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Extracellular matrix as a driver for lung regeneration

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Introduction

Cells within connective tissues reside in a dynamic microenvironment that provides both a three-dimensional scaffold “housing” and a milieu of biophysical signals. This scaffolding, termed the extracellular matrix (ECM), is primarily composed of basement membrane and interstitial connective tissue and actively stimulates resident cells on both the macroscopic (global) and the microscopic (local) level. Within the context of the lung, macroscopic regulatory roles include providing structural support to facilitate gas exchange and prevent airway collapse; developing a hierarchical, branched tissue architecture separating the epithelium, endothelium and interstitium; and also providing a scaffold capable of imparting global mechanical forces onto resident cells (e.g., stretch from breathing)[82]. On the cellular level, the ECM also provides a “biochemical and mechanical language” that governs fundamental processes such as cell signaling pathways[10], cell shape and function[2], changes in cytoskeletal organization and differentiation[15], formation of focal adhesions and stress fibers[14], alterations of proliferation and migration[90], stimulation of polarity and gene expression[94], induction of metastatic activity[58], response to growth factors[16], and information regulating appropriate location of specific cell contingencies within the matrix[11]. Further, cell-matrix interactions play critical roles throughout life: during embryonic development and organogenesis[21, 79], angiogenesis[23], wound healing[74], disease and metastasis[62].

The importance of matrix biology in the context of regenerative medicine has resulted in substantial efforts to define the scope of matrix effects on cells, determine the underlying mechanisms of this regulation, and harness this relationship to facilitate the production of tissue engineered organs. One notable advancement has been the development of decellularized scaffolds[20]. These “repurposed biomaterials” serve as an attractive source of microscopic, tissue-specific matrix constituents and a means to recreate physiological conditions for *in vitro* or *ex vivo* studies. Decellularized matrix is currently used in 2D cell culture systems, as a model for organ development or disease, and as a potential platform for the creation of organ replacements.

A brief overview of lung matrix composition

Lung matrix is generally composed of collagen and elastin fibers that are interwoven with glycosaminoglycans (GAGs), fibronectin fibrils, proteoglycans (PGs), and water sequestered by PGs and GAGs. (For a more thorough review of lung ECM composition please refer to Dunsmore *et al*[22].) Other essential ECM constituents include laminin, heparan sulfate, nidogen/entactin, hyaluronate, chondroitin sulfate and matricellular proteins such as thrombospondin, tenascin X, and tenascin-C[9, 22]. Given that our knowledge of ECM composition in the lung is still evolving, it is highly likely that some variants of these common matrix structural proteins have not yet been characterized.

The major collagen subtypes that populate the lung are types I, III, IV, and V. Of these subtypes, the interstitial collagens (I and III) play the principle load-bearing role in the parenchyma, while type IV is a key basement membrane component, and assists in barrier function[22]. Elastin, the matrix component that is largely responsible for the intrinsic recoil property of lung tissue, is a highly flexible and crosslinked protein that can withstand up to 200% strain[29]. Elastin is particularly resilient and has a half-life that approximates the life expectancy of the organism (80 or more years for humans)[75]. PGs are proteins that are located on the surface of cell membranes, and also within intracellular vesicles, and are incorporated throughout the ECM[22]. These proteins are composed of glycosaminoglycans (GAGs), a family of highly charged polysaccharides, and a protein core. PGs, with their attached GAGs, sequester water, ions, growth factors, and directly control macromolecular and cellular movement across the basal lamina[63]. The mechanical nature of the lung, characterized by viscoelastic stress-strain patterns and elastic recoil, enables ventilation and ultimately gas exchange. (For an excellent review of lung matrix mechanics please refer to reviews by Suki and colleagues[82, 83], and for a thorough review of vascular composition and mechanics refer to Mecham and colleagues[91].) These properties are not simply a reflection of the summation of individual protein contributions, but rather a combination of coupled interactions between matrix components, and regional organization of the tissue. Therefore the depletion or damage of one matrix protein (e.g., elastin depletion as seen in emphysema) has an impact on the function of the neighboring matrix proteins as well[29, 95].

Although fibronectin and laminin may not contribute greatly to the mechanical nature of the lung, they are essential proteins for cell adhesion and survival[85]. Fibronectin is a cell-adhesive glycoprotein that is of particular importance for the adherence of a variety of pulmonary cell types to the extracellular matrix[88] and interacts with cells to impact their morphology, motility, and differentiation[31, 35, 42]. Fibronectin is also important for growth factor storage, a feature of particular importance during states of remodeling[74].

