Curr Stem Cell Rep (2017) 3:55–67
DOI 10.1007/s40778-017-0082-8

ARTIFICIAL TISSUES (A ATALA AND JG HUNSBERGER, SECTION EDITORS)

Engineering Bioartificial Lungs for Transplantation

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Published online: 11 May 2017

Abstract
Purpose of Review The purposes of this study were to review current progress being made in engineering bioartificial lungs for transplantation and identify tangible benefits to the implementation of this research into development of 4D lung models for disease modeling, drug screening, and personalized medicine that could be realized in the next 5 years.
Recent Findings We focused primarily on the generation and analysis of decellularized lung scaffolds, the repopulation of acellular lung scaffolds by primary cells and pluripotent stem cells-derived cells, and current disease modeling approaches.
Summary Bioengineered lung has significant potential in transplantation and facilitates future biomedical research by establishing of 4D cell culture model on the scaffold; it will benefit the implementation of the development of disease modeling, drug screening, and personalized medicine.

Keywords Perfusion decellularization · Lung scaffold · Primary cells · iPSCs · Regeneration · Disease modeling

Introduction

The stark contrast between patients suffering from end-stage lung diseases and the shortage of donor organs has fueled research to develop therapeutic alternatives to solid organ transplantation. Building on the principles of tissue engineering, organ engineering based on perfusion decellularized native matrix has enabled the formation of tissue grafts capable of performing higher level functions. While some early milestones up to transplantation in small and large animal models have been met, many questions remain. Among other topics, several groups focus on better characterization of the acellular scaffold, various cell types required for repopulation, guiding interaction between cells and scaffold, and the functional analysis of regenerated organs. To better appreciate the importance of continuing research efforts to address these problems and ultimately improve organ regeneration, it is essential to delineate the major achievements in lung bioengineering the past several years.

This article is part of the Topical Collection on *Artificial Tissues*

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Native Lung Scaffolds Generated by Decellularization

Perfusion Decellularization of Whole Lung

Perfusion decellularization of whole cadaveric rat hearts was first reported in 2008 by antegrade coronary perfusion with detergents [1•]. The resulting acellular scaffolds could be recellularized with neonatal cardiac cells, thereby generating a new model of bioengineering on a whole organ level in vitro [1•]. Since then, several groups reported on perfusion decellularization and recellularization of whole rodent lungs, which highlighted the platform character of this technology, and helped to expand the field of lung tissue engineering [2, 3••, 4, 5•]. More recently, successful decellularization of porcine, primate, and human lungs were reported accompanied by the development of human-sized bioreactor for decellularization and recellularization [6, 7•, 8–11], showing the scalability of this organ engineering approach to human size grafts.

In contrast to previously reported immersion decellularization, perfusion decellularization has demonstrated preservation of the native organ architecture. Imaging and microscopic analysis of pulmonary vasculature and airways in acellular lung rodent scaffolds has confirmed that capillary microstructures and distal airspaces were intact [3••, 12]. Preserved architecture of the lung scaffold provides researchers with the opportunity to reintroduce specialized epithelial and endothelial cells in its native anatomic location and also makes perfusion and ventilation of the regenerated lung possible.

Although enzymatic decellularization has been historically applied on tissue engineering, nowadays, most researchers prefer whole lung perfusion or ventilation decellularization using detergents. Various detergents have been used during perfusion or ventilation-based whole lung decellularization by different investigators and for different animal species (summarized in Table 1). The first mouse whole-lung perfusion decellularization was performed by Triton X-100, sodium deoxycholate (SDC), and porcine pancreatic DNAse [4]. For the first rat whole-lung perfusion decellularization, one group used sodium dodecyl sulfate (SDS) and Triton X-100 as the detergents, while others used 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium chloride (NaCl), and ethylenediaminetetraacetic acid (EDTA) [2, 3]. Perfusion decellularization methodology has been upscaled to porcine, primate, and human whole lungs by using mainly ionic detergents, like SDS, instead of zwitterionic detergents, like CHAPS, indicating that ionic detergents might be more effective in cell removal and preservation of scaffold architecture [6, 7•, 8–11, 17].

