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## Can Stem Cells be Used to Generate New Lungs? Ex Vivo Lung Bioengineering with Decellularized Whole Lung Scaffolds

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### Abstract

For patients with end-stage lung diseases, lung transplantation is the only available therapeutic option. However, the number of suitable donor lungs is insufficient and lung transplants are complicated by significant graft failure and complications of immunosuppressive regimens. An alternative to classic organ replacement is desperately needed. Engineering of bioartificial organs using either natural or synthetic scaffolds is an exciting new potential option for generation of functional pulmonary tissue for human clinical application. Natural organ scaffolds can be generated by decellularization of native tissues; these acellular scaffolds retain the native organ ultrastructure and can be seeded with autologous cells toward the goal of regenerating functional tissues. Several decellularization strategies have been employed for lung, however, there is no consensus on the optimal approach. A variety of cell types have been investigated as potential candidates for effective recellularization of acellular lung scaffolds. Candidate cells that might be best utilized are those which can be easily and reproducibly isolated, expanded *in vitro*, seeded onto decellularized matrices, induced to differentiate into pulmonary lineage cells, and which survive to functional maturity. Whole lung cell suspensions, endogenous progenitor cells, embryonic and adult stem cells, and induced pluripotent stem (iPS) cells have been investigated for their applicability to repopulate acellular lung matrices. Ideally, patient-derived autologous

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cells would be used for lung recellularization as they have the potential to reduce the need for post-transplant immunosuppression. Several studies have performed transplantation of rudimentary bioengineered lung scaffolds in animal models with limited, short-term functionality but much further study is needed.

## Keywords

lung; tissue engineering; decellularization; recellularization; stem cells

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## Introduction

Many devastating lung diseases including chronic obstructive pulmonary diseases (COPD), idiopathic pulmonary fibrosis (IPF), and cystic fibrosis, among others, have no cure and cause significant morbidity and mortality. Further, unlike other major diseases, lung diseases, notably COPD are increasing in prevalence and COPD is predicted to be the third leading cause of death worldwide by the year 2020<sup>1,2</sup>. Currently, patients with end stage lung diseases are limited to lung transplantation as their only treatment option. Unfortunately, there are few available lungs for transplant, 5 year survival after lung transplantation is only approximately 50%, and transplantation recipients require lifelong immunosuppression<sup>1,2</sup>. New options are desperately needed.

A promising and rapidly growing area of investigation is that of *ex vivo* bioengineering of functional lung tissue that could then be implanted into patients with diseases such as COPD or IPF. This could be accomplished by utilizing either biologically-derived or fabricated 3 dimensional (3D) matrices or other artificial scaffolding seeded with autologous stem, progenitor, or other cells obtained from the eventual transplant recipient. The use of autologous cells would eradicate the need for lifelong immunosuppressive drugs. These approaches have been successfully utilized in regeneration of other tissues including skin, vasculature, cartilage, bone, and trachea and more recently more complex organs including heart and liver<sup>3-15</sup>. Synthetic constructs offer one option and a number of different synthetic scaffold materials and manufacturing technologies have been evaluated for use to produce matrices for *ex vivo* lung parenchymal development and for the study of growth factors and mechanical forces on lung remodeling<sup>16-21</sup>. These studies have included implantation of various scaffolds impregnated with stem or other cells in order to produce functioning lung tissue<sup>22-25</sup>. Comparable approaches have been utilized to study creation of pulmonary vascular networks from synthetic scaffolds and to investigate effects of vascular endothelial cells on development of airway and alveolar epithelial tissues<sup>26,27</sup>. However, current state-of-the-art manufacturing technologies are unable to recapitulate the complex 3- dimensional architecture of the lung and, further, robust schemes for successful implantation and clinical use of synthetic lung scaffolds remain unknown.

An alternative approach is to utilize whole lungs in which all cells and cellular materials are removed leaving an intact 3-dimensional scaffold comprised of innate extracellular matrix (ECM) proteins in a bio-mimetically similar 3-dimensional architecture. This approach, termed decellularization, preserves native airway and vascular structure and provides an acellular matrix for cell seeding and functional recellularization<sup>3,28-30</sup>. This approach also provides a novel culture system to study cell-matrix interactions and environmental factors such as mechanical stretch on lung cell growth and development. This technique was originally described many years ago, one classic example is by Lwebuga- Mukasa and colleagues in 1986 in which a decellularized rat lung was utilized to study the effect of the basement membrane on growth of type II alveolar epithelial (AEII) cells<sup>30</sup>. The technique

was re-invigorated in 2010 and a number of laboratories are currently exploring this approach (Table 1)<sup>31–44</sup>.

In this review, we will address some of the critical factors involved in the theoretical and practical considerations for use of decellularized whole lungs (alternatively referred to as acellular) for *ex vivo* lung regeneration. These include decellularization and recellularization procedures as well as consideration of the potential immunogenicity of the scaffolds (schematic in Figure 1). We will also speculate as to the logistics involved in implementation of this approach for lung diseases. Finally, we discuss the feasibility of employing acellular scaffolds for ‘repopulation assays’ of stem-progenitor cells.

## Review

### Decellularization

**Methods of Decellularization**—Creation of organ scaffolds requires removal of the native cell population while minimizing alterations to the dimensions and mechanical characteristics of the organ, the structural support for the airway, vascular and lymphatic networks, and to the composition of the native matrix including important cell binding ligands<sup>3</sup>. Common methods for decellularization of lung tissue pieces include sonication, sieving, and extraction of thin pieces of lung tissue and digestion with acetic acid followed by sonication. While useful techniques for developing *in vitro* systems to study lung biology, these methods did not preserve the 3 dimensional architecture of the lung. Recently, several techniques have emerged for decellularizing whole lungs which retain the 3 dimensional architecture as well as key extracellular matrix proteins (Table 2, Figure 2). These techniques vary significantly with use of different combinations of physical, ionic, chemical and enzymatic methods and procedure times that vary between 2 hours to 7 weeks. Detergent-based decellularization is a frequently utilized approach and commonly used detergents include Triton X100, sodium deoxycholate (SDC), sodium dodecyl sulfate (SDS), and CHAPS in addition to hypertonic lysis of cells with NaCl as well as a DNAase and/or RNAase. Furthermore, some investigators employed both vascular and airway perfusion with these agents while others have only perfused through the vasculature. As such, significant differences in histologic appearance of the decellularized lungs and in content of both ECM and other retained proteins occur in the various published works evaluating the quality of decellularized lungs. How these differences might affect recellularization or potential immunogenicity of the implanted scaffold are still poorly understood. Some proposed criteria for optimal decellularization include complete absence of visible cellular or nuclear material on histological examination, less than 50ng of dsDNA per 1 mg of dry weight of the extracellular matrix scaffold and remnant DNA molecules shorter than 200 bp<sup>3,40</sup>. However, further study is needed to understand and define optimal endpoints for decellularization. Other criteria such as retention of specific ECM components and maintenance of macro and micro-mechanical properties are likely critical parameters in defining optimal decellularized scaffolds.

**Residual Extracellular Matrix and Other Proteins**—The lung is composed of a variety of cells and associated extracellular matrix (ECM). The ECM has an essential role in prenatal development, postnatal maintenance of normal function, and is known to be an inductive scaffold in directing the remodeling response after injury<sup>45–49</sup>. The ability of cells to receive organotypic signals from native ECM makes decellularized scaffolds a seemingly better choice than synthetic constructs for tissue engineering. Therefore, retention of key ECM components is essential in the decellularization process. Which combination of ECM proteins must be retained to maintain critical cues for cell functions remains unknown. Further, the detergents utilized during the decellularization process can activate matrix metalloproteinases and thus potentially exacerbate degradation of critical binding epitopes

on the remaining ECM proteins<sup>41</sup>. The major structural and functional molecules in the ECM include glycosaminoglycans (GAGS) and the collagens, elastin, fibronectin, laminin, and vitronectin. Collagens are important structural components of the lung and are responsible for overall mechanical strength. Elastin is an important ECM protein for providing the reversible distension and intrinsic recoil properties of lung tissue. GAGs help control macromolecular and cellular movement across the basal lamina and may play a role in the mechanical integrity of the lung. These matrix molecules are generally highly conserved proteins in eukaryotic organisms and theoretically may explain the absence of an adverse immune response after xenotransplantation as seen with some other decellularized organ transplants such as the tracheal patch<sup>14,15</sup>. However, some ECM proteins, for example collagen V, are postulated to play significant immunogenic roles in rejection of lung transplantation<sup>50</sup>. Laminin, fibronectin, elastin, and collagens I and IV have also been found to play a role in trans-membrane cell signaling, cellular differentiation, respiratory mechanics and other pulmonary-specific functions<sup>46-49</sup>.

The ECM components remaining in decellularized lung scaffolds can be evaluated using a combination of histologic, immunohistochemical, and Western blotting techniques (Table 1). In most instances, the decellularization process largely preserves collagen but results in a moderate loss of elastin<sup>31-33,38-43</sup>. In one study, comparison of different detergents in the decellularization process revealed that SDS was associated with a greater loss of type 1 collagen and elastin when compared with CHAPS<sup>44</sup>. Another study demonstrated that different detergent based de-cellularization protocols result in significant differences in histologic appearance, gelatinase activation, distribution of ECM components, and lung mechanics<sup>41</sup>. However, despite these differences in composition of the lungs, inoculated cells appeared to attach and recellularize the lung regardless of the decellularization protocol utilized<sup>41</sup>. Furthermore, recent investigations of relevant clinical parameters including effect of donor age, time to necropsy, prior lung injury, length of scaffold storage, and sterilization method demonstrate that each of these parameters can affect the outcome and histology of the decellularized scaffold<sup>42-43</sup>. Therefore, the choice of the lung utilized, method of decellularization, and clinical storage conditions must, at a minimum, preserve appropriate ligands to allow adherence, spreading, polarization (if appropriate), and in some cases, proliferation of cells.

Mass spectrometry is increasingly utilized to identify a broader range of residual ECM proteins, including isoforms not readily distinguished by the other analytical techniques<sup>38,39,41-43</sup>. This approach also demonstrates that a wide range of other non-ECM proteins are retained in decellularized scaffolds with the current techniques including intracellular, cytoskeletal, and cell membrane-associated proteins. This suggests that cellular proteins are not all removed by decellularization protocols, presumably due to tight anchoring of transmembrane proteins to ECM ligands. Importantly, the spectrum of retained cellular (non-ECM) proteins differs depending on the decellularization protocol utilized<sup>41</sup>. The effects of these cellular proteins on recellularization and potential immunogenicity of the scaffold is unknown at present but several retained proteins (e.g. histones) are known to be immunogenic<sup>51</sup>. Moreover, the range of different GAGs that are retained which may be important for recellularization and contribute to potential immunogenicity has not been well explored<sup>52</sup>. These are important areas for future study.

**Species differences in decellularized lungs**—Published data to date includes decellularization of mouse, rat, sheep, macaque, and human lungs. Our collaborative group has also accumulated extensive data on decellularization of pig and cadaveric human lungs (manuscripts in preparation). While there are no obvious substantive differences in the final decellularized lungs from each species, differences in lung and pleural anatomy may significantly affect the decellularization process. Further, decellularizing larger lungs

requires practical modifications of detergent-based techniques including close attention to rate and volume of solutions used to perfuse and wash the more cumbersome larger lungs. Special consideration needs to be taken for decellularizing human lungs under appropriate containment conditions including use of appropriate antimicrobial agents and an appropriate post-decellularization sterilization protocol.

**Functionality of the Decellularized Scaffold**—*In vitro* evaluation of the “function” of the decellularized scaffolds can be challenging. It is not clear what type of functional assessments best reflect the ability of decellularized lung scaffolds to support recellularization and ultimately development of functional lung tissue. Investigators have explored mechanical function including assessment of lung mechanics<sup>39–41</sup> as well as force tension relationships in linear strips of decellularized lungs<sup>44</sup>. These methods give an indication of the elastance, compliance, resistance, and diffusion properties of the scaffolds. However, in the absence of cells and surfactants, the decellularized lungs are stiff, a factor that must be taken into account for recellularization schemes using bioreactor technology. One potential bioassay to follow over time as the lungs are recellularized is the decrease in elastance (increase in compliance) due to growth and maintenance of a functional population of surfactant-producing cells<sup>39</sup>. Total lung water, normally controlled by endothelial, lymphatic, and type I alveolar epithelial cells might also be important to monitor but no guidelines have been established for these endpoints.

### **Recellularization of acellular scaffolds for bioengineering and assays of organotypic repopulation**

The lung is comprised of many (>40) cell types that are replenished by resident stem or progenitor cells following injury. However, there is much debate concerning the identification and nature of different types of endogenous lung stem/progenitor cells that can function in repair<sup>53–55</sup>. Notably, this area of research has been hampered by the lack of robust assays of endogenous stem/progenitor cell function including an accurate repopulation assay. Repopulation assays are critical for verifying the capacity for multipotency, self-renewal (i.e. stemness), and for establishing the heterogeneity of stem, progenitor, and progeny cells, and characterization of cell niches, as evidenced by their long-standing application to studies of hematopoiesis. Repopulation studies using single cells, clonally-derived populations, or mixed cell populations further provide an understanding of their spatial (i.e., homing, niche), functional (secretory, paracrine, matrix synthesis, self-renewal, differentiation), and population (kinetics, heterogeneity) characteristics. As such, one potential novel and important use of decellularized lung scaffolds is as a matrix for repopulation assessments. This further allows unique opportunities to study abnormal matrices obtained from diseased lungs<sup>43,56</sup>.

Seeding decellularized lung scaffolds with whole lung fetal or post-natal cell suspensions has the potential advantage of providing a model of spontaneous self-assembly and facilitating natural cell-cell interactions that could potentially improve organotypic growth. When fetal rat (E17) whole lung suspensions were delivered intratracheally to decellularized rat lungs and cultured in a bioreactor, cells adherent in the alveolar compartment expressed markers of ATII (CK18 and pro-surfactant C (proSPC)); markers of type I alveolar epithelial (ATI), endothelial, mesenchymal, or club cells (formerly known as Clara cells) were absent<sup>31</sup>. E17 lung cells are enriched for ATII cells so it is possible that this observation relates to the difficulty to induce ATII cells to convert to ATI cells under these conditions. In contrast, seeding with rat fetal lung cells (E17–20) resulted in multilineage engraftment again, mainly in the alveolar compartment, with expression of markers of ATII cells such as pro-surfactant A (proSPA), proSPC, thyroid transcription factor (TTF-1/Nkx2.1), ATI (T1 $\alpha$ ), and fibroblasts (vimentin); concurrent inoculation with human umbilical vein



endothelial cells (HUVEC) by way of the pulmonary artery showed retention along the entire vasculature and close apposition of endothelial and alveolar epithelial cells suggestive of perfusion of distal lung<sup>33</sup>. Larger airways were only sparsely engrafted in this model. Neonatal (P7) whole lung cell suspensions exhibited similar multilineage engraftment including evidence of distal airway repopulation with club cells (club cell secretory protein (CCSP+,CC10)) and basal cells (CK14+), as well as alveolar engraftment of ATII cells (proSPC+) and ATI cells (Aqp5+); endothelium engrafted the pulmonary vasculature and formed tight junctions<sup>32</sup>. While it is difficult to ascertain the precise geospatial distribution of cells, it is clear that fetal or post-natal lung homogenates can repopulate scaffolds with cells exhibiting a wide range of phenotypes with preferential distribution to the alveoli and distal airways.

However, fetal lung cells and transformed cell lines are not practical for clinical use in recellularizing lung scaffolds; therefore, the question arises as to which cells are appropriate for lung bioengineering applications. While there is still only limited experience, the ability of other cell types to home or engraft in specific regions of the lung is emerging (Table 3). Cell types that might be best utilized are those which can be easily and reproducibly isolated, expanded *in vitro*, seeded to decellularized matrices, induced to differentiate into pulmonary lineage cells, and survive to functional maturity. Each of these criteria requires careful optimization in order to recapitulate the natural tissue environment. In particular, the human lung is comprised of a complex mix of airway and glandular epithelial cells, mucoid cells, neuroendocrine cells, and endogenous progenitor cells; all of which exist in a specific gradient of niches polarized from the proximal to distal airway<sup>53</sup>. As such, it is logical that recellularization of decellularized lung scaffolds will require multiple cell types to be seeded at various stages of differentiation and at specific locations throughout the airway scaffold for successful tissue regeneration.

Ideally, patient-derived autologous cells would be used for lung recellularization as they have the potential to reduce the need for post-transplant immunosuppression. Terminally differentiated cells derived from adult lungs are highly specialized and lose their proliferative ability by the time they reach this state; therefore, primary airway or vascular cells, while offering the most functionally diverse options for lung tissue engineering, may not be capable of long-term success without a source of progenitor cells for maintenance and repair. A more desirable strategy is one in which stem or progenitor cells are differentiated along pulmonary lineages following programs that mimic fetal lung development. The ideal candidate cells must be easily isolated, expanded in culture, and sustained stably while undergoing tissue-specific differentiation.

Embryonic stem cells (ESCs) derived from the inner mass of *in vitro*-fertilized embryonic blastocysts have the potential to differentiate into mature cells of all three germ layers. Directed differentiation of ESCs results in the production of lineage-specific progenitor cells that may potentially be used in therapeutic or regenerative applications<sup>57</sup>. In the laboratory, lineage specification of ESC has been accomplished by recapitulating the developmental environment *in vitro*. Murine ESCs have been induced to express markers of lung epithelial phenotype including alveolar (TTF-1, SPA and SPC) and airway (CCSP) airway epithelium after specification of definitive endoderm using Activin A to mimic the Nodal signaling pathway followed by adherent cell culture in small airways growth medium or use of more selective differentiation reagents<sup>45,58-61</sup>. Similar findings using human ESCs have also been reported<sup>62,63</sup>. Induced pluripotent stem (iPS) cells, an alternative cell type similar to ESCs with less ethical controversy, are derived by re-programming somatic cells to a stem-like state by inducing simultaneous expression of combinations of the transcription factors Oct4, Klf4, Sox2, and cMyc<sup>64,65</sup>. iPS cells re-establish pluripotency and, like ESCs, with appropriate stimulation, can differentiate into lineages of all three germ layers including

those from which the host cell was not derived. A limitation of iPS cells is that they are not free from age-, environment-, and tissue-associated epigenetic modifications; therefore, there is some question as to whether iPS cells will respond developmentally and functionally as do ESCs. Further, iPS cells, like ESCs carry risk of teratoma formation. Nonetheless, iPS cells have been shown to be responsive to developmental stimuli for the specification of anterior foregut endoderm and further differentiation into early lung progenitor populations<sup>60,66</sup>. An attractive feature of iPS cell generation is that they may be derived autologously from a patient, thereby eliminating the need for allogeneic cells and avoiding much of the controversy associated with ESCs. However, it is unknown as to whether the genetic manipulation required for creating iPS cells or the epigenetic modifications inherent in the initial host cells will have any bearing on the ability of iPS cells to create fully functional tissues that can be used as transplantable substitutes for diseased tissues<sup>67</sup>.

ESCs or iPS cells or their endoderm derivatives might be expected to reconstitute acellular lung scaffolds efficiently with progeny upon exposure to instructive cues embedded in the matrix. Seeding acellular rat scaffolds with mouse ESCs resulted in both greater survival compared to cells seeded onto non-lung matrices (Matrigel, gelfoam, or collagen) and also apparent differentiation toward multiple lineages that exhibited region-specific distribution, including club cells (CC10+), ATII cells (CK18+, proSPC+), endothelial cells (CD31+), and mesenchymal cells (PDGFR $\alpha$ +)<sup>34</sup>. Thus it is theoretically possible to observe differentiation of the most primitive of stem cells along the lines of development simply by seeding them on acellular scaffolds. However, the efficiency of this system was not explicitly evaluated. Seeding of decellularized mouse lungs with definitive endoderm derived from ESCs and subsequent culture of lung slices resulted in spontaneous differentiation to elongated type I alveolar epithelium expressing T1 $\alpha$  (podoplanin) that distributed along the alveolar septae; in contrast, inoculation with parent ESCs produced hypercellular sheets of disorganized cells lining both the alveoli and some ciliated cells along airways<sup>61</sup>. Similarly, mouse ESCs differentiated to Nkx2.1+, proSPC+ ATII-like cells directly seeded onto mouse acellular scaffolds that were implanted subcutaneously distributed to airways (FoxJ1pos) and alveolar regions (proSPC+ or PDGFR $\alpha$ + cells) and maintained phenotype expression for 14 days<sup>40</sup>. In parallel, host-derived endothelial cells infiltrated the scaffolds suggesting that functional vascularization might occur. These studies also demonstrated that Matrigel as a vehicle for seeding increased the frequency of proSPC, TTF-1, PDGFR $\alpha$ , and FoxJ1 positive cells after 14 days, implying that biomimetic basement membranes may preserve heterogeneity in populations of lung endoderm derived from ESC or iPS cells, particularly in the airway epithelial fractions. However, no data is as yet available examining the behavior of iPS cells at any stage of differentiation in decellularized lung scaffolds.

The use of more committed cells should better define the fidelity of repopulation including region-specific distribution. To date, only a limited number of cell types have been employed, and almost all of these cell types normally inhabit the alveolar compartment. In one study, ATII cells converted to ATI-like cells (flattening, loss of lamellar bodies, formation of tight junctions, and synthesis of pinocytotic vesicles) when cultured on acellular lung but not amniotic matrices, again supporting the specific role of lung matrices to instruct differentiation of progenitor cells<sup>30</sup>. In a more recent study, primary or passaged ATII cells seeded directly onto acellular lung for 22 days maintained proSP-C and mature SP-C expression with some cells developing expression of Aqp5 and T1 $\alpha$ <sup>36</sup>. Moreover, fresh ATII cells seeded on lung scaffolds showed CC10 expression although the specific distribution of these cells was not defined. In other studies, seeding decellularized mouse lungs with immortalized non-neoplastic mouse ATII cells (C10 cells) resulted in widespread distribution including predominantly small but also large airways as well as alveolar regions<sup>41-43</sup>. This suggests that transformed, and likely also neoplastic, cell lines will not home specifically to the region inhabited by their cell of origin (*i.e.* ATII cells) and may



exhibit atypical morphologies. While these data do not completely resolve the specificity of homing and engraftment of ATII cells, they suggest that these events are promoted by the acellular scaffolds acknowledging that the composition and quality of the scaffold is a critical factors in retention, survival, phenotype, and function during recellularization. Data concerning regio-specific repopulation of acellular lung scaffolds with specific populations of basal cells, club cells, neuroendocrine cells, submucosal glandular cells, bronchioalveolar stem cells (BASC), ciliated or non-ciliated airway epithelial cells, lung fibroblasts, and microvascular endothelium are lacking.

While the existence of adult endogenous lung epithelial progenitor cells is a topic of great interest to pulmonary biology, it is unclear how acellular scaffolds will influence the phenotype and function of these cells. Importantly, our understanding of the specific features of cellular niches in the lung is limited, and therefore it is unknown how acellular scaffolds succeed or fail to recapitulate those microenvironments. In some areas of lung (e.g. basal cell layer) the features of a niche are better understood than in others (e.g. alveolus). Until the features of a niche are known, it may be impossible to distinguish or direct homing after recellularization to a specific niche (i.e., true 'repopulation') from stochastic events. Exploiting endogenous stem-progenitor cell populations for bioengineering purposes will be further constrained due to the relatively small number of stem-progenitor cells that inhabit the lung, and the challenges of isolation, culture, and expansion of those cells while preserving their native characteristics. Furthermore it remains unknown as to whether endogenous epithelial progenitor cells obtained from diseased lungs, when placed into an epigenetically new environment (i.e. acellular scaffold) will adapt to these changes by restoring function. Thus while many opportunities exist to study fundamental mechanisms of stem-progenitor cell-scaffold interactions in decellularization-recellularization models, it will be critical to develop acellular scaffolds and culture conditions that replicate normal 'homeostatic' conditions before they are used for bona fide 'repopulation' assays akin to those assays employed for study of hematopoiesis.

The lung also harbors non-endodermal (mesenchymal) sources of progenitor cells. Tissue stroma in particular appears to be robust sources of mesenchymal stem-like cells that may participate in tissue maintenance, repair, and immune regulation<sup>68</sup>. Lung-resident mesenchymal stem/stromal cells (L-MSCs) have been identified in mice and humans by fluorescence-activated cells sorting (FACS) for vital dye efflux as well as by adherent cell culture from bronchioalveolar lavage and lung tissue explants<sup>69-71</sup>. Recent reports demonstrate that L-MSCs contributed to lung repair after bleomycin-induced lung injury and in elastase-mediated injury in murine and ovine models of experimental emphysema<sup>70-72</sup>. Co-culture of L-MSCs with ATII cells induced the expression of CK18, CK19, occludin, and SPC in L-MSCs suggesting that these lung-resident stromal cells have the ability to differentiate into alveolar-like cells *in vitro*; however, little is known about whether they accomplish this *in vivo*<sup>73</sup>. Like bone marrow-derived MSC, L-MSCs also exhibit the ability to differentiate into endothelial-like cells that take up acetylated-LDL when cultured in endothelial growth medium on appropriate substrates<sup>70</sup>. L-MSCs may thus be useful in lung bioengineering strategies as they exhibit several desirable characteristics including tissue support, regulation, repair, and potentially regeneration<sup>70,71,74,75</sup>. For example, ovine L-MSCs were shown to promote epithelial growth in co-culture, engrafted and synthesized provisional matrices (laminin, fibronectin, collagen IV) on acellular sheep lung scaffolds, and promoted tissue healing in a sheep model of emphysema<sup>74,75</sup> and murine L-MSCs reversed elastase-induced injury<sup>70</sup>.

Just as resident mesenchymal stem/stromal cells from the lung may be exploited for bioengineering, more accessible MSCs isolated from bone marrow (BM-MSCs) and other sources including adipose and placental tissues may also be potentially utilized in used in

this context. While it has been demonstrated that BM-MSCs and cord blood-derived MSCs can differentiate into pulmonary-like cells *in vitro* when cultured in specialized media or co-cultured with airway epithelial cells<sup>76,77</sup>, *in vivo* engraftment is currently felt to be a rare phenomenon of no likely physiologic or therapeutic significance<sup>54,77-79</sup>. However, MSCs may hold more potential for recellularizing organ scaffolds and have been utilized in bioengineering schemes utilizing both synthetic and decellularized tracheas<sup>14,15</sup>. Our collaborative group has investigated the initial interactions of BM-MSCs with decellularized lung parenchyma in both murine and non-human primate models to test their applicability toward regeneration of functional pulmonary tissue<sup>38,39,41-43</sup>. As with L-MSCs, MSCs from other sources will most likely have a role to provide a stroma and participate in formation of niches for epithelial and endothelial cells<sup>80,81</sup>.

To best develop repopulation assays using recellularization of acellular scaffolds, it will be crucial to characterize stem-progenitor cell 'niches' in the lung, including the role for supportive stromal cells and the identity of extracellular matrix ligands that presumably control the fate and function of cells within and upon release from the niche. In some areas of lung (e.g. basal cell layer) the features of a niche are better understood than in others (e.g. alveolus). Until the features of a niche are known, it may be impossible to distinguish or direct homing to a niche ('repopulation') from stochastic phenomena.

### Implantation of Recellularized Scaffolds

Several investigators have performed xenotransplantation of decellularized scaffolds into rodents as well as larger animals. Using decellularized rat lung scaffolds re-epithelialized with a mixture of rat fetal lung homogenate and A549 lung carcinoma cells, and re-endothelialized with human umbilical vein endothelial cells, two groups were able to transplant the repopulated scaffold into rats that had undergone previous pneumonectomy<sup>32,33</sup>. Prior to implantation, the repopulated scaffolds were able to maintain adequate oxygenation, carbon dioxide exchange, and appropriate pressure/volume relationships. However, once implanted, the grafts developed significant pulmonary edema and/or hemorrhage resulting in respiratory failure after several hours. In a follow-up study, survival for fourteen days was achieved after implantation but graft function progressively declined and the histologic appearance of the graft at necropsy demonstrated significant fibrosis<sup>37</sup>. Though these studies are technically innovative and provide proof of concept that acellular matrices can be repopulated, transplanted, and maintain a degree of function, they are not yet clinically translatable. In order to have a clinically translatable model, adequate gas exchange, re-creation of intact alveolar and vascular compartments, unidirectional mucociliary clearance, immune surveillance, clearance of infection, and maintenance of physiologic airway pressures and volumes. Thus far, there has been a compartmentalized approach to the respiratory system, separating the vasculature, proximal airways, and distal lung. A successful translatable implantation animal model has not yet been created, but will need to balance all of these requirements. It will likely be a number of years before this is achieved.

### Immunogenicity of Implanted Scaffolds

A critical assumption for clinical use of decellularized lung scaffolds is that they will be relatively non-immunogenic and minimize any detrimental host response following implantation. However, ECM and other proteins remaining in decellularized scaffolds can provoke immune responses<sup>82-85</sup>. Interestingly, some of these may be beneficial as growing literature suggests that decellularized scaffolds can polarize macrophages to anti-inflammatory M2 phenotype with subsequent permissive effects on implanted scaffolds<sup>86-88</sup>. With respect to lung, proteomic assessments utilizing mass spectrometry and/or western blotting demonstrate that a wide range of residual proteins, including

intracellular, nuclear, cytoskeletal, and others can remain in the lungs, despite apparent effective de-cellularization<sup>38,39,41-43</sup>. Whether these residual proteins provoke immune responses is currently the focus of intense inquiry. Theoretically, despite conservation of ECM proteins, any denuded basement membrane may provoke an immune response, in part to mobilize the necessary cells to cover the “damaged” membrane<sup>50</sup>. Whether cells inoculated into decellularized scaffolds will secrete ECM and other proteins and remodel the scaffold accordingly is also the subject of intense current investigation<sup>39</sup>.

### Mechanical Factors in *Ex Vivo* Lung Regeneration

In addition to utilizing the proper cell type(s) to inoculate into the scaffolds and using appropriate growth factors, environmental cues such as mechanical stimuli may also play a critical role. There is a large and growing body of literature that delineates the importance of various mechanical stimuli on regulating development as well as normal and diseased tissue homeostasis *in vivo*. For example, mechanical stretch is known to induce overexpression of SPC mRNA and protein expression in ATII cells, while shear stress on endothelial cells is critical for VEGF expression. Several studies have examined the biological consequences of mechanotransduction on fetal or adult lung cells *in vitro*<sup>85-88</sup> but there is no available information on effects of stretch on development of lung epithelial tissue from embryonic or adult stem cells or from endogenous lung progenitor cells. It is likely that in addition to derivation of an optimal decellularized scaffold, precise control of the mechanical environment with bioreactor technologies (*ie.* stimuli mimicking stretch from breathing and shear stress induced by blood flow) will be necessary for a successful regeneration scheme. Other environmental factors such as oxygen tension will likely also play critical roles in recellularization schemes.

### Summary

The challenges in developing complex 3-dimensional functional lung tissues *ex vivo* will be in recapitulating the normal dynamic integrated network of component cells, orientation and function of the fiber network, perfusion ventilation relationships, and immune surveillance, all of which are vital for proper function (92). Whether decellularized lung constructs or synthetic 3-dimensional lung scaffolds achieve these goals is an area of intense excitement and study.

### Biographies

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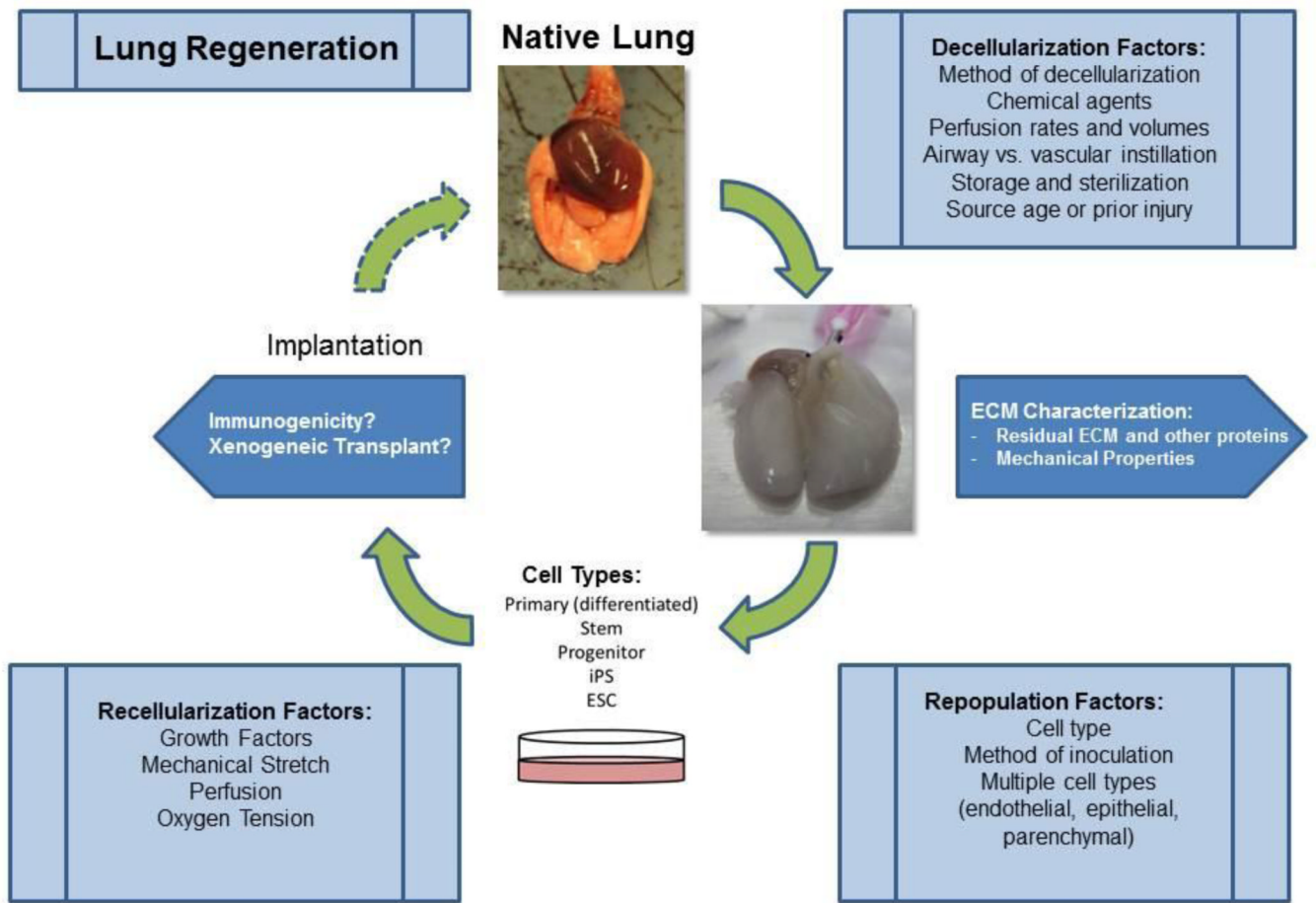
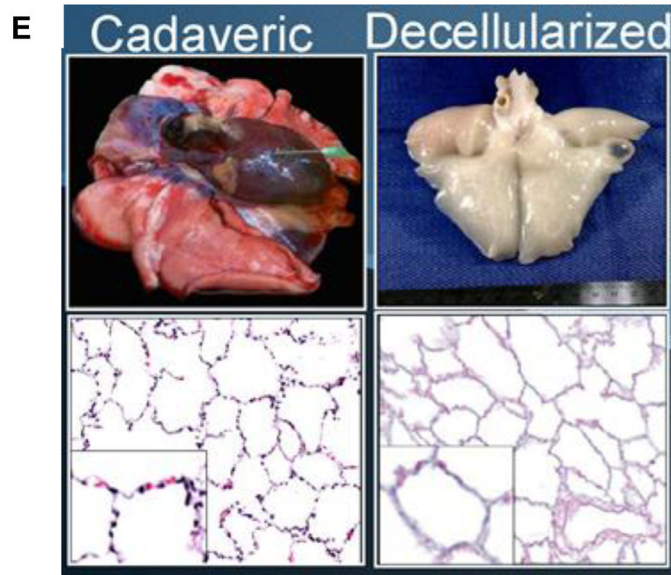
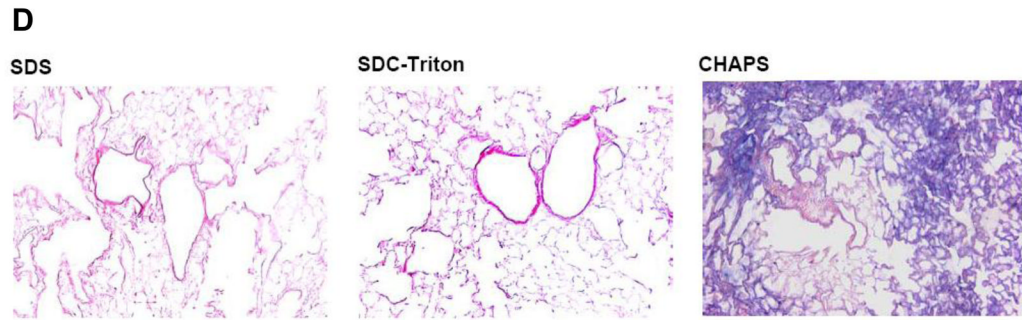
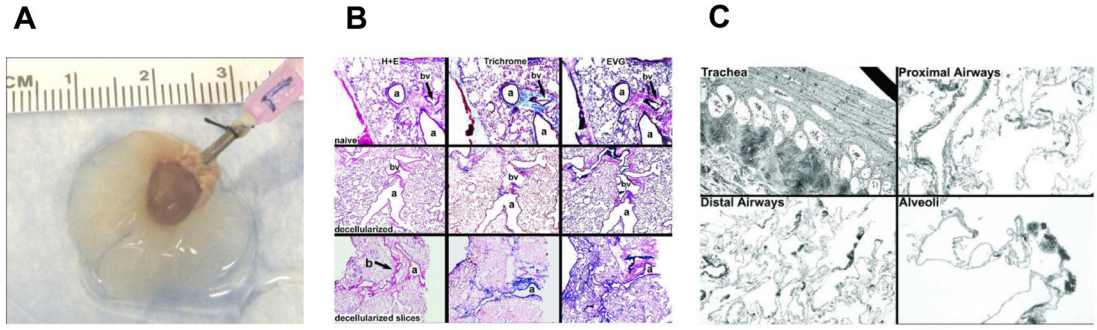


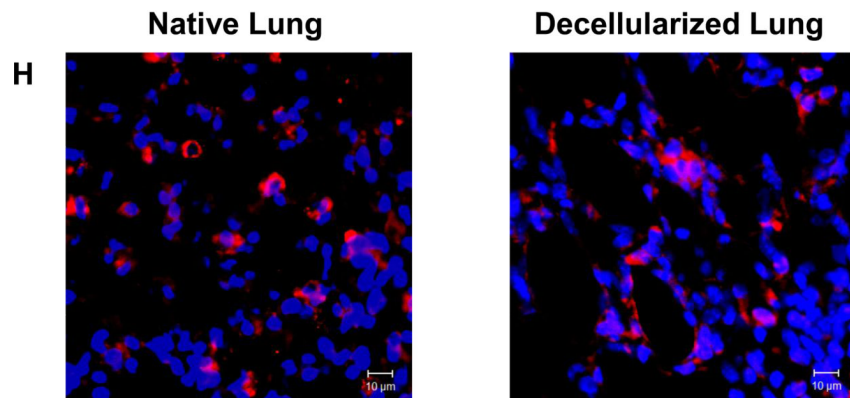
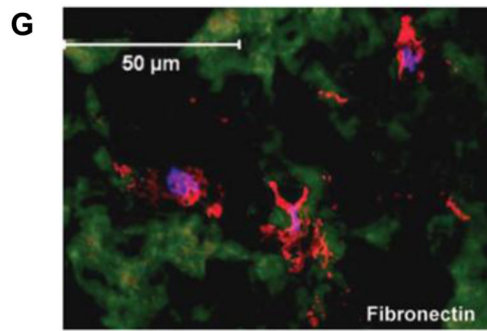
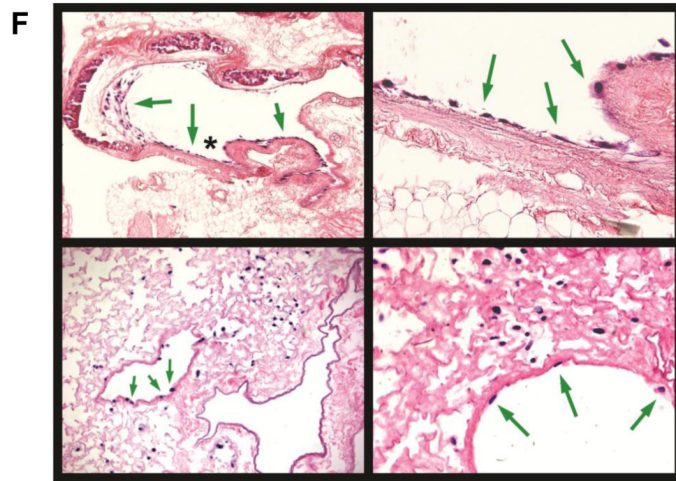
Figure 1. Schematic for optimal decellularization, recellularization, and implantation

**A: Decellularization**

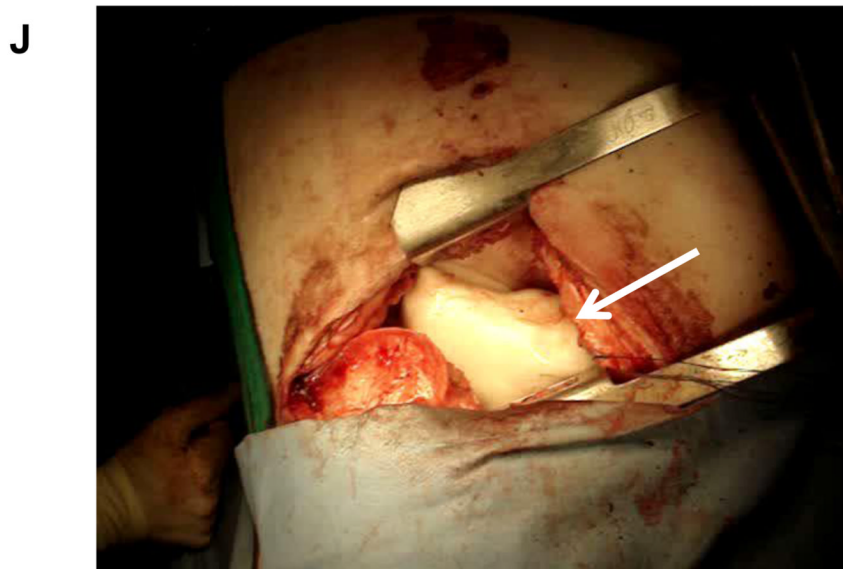
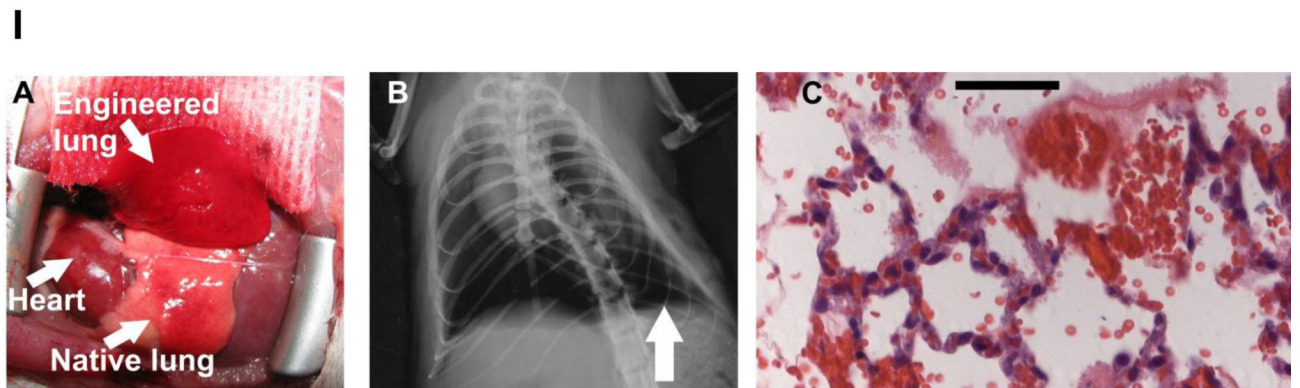




B: Recellularization



## C: Implantation



**Figure 2. Representative images depicting decellularization, recellularization, and implantation of decellularized lung scaffolds**

**A)** Whole de-cellularized mouse heart-lung bloc. The trachea is cannulated with a 18 gauge blunted needle. Reprinted with permission from Daly et al., Initial Binding and Re-Cellularization of De-Cellularized Mouse Lung Scaffolds with Bone Marrow-Derived Mesenchymal Stromal Cells Tissue Engineering Part A, Vol. 18, No. 1–2: 1–16, 2012 (39).

**B)** H and E, Masson's Trichrome collagen, and Verhoeff's Van Gieson staining of native mouse lungs, de-cellularized whole mouse lungs, and approximately 1 mm thick slices of mouse de-cellularized lungs. Original magnifications: 100 X. a = airway, bv = blood vessel. Reprinted with permission from Daly et al., Initial Binding and Re-Cellularization of De-



Cellularized Mouse Lung Scaffolds with Bone Marrow-Derived Mesenchymal Stromal Cells Tissue Engineering Part A, Vol. 18, No. 1–2: 1–16, 2012 (39).

**C)** Transmission electron micrograph images of different regions of a representative decellularized whole mouse lung are shown. Original magnifications A) 600X, B) 1,000X, C) 1,000 X, D) 3,000X. Reprinted with permission from Daly et al., Initial Binding and Re-Cellularization of De-Cellularized Mouse Lung Scaffolds with Bone Marrow-Derived Mesenchymal Stromal Cells Tissue Engineering Part A, Vol. 18, No. 1–2: 1–16, 2012 (39).

**D)** Histologic assessment of H and E stained whole mouse lungs decellularized using different detergent-based protocols demonstrates significant differences in resulting histologic architecture notably loss of detail and parenchymal structure when using CHAPS. a = airway, bv = blood vessel, Original magnifications 100X. Reprinted with permission from Wallis et al., Comparative assessment of detergent-based protocols for mouse lung de-cellularization and re-cellularization. Tissue Eng Part C Methods 2012; 18: 420–432 (41).

**E)** Representative gross and histologic images of native and decellularized macaque lungs. Reprinted with permission from Bonvillain et al., A nonhuman primate model of lung regeneration: detergent-mediated decellularization and initial in vitro recellularization with mesenchymal stem cells. Tissue Eng Part A 2012; 18: 2437–52 (38)→.

**F)** Intratracheally inoculated MSCs cultured up to one month in both basal MSC media and in SAGM grow in parenchymal and airway regions of de-cellularized whole mouse lungs. Representative photomicrographs depict MSCs in large airways (upper row) and in parenchymal lung regions (lower row). Green arrows highlight cells growing in large airways, and the asterisk in the upper left-hand image show the region magnified in the upper right-hand image. Original mags are 100X, 400X, 400X and 200X. Reprinted with permission from Daly et al., Initial Binding and Re-Cellularization of De-Cellularized Mouse Lung Scaffolds with Bone Marrow-Derived Mesenchymal Stromal Cells Tissue Engineering Part A, Vol. 18, No. 1–2: 1–16, 2012 (39).

**G)** Lung-derived MSCs (L-MSCs) inoculated into decellularized sheep lung scaffolds express and grown for 2 weeks express a variety of ECM proteins (fibronectin is depicted) that may help to remodel the scaffold. Reprinted with permission from Ingenito et al., Autologous lung-derived mesenchymal stem cell transplantation in experimental emphysema. Cell Trans 2012; 21: 175–189 (75).

**H)** Mouse embryonic stem cells (ESCs) pre-differentiated in routine tissue culture to express pro-surfactant protein C (pro-SPC, red stain) maintain pro-SPC expression after culture in decellularized mouse lung scaffolds. Depicted are images of native mouse lung and decellularized mouse lung 1 week after inoculation with SPC-expressing murine ESCs. Original magnification 200X. Reprinted with permission from Jensen et al., A rapid lung de-cellularization protocol supports embryonic stem cell differentiation in vitro and following implantation. Tissue Eng Part C Methods 2012; 18: 632–46 (40).

**I)** Tissue-engineered left lung was implanted into adult Fischer 344 rat recipient and photographed ~30 minutes later. (B). X-ray image of rat showing the implanted engineered left lung (white arrow) and the right native lung. (C) H and E stain of explanted lung. Red blood cells perfusing septa are evident, and some red blood cells are present in airspaces. Scale bar 50  $\mu$ m. Reprinted with permission from Petersen et al., Tissue-Engineered Lungs for in Vivo Implantation. Science, 2010 329:538–541 (32).

**J)** Decellularized sheep lung lobe (white arrow) implanted into an adult sheep establishing both airway and vascular anastomoses with appropriate inflation and vascular perfusion. Hoffman, Finck, Weiss unpublished data.

**Table 1**  
Compiled Studies of Ex Vivo Lung Bioengineering Using Decellularized Whole Lung Scaffolds

Reference	Scaffold	Objective	Method Of Decellularization	Timing Of Decellularization Process	Endpoints
<b>Kuttan Lung 1981 (29)</b>	Alveolar Basement Membrane (Calf, Dog, Rabbit, Adult/Newborn Rat)	Study Basement Membrane	Filtered Distal Lung Homogenate, Saline, 4% Triton-X100 With Protease Inhibitors, NaHCO <sub>3</sub> Rinse, Distilled H <sub>2</sub> O Rinse.	26–52 Hours Depending On Homogenate Volume	Histology, Immunofluorescence, Electron Microscopy, Amino Acid Analysis, Carbohydrate Analysis
<b>Lwega Mukasa et al. Exp Lung Res 1986 (30)</b>	Acellular Alveolar Versus Amniotic Basement Membranes	Differentiation Pattern On Different Basement Membranes	Distilled H <sub>2</sub> O, 0.1% Triton X100, 2% SDC, NaCl, Pancreatic DNase Type 1S	> 2 Days	Cell Attachment And Morphology
<b>Price et al Tissue Engineering Part A 2010 (31)</b>	Mouse (Female C57/BL6) Acellular Lungs	Effect Of Matrix On Geospatial Engraftment Of E17 Fetal Lung Homogenate	Airway And Vascular Perfusion: Distilled H <sub>2</sub> O, 0.1% Triton X100, SDC, NaCl, Porcine Pancreatic DNase	3 Days (Approximately 63 Hrs)	Histology, Quantification Of ECM Proteins, Immunofluorescence, SEM, Function With Flexivent, Bioreactor With Fetal Type II Cells
<b>Petersen et al Science 2010 (32)</b>	Rat Acellular Lungs (Male Fischer 344)	Development Of Bioartificial Lung For Orthotopic Transplantation	Vascular Perfusion Only (1–5ml/Min With Less Than 20 Mmhg Arterial Pressure) CHAPS, NaCl, EDTA, PBS	4 Hours	Histology, Immunofluorescence, DNA Quantification Assay, Collagen Assay, GAG Assay, Western Blots, SEM, TEM, Micro-CT Imaging
<b>Cortiella et al Tissue Eng Part A 2010 (34)</b>	Rat Acellular Lung (Sprague Dawley)	Comparison Of Matrices Including Decellularized Rat Lung In Ability To Support mESCs	Fast Freeze/Thaw Cycles, 1% SDS, Dnaase, Rnaase, PBS, Penicillin/Streptomycin, Amphotericin, DMEM	>6weeks	Quantification Of DNA, Immunohistochemistry, Confocal Microscopy, Flow Cytometry, 2 Photon Microscopy, Presence Of Spa

Reference	Scaffold	Objective	Method Of Decellularization	Timing Of Decellularization Process	Endpoints
Ott et al Nature Med 2010 (33)	Rat Acellular Lung (Sprague Dawley)	Development Of Bioartificial Lung For Orthotopic Transplantation	Vascular Perfusion Only: Pulmonary Artery Pressure Kept Constant At 80cmh2o, Heparinized PBS With 0.1% SDS, Deionized Water, Triton X100, And PBS With Penicillin, Streptomycin, Amphoterin B	3 Days (Approximate y 75 Hrs) Including Incubation With Antibiotics	Histology, Morphology, Mechanical Function, Fluorescopy, Gas Exchange, Transplantation, Protein Analysis
Song, Ott et al. Ann Thorac Surg 2011 (37)	Rat Acellular Lung (Sprague Dawley)	Orthotopic Transplantation	Vascular Perfusion Only: Pulmonary Artery Pressure Kept Constant At 80cmh2o, Heparinized PBS With 0.1% SDS, Deionized Water, Triton X100, And PBS With Penicillin, Streptomycin, Amphoterin B	3 Days (Approximate y 75 Hrs) Including Incubation With Antibiotics	Histology, Immunohistochemistry, Morphology, Fluorescopy, Functional Analysis, Transplantation Seeded Lungs With Fetal Pulmonary Cells And Pulmonary Artery And Vein With Endothelial Cells
Shamis et al. Tiss Eng Part C 2011 (36)	Rat Acellular Liver And Lung (Lewis)	Cellular Differentiation On 3D In Vitro Scaffold	Lung Lobes Cut Into 300 Micron Thick, 0.5% Tritonx100, 10mm Ammonia, Mechanical Disruption, PBS, Distilled Water	N/A	Histology, TEM, Environmental Scanning, PCR, Immunohistochemistry, Liquid Chromatography With Tandem Mass Spectrometry
Daly et al Tissue Eng Part A 2011 (39)	Mouse Acellular Lung (C57BL/6; BALB/C)	Initial Binding And Recellularization Of Mses In Acellular Scaffold; Directed Seeding With Integrin Blocking	Airway And Vascular Perfusion: Distilled H2O, 0.1% Triton X100, 2% SDC, NaCl, Pancreatic DNase Type 1S	3 Days (Approximate y 72 Hrs)	Histology, Immunofluorescence, EM, Perfusion To Assess Vascular Continuity, Mass Spectrometry, Western Blot, Lung Mechanics With Flexivent, Inoculation Of Bone Marrow Derived MSCs
Wallis et al. Tissue Engineering Part C 2011	Mouse Acellular Lung And Lung Slices (BALB/C)	Comparison Of Detergent-Based Decellularization Protocols	Airway And Vascular Perfusion. 3 Different Protocols Tested: 1) H2O, 0.1% Triton	3 Days (Approximate y 72 Hrs)	Immunohistochemistry, Mass Spectrometry, Western, Mechanical

Reference	Scaffold	Objective	Method Of Decellularization	Timing Of Decellularization Process	Endpoints
(41)			X-100, 2% SDC, NaCl, Porcine Pancreatic DNAase; 2) PBS, 0.1%SDS, 0.1% Triton X-100; 3) PBS, CHAPS, NaCl, EDTA, DNAase, FBS		Analysis, Gelatinase, Dnaase, Rnaase, Comparative Recellularization With MSCs And C10 Epithelial Cells
<b>Bonvillian et al. Tissue Eng Part A 2012 (38)</b>	Normal Rhesus Macaque Acellular Lung	Initial Binding And Recellularization Of MSCs In Acellular Scaffold	Airway And Vascular Perfusion: PBS, EDTA, Penicillin/Streptomycin At Initial Harvest: Pulmonary Artery: PBS+Heparin+Sodium Nitroprusside With Pressures 25-30mmHg; Then Trachea And Vasculature: Deionized H2O, 0.1%Tritonx100, 2%SDC, NaCl, Bovine Pancreatic DNAase	2-3 Days (Approximately 48-72 Hrs)	Histology, Morphology, Immunohistochemistry, Western Blot, Genomic DNA, Proteomics, Seeding With Bone Marrow And Adipose Derived Rhesus MSCs
<b>Longmire et al. Cell Stem Cell 2012 (61)</b>	Mouse Acellular Lung And Lung Slices (C57/BL6)	Seeding With And Differentiation Of mESCs-Derived Endodermal Lung Precursors	Airway And Vascular Perfusion: Distilled H2O, 0.1% Triton X100, 2% SDC, NaCl, Pancreatic DNAase Type 1S	3 Days (Approximately 72 Hrs)	Evaluation Of Ability To Differentiate mESCs Into Lung Precursor Cells
<b>Jensen et al. Tissue Eng Part C 2012 (40)</b>	Mouse Acellular Lung (C57BL/6)	Comparison Of Timing Of Decellularization, Coating Of Decellularized Matrices, And Support Of mESCs Differentiated Into Alveolar Epithelial Cells	Airway And Vascular Perfusion: 0.1% Triton X100, 2%SDC, NaCl, Porcine Pancreatic DNAase, PBS	1 Vs 3 Days (Approximately 24 Hours Vs 50 Hours)	Histology, Morphology, EM, Western Blot, Gelatinase Assay, Immunofluorescence, Mechanical Properties With Flexivent, Support Of Differentiated mESCs Within Scaffold, Subcutaneous Implantation Of Scaffold

Reference	Scaffold	Objective	Method Of Decellularization	Timing Of Decellularization Process	Endpoints
<b>Petersen et al. Cells Tissues Organs 2012 (44)</b>	Rat Acellular Lung (Fisher 344)	Comparison Of Different Detergent-Based Decellularization Protocols	2 Approaches: 1- Vascular Perfusion CHAPS, NaCl, EDTA, PBS; 2- NaCl, EDTA, SDS	4 Hours	Histology, Collagen Assay, Elastin Assay, GAG Assay, DNA Assay, Mechanical Testing With Linear Strips
<b>Booth et al. Am J Resp Crit Care 2012 (56)</b>	Human Normal And Fibrotic Acellular Lungs	Development Of An <i>In Vitro</i> System For Normal And Fibrotic Matrices	Airway And Vascular Perfusion: Distilled H <sub>2</sub> O, 0.1% Triton X100, PBS, 2%SDC, NaCl, DNAase, Mgso <sub>4</sub> , Cacl <sub>2</sub> ; 0.18% Paracetic Acid/4.8% Ethanol	3 Days (Approximate y 72 Hrs)	Histology, Western Blot, PCR, AFM, Mass Spectrometry, EM; Fibroblasts Were Seeded In To Normal And Fibrotic Lungs And Assayed For Gene And Protein Expression Changes
<b>Bonenfant et al Biomaterials 2013 (42)</b>	Mouse Acellular Lung And Lung Slices (C57BL/6)	Effect Of Time To Necropsy, Length Of Storage, And 2 Different Methods Of Sterilization Of Construct	Airway And Vascular Perfusion: Distilled H <sub>2</sub> O, 0.1% Triton X100, 2% SDC, NaCl, Pancreatic Deoxyribonuclease Type 1S, Mgso <sub>4</sub> , Cacl <sub>2</sub> , Penicillin, Streptomycin	3 Days (Approximate y 72 Hrs)	Histology, Immunohistochemistry, Morphology, Mass Spectrometry, Seeded Lungs With MSCs And C10 Epithelial Cell Line
<b>Sokocevic et al Biomaterials 2013 (43)</b>	Mouse Acellular Lung And Lung Slices (C57BL/6)	Effect Of Recipient Age And Elastase, Or Bleomycin Injury On Decellularization And Recellularization	Airway And Vascular Perfusion: Distilled H <sub>2</sub> O, 0.1% Triton X100, 2% SDC, NaCl, Pancreatic Deoxyribonuclease Type 1S, MgSo <sub>4</sub> , CaCl <sub>2</sub> , Penicillin, Streptomycin	3 Days (Approximate y 72 Hrs)	Histology, Immunohistochemistry, Mass Spectrometry, Inoculation With MSCs And C10 Cells

**Abbreviations Used:**

AFM- Atomic Force Microscopy  
CaCl<sub>2</sub>-Calcium Chloride  
CHAPS-3- [(3-Cholamidopropyl)Dimethylammonio]-1-Propanesulfonate Hydrate  
cmh<sub>2</sub>o: Centimeters of Water (Pressure)  
CT: Computed Tomography  
DMEM: Dulbecco's Modified Eagle's Medium  
DNAase: Deoxyribonuclease  
DNA- Deoxyribonucleic Acid  
E17- Embryonic Day 17

ECM- Extracellular Matrix  
EDTA- Ethylenediaminetetraacetic Acid  
FBS: Fetal Bovine Serum  
GAG- Glycosaminoglycan  
H<sub>2</sub>O- Water  
mM: Millimolar  
mESC: Embryonic Stem Cells  
MgSO<sub>4</sub>- Magnesium Sulfate  
mmHg: Millimeters Of Mercury  
MSC: Mesenchymal Stem Cells  
NaCl- Sodium Chloride  
NaHCO<sub>3</sub>- Sodium Bicarbonate  
PBS- Phosphate Buffered Saline  
PCR: Polymerase Chain Reaction  
RNAase: Ribonuclease  
SDC- Sodium Deoxycholate  
SEM- Scanning Electron Microscopy  
SDS: Sodium Dodecyl Sulfate  
SpA: Surfactant Protein A  
TEM- Transmission Electron Microscopy



**Table 2****Agents Commonly Used During De-Cellularization**

<b>Agent</b>	<b>Properties</b>
Triton X-100	Nonionic detergent used to solubilize proteins; mild non-denaturing detergent
Sodium Deoxycholate (SDC)	Water soluble ionic detergent used for disrupting and dissociated protein interaction
Sodium Dodecyl Sulfate (SDS)	Anionic surfactant used for lysing cells and unraveling proteins
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Non-denaturing zwitterionic detergent used to solubilize proteins
Ethylenediaminetetracetic acid (EDTA)	Chelating agent that binds to calcium and prevents joining of cadherins between cells, preventing clumping of cells grown in liquid suspension, and detaching adherent cells. Can also be used to inhibit metalloproteinases.
Antibiotics	Typically Penicillin, Streptomycin, and an anti mycotic Amphotericin
Other	DNAase, RNAase, and heparin

Table 3

Distribution and phenotype of cells seeded onto acellular scaffolds.

Reference	Cells used for seeding	Scaffold	Route	Duration	Distribution	Final phenotype
Lwega-Mukasa et al. Exp Lung Res 1986 (30)	AECII	Acellular alveolar vs. amniotic basement membranes	Direct seeding	8 days	N/A	Alveolar matrices: AECI; amniotic membranes AECII
Cortella et al. Tissue Eng Part A 2010 (34)	mESC	Rat (Sprague Dawley) acellular lung	Trachea	21 days	Proximal-distal regiospecific CC10, proSP-C expression)	tracheobronchial: CC10, CK18; distal lung: proSP-C, CD31, PDGFR $\alpha$ .
Ott et al. Nature Med 2010 (33)	HUVEC (DsRed)	Rat acellular lung	Pulmonary artery	9 days	All vessels	endothelial cells
	A549	Rat acellular lung	Trachea	9 days	Airways/alveoli	airway / alveolar epithelium
	HUVEC (DsRed)	Rat acellular lung	Pulmonary artery	9 days	Entire vasculature	endothelial cells
	Rat fetal lung cells (GD19–20)	Rat acellular lung	Trachea	9 days	Airways/alveoli	proSP-A, proSP-C, Ttf-1/Nkx2.1 (AECII); T1 $\alpha$ (AECI); Vimentin (fibroblast)
Petersen et al. Science 2010 (32)	Neonatal (7d) lung epithelial cells (rat)	Rat acellular lungs (Fischer 344)	Trachea	8 days	Alveolar, small airways	CCSP (Clara cell), proSP-C (AECII), Aqp5 (AECI), CK14(basal cell)
	Lung vascular endothelium (rat)	Rat acellular lungs (Fischer 344)	Pulmonary artery	7 days	Microvascular	CD31
Price et al. Tissue Engineering Part A 2010 (31)	Fetal lung (E17)	Ms acellular lungs	Tracheal	7 days	Alveolar	CK18+/proSP-C+ (AECII); no CD11b, aquaporin-5, CCSP, CD31, or vimentin
Daly et al. Tissue Eng Part A 2011 (39)	mBM-MSCs	Ms acellular lung	Trachea	28 days	Parenchymal>airway (squamous)	MSCs: No evidence for transdifferentiation

Reference	Cells used for seeding	Scaffold	Route	Duration	Distribution	Final phenotype
	C10 - hAECII (non-tumorigenic)	Ms acellular lung	Trachea	28 days	Parenchymal	N/A
Ott et al Ann Thorac Surg 2011 (37)	Rat fetal (GD17-20) pneumocytes	Rat acellular lung	Trachea	14 days	Alveolar/distal bronchioles > trachea/bronchi	CCSP (airways); TTF-1, proSP-C (alveolar)
	HUVEC	Athymic nude rat	Pulmonary artery	14 days	Proximal to distal vasculature	CD31 <sup>pos</sup>
Shamis et al Tissue Eng Part C 2011 (36)	Ms AECII (primary or P2)	Acellular lung micro scaffold	Direct seeding	22 days	Alveolar	proSP-C/SP-C (AECII-like, from primary or cultured AECII); App5, Pdpn (AECI); CCSP (primary)
Wallis et al Tissue Engineering Part C 2011 (41)	mBM-MSCs	Ms acellular lung	Trachea	14 days	Alveolar	MSCs
	C10 - hAECII (non-tumorigenic)	Ms acellular lung	Trachea	14 days	Large and Small Airways	squamous morphology
Bonvillain et al, Tissue Eng Part A 2012 (38)	Rhesus BM-MSCs	Rhesus macaque	Secondary bronchus	7 days	Alveolar septae, terminal bronchioles, respiratory bronchioles	MSCs phenotype
	Rhesus AD-MSCs	Rhesus macaque	Secondary bronchus	7 days	Alveolar septae, terminal bronchioles, respiratory bronchioles	MSCs phenotype
Jensen et al Tissue Eng Part C 2012 (40)	mESCs diff. to Ttf1 <sup>pos</sup> /proSP-C <sup>pos</sup>		Immersion	14 day	Alveolar	Ttf1/Nkx2.1, proSP-C (alveolar); PDGFR $\alpha$ (mesenchymal)
Longmire et al. Cell Stem Cell 2012 (61)	mESCs	Ms acellular lung	Trachea	10 days	Hypercellular sheets (alveolar)	ciliated cells (airways); T1 $\alpha$ <sup>neg</sup> (alveoli)
	Nkx2.1 <sup>GFP</sup>	Ms acellular lung	Trachea	10 days	Alveolar	Nkx2.1/T1 $\alpha$ (alveoli)

Reference	Cells used for seeding	Scaffold	Route	Duration	Distribution	Final phenotype
Bonenfant et al Biomaterials 2013 (42)	Mouse BM-MSCs	Ms acellular lung	Trachea	28 days	Alveolar	N/A
	C10 -AECII (non- tumorigen ic)	Ms acellular lung	Trachea	28 days	Alveolar	N/A
Sokoce vic et al Biomaterials 2013 (43)	mBM- MSCs	Ms acellular lung	Trachea	28 days	Alveolar	N/A
	C10 - hAECII (non- tumorigen ic)	Ms acellular lung	Trachea	28 days	Alveolar	N/A