1) Tissue elasticity

Over the last several decades it has become increasingly clear that matrix stiffness, a mechanical property inherit to the ECM, has an influential role in numerous cell functions and is as important as chemical composition in regulating cell behaviors such as migration (durotaxis)[46], formation of focal adhesions[84], cell proliferation[4], apoptosis[56], growth factor or surfactant production[4], and stem cell differentiation[25]. Culturing type II alveolar cells on stiffer substrates results in enhanced laminin and fibronectin assembly, upregulation of the

α3 laminin subunit, and a change in morphology from the rounded to a flattened shape that is more typical of type I cells[24]. Matrix stiffness can also underpin pathological outcomes; interstitial cells placed on very stiff surfaces have been shown to differentiate into the contractile and synthetic cell types associated with lung fibrosis [4, 44].

Lung matrix stiffness varies dramatically on a global and local scale due to regional differences in matrix configuration, and because of intrinsic differences in individual protein properties[81]. On a tissue level, normal lung stiffness ranges from 0.5 to 15 kPa, depending on the location in the lung and the method of stiffness measurement[45, 83]. On the matrix nano-level, collagen type I is at least 2 orders of magnitude stiffer than elastin[29]. It has been suggested that these local differences, on a nano and micro scale, of the lung parenchyma could be important in regulating the spatial distribution, differentiation, and function of cells[48].

2) Matrix composition

The lungs vary regionally and temporally in ECM composition (Figure 1)[17, 22]. For example, collagen type I is typically found in the large bronchi, blood vessels, and irregularly placed throughout the interstitium of the alveolar septae[5, 51]. Collagen II is typically found only in bronchial and tracheal cartilage, whereas collagen type III is found in the large bronchi, perivascular, and interstitum of the alveolar septa[5, 51]. Collagen IV is primarily found in to co-distribute with collagen V in linear patterns in both the alveolar and capillary basement membranes, and collagen type VI is found co-distributed in blood vessels with collagen types I and III[51]. In addition to subtype, total collagen also varies regionally throughout the matrix. In a study analyzing collagen I and III distribution in rat lungs, collagen concentrations were the highest in the pulmonary artery (50 ug/ug dry tissue), followed by small airways, arteries, main intrapulmonary bronchus, and parenchyma (25 ug/ug dry tissue)[39]. In contrast to collagen content, elastin content is highest in the lung parenchyma, followed by blood vessels and bronchi[41, 68].

The composition of ECM in fetal, neonatal and adult tissue is markedly different, and has been shown to temporally regulate resident cells in terms of morphology, migration, differentiation, response to mechanical factors, growth factor production, and enhanced wound healing capacity[18, 72]. During embryonic development of the murine lung, all five laminin α chains are present. However, the normal adult lung tissue contains primarily laminins α3, α4, and α5.[57, 59, 77]. Fetal lung tissues also contain more total GAGs and PGs throughout the organ, and also more collagen I and III in the pleura and the alveolar septae than adult tissue[5]. Since fibronectin binds more readily to collagen type III than to collagen type I (or type IV)[26], the enhanced collagen III promotes fibronectin sequestration and provides a mechanism for enhanced platelet aggregation[6]. Matrix components are also responsible for inducing differentiation of many cell types. Highly sulfated PGs are responsible for the spreading of cells during transdifferentiation of type II cells to mature type I cells[43]. The alveolar basement membrane below type I cells is also highly sulfated as compared with the matrix below type II cells [71, 89].

A wide range of matrix molecules is known to mediate cell attachment and survival in a cell type-specific manner. These include collagen (I and IV), laminin, fibronectin, and

vitronectin. Each cell type has a unique integrin profile that is reflective of their matrix, function, and differentiation state[28, 52]. As ECM changes along the proximal or distal regions of the lung, the matrix-specific integrins also change and presumably play a role in location-specific adhesion, homing, and proliferation. For example, alveolar type II cells preferentially adhere to fibronectin, and adhere significantly less (~ 50% or greater) to alterative matrix proteins such as laminin, vitronectin, or collagens I, III, or IV[15]. Fibronectin alters alveolar gap junction intercellular communication[2] and enhances cell migratory behavior[42], suggesting that alveolar type II cells may migrate in the direction of fibronectin, adhere preferentially, and form stable cell-cell junctions. In the vasculature, endothelial cells prefer to co-localize with fibronectin over tenascin-C, collagen type I, collagen type VI, collagen type IV, decorin, or versican[78]. In contrast, both fibroblasts and smooth muscle cells adhere non-discriminately to collagen (types I and IV)[58], although attachment to polymeric collagen has been well documented to inhibit proliferation of these cell types[33, 40, 73]. Interestingly, monomeric collagen has been shown to induce mesechymal proliferation[40]. Given that uncontrolled proliferation of fibroblasts is contributory to lung fibrosis, the maintenance of intact polymeric collagen is likely important for the construction of functional engineered lungs.

Building the Acellular Scaffold

Over the past several years, advancements have been made with regard to producing decellularized scaffolds from native lungs. Detergents that are commonly used in the decellularization process include Triton-X 100, sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), and 3-[(3-cholamindopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). These detergents solubilize cell membranes, disengage cytoskeletal proteins from cells, and detach DNA and DNA remnants from proteins[20]. As reported by many groups, these scaffolds often retain many of the essential proteins present in the original organ[32, 60, 61, 92]. Ideally, this matrix would retain sufficient structural proteins within the airways and interstitium, the basement membrane, adhesive proteins required for cell engraftment, growth, and differentiation, and growth factors required for proper cell adhesion, survival and proliferation[22].

In a study by White and colleagues, normal and fibrotic decellularized lung matrix was reseeded with normal healthy lung fibroblasts. Fibroblasts seeded onto decellularized tissue from fibrotic lungs demonstrated a pro-fibrotic response – these cells underwent enhanced myofibroblast differentiation and increased TGF-β1 growth factor production, whereas fibroblasts seeded onto “healthy” acellular tissue did not[8]. In a study examining the impact of emphysema on matrix, lung tissue from healthy and emphysetamous patients was decellularized and reseeded with a variety of cell types. Although the healthy tissue supported engraftment of all cell types for one month, the decellularized emphysetamous tissue did not[92]. Although decellularized lung tissue can provide a model of matrix degradation as seen in aging or lung disease[8, 76], it is important to recognize that many decellularization protocols render a depleted scaffold and may not be optimized for long-term cell culture.

Factors Affecting Scaffold Quality

In the very long term, acellular lung matrices may serve as a platform for regeneration of functional lung tissue. To be functional, a regenerated lung should minimally be able to 1) maintain lung-specific cells: i.e., cells that produce surfactant, growth factors, have cilia, etc; 2) provide a barrier to separate blood from air, along with functional alveolar epithelial and microvascular endothelial cells; 3) have a hierarchical branching geometry that provides high surface area for gas exchange, 4) contain a perfusable microvasculature that is resistant to thrombosis, and 5) be sufficiently mechanically robust to allow for ventilation and physiological mechanical stresses[13]. To date, although significant progress has been made towards the production of such an organ[7, 32, 61, 65, 69], these functional criteria have not yet been achieved. The quality of the underlying matrix scaffold will drive success or failure for many of these critical design functions.

1) Impact of tissue age on matrix characteristics

The composition and of lung tissue changes dramatically as we age[34, 68]. For example, studies of GAG content in rabbit lung parenchyma at different states of development have shown that fetal lungs contain a high proportion of chondroitin 4-sulfate, while older animals predominantly retain dermatan sulfate, heparan sulfate, and heparin[34]. Although all GAG production was low in older rabbits, there was a relatively greater synthesis of dermatan sulfate and heparin [34]. In humans, the collagen content in young parenchyma, pleura, and arterial wall is higher than in older lungs. For example, collagen comprises 16% of the pulmonary artery wall in young adults, and decreases to 10% in individuals over 80 years old[12, 50, 67]. These differences are due to both decreases in new collagen synthesis, and to increases in collagen degradation. In the rat lung, collagen synthesis decreases with age – from 13% per day at one month of age to 1% per day at 2 years. The degradation rate of newly synthesized collagen rats increases from 28% to 62% over the same time frame (1 month to 2 years)[53]. In contrast to collagen, the elastin content of the lung parenchyma does not change over the same time span - less than 1% of the total body elastin pool turns over per year. The next effect is one of decreasing the collagen-to-elastin ratio[68] and changing the biochemical make-up of the extracellular matrix of the lung as the animal ages. Presumably, a change in quality and content of native scaffolds from age or exposure to environmental factors will impact the matrix outcomes after decellularization. In a study examining the impact of age on ECM retention of decellularized mice lungs, tissues that were produced from older animals had reduced ECM contents[76]. Therefore, decellularized tissues of older animals may have diminished biologic cues that could lead to inferior recellularization and remodeling outcomes.

2) Species-Specific effects

There are intrinsic differences between species with respect to organ size, alveolar size and number, tissue density, cell numbers and composition. Taking for example differences between rat lungs and human lungs, the rat has 19.7×10^6 alveoli, each having diameters of ~70 μm, whereas the human has 494×10^6 alveoli, with diameters of ~ 200–400 μm[19, 55, 80]. Tissue composition between species is also markedly different - collagen and elastin fiber densities are 3–4 fold higher in human parenchyma than in rat parenchyma[54]. Additionally,

the alveolar septal walls in humans are three fold thicker than in rats, and about 50% thicker than in monkeys[55], and elastin fibers penetrate significantly deeper into the alveolar septal walls of human lungs than in rat lungs[54]. These spatial differences in matrix result in a dramatically different matrix compositions between species, and possibly different outcomes following decellularization, though investigations have not yet been published that specifically address species differences for lung decellularization.

In a study that compared the cellular composition in the alveolar regions of rats, baboons and humans, the percentage of resident cell types varied dramatically between species[19]. Although the total alveolar epithelial percentages did not change for type I and type 2 alveolar epithelial cells, the endothelial cell number as a percentage of total lung cells. In rat, baboon and human, endothelial cells were 46%, 36%, and 30% respectively. Although the fraction of alveolar cells remains similar across species, the total alveolar cell number increases logarithmically with body weight (larger lungs from larger species increase in total cell number)[80]. Given these differences in cell percentages, it is entirely possible that different methods of decellularization (perfusion of detergent via vasculature, as opposed to administering via airways) will differentially impact the matrix outcomes and amounts of cellular residuals, dependent on the species.

Although it is well established that there are intrinsic differences between species in terms of matrix and cellular composition, there is surprisingly little information regarding species-dependency on the decellularization efficiency, quality and composition of acellular lung matrices. In a study comparing decellularization efficiency on human and porcine heart valves, porcine valves had substantially more residual protein as compared to human tissue on a per gram basis, and were reportedly more difficult to decellularize than human tissues[70]. In a study comparing human versus porcine decellularized myocardial matrix tissue for the use of producing acellular hydrogels, porcine-derived decellularized matrix retained collagens II, V, and VI and fibulin-3, while the human decellularized tissue did not[37]. In contrast, human tissue retained collagen XII, fibulin-2, heparin sulfate, and periostin after decellularization, while porcine tissues were depleted of these proteins. Although these studies only investigated human and pig tissue, they highlight the need for consideration of species differences in decellularization studies.

Decellularization is a delicate balance between effective cell removal and preservation of critical matrix components. As detergents remove cellular debris, these solutions simultaneously extract and damage components of the ECM that are essential to lung function and cell adhesion, including various glycoproteins and PGs[27]. To date, an analysis of various decellularization protocols applied to porcine lungs has demonstrated matrix deterioration that is ranging in severity. Reported matrix damage includes loss of collagen[32, 60], elastin[32, 60, 61], laminins[60], fibronectin[60], and sulfated glycosaminoglycans (GAGs)[61, 65]. The functional consequences of specific protein removal can range from impaired cell engraftment due the loss of fibronectin,[87] to substantial loss of tissue strength from loss of collagen type I[64]. Comparative studies investigating decellularization of lungs with Triton-SDC, SDS or CHAPS have found conflicting results with respect to protocol-dependent matrix loss. For example, Weiss and colleagues found comparable levels of retained collagen in Triton-SDC and SDS decelled lungs[93] while CHAPS treated lungs were

severely depleted, Ott and colleagues have found the most retention of collagen with SDS[32] and significant loss with SDC and CHAPs treated lungs, and Vunjak-Novakovi and colleagues have found no significant differences between detergents in total collagen retention[61]. It should be noted that these studies were performed using mouse[93], rat[32], or porcine and human lungs[61], and the differences in species could be a cause for the discrepancy in results.

Cell and host-Scaffold interactions

Successful decellularization should entail the removal of cell membrane epitopes, damage-associated molecular pattern (DAMP) molecules, and DNA remnants from the scaffold, since these components are known to induce inflammatory reactions[47, 96]. Host responses to acellular matrices include the activation of M1 (pro-inflammatory) or M2 (pro-constructive remodeling) responses[3, 38]. The threshold level of nuclear material that induces negative, pro-inflammatory responses has not yet been determined, and hence acceptable levels of decellularization for various organs are simply not known[30, 38]. As a consequence, reports in the literature of what is described as an “acellular” or “decellularized” lung matrix ranges anywhere from 75% to up to 98% donor DNA removal[7, 32, 61, 65, 69]. Despite the lack of clear benchmarks for what constitutes “decellularized”, it has been generally established that DNA fragments that are less than 300 bp in length will not elicit an adverse remodeling responses[30]. Therefore, it is possible that if residual DNA is broken into small enough fragments during the decellularization process, the negative consequences of residual DNA could be minimized. In terms of the impact of non-nuclear donor material on adverse immune responses, it remains unclear if protein sources of cell debris are problematic. Currently, there are multiple reports of decellularized tissues with detectable cytoskeletal debris, such as β-actin,[124, 144], although the functional consequences of these remnents are not known. Macchiarini and colleagues reported that despite retaining some pre-existing non-nuclear intracellular elements in the cartilaginous regions of decellularized trachea, the implanted material did not incite inflammation and did not require immunosuppressive drugs. In fact, they go on to speculate that these residual elements from the donor tissue may even provide helpful signals to host cells[49]. Lastly, the impact of xenogeneic matrix in the field of lung transplantation has not even been addressed as yet. Though many groups are working to decellularize porcine lungs, the immunogenicity of the resulting matrix has not been reported.

Extracellular matrix breakdown is an important contributor to the progression of several lung pathologies[97]. Bioactive fragments of extracellular matrix, or matrikines, are known to be mediators of inflammation and immune response[1]. For example, collagen breakdown has been shown to promote neutrophil migration [66]. Additionally, exposed native type V collagen - a “sequestered” collagen that typically does not come into direct contact with airway epithelium - is a major risk factor for bronchioloitis obliterans syndrome [36]. Although it is well established in other tissues that “under-decellularizing” tissue can result in detrimental immune reactions, “over-decelluarization” can result in shattered, partially degraded or matrix devoid of components that can induce immune response. One study investigating the impact of pH on decellularized lung determined that high pH (≥10) during decellularization can induce matrix damage that stimulates an inflammatory response in

vivo [86]. It is very likely that degraded matrix has a larger role in immune and inflammatory response than previously anticipated and should be considered, along with adequacy of cellular removal, in efforts to minimize negative host responses after implantation.

Future directions

Currently, decellularized matrix holds great promise as means to generate lung replacements. In terms of creating readily available, patient-specific lung equivalents, the current limitation in terms of scaffold production include assessing the feasibility of xenographic sources, determining which cues from the “matrix footprint” are critical in cell adhesion and viability, and the careful characterization of host responses to matrix constituents and residual cell debris. Future studies should also include determining which pulmonary cell types are required to restore tissue functionality, how to reintroduce these cells into their proper location through seeding methods or induced migration, and how to acquire these patient-specific cells.

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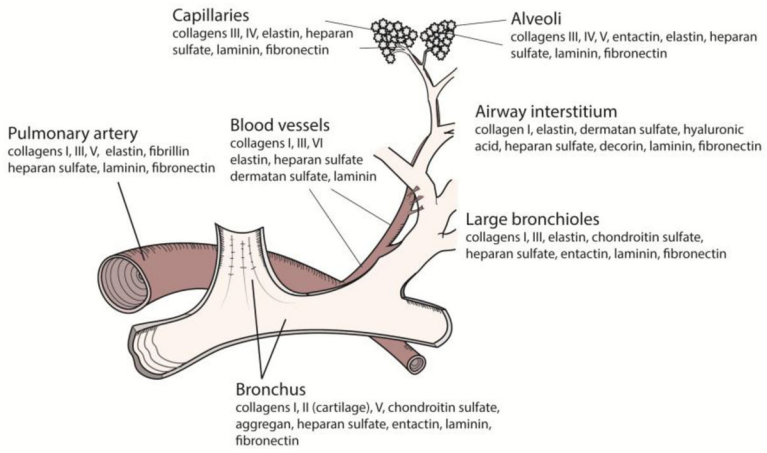

Figure 1.

Regional ECM composition in a human lung acini. This schematic highlights some of the structural and adhesive major components and how they are distributed throughout the lung.