Revascularization of decellularized lung scaffolds: principles and progress

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Stabler CT, Lecht S, Mondrinos MJ, Goulart E, Lazarovici P, Lelkes PI. Revascularization of decellularized lung scaffolds: principles and progress. Am J Physiol Lung Cell Mol Physiol 309: L1273–L1285, 2015. First published September 25, 2015; doi:10.1152/ajplung.00237.2015.—There is a clear unmet clinical need for novel biotechnology-based therapeutic approaches to lung repair and/or replacement, such as tissue engineering of whole bioengineered lungs. Recent studies have demonstrated the feasibility of decellularizing the whole organ by removal of all its cellular components, thus leaving behind the extracellular matrix as a complex three-dimensional (3D) biomimetic scaffold. Implantation of decellularized lung scaffolds (DLS), which were recellularized with patient-specific lung (progenitor) cells, is deemed the ultimate alternative to lung transplantation. Preclinical studies demonstrated that, upon implantation in rodent models, bioengineered lungs that were recellularized with airway and vascular cells were capable of gas exchange for up to 14 days. However, the long-term applicability of this concept is thwarted in part by the failure of current approaches to reconstruct a physiologically functional, quiescent endothelium lining the entire vascular tree of reseeded lung scaffolds, as inferred from the occurrence of hemorrhage into the airway compartment and thrombosis in the vasculature in vivo. In this review, we explore the idea that successful whole lung bioengineering will critically depend on 1) preserving and/or reestablishing the integrity of the subendothelial basement membrane, especially of the ultrathin respiratory membrane separating airways and capillaries, during and following decellularization and 2) restoring vascular physiological functionality including the barrier function and quiescence of the endothelial lining following reseeding of the vascular compartment. We posit that physiological reconstitution of the pulmonary vascular tree in its entirety will significantly promote the clinical translation of the next generation of bioengineered whole lungs.

lungs, otherwise unsuitable for transplantation, or even individual lung lobes, might serve as a source for engineering replacement lungs and/or as organotypic scaffolding material (13, 28). “Whole organ engineering,” which entails removal of cellular constituents (decellularization) and the subsequent recellularization, i.e., reseeding with the appropriate cocktail of tissue-specific cells, has the potential for increasing the number of available organs for clinical use, provided the functionality of the recellularized organ can be restored (6, 7, 19, 104, 131). During the decellularization process, a combination of reagents, usually detergents, is used to ablate the cells of a given organ, leaving behind an endogenous three-dimensional scaffold composed of insoluble extracellular matrix (ECM) proteins. Among the unresolved issues of the decellularization process is the incomplete removal of cellular components, which leaves behind residual fragments of cytosol, organelles, nuclei, and cell membranes, all of which can be immunogenic (8, 69). For a recent critical review of decellularization and some of the related issues see Ref. 47. The feasibility of the decellularization/recellularization (D/R) approach and its in vivo applicability was demonstrated in the initial in vivo studies that revealed a limited functionality of tissue-engineered left lung lobes in rodent models (29, 74, 80, 103).
Following D/R of whole rat lungs, the transplanted bioengineered left lobes were able to contribute to pulmonary gas exchange for up to 7 days in vivo. Some of the serious complications observed in these studies, such as pulmonary hemorrhage and edema, might have been caused in part by the incomplete recellularization of the vasculature and by the immune rejection of the engineered constructs. This suggests the occurrence of a graft-vs.-host response toward reseeded cells and residual ECM components, including residual fragments from the cells of the donor tissue. Hence at this stage the D/R approach is still far from being useful for chronic support or replacement of a failing lung in an animal model or, even less so, clinically. Although the major hurdles of complete pulmonary vascular/airway compartment recellularization are of major concern for the a short-term (days-weeks) survival of the implanted D/R organs, we anticipate that the long-term survival (months to years) of the D/R lungs in vivo will require future studies on the use of additional surgical techniques, such as BAR, owing to their beneficial effect on the survival of transplanted intact lungs (112). As an alternative to their anticipated use in the clinic, D/R lungs could also be utilized, already now, to construct improved tissue models in vitro that might accelerate pulmonary differentiation of stem cells (99) or the discovery of better pharmaceuticals/biologics for lung diseases (86).

The partial, time-limited restoration of respiratory function in these engineered whole organs in vivo may, in addition to the potential causes listed above, also be due to damage to the ECM during decellularization, incomplete recellularization of the vascular and/or airway compartments, lack of interstitial cells, and absence of autonomic innervation of the transplanted bioengineered lung. Innervation of bioengineered organs, e.g., the lung, is a very complex process that has yet to be addressed adequately and is beyond the scope of this review (5).

The majority of the published work on lung tissue engineering, especially on recellularization of the decellularized lung scaffolds, has focused on the airway compartment (14). For recent comprehensive reviews on recellularizing the airways, see Refs. 94, 114, 121. By contrast, D/R of the vascular compartment in combination with in situ maturation of the seeded cells and ECM remodeling, cumulatively defined here as revascularization, lacks detailed systematic bioengineering studies. To the best of our knowledge, there is only one study focusing solely on assessing via corrosion casting the effect of recellularization on microvascular morphology and integrity (64).

In this review we will discuss the current state of lung decellularization mainly from the vantage point of the vasculature with emphasis on the maintenance of the integrity and composition of the vascular ECM and address emerging approaches for lung revascularization. Restoration of a functional pulmonary vasculature will require reestablishing intact, i.e., quiescent and nonthrombogenic, endothelial cell monolayers lining the entire vascular tree throughout the complex decellularized scaffold (17, 95). We posit that successful bioengineering of functional lungs and their clinical usefulness will depend on innovative approaches to restoring a functional pulmonary vasculature.