Despite the different methods of decellularization, there are several elements to examine in order to ensure the quality of the scaffold:

1. The loss of cellular component

Confirming that the decellularized tissue is completely free of cells is important as remaining cells may impair regeneration of the scaffold. For this purpose, hematoxylin and eosin (H&E) staining and 4′,6-diamidino-2-phenylindole (DAPI) staining are generally performed on sliced tissue. DNA content in the scaffold indicates the residual cellular material. DNA extracted from the matrix could be directly measured [2], or measured by DNA quantification assays such as PicoGreen, or by electrophoresis [18].

2. The composition of extracellular matrix (ECM) component

ECM is composed of fibrous proteins (collagens, elastins, laminins, etc.) and proteoglycans (glycosaminoglycan) [19], which have regulatory roles on cellular functions. Immune staining, western blot, and various quantitative protein assays are being used to identify the existence and amount of certain ECM proteins. Mass spectrometry provides a more comprehensive understanding of the remaining proteins on decellularized matrix [8].

3. The preservation of lung architecture

Preservation of the airway and vasculature architecture on decellularized lung significantly facilitates organ regeneration. Scanning electron microscopy (SEM) reveals the alveolar structure while micro-CT shows the arterial tree and microvasculature [3••, 20]. Casting and fluorescent bead infusion of the whole decellularized lung indicate if the integrity of the architecture is comparable to native lung [12, 21••].

4. Mechanics of the matrix

Different decellularization protocols affect the mechanical properties of the matrix in various ways. The inspiration/expiration capability of the regenerated lung needs to be preserved. Mechanical ventilation is being used to measure the airway elastance and resistance [22] and tensile testing shows the strength of the matrix by generating the stress/strain curve [15].

From a tissue engineering perspective, the ability of the matrix to support growing cells and functional tissue is the most essential criterion to assess whether decellularization is successful or not. Therefore, more in-depth analyses of the morphology, structure, marker protein expression, and function of the regenerated lung are needed for a successful future clinical transition.

Structure and Components of Whole Lung Scaffold

To further characterize the ECM and assess the mechanics of decellularized lung scaffolds, proteomic and mechanical analyses of the ECM have been performed [8, 13, 14, 23, 24]. Although it was hypothesized that different detergents used for lung decellularization will result in constructs consisting of different ECM components, proteomic analysis of decellularized mouse lung scaffolds following CHAPS-, SDS-, and SDC-based solutions showed relatively equivalent preservation of prominent ECM proteins. However, when architectural preservation was assessed, SDS and SDC performed better than CHAPS [14]. In rats, lung scaffolds have been shown to retain more collagen and demonstrate better mechanical properties following CHAPS-based decellularization when compared to SDS [15]. Microcapillary liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis indicated that SDS-based decellularization resulted in preservation of most ECM components among all detergent-based protocols [8]. Another group reported a 35% reduction in stiffness of rat lung scaffold when CHAPS-based decellularization protocol was

Table 1 Different methods of whole lung perfusion decellularization and the ECM components

Reference	Type of scaffold	Decellularization solutions	Method of analysis	Predominant ECM components
Ott et al. 2010 [2]	Rat lung	PBS, 0.1% SDS, DI water, 1% Triton X-100, PBS	Immune-histochemistry staining	Collagens I, III, IV, laminin, elastin, fibronectin, GAGs
Peterson et al. 2010 [3••]	Rat lung	8 mM CHAPS + 1 M Nacl + 25 mM EDTA in PBS, PBS	Immune-histochemistry staining Quantitative protein assays	Fibronectin, collagens I, IV, elastin, laminin, GAGs (less than 10% of native lung)
Price et al. 2010 [4]	Mouse lung	DI water, 0.1% Triton X-100, 2% SDC, 1 M NaCl, porcine pancreatic DNase, PBS	Immune-histochemistry staining Zymography	Elastin, collagen, laminin, GAGs No MMP2 or MMP9
Daly et al. 2012 [13]	Mouse lung	DI water, 0.1% Triton X-100, 2% SDS, 1 M NaCl, DNase solution, PBS	Immune-histochemistry staining Mass spectrometry Western blot	Collagens I, IV, fibronectin, laminin, GAGs (substantially loss), myosin, tubulin, actin et al.
Jensen et al. 2012 [5•]	Mouse lung	0.1% Triton X-100, 2% SDC, 1 M NaCl, DNase solution, PBS	Immune-histochemistry staining Western blot Quantitative protein assays	Collagens I, IV, fibronectin, elastin, laminin, myosin
Wallis et al. 2012 [14]	Mouse lung	(1) DI water, 0.1% Triton X-100, 2% SDC, 1 M NaCl, DNAse solution, PBS (2) PBS, 0.1% SDS, 1 M NaCl, DNAse solution, PBS (3) PBS, 8 mM CHAPS + 1 M Nacl + 25 mM EDTA in PBS, DNAse solution, 10% FBS, PBS	Immune-histochemistry staining Mass spectrometry Western blot Quantitative protein assays	Comparative analysis of different decellularization methods Common components: collagens I, IV, laminin, fibronectin, myosin, tubulin, actin, et al. No MMP2 or MMP9
Peterson et al. 2012 [15]	Rat lung	(1) 8 mM CHAPS + 1 M Nacl + 25 mM EDTA in PBS, PBS (2) 1 M NaCl + 25 mM EDTA + 1.8 mM SDS in PBS, PBS	Immune-histochemistry staining Quantitative protein assays	Comparative analysis of different decellularization methods Common components: collagen, elastin, GAGs
Nichols et al. 2013 [7•]	Pig and human lungs	1% SDS, DI water, PBS	Immune-histochemistry staining	Collagens I, IV, fibronectin, elastin, laminin
Gilpin et al. 2014 [8]	Rat lung, pig and human lungs	Rat lungs: (1) PBS, 0.1% SDS, DI water, 1% Triton X-100, PBS. (2) PBS, 0.1% Triton X-100, 2% SDC, 1 M NaCl, DI water, PBS (3) PBS, 8 mM CHAPS + 1 M Nacl + 25 mM EDTA in PBS, DI water, PBS Pig and human lungs: PBS, 0.5% SDS, DI water, 1% Triton X-100, PBS	Immune-histochemistry staining Quantitative protein assays Mass spectrometry	Comparative analysis of different decellularization methods Common components: collagen, elastin, laminins, GAGs, myosins, et al.
Weymann et al. 2015 [10]	Pig lung	PBS, 4% SDC, 0.1% Triton X-100, PBS	Western blot	Collagen, elastin
Balestrini et al. 2015 [11]	Pig lung	PBS, 0.0035% Triton X-100, PBS, 0.01% SDS + 5 mM	Immune-histochemistry staining Quantitative protein assays	Collagens I, IV, fibronectin, elastin, laminin, GAGs

Table 1 (continued)

Reference	Type of scaffold	Decellularization solutions	Method of analysis	Predominant ECM components
		EDTA + 1 M NaCl, PBS, 0.05% SDS + 5 mM EDTA, 0.1% SDS + 5 mM EDTA, PBS, 0.5% Triton X-100 + 5 mM EDTA, PBS	Western blot	
Platz et al. 2016 [16•]	Native ad a-gal KO pig lung	PBS, 0.1% Triton X-100, 2% SDC, 1 M NaCl, DNAse solution, PBS	Immune-histochemistry staining Mass spectrometry	Comparative analysis of different scaffold Common components: collagens I, IV, fibronectