelium. During the canalicular stage of development, the capillaries and respiratory acini are brought into closer proximity to one another, in large part because of condensation of the extracellular matrix [145]. Loss of collagen VI may hinder this process, as one of the functions of collagen VI is thought to be as a tether between cells adjacent to the basement membrane and the underlying matrix [47]. This may be a particularly important function in the region between the alveoli and respiratory microvasculature, where the basement membrane is shared. Late vascular development is thought to occur in tandem with alveolar development, and it has been suggested that there is crosstalk between the alveolar epithelium and the capillary endothelium [139]. Disruption of the shared basement membrane could affect the proximity of these cells and their ability to communicate during these processes, potentially negatively impacting vascular development. Experiments in the field of cancer biology have identified that melanoma tumors implanted in the *Col6a1*$^{-/-}$ mouse brain develop smaller vessels, which have increased leakiness. In addition, the vessel pericytes do not mature properly, and endothelial cell survival is reduced [198]. The improper maturation of pericytes may result in aberrant repair of the vasculature in an injury scenario. While we did not test for vascular leak, the maintenance of the air-blood barrier is critical for efficient gas exchange and the homeostasis of the lung. Increased vascular leak can lead to pulmonary edema, and infiltration of immune cells, leading to increased tissue damage [199].

CDC42 activity as a regulator of branching morphogenesis and epithelial cell organization

Studies examining the role of CDC42 in lung development have identified critical roles in branching morphogenesis and epithelial cell organization. The loss of CDC42 in mice results in enlarged airways, with disorganized buds at the tips. In addition, the distal airway epithelial cell layer is disorganized, with a pseudostratified appearance, rather than a simple columnar layer [200]. The airway epithelial cells do not properly polarize in the absence of CDC42, and it is hypothesized that this is the reason for the disorganization of the epithelial layer and impaired branching morphogenesis [200,201]. The airway epithelial cell layer in *Col6a1*$^{-/-}$ mouse lungs is disorganized, with what appears to be a large number of nuclei not residing on the basal side of the cell, near the basement membrane [137]. This is reminiscent of a pseudostratified epithelial layer. In addition, the cell density is significantly increased *in vivo*, in *Col6a1*$^{-/-}$ airways, and *in vitro*, in cells plated on

matrices not containing collagen VI [56,137]. It has been observed that CDC42 activity is a key component of the enhanced spreading and wound-healing response to collagen VI in airway epithelial cells. Inhibition of CDC42 and PI3K results in increased airway epithelial cell density on collagen VI, nearly to the cell density observed on Matrigel or collagen I [56]. It is possible that in the absence of collagen VI, the branching epithelium is unable to properly polarize, leading to disorganization of the epithelial layer, and irregular branching. This may be due to a reduction in paxillin and PI3K signaling, resulting in reduced activation of CDC42. Further examination of the polarization of airway epithelial cells in the presence and absence of collagen VI could provide insight into the developmental origin of the structural defects observed in the *Col6a1*$^{-/-}$ mouse lung.

Collagen VI variants in human disease

A study by Hamvas et al. examined exome sequencing data from subjects with and without BPD. This dataset was examined for variants in collagen VI that were significantly associated with BPD. Analysis of this data identified a common variant in COL6A5 that is significantly associated with BPD (EMMAX $p < 0.05$). The variant (rs11917356) is a guanosine (minor allele) substitution for adenosine (ancestral allele) and has a frequency of 23.9% among no BPD subjects and 32.1% among BPD subjects [202]. This is a missense variant, causing the substitution of an asparagine residue with a glycine residue within an α-helical region of one of the n-terminal VWF-A domains of the α5(VI) chain (Fig. 3). In contrast to common variant analysis, which examines specific loci, FFB-SKAT analysis combines all variants (rare and common) identified in each gene and determines the association of that set of variants with the phenotype. When performing variant analysis of subjects with and without BPD by FFB-SKAT, the *COL6A6* gene was identified as having significant differences between the two groups. A total of 52 common and rare variants were identified across this gene (FFB-SKAT $p < 0.05$) (Supplementary Table 1).

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The collagen VI human sequence variants identified in association with BPD have the potential to affect the formation or function of collagen VI protein. Even in the absence of changes in gene expression, variants have the ability to affect protein production and function, and similar variants in other collagen proteins are known to impact epithelial cell adhesion and wound healing, leading to deleterious pathologies [203]. The common variant in *COL6A5* associated with BPD causes an amino acid substitution of glycine for asparagine (Fig. 3). Asparagine is polar, is commonly involved in active or binding sites, and is frequently modified post-translationally. The substitution of a small, flexible, uncharged glycine in place of an asparagine may affect secondary, tertiary, or quaternary protein structure by changing any of these functions [204,205]. This substitution is generally thought to be neutral, not favored or disfavored, though in some proteins this can be a highly unfavorable substitution [206]. This variant, therefore, may impact the overall structural organization or function of the complex collagen VI tetramer or beaded filament. Similar mutations affecting glycine residues in the highly homologous *COL6A3* gene have been identified as causal mutants in Bethlem Myopathy and Ullrich congenital muscular dystrophy cases. These mutations include multiple Glycine → Asparagine substitutions [207]. This supports the hypothesis that the *COL6A5* mutation may negatively impact its function, or the formation of properly functioning collagen VI monomers. A variant altering the function of one of the collagen VI chains could lead to reduced production of functional collagen VI or the destabilization of its structure once in the extracellular matrix. This would occur if the variant affected the interaction of the fully-formed collagen VI hererotetramers, preventing the formation of beaded filaments, or by blocking a binding site required for interaction with cells or other matrix components. Depending upon the way in which the mutation alters collagen VI formation or function, there is a potential for a large proportion of the collagen VI produced to be rendered nonfunctional, even without alterations to gene expression.

The combined variants identified in *COL6A6* are less understood. There were 52 total variants identified in this chain, any one of which has the potential to be silent, or highly deleterious to the chain. Further analyses of the exome sequencing

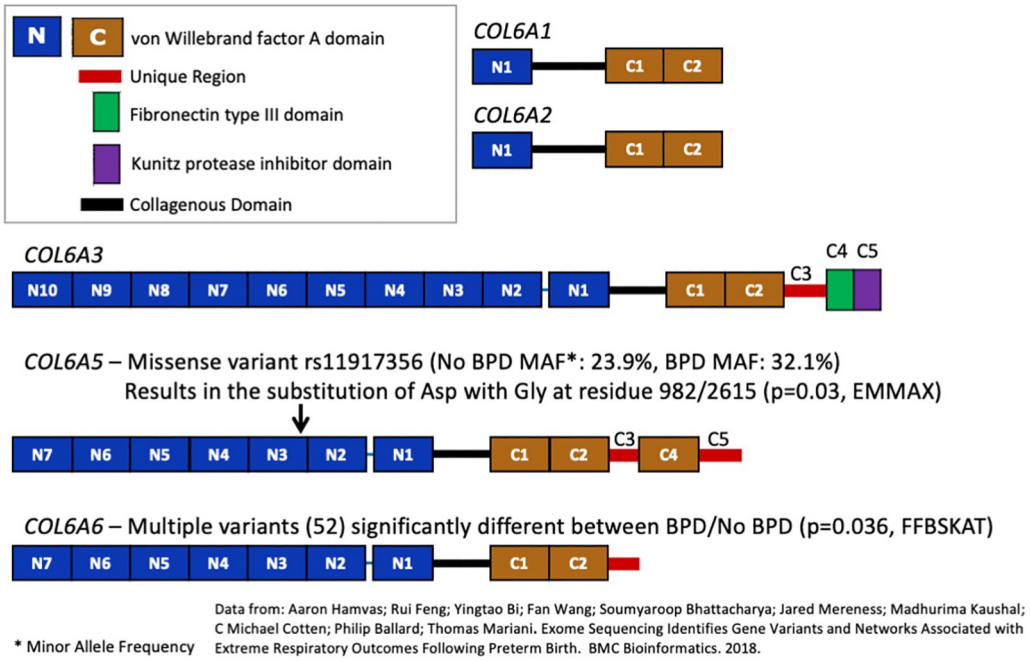

Fig. 3. Exome sequence variants identified in collagen VI having significant association with BPD. Schematic representation of general protein chain structure for all collagen VI genes expressed in humans. (a) Description of common exome sequence variant identified in *COL6A5* with significant association with BPD. (b) Analysis of combined common and rare variants across genes identifies significant differences between BPD cases and No BPD controls in multiple *COL6A6* chain variants.

data are necessary to fully characterize the variations in *COL6A6* and their potential impact to the α6(VI) protein chain. Even though the α3(VI), α5(VI), and α6(VI) chains are potentially redundant, a variant-containing chain expressed at low levels still has the potential to impact functional collagen VI tetramers due to the extensive structural organization that occurs in the formation of each functional collagen VI subunit. Previous work has shown that the loss of collagen VI in the basement membrane may impact pulmonary function and epithelial homeostasis [50,51]. It follows, that loss of functionality of collagen VI may cause similar alterations to the lung.

Common variants, like the *COL6A5* mutation and any common variants among those identified in *COL6A6*, are, by definition, present in a relatively large portion of the population (including controls) [202]. If these mutations caused severe deleterious effects to the respiratory system, they would likely not be present in individuals without respiratory problems. Therefore, the effects of the mutations are likely subtle. It is possible that they may cause mild defects in lung structure, or result in delayed development of the lung structure. When coupled with premature birth, this could result in a lung that is more premature than gestational age might suggest, which could leave the subject at higher risk of aberrant lung development or damage by oxygen exposure. Hyperoxia exposure in mice resulted in reduced expression of multiple collagen VI chains [208]. It is possible that this occurs in newborns exposed to supplemental oxygen. Coupled with a mutation that reduces the function of collagen VI, this may reduce the amount of functional collagen VI to some critical point that it causes developmental arrest or impairs injury repair. These mechanisms causing reduced collagen VI function could impact lung development as outlined previously in this chapter. In this fashion, common variants, like those identified in *COL6A5* and *COL6A6* could increase susceptibility to the development of BPD in individuals born prematurely.

The association of mutations in the *COL6A5* and *COL6A6* genes with the development of BPD raises an interesting question: why don't individuals with BPD develop a myopathy or muscular dystrophy phenotype? First, a majority of BPD patients likely do not carry mutations in collagen VI genes. For example, the *COL6A5* variant is only present in 32.1% of BPD subjects. While mutations in collagen VI genes may convey susceptibility to individuals that carry them, collagen VI is likely one of many genes that influence susceptibility to BPD. For BPD patients that do carry mutations in collagen VI genes, the lack of muscular phenotype may be due to tissue-specificity in the expression of the α5(VI) and α6(VI) chains. According to expression data from the BioGPS Barcode on normal tissues, the α1(VI), α2(VI), and α3(VI) chains, which are known to be mutated in the collagen VI muscular dystrophies, are highly

expressed in the muscle and other tissues where collagen VI is present. However, the α5(VI) and α6(VI) chains are expressed at very low levels in skeletal muscle, while they are expressed at much higher levels in the lung [209–211]. Since these chains are thought to substitute for the α3(VI) chain, mutations in these chains may lead to effects in the lung, while leaving the muscle unaffected.

While mutations of the α5(VI) and α6(VI) chains may lead to lung-specific alterations in collagen VI production or function, due to their more ubiquitous expression, mutations in the α1(VI), α2(VI), and α3(VI) chains are likely to have much more broad effects. Due to the expression patterns of the collagen VI chains, the lung has the potential to be impacted by any mutant. There is some evidence that lung structure may be altered in individuals with known mutations in the α1(VI), α2(VI), or α3(VI) chains, presenting with collagen VI-related muscular dystrophy [136]. These individuals are susceptible to pneumothorax and blebbing of the lung parenchyma, indicating possible loss of the structural integrity of the lung tissue. This supports the hypothesis that lung structure has the potential to be impacted by mutation of the collagen VI chains. It is possible that individuals with mutations in collagen VI that would cause myopathy may also be susceptible to developing BPD, should they be born prematurely. However, this is likely to be a rare event that would be difficult to study, as evidence of a myopathy may not present until many years after. Additional studies examining the involvement of the lung tissue in collagen VI muscular dystrophies would be highly informative to the potential effects of collagen VI loss of function on human lung structure, as well as provide a better understanding of the respiratory insufficiencies developed in a majority of collagen VI-related muscular dystrophy cases. Examination of lung structure in collagen VI muscular dystrophy cases could also provide insight into the potential role of collagen VI as a factor in BPD susceptibility.

Areas for future study

The work described here opens up several questions for future study. The characterization of the developmental roles of collagen VI is necessary to elucidate the mechanism of structural changes in the absence of collagen VI. Conversely, the determination of any degenerative effects of collagen VI loss would also be critical to the interpretation of data on changes in lung structure. In addition, developmental and potentially degenerative effects of collagen VI loss of function would provide insight into the ontogeny of respiratory insufficiencies in collagen VI muscular dystrophies, and potentially BPD. Further study of the *Col6a1*$^{-/-}$ mouse model may have potential use in the study of BPD pathology and susceptibility, particularly in combination with known environmental influences associated with BPD. Examination of the paxillin signaling pathway and its involvement in the Integrin-PI3K-CDC42 signaling mechanism in response to collagen VI would provide a more clear mechanism of collagen VI signaling, and may provide an additional target for manipulation to mitigate its absence.

Developmental and degenerative effects of collagen VI loss

We hypothesize that the structural abnormalities observed in the lungs of *Col6a1*$^{-/-}$ mice are developmental in origin. Examination of lungs from *Col6a1*$^{-/-}$ mouse lungs at earlier time points representing different stages of lung development is necessary to fully understand the developmental role of collagen VI in the lung.

The Col6a1$^{-/-}$ mouse as a potential model of BPD pathophysiology

We hypothesize that the loss of collagen VI may result in increased susceptibility for developing BPD-like pathology. To test this hypothesis, the loss or reduction of functional collagen VI could be combined with hyperoxia exposure, which is known to contribute to the development of BPD. It is known that the extent of oxygen exposure is a major risk factor in the development of BPD due to oxidative damage to the delicate immature lung tissue [212,213]. Because the need for oxygen is the major clinical criteria for diagnosing BPD, all patients with a BPD diagnosis have, therefore, been exposed to some level of supplemental oxygen [150,214]. Mice exposed to high levels of oxygen during the postnatal period have been used extensively to model BPD pathophysiology, as they develop structural defects in the lung similar to what is observed in BPD [215–217].

As identified in a transcriptomic study by Bhattacharya et al., there is significant reduction of the expression of multiple collagen VI genes in lung tissue from mice exposed to hyperoxia [208]. This may be one mechanism by which the structural abnormalities arise in this model. It is possible that variants resulting in reduced collagen VI function or lower basal levels of collagen VI production in an individual may make them more susceptible to the damage caused by hyperoxia exposure, or reduce the ability to properly repair the lung after injury. The hyperoxia-exposed mouse model does not necessarily represent a collagen VI loss-of-function scenario, and the combination of these two models is likely to result in disruption of a broader range of genes and pathways. This could potentially impact the lung to a greater, or broader extent than either condition alone, possibly providing a more ideal model for the study of BPD, including both genetic and environmental influences. Of particular interest are the effects of hyperoxia on

heterozygous *Col6a1*$^{+/-}$ mice. While these mice do not show significant alteration in collagen VI protein levels relative to WT mice in unstressed conditions, their response to hyperoxia may result in moderate reductions in collagen VI protein levels. This would provide a model in which to compare the effects of complete loss of collagen VI with a partial reduction. Finally, transcriptomic analysis would allow for the interrogation of potential synergistic effects caused by the combination of collagen VI loss and hyperoxia exposure.

Paxillin as a potential effector of collagen VI signaling

Paxillin is known to act upstream of many other signaling molecules, including PI3K and CDC42. It has known effects on actin cytoskeletal dynamics, influencing events like cell-spreading and migration. We hypothesize that paxillin may be involved in the Integrin-PI3K-CDC42 signaling pathway that appears to promote enhanced pulmonary epithelial cell autonomous function on collagen VI. Examination of the effects of paxillin on epithelial cell-spreading, wound-healing and steady-state cell density in response to collagen VI could be used to test this hypothesis. There are no commercially available inhibitors that act directly on paxillin. Therefore, knockdown by siRNA or lentiviral shRNA expression is likely to be required in order to examine paxillin loss-of-function. In addition to effects on spreading, wound-healing and confluent cell density, additional work determining the effects of paxillin on PI3K and CDC42 would be necessary to determine whether it acts as a part of the previously determined signaling mechanism. *In vitro* assays of PI3K phosphorylation, and CDC42 activation in cells on collagen VI with or without paxillin loss-of-function would be useful endpoints in understanding whether paxillin acts upstream of PI3K and CDC42 in response to collagen VI.

Involvement of lung tissue in respiratory insufficiency associated with collagen VI muscular dystrophies

Current knowledge of lung tissue defects in collagen VI muscular dystrophies is based on only a few case studies [136]. However, they provide compelling evidence that the respiratory insufficiency developed in many collagen VI muscular dystrophy cases is not simply due to effects on respiratory musculature. We hypothesize that there may be structural changes in the lungs of collagen VI-muscular dystrophy patients, which may contribute to respiratory insufficiency in these individuals. Studies of lung tissue samples from patients with collagen VI muscular dystrophies would be highly beneficial in examining the lung structure in these individuals. However, due to the potential difficulties in obtaining such samples, examination of chest commuted tomography (CT) data from additional subjects may be the most practical approach. This data could help to determine whether the changes in parenchymal structure, originally observed by Fraser et al., ERJ Open Research, 2017, are common throughout the collagen VI muscular dystrophies, or only observed in a small subset of individuals. Of particular interest would be structural data from younger collagen VI muscular dystrophy patients. The presence of structural abnormalities, for example, alveolar simplification, airway disorganization or hyper-branching, or blebbing, may be indicative that there are developmental defects in the lung that are present very early in the myopathy. In addition, this would provide further evidence that collagen VI mutations negatively impact lung development, and would support the idea that collagen VI mutations may convey susceptibility to BPD when combined with additional stressors, including premature birth and oxygen exposure. Pulmonary function testing in pediatric subjects could also provide insight into the development of respiratory insufficiency in subjects with collagen VI-related muscular dystrophies. The respiratory insufficiency in these individuals has been attributed to restrictive ventilatory defects, caused by weakness and contractures of the respiratory musculature [133,189]. While this is likely to be a major contributor to respiratory insufficiency in these patients, obstructive respiratory disease, caused by structural changes in the lung tissue, may also be present. It is possible that a more subtle, obstructive defect may be detectable prior to the onset of significant restrictive defects caused by degeneration of the respiratory musculature.

Conclusions

The findings that collagen VI plays a role in the structure and organization of the lung and is a regulator of critical pulmonary epithelial cell functions, are of potential importance to the fields studying collagen VI muscular dystrophies, developmental biology, and chronic lung disease. The studies included here have greatly enhanced our understanding of collagen VI as an important component of lung structure, and potential regulator of pulmonary epithelial cell function. We have identified several roles for collagen VI in the structural organization of the lung. In addition, we have identified an intracellular signaling mechanism that mediates pulmonary epithelial cell responses to collagen VI. The structural defects associated with collagen VI loss are indicative of a potential developmental role. It is possible that defects in collagen VI may lead to structural abnormalities of the lung in individuals with collagen VI muscular dystrophies, or in premature infants who go on to develop BPD. This work has generated additional questions encompassing multiple fields of clinical and basic research that

could significantly benefit our knowledge of multiple diseases and developmental processes.

The following is the supplementary data related to this article.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mbplus.2021.100058.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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