Pulmonary Vascular Organization

The lung contains two parallel conducting systems, the airway tree and the vascular tree (133). The human pulmonary airway tree is composed of 23 bifurcations, also termed “generations,” that represent airways with decreasing length and diameter as they progress deeper into the lung terminating in the alveoli, i.e., the basic functional unit of the distal lung that provides the gas exchange with the pulmonary vasculature across an ultrathin basement membrane (less than 1 μm in healthy human lungs) separating alveoli and capillaries (111, 124, 133). The human pulmonary arterial vascular tree bifurcates from the large diameter pulmonary artery into segmental and subsegmental branches. The bifurcations of the subsegmental branches vary in both number and location but terminate in an extensive network of capillaries enrobing the distal alveoli. The diameters of the vessels decrease stepwise as they extend distally through a network of bifurcations. Thus the pulmonary vasculature generates a very large heterogeneous “plumbing system” that can be roughly characterized by size (macro vs. micro), location (arterial vs. venous), and function (45, 106). Large “elastic” pulmonary arteries (~1,000–5,000 μm in diameter) entail multiple concentric elastic laminae separated by layers of smooth muscle and collagen fibers, with a proteoglycan basement membrane altogether creating three distinct layers (tunica intima, tunica media, tunica adventitia). Medium-sized “muscular” pulmonary arteries (ca. 500–100 μm in diameter) possess a smaller tunica media positioned between the internal and external elastic laminae (36). Large and medium diameter pulmonary veins mainly differ from the large elastic and medium muscular pulmonary arteries by their thinner elastic laminae and location closer to the connective tissue between secondary lobules (16). Pulmonary arterioles (ca. 25–100 μm in diameter) lack the well-defined muscular layer along the proximal-distal axis but still maintain a multi-layer morphology of endothelial and smooth muscle cells, though less distinct than large and medium sized vessels. Distal lung capillaries (<25 μm in diameter) that enrobe the alveoli are comprised of only a single monolayer of flattened endothelial cells (ECs) supported on the ablumenal side by pericytes and fibroblasts. Pulmonary venules are virtually indistinguishable from pulmonary arterioles on the basis of histological morphology alone (61).

The vascular endothelium of the lung has some degree of innate functional heterogeneity and vascular niche-specific plasticity (1, 106). For example, vascular segment-specific differences in fluid permeability exist between the pulmonary macrovasculature (arterial or venous) and the microvasculature (capillaries) (77): the barrier function of the capillary endothelium in the distal lung is 58 times more restrictive than that of the arterial endothelium and 26-fold higher than that of the venous endothelium (77). Microvascular ECs differ from the macrovascular ECs in terms of surface glycosylation: for example, microvascular ECs express glycoproteins that are preferentially recognized by Griffonia Simplicifolia I lectin (binds to galactose), whereas macrovascular ECs preferentially bind Helix Pomatia lectin (binds to α-N-acetylgalactosamine) (50). Moreover, pulmonary capillary ECs lack Weibel-Palade bodies, which are ultrastructural hallmarks of other subtypes of endothelial cells, such as pulmonary arteries and arterioles (72). The phenotypic differences of the bronchial circulation...
endothelium to the pulmonary endothelium reflects differences in function, for example increased constitutive expression of E-selectin and decreased tight junction formation, both of which reflect the natural immune response of the bronchial endothelium toward inhaled pathogens (2). However, the lack of a bronchial circulation in the rodents limits the ability to study this vascular bed in the current models of decellularized rodent lungs.

Lymphatic ECs differ from vascular ECs, for instance, by the unique expression of lymphatic vessel hyaluronan-1 receptor-1 during development and the transcription factor Prox1 in the adult. Lymphatic ECs lack tight barrier function, and exclusively respond to lymphangiogenic factors, e.g., VEGF-C (4). The availability of protocols for the isolation/purification of ECs from different vascular beds (32) or the lymphatics (54, 109) may enable their targeted use in bioengineering different segments of the pulmonary vascular, the bronchial circulation, or the lymphatic tree. A review of the lymphatic endothelium, however, as important as it is for lung function during development (39) and in the mature organ, is beyond the scope of this review.

Successful bioengineering of a revascularized lung will recognize and leverage the endogenous endothelial heterogeneity to restore vascular functionality in a region-specific manner. Past approaches to lung revascularization used primary isolates of adult macro-micro-vascular ECs (80, 103, 119) or less-well-characterized stem cell-derived ECs (29), ignoring the need for regional heterogeneity across the pulmonary vasculature. We speculate that this issue will be critical for successful functional revascularization of decellularized lungs, which will more faithfully mimic the complex physiology of the organ.

Extracellular Matrix Composition Relevant for Lung Vascular Tissue Engineering

All pulmonary ECs, whether macro- or microvascular, produce their own underlying basement membrane (BM), while the vessels themselves are all embedded in or surrounded by a stromal/interstitial ECM. The pulmonary vascular ECM is comprised of distinct proteins (40) that interact with and regulate the behavior of the ECM-resident cells. It modulates vascular cell proliferation, migration, and attachment; sequesters growth factors; and aids proper physiological vascular functions (110). Both ECM and BM are heterogeneous in terms of anatomical location-dependent composition and ratio of their components (12).

Among the many collagen subtypes found in the lung matrix, collagens type I (Col-I), II, III, IV, V, and XI are the most abundant (43), constituting 15–20% of the lungs’ dry weight (12, 81). As for the fibrillar collagens, Col-I and III confer tensile strength to the alveolar interstitium, the pulmonary blood vessels, the visceral pleura and to the connective tissue that surrounds the tracheobronchial tree, whereas types II and XI determine the mechanical strength of the bronchial and tracheal cartilage (12). The most abundant nonfibrillar network-forming collagen in the lung is Col-IV, which constitutes part of the ultrathin BM separating capillaries and the alveolar epithelium, and provides stability and tensile strength to the blood-gas barrier as well as to the alveoli and the pulmonary capillaries (126). Following decellularization, residual Col-I and Col-IV are critical ingredients of engineered lungs, which, once implanted, will require both the tensile strength of fibrillar Col-I to withstand the strain of breathing as well as some of the unique cell-binding domains of Col-IV [i.e., the trimeric cyanogen bromide-derived fragment CB3 (117)] to support epithelial and EC homeostasis.

Laminins (LNs) are a multimember family of large structural heterotrimeric glycoproteins composed of a combination of α, β, and γ chains. Together with Col-IV, LNs play a significant role in the assembly, integrity, architecture, and regulation of lung vascular BM but also more generally in the modulation of different cellular and molecular effects, such as expression of tissue factor (35) and guidance of capillaries into preexisting alveolar clefts (63). The heterogeneity of LN expression in the lung is mainly attributed to variations in the α-chain. For example, laminin isoforms 511 and 521 are essential for normal lobar seption in early lung development as well as for normal alveolarization and distal epithelial cell differentiation and maturation in late lung development (68). Additional studies are required for more insight into the regional distribution of the LN α chains in the lung vascular bed in the native lung and for understanding how this distribution pattern is retained after decellularization.

Elastins (ENs), a family of structural proteins (89), are secreted mainly by interstitial fibroblasts (62) and represent 3–7% of the human lungs’ dry weight (60). Tropoelastin is the soluble precursor of the insoluble fibrillar elastin in situ (129). EN is an important protein that impacts lung development predominantly during the alveolar stage. EN is found in the conducting airways, vasculature, and parenchyma of the lung, where it is a primary contributor to airway recoil, patency, and parenchymal tethering (85) and also to the elasticity of the arteriole and venule pulmonary vascular beds, as characterized by physiological compliance coefficients (132). EN degradation is a key step in the pathogenesis of lung disorders such as chronic obstructive pulmonary disease (97). Current methods of decellularization lead to significant and indiscriminate loss of EN in the airways and vasculature that has a direct impact on the vessels’ ability to recoil following distension from blood flow (79).

Fibronectin (FN) is a regulatory multidomain glycoprotein that binds simultaneously to several integrin and nonintegrin receptors, collagen, and proteoglycans (PGs) (88). During embryogenesis FN is deposited around developing airways, predominantly at cleft sites, and is critical for guiding branching morphogenesis (88, 92). In the adult human lung FN is found around capillaries and associated with the BM of the alveolar walls and conducting airways (53, 105). FN plays a critical role in mediating the adhesion of cells to engineered constructs such as decellularized lung scaffolds (DLS) (52) and is important for the subsequent stimulation of ECM production by the seeded cells (9, 52). The current crude methods to decellularize the lung remove a significant portion of FN from the ECM. The remaining FN is either denatured (118) and/or fragmented, as seen by Western blotting (122), therefore significantly reducing the adhesive and instructive cues attributed to FN within the vasculature.

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains composed of repeating disaccharide units, whereas PGs are composed of mostly anionic GAG chains covalently linked to a core protein (3). Specific GAGs and PGs, such as
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heparan sulfate, play important roles in EC migration (51) and adhesion (127). Importantly, GAGs bind and serve as local reservoirs for heparin binding growth factors, such as basic fibroblast growth factor (91, 102), in both embryonic and adult lung. Mice deficient in heparan sulfate 6-O-sulfotransferase-1, a key enzyme in the biosynthesis of PGs, reveal aberrant lung morphology indicating the crucial role of these ECM constituents in lung development and maintenance (31). With the current techniques for decellularization, however, the GAGs are the most widely lost ECM component of the whole lung including in the vascular tree (58), which will likely limit the induction of angiogenesis upon reendothelialization (76).

**Extracellular Matrix Composition of the Decellularized Pulmonary Vasculature**

Following decellularization the integrity of the remaining ECM in the DLS varies with different decellularization protocols (19). Table 1 summarizes the few published studies that globally assess the remaining ECM within both the vascular and airway compartments. Although separate dedicated studies on the two distinct compartments have yet to be performed, we can deduce the effects of decellularization on the vascular compartment from current analyses of whole decellularized lungs. Furthermore, experience gained from studying D/R of isolated large blood vessels, including pulmonary arteries, can add to the knowledge of the residual ECM composition of the macrovasculature of the DLS (130). Comparing the various modes of decellularization, the type of detergents used, and the method of their application appear to be the two major factors that determine the quality and quantity of the residual ECM (Table 1). The effects of detergents can be classified in general according to the histology, biochemistry, and biomechanics of the DLS. Each of these categories can be assessed according to three different ratings (poor, marginal, good), operationally defined as follows:

- **Poor (i.e., pathological).** 1) Morphology that shows significantly altered alveolar size and/or septa that surround the vasculature [i.e., as measured by the mean line intercept parameter and/or percent parenchymal volume (73)]; macroscopically the decellularized organ is visually different than the normal lung (115). 2) ECM composition that shows differential loss (in the range of 60–95%) of either collagens, ENs, or GAGs as evaluated by quantitative histological analysis including attention to the vascular walls (79). 3) The mechanics of the whole decellularized tissue [resistance, elastance, and compliance measured by tensile testing or atomic force microscopy (107)] is weakened compared with normal lung [e.g., stiffness of normal human lung is ~2 kPa while that of the DLS is ~1.6 kPa (11)].

- **Marginal (i.e., slightly pathological).** 1) Morphology shows significant alterations in either the alveolar size or parenchymal volume surrounding the vasculature, but the decellularized organ macroscopically resembles the normal lung tissue (9). 2) Histological analysis shows insignificant loss of collagens but 30–60% loss of elastin and/or GAGs (58, 69). 3) The mechanics of the entire decellularized tissue shows no change in the dynamic compliance and static compliance, while the specific compliance and other parameters, such as the Young’s modulus or the elastance, are less than those of normal lung (84).

**Table 1** Summary of Published Studies on the ECM Composition of the Decellularized Pulmonary Vasculature

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Collagens</th>
<th>ENs</th>
<th>GAGs</th>
<th>ECM Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX/CHAPS</td>
<td>~70%</td>
<td>~70%</td>
<td>~50%</td>
<td>Good</td>
</tr>
<tr>
<td>TX/SDC</td>
<td>~50%</td>
<td>~70%</td>
<td>~60%</td>
<td>Good</td>
</tr>
<tr>
<td>CHAPS</td>
<td>~70%</td>
<td>~70%</td>
<td>~50%</td>
<td>Good</td>
</tr>
<tr>
<td>Zwitterionic</td>
<td>~50%</td>
<td>~70%</td>
<td>~60%</td>
<td>Good</td>
</tr>
<tr>
<td>Denaturing</td>
<td>~70%</td>
<td>~70%</td>
<td>~60%</td>
<td>Good</td>
</tr>
<tr>
<td>Nondenaturing</td>
<td>~70%</td>
<td>~70%</td>
<td>~50%</td>
<td>Good</td>
</tr>
</tbody>
</table>

**Good (i.e., physiological).** 1) Morphology with similar alveolar size, or parenchymal volume surrounding the vasculature, and the decellularized organ resembling the normal lung macroscopically (103). 2) ECM levels of Col-I, Col-IV, EN, LN, FN, and GAGs in the DLS similar to those of the intact organ, also within the vascular compartment (9). 3) No significant difference seen in compliance or elastance of the DLS compared with native lung (74).

For example, following perfusion of a pig or human lung with a nonionic, nondenaturing detergent, such as Triton X-100 (TX), followed by an anionic detergent, such as sodium deoxycholate (SDC), the morphology of remaining ECM was marginal, whereas the composition was good, (120). Unfortunately the mechanics of the DLS were not investigated (Row 1, Table 1; Ref. 120).

Use of TX in combination with SDC preserves the thickness of the natural alveolar septa, whereas zwitterionic detergents, such as 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), or a denaturing anionic detergent, such as sodium dodecyl sulfate (SDS), yield septa that are significantly thicker than the natural alveolar basement membrane barrier in healthy rodent lungs (122). As an example of how excessive alveolar septa thickness negatively affects gas exchange, in the pathological rodent model of bleomycin-induced fibrosis the thickness of the alveolar-capillary interface increases to ~5 μm (23) from less than 1 μm in healthy lungs (123), leading to significant reduction in the efficacy of gas exchange. This observation suggests that preservation of a thin basement membrane layer in the DLS is mandatory for proper function following recellularization.

Treatment with a combination of TX/SDC generates DLS with higher elasticity than other detergents, allowing for a more biomimetic engineered lung scaffolds. However, the same combination of TX and SDC consistently depletes ENs and GAGs (58). Comparing the studies listed Table 1 we conclude that nondenaturing, nonionic detergents (i.e., TX/SDC) have the least damaging effect on collagens, ENs, and LNs, whereas denaturing and zwitterionic detergents (i.e., SDS and CHAPS) deplete these critical structural ECM/BM proteins in both the vascular and airway compartments. Recently other detergents have been used for lung decellularization, i.e., sulfobetaine-10 (SB-10) (64) and sodium lauryl ester sulfate (46), which seem to overcome the detrimental effects of zwitterionic detergents, such as SDS, on the decellularized vascular ECM. For example, comparing the effects of SB-10 and TX alone or in combination, analysis of decellularized pulmonary vasculature via a quantitative corrosion casting method revealed that the least amount of extravasation of the casting solution (i.e., vascular damage) occurred with SB-10 (64). Taken together, decellularization protocols still need to be refined and standardized to better maintain the integrity of the pulmonary vasculature, in particular that of the delicate capillary beds, to decrease the potential for hemorrhage. Given the nature of the detergents used to date and the global routes of their administration, removal (at least in part) of critical ECM components throughout all lung compartments appears unavoidable. Hence, rather than in lieu of optimizing the decellularization techniques, replenishment of essential, cell-instructive matricellular ECM components might be an alternative approach for countering ECM loss and improving functional recellularization (52).
### Table 1. Summary of lung D/R approaches and their functional outcomes

<table>
<thead>
<tr>
<th>Approach</th>
<th>Detergent Type</th>
<th>Species</th>
<th>Morphology</th>
<th>Composition</th>
<th>Mechanics</th>
<th>Cell Delivery</th>
<th>Culture Approach</th>
<th>Culture Approach</th>
<th>Cell Types</th>
<th>Airways</th>
<th>Vasculature</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Perfusion^A</td>
<td>Nonionic nondenaturing + anionic^H</td>
<td>Pig, Human</td>
<td>Marginal</td>
<td>Good</td>
<td>NA</td>
<td>Suspended in media &amp; instilled manually^A</td>
<td>Sections in static condition^G</td>
<td>HBE, HLF, hMSC, CBF, LL</td>
<td>Adequate Favoring</td>
<td>Parenchyma</td>
<td>Adequate Favoring</td>
<td>120</td>
</tr>
<tr>
<td>2 Perfusion^B</td>
<td>Anionic nondenaturing + Nonionic nondenaturing^G</td>
<td>Rat</td>
<td>Good</td>
<td>NA</td>
<td>Good</td>
<td>Suspended in media &amp; instilled by gravity^B</td>
<td>Sections in static condition^G</td>
<td>rFLC, HUVEC^C</td>
<td>Good</td>
<td>Good</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>3 Perfusion^D</td>
<td>Nonionic nondenaturing + zwitterionic</td>
<td>Rat, Human</td>
<td>Good</td>
<td>Marginal</td>
<td>Good</td>
<td>Suspended in media &amp; instilled by gravity and pump</td>
<td>Sections in static condition^G</td>
<td>A549, HUVEC</td>
<td>Good</td>
<td>Good</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>4 Zwitterionic</td>
<td>Suspended in media &amp; dropped onto section^C</td>
<td>Human, Rat</td>
<td>Good</td>
<td>NA</td>
<td>NA</td>
<td>Suspended in media &amp; dropped onto section^C</td>
<td>Sections in static condition^G</td>
<td>hPSC, hACE, hAEC</td>
<td>Indiscriminate Attachment</td>
<td>Indiscriminate Attachment</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>5 Perfusion^A</td>
<td>Zwitterionic nondenaturing^G</td>
<td>Rat</td>
<td>Good</td>
<td>NA</td>
<td>NA</td>
<td>Suspended in media &amp; dropped onto section^C</td>
<td>Sections in static condition^G</td>
<td>rASC</td>
<td>Good</td>
<td>Marginal</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>6 Perfusion^E</td>
<td>Nonionic nondenaturing + anionic^H</td>
<td>Rat</td>
<td>Marginal</td>
<td>Good</td>
<td>NA</td>
<td>Suspended in media &amp; instilled by gravity^B</td>
<td>Sections in static condition^G</td>
<td>rMIC</td>
<td>Good</td>
<td>Adequate Favoring</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>7 Immersion^G</td>
<td>Nondenaturing</td>
<td>Monkey</td>
<td>Good</td>
<td>NA</td>
<td>NA</td>
<td>Suspended in media &amp; instilled manually</td>
<td>Sections in static condition^G</td>
<td>rMESC^7</td>
<td>Good</td>
<td>Adequate Favoring</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>8 Perfusion^F</td>
<td>Nonionic nondenaturing + anionic</td>
<td>Human</td>
<td>Good</td>
<td>Good</td>
<td>NA</td>
<td>Suspended in media &amp; instilled by gravity and pump</td>
<td>Sections in static condition^G</td>
<td>HBE, HLF, CBF</td>
<td>Indiscriminate Attachment</td>
<td>Indiscriminate Attachment</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>9 Immersion^G</td>
<td>Anionic denaturing^K</td>
<td>Pig, Human</td>
<td>Marginal</td>
<td>Marginal</td>
<td>Marginal</td>
<td>Suspended in media &amp; dropped onto section^C</td>
<td>Sections in static condition^G</td>
<td>HLF, hNAEC, hMSC^K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D/R, decellularization/recellularization. ^AIntermittent perfusion through the trachea and/or pulmonary artery without record of pressure and/or flow rate; ^BContinuous perfusion through both the trachea and the pulmonary artery at 0.6 ml/min without record of pressure; ^CPerfusion continuously through pulmonary artery at pressure 60 mmHg which is 3-fold over physiological (99); ^DPerfusion continuously through pulmonary artery alone at a physiological pressure of 15–30 mmHg; ^EIntermittent perfusion through main lobar bronchi or pulmonary artery trunk at 0.5–2 l/min (for pig or human lung) without record of pressure; ^FImmersion in decellularization media at 4–37°C; ^GFirst 0.1% Triton X-100 (TX; 1-2 days), then 2% sodium deoxycholate (SDC; 1-2 days), then 1 M NaCl for 1–2 h; ^HHuman primary bronchial epithelial cells; ^IHuman primary lung fibroblasts; ^JHuman primary bone marrow mesenchymal stem cells (MSCs); ^KHuman endothelial colony-forming cells; ^MHuman primary fetal pneumocytes isolated from rats of gestational days 17 to 20; ^NHuman primary umbilical vein endothelial cells (ECs); ^OHuman alveolar basal epithelial adenocarcinoma cell line; ^PPrimary lung microvascular ECs; ^QHuman induced pluripotent stem cells (iPSCs); ^RHuman primary alveolar epithelial type 2 cells; ^SPrimary adipose-derived stem cells; ^THuman primary bone marrow MSCs; ^UHuman small airway epithelial cells; ^Vefficacy of the recellularization process based on cell attachment is presented on a scale of 3 semiquantitative values (morphology, ECM composition, and mechanics), grading their effect as good, marginal, or poor, as described in the text; ^WMethods of these columns contain no record of airways by gravity perfusion and through the pulmonary artery and pulmonary vein by anterograde arterial gravity perfusion; ^XSections of tissue seeded with cells were cultured within a multiwell cell culture plate batch fed with media; ^YWhole lung organ cultured in a bioreactor that allows for perfusion of media through the pulmonary artery via a peristaltic pump; ^ZHuman primary bronchial epithelial cells; ^AHuman primary lung fibroblasts; ^BHuman primary bone marrow mesenchymal stem cells (MSCs); ^CHuman endothelial colony-forming cells; ^DDrived by 10.220.32.246 on June 22, 2017 http://ajplung.physiology.org/ Downloaded from
The general maintenance of the morphology of the lung vascular ECM network following decellularization has been reported, especially for the macrovasculature (10, 21, 74, 83, 93, 119). As detailed earlier, in intact lung tissue one can utilize histological stains such as Verhoeff’s van Gieson, which stains elastic fibers, to identify large diameter arteries from large-diameter veins and airways by the presence of a thick internal elastic lamina in the arteries and its low abundance in the veins and airways (61). Similarly, arterioles and venules of 100–500 μm can be distinguished on the basis of the thickness of the subendothelial elastic fibers (98). Unlike the vessels with diameter greater than 100 μm, the structure of small diameter arterioi and venules is similar and difficult to distinguish without highly specific histological techniques (61). Although proteomic analysis of the whole DLS has shown a significant loss of EN (79) following decellularization, histological analysis focused on the vascular compartment suggests that this loss occurs mainly from the airways, pleura, and parenchyma, but not from the vascular compartment: in rodent (21, 74, 83, 93), nonhuman primate (10), and human (119) DLS, the internal elastic lamina of large diameter arteries is maintained. Morphological analysis of the DLS by scanning electron microscopy has shown persistence of hollow tubular structures resembling macrovasculature and, to a lesser extent, also the microvasculature down to the capillary level, thus leaving conduits for blood perfusion largely intact morphologically, yet unable to prevent leakage/hemorrhage (58). Transmission electron microscopic studies following lung decellularization revealed thin, dense ECM structures resembling the respiratory basement membrane as well as cross sections of collagen fibrils supporting the alveoli, indicating fairly faithful preservation of ECM architecture (113). However, the physiological impact of changes in the precise composition and micromechanical properties of the decellularized scaffolds on the functionality of decellularized engineered organs remains poorly understood and will require further mechanistic studies looking at survival, proliferation, differentiation, and functionality of the cells seeded into the various DLS (113). Decellularized rodent lungs can be perfused throughout the entire pulmonary vascular tree, albeit leakage is observed at all levels of the vasculature, as shown with vascular casting visualized with micro-CT imaging (80, 93) or dye perfusion visualized with real-time imaging (Evans blue dye (21) or Trypan blue dye (58)). Current analysis methods such as Western blotting, high-pressure liquid chromatography, and mass spectroscopy do not distinguish between the ECM/BM of airways and that of the vascular compartments but rather provide global assessments of the lung ECM protein content and localization. However, novel methods for quantitative mass spectroscopy might help in quantifying the effects of different decellularization protocols on the residual proteins (33). Among the possible new approaches to analyzing specific compartments individually, correlative fluorescence immunohistochemistry and electron microscopy (56) might provide more specific means for differential quantitation of ECM protein distribution and ultrastructure/permeability in defined locale, such as the basement membranes surrounding the vasculature and the airways, respectively.

Analysis of the various decellularization methods used (Table 1; Ref. 19) suggests that a number of parameters beyond just the use or combination of specific detergents are important for maintaining the lung ECM. For example, the distinct effects of the detergent application method (i.e., continual vs. intermittent perfusion vs. immersion, flow rate/pressure, duration of decellularization, etc.) remain to be studied systematically for both vascular and airway compartments. Monitoring/controlling perfusion mechanics throughout the decellularization process should result in improved maintenance of the pulmonary vascular ECM (20). In the native lung, the pulmonary capillary pressure is very low, varying from ~6 to ~8 mmHg (26). This pressure can significantly increase under harsh and stress conditions (such as perfusion decellularization), causing perforation of the fragile respiratory membrane separating alveoli and capillaries (125). We hypothesize that high perfusion pressure through the pulmonary vasculature during decellularization will be detrimental to the residual microvasculature ECM especially for the thin BM of the respiratory membrane. Preliminary experiments from our laboratory (Stabler CT, unpublished data) indicate that decellularization of whole rodent lungs by controlled low-pressure perfusion through trachea and pulmonary artery using nonionic, nondenaturing, and anionic detergents leads to excellent preservation of the histotypic morphology of the ECM of both the lung parenchyma and in particular the vasculature, on the micro- and nanoscale level.

**Lessons To Be Learned from Decellularizing Peripheral Blood Vessels**

Decellularization of blood vessels is not a new approach. Specifically, we suggest that some of methodologies used to decellularize large- and small-diameter peripheral blood vessels might also be applied for the vascular compartment of whole lungs. In the periphery, these methods yielded excellent preservation of ECM in, e.g., bovine femoral arteries (24), ovine carotid arteries (59), porcine aortic roots (75), and porcine carotid arteries (38). Tissue-engineered vascular grafts made from decellularized peripheral blood vessels have the benefit of containing native vascular ECM proteins but must still overcome problems of mechanical strength and immunogenicity (65). In some of these studies, decellularization with either 0.1% SDS, autologous/allogeneic serum (i.e., endogenous proteolytic activity from the complement system and endonucleases), or 1% TX resulted in differential preservation of ECM components, higher elastic moduli, and lower compliance of the decellularized vascular grafts compared with native vessels (24, 38, 59, 75). Preservation and/or ease of restoration of the elastance and compliance of the vascular compartment in the lung will have to be integral considerations for future pulmonary decellularization protocols.

As a caveat, leakage of decellularization solutions from the vascular compartment through the interstitium into the airway compartment and vice versa might limit the ability to apply distinct protocols to the vascular and airway trees. Instead, limiting the concentration of detergents in “whole lung decellularization solutions,” while maintaining physiological perfusion pressures, and/or use of autologous serum, as described with porcine carotid arteries (38), may also be beneficial for preserving ECM composition in the pulmonary vasculature. Using low concentrations of SDS as the main decellularization agent has shown good maintenance of collagens as well as GAGs in decellularized human common femoral arteries (128). In these studies, agitating human common femoral arteries in...
solution containing 0.1% SDS resulted in loss of only 1.8% of collagen and 10% of sulfated PGs (128), compared with a loss of ~80% of collagen and 90–95% of GAGs in decellularized lungs perfused with 0.1% SDS (79, 80). The walls of these large arteries are made of a thick layer of dense cells and copious amounts of ECM, while the majority of the lung is composed of very thin single cell sheets and basement membrane, and thus lung decellularization may require lower concentrations of detergents (e.g., <0.1% SDS) than what has been used for the decellularization of large vessels. In addition, maintaining physiological perfusion pressure will be critical for preserving collagens and EN within the pulmonary vascular tree of bioengineered DLS. Future protocols may also consider significantly shortening the time of detergent exposure, e.g., using intermittent flow regimen (58) in combination with the use of protease inhibitors (128) and/or autologous serum (38), to decrease the loss of critical matrix components within the pulmonary vasculature.

Recellularizing the Pulmonary Vasculature

Following decellularization, different cell populations have been used to recellularize the pulmonary vasculature, including ECs derived from human umbilical veins (103), rat lung microvessels (80), human endothelial colony-forming cells (119), or induced pluripotent stem cells (iPSCs) (29). These various types of cells have yet to show physiological functionality (e.g., generation of a restrictive barrier) after being seeded in the DLS. Other than expanding autologous microvascular ECs isolated from human lung biopsies (55), pluripotent stem cells [e.g., embryonic stem cells (ESCs) or iPSCs] are a viable option as a cell source that can be differentiated toward an EC lineage (44). Patient-specific iPSC-derived ECs are regarded as autologous upon reimplantation into the donor, thus avoiding graft vs. host responses and regulatory complications. However, the ability of these cells to adapt to the distinct pulmonary locations remains to be verified. The inclusion of supporting cells of the vasculature (e.g., smooth muscle cells, pericytes, fibroblasts) is seen as necessary for long-term stability of the reendothelialized vessels. For example, when included in a 3D microfluidic assay of endothelial tube formation, pericytes are able to stabilize the endothelial tubes in vitro by decreasing their diameter, increasing number of branches and decreasing permeability (49). To date there have been no attempts to include these cell types in the recellularization of the decellularized pulmonary vasculature, but such an approach may be paramount for long-term viability and functionality of the reconstituted vasculature in the DLS.

The use of several distinct cell types of the vascular wall such as ECs and pericytes will likely require sequential seeding, e.g., first of the supporting cell type followed by the endothelial cells, or a reliance on ability of the supporting cell type to migrate from the airways to the vascular compartment. As an alternative approach, functional recellularization of the pulmonary vasculature might also emulate prior studies by Y. Noishiki and his colleagues who demonstrated that a mixed population of vascular cells can spontaneously organize in the proper tunica layers in tissue-engineered vascular grafts when exposed to physiological perfusion conditions (70). The method of using mixed vascular cell population in a DLS will need to be adjusted in accordance with the complexity of the decellularized pulmonary vascular tree. In this context, we surmise that pluripotent (ESC or iPSC) or multipotent cells [mesenchymal stem cells (MSCs), adipose-derived mesenchymal stem cells (ASCs)], either predifferentiated into progeni-
tors of vascular cells, or terminally differentiated into endothelium, etc., might serve as the one-stop source of mixed populations of vascular wall cells required for reseeding the vascular tree of the DLS. To date, there have been no attempts at recellularizing the bronchial circulation or lymphatic tree and therefore will not be addressed here.

Current preclinical studies of D/R-based lung organ engineering contain three in vitro experimental approaches of increasing complexity (Fig. 1; Table 1, Cell Delivery Approach).

In the first approach (Arrow 1, Fig. 1; Refs. 11, 57), the goal is to test the suitability of the DLS for seeding with vascular, airway, and/or somatic cells. For this purpose the DLS is sectioned transversely, thus exposing all compartments of the lung (i.e., airways, vasculature, lymphatics, pleura, etc.). Thereafter, these sections are then seeded randomly with various types of cells including embryonic (67), mesenchymal (71), or induced pluripotent stem cells (27) or primary somatic cells (11) and cultured in vitro for various periods of time. In all these studies the seeded cells indiscriminately attached to the various compartments/regions of the DLS slices. This approach is limited to studying interactions of the seeded cells with the residual lung ECM and obviously not suitable for organ bioengineering.

In the second approach (Arrow 2, Fig. 1; Ref. 9) the goal is to assess directed cell attachment to and interactions with individual DLS compartments (i.e., airway vs. vascular). For this purpose, MSCs (9), primary somatic cells (120), or ESCs (69) were delivered either via the airway or vascular network and left in situ under static conditions in the absence of perfusion for no more than 24 h. Thereafter the tissue was sectioned and further cultured in vitro to assess anatomical reconstitution of the two physiological compartments. The whole DLS was seeded via tracheal instillation or vascular perfusion, each driven by gravity (27) or by hand (93). Instillation by gravity allows for control of perfusion pressure and flow rate into the lung, whereas instillation by hand (referred to in Table 1 as “manually”) lacks such control and therefore could lead to high variability in cell distribution throughout the lungs. Using these approaches the published studies showed initial restriction of the seeded cells to the vascular or airway compartments, respectively, therefore providing a tool for fine-tuning of whole lobe recellularization in terms of cell density, perfusion pressure, etc. For example, Scarrett et al. (93) resuspended ASCs in low-melting agarose and perfused the mixture throughout the airways or vasculature of single lower lobes of nonhuman primate DLS. Following cell seeding and rapid solidification of the agarose, the perfused lung lobe was sectioned into slices that were then cultured for up to 14 days separately in tissue culture plates (93). This study indicated the feasibility of both pulmonary vascular and airway recellularization, since the cells were targeted to a specific anatomical compartment. As a caveat, the recellularized organs were kept for a short time only before sectioning (until the seeded cells attached) and without exposure to the mechanical cues of ventilation or vascular perfusion of the whole lung.

In the third approach (Arrow 3, Fig. 1; Refs. 74, 80) the goal is to study the maturation of recellularized lung constructs in situ to generate a more suitable, functional engineered organ, which will reconstitute the natural barrier properties of both the vasculature and the airway compartments. For this purpose the cells are delivered individually into either the airway and/or the vascular network and maintained for various periods of time under perfusion through the pulmonary artery with or without ventilation through the trachea. Subsequently the recellularized organ is then cultured in an ex vivo bioreactor for functional assessment of gas exchange, surfactant production, and endothelial barrier function. For example, human iPSC-derived ECs were seeded into the pulmonary artery of decellularized rodent lungs, which were then cultured in an ex vivo-perfused bioreactor for 10 days. Histological analysis indicated limited EC attachment in the microvasculature (29). This approach proves the feasibility of generating biomimetic cultures of the cells in a three-dimensional environment. Novel developments in the field have considered and exploited the physiological blood flow distribution within the lungs, which favor the right upper lobes in vivo (25), to control endothelial cell delivery within the lobes of the DLS (Stabler CT et al., unpublished data).

Although adhesion and distribution of ECs are not the only factors required for proper reendothelialization of the DLS, it is an important starting point in the field of DLS revascularization, which to date has not advanced further than this point. In analogy to classifying the quality of decellularization, we can operationally define the categories for recellularization of the vascular compartment as follows:

**Good.** Cells seeded directly into the vasculature showed attachment to both macrovasculature as well as microvasculature, with no infiltration into the airways or parenchyma.

**Adequate favoring distal or proximal regions of the vasculature.** Cells seeded directly into the pulmonary artery favored either the microvasculature (distal) or the macrovasculature (proximal), but not simultaneously, with no infiltration into the conducting airways or parenchyma.

**Poor.** Cells seeded directly into the pulmonary artery showed infiltration into the conducting airways, parenchyma, and macro- and microvasculature.

**Indiscriminate attachment.** Cells in homogeneous suspension seeded onto sections of tissue showed attachment to conducting airways, parenchyma, and macro- and microvasculature.

Similar categories based on selective distribution and efficacy of cell adhesion within the airway compartment, compared with the vascular, have been detailed in Table 1. The studies analyzed in Table 1 prove the concept that vascular recellularization of DLS is feasible across various methods as assessed by attachment to the macro- and microvasculature with no migration into the airway compartment. The efficacy of the reseeding in terms of the percentage coverage of the decellularized pulmonary vascular wall by the seeded cells has not yet been described in any published studies, presumably because of the technical difficulties of such an analysis. Future studies, beyond merely tracking the adhesion and localization of the cells in the vascular compartment of the DLS, should include a time-dependent analysis of the coverage of the vessel walls with seeded cells as they spread and migrate using 3D reconstructions of multiphoton images, as well as functional assessment of the recellularized organ, specifically the regeneration and intactness of the vascular permeability barrier and the efficacy of gas exchange across the respiratory membranes at the capillary/alveolar interface.
Reconstituting the Pulmonary Vasculature ECM

The decellularization process of the lungs unavoidably damages the ECM to some degree, and therefore the reconstitution of this damaged ECM is crucial for full functionality of the organ. A critical tenet for functional reconstitution of the pulmonary vasculature is that the cells used for reseeding the decellularized vascular tree will regenerate the damaged ECM of the decellularized lung through their innate capacity to produce their own ECM. In other words, the cells, such as endothelial cells, pericytes, MSCs, etc., used to reconstitute the pulmonary vasculature are expected to repair/rebuild the ECM [i.e., remodeling (22) in the vascular compartment] and further generate capillaries (e.g., neovascularization by ECs or MSC-derived EC progenitors, EPCs). This has indeed been demonstrated in the peripheral vasculature. For example, following implantation of tissue-engineered vascular grafts repopulated with murine MSCs in an inferior vena cava interposition model, the seeded cells produced Col-I, III, and IV, elastin, fibrillin and GAGs over a 4-wk period (66). Similarly, ex vivo studies have demonstrated that, following lung decellularization, the residual ECM retains certain integrin-recognition motifs necessary for cell adhesion, migration, and proliferation of different cell types (ESCs, fibroblasts) (52, 108). Preseeding of the DLS with MSCs could have multiple beneficial effects, e.g., by providing an adhesive surface for subsequent seeding with ECs. Further in-depth studies are required to elucidate the distinct biological mechanism that lead to remodeling/regeneration of the ECM in situ (52, 108), and more specifically the effects of surface chemistry of the DLS on vascular endothelial cell adhesion, migration, and ECM secretion (96).

Another perspective is to reconstitute/repair the potentially damaged ECM of the DLS by exogenous supplementation with defined or cell-derived ECM proteins prior to cell seeding. For example, Jensen et al. (41) attempted to compensate for the loss of damage to ECM/BM proteins by precoating the decellularized scaffolds with defined adhesion promoting proteins, such as Col-I or Matrigel. More recently we have shown that manipulation of ECM protein epitopes available for cell adhesion will significantly affect the cell reseeding efficacy and the pattern of cell distribution on a macro scale, which is relevant for whole organ bioengineering (52). For these studies we used as a proof of concept the secretome of cultured human lung adenocarcinoma-derived A549 cells. This secretome, which contains copious amounts of lung-specific ECM proteins especially lamin 511, may serve to reconstitute the natural lung niche for promoting growth and differentiation of embryonic stem cells (52) or their lung-specific derivatives (90, 100) after recellularization. As a next step toward clinical translation, lung ECM functionality might be restored by precoating the DLS with defined human recombinant proteins, such as purified lung-specific laminins typically used for culture of ESCs (87). Importantly, manipulation of ECM protein epitopes available for vascular cell adhesion will depend on the integrin repertoire of the cells to be seeded (82).

Partial Functionality of Bioengineered Lung In Vivo

The field of whole lung engineering was propelled to prominence in 2010, when several research groups reported nearly simultaneously that recellularized rodent lungs, surgically implanted in rats after left lobectomy, were capable of providing limited gas exchange and ventilation for 45 min (103) (Arrow 4, Fig. 1; Ref. 103). Although in itself this is an exciting development, the clinical applicability of D/R-engineered lungs is still a remote option, given the numerous serious complications associated with the current state of this technology. For example, the first studies reported significant hemorrhage into the interstitial tissue and the airways as early as 45 min postimplantation. The appearance of red blood cell infiltrates in the airways, as observed in histological sections, indicates a dysfunctional vascular/airway barrier caused by either incomplete cell seeding and maturation or rupture of small vessels due to an inability to handle the perfusion pressure of the right ventricle after just 45 min postimplantation (80). This problem has not been resolved in subsequent studies (29). Furthermore, measuring partial oxygen (P02) and carbon dioxide (Pco2) pressures, from the pulmonary artery as well as the right (control, intact) and left (D/R implant) pulmonary veins after 45 min to 2 h postimplantation, showed that the P02 of the left pulmonary vein was nearly three times less than that of the right lung, and the Pco2 was only half that of the right (80). These results indicated a limited function of the engineered lung with some degree of gas exchange between the inhaled air and the circulating blood across the respiratory membrane. The data from the longest published surviving rodents receiving bioengineered lungs showed that the implants provided significantly more gas exchange than measured in pneumonectomized controls, at least for up to 7 days (103). However, by day 14 the engineered lung had lost its functionality and that from that time point forward the sole contributor to the P02 was the right (intact/control) lung (103). These studies serve an exciting proof of principle that, with further significant improvement, bioengineering whole lungs by D/R may in the future yield functional organs for transplantation.

Loss of physiological function in the preclinical models may have many reasons, including (but not limited to) insufficient/inhomogeneous recellularization of airways and vasculature leading to malfunction of the alveolar barrier followed by hemorrhage into the airways, lack of lymphatic drainage, ventilation trauma, insufficient amount of surfactant production, insufficient amount of columnar cells, and/or the differentiation of the seeded cells (80). As mentioned earlier proper gas exchange requires functional regeneration of both sides (vascular and airways) of the air-blood barrier and of the respiratory membrane in between. To date, most if not all published studies have focused on recellularization and enhancement of the airway compartment. New studies are required to determine whether the vascular permeability barrier can be regenerated and remains intact at all levels of the vascular tree following recellularization, e.g., by measuring electrical resistance or permeability of fluorescent markers of different sizes and molecular weights. Published studies suggest that the mechanics of the pulmonary microvasculature was maintained following decellularization on the basis of vascular casting of the microvessels (64) as well as the vessels ability to withstand the majority of the cardiopulmonary output in vivo (103), but further studies are required to elucidate the effect of decellularization of the mechanics of the vascular compartment alone (64).

We surmise that the next critical steps in recellularization of whole lungs will focus on improving the effective and complete regeneration of the entire vascular compartment, from the
three-layered large vessels down to the capillaries. Although the rodents lack bronchial circulation and easy access to the lymphatics, we surmise that the reconstitution of these compartments will be critical in human studies. The next methodological steps will include further optimization of decellularization process, leaving the ECM/BM as intact as possible, improvements to procedures for efficient and complete recellularization, and standardization of the maturation and conditioning of bioengineered lungs ex vivo in perfused/ventilated bioreactors.

It remains to be determined whether a recellularized DLS needs to be fully matured outside of the body or whether it can be partially conditioned/matured in a biomimetic bioreactor and then implanted and allowed to mature in situ/in vivo. The latter strategy, recently proposed for tracheal implants (42), might work for simple tubular structures but needs more detailed studies for a complex organ as a whole lung. Among the next steps toward clinical translation is the development of more sophisticated bioreactors that more closely mimic the natural environment of the lung, allowing for independent D/R of the airway and the vascular compartments and subsequent dynamic ex vivo culture of the engineered organ with independent control of physiological fluid flow and ventilation parameters (15, 18, 48, 69, 78, 84).

Future Directions

The hemorrhage observed in the bioengineered lungs upon orthotopic transplantation in rodent models highlights the importance of establishing and maintaining an intact barrier between the vascular and airway systems. Bronchial circulation and lymphatic system repopulation will be necessary for long-term (months to years) viability of the circulation and lymphatic system repopulation will be necessary for long-term (months to years) viability of the human bioengineered lung but currently remain unfeasible in rodent studies. Focusing on the pulmonary vasculature, some of the key questions include but are not limited to 1) what is the quality and quantity of the BM and the ECM of the decellularized pulmonary vasculature as a distinct compartment? 2) Which cells are capable of reconstituting the phenotypically and functionally heterogeneous pulmonary vasculature (e.g., ESCs, iPSCs, lung-specific stem/progenitor cells, adult endothelial progenitors, terminally differentiated autologous adult endothelial cells, or a mixed population)? 3) How can the phenotypic heterogeneity of the pulmonary vasculature be attained or restored, from the microvasculature to macrovasculature? 4) How can the cells be optimally delivered and distributed to allow for homogeneous reseeding and establishment of a functional endothelial monolayer throughout the entire pulmonary vascular bed? 5) How will the supporting cells of the vascular compartment (e.g., SMC and pericytes) be delivered to the appropriate niche and be made functional? 6) What kind of ex vivo conditioning in bioreactors will be required to prepare the engineered lungs for transplantation, and how will the conditioning promote/sustain differentiation of the seeded cells and remodel the ECM, especially the respiratory membrane?

Given that progress continues to be made in resolving these questions, we are optimistic that it is a question of when, and not if, whole lung bioengineering can be translated into the clinic as a promising life-saving biotechnological solution for a variety of otherwise fatal lung pathologies.

NOTE ADDED IN PROOF

After the final version of the manuscript for this review was submitted and accepted, Ren et al., published a seminal paper (Ren X, Moser PT, Gilpin SE, Okamoto T, Wu T, Tapia LF, Mercier FE, Xiong L, Ghatari R, Scadden DT, Mathisen DJ, Ott HC. Engineering pulmonary vasculature in decellularized rat and human lungs. Nat Biotechnol. 2015 Sep 14. doi: 10.1038/nbt.3354. [Epub ahead of print]) in which they described improved methods for reseeding decellularized lung scaffolds with iPSC-derived endothelial and perivascular cells, accompanied by enhanced maturation of the constructs and dual stage of culturing, all of which contributed to the partial regeneration of a functional pulmonary vasculature in engineered rat and human lungs.

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AUTHOR CONTRIBUTIONS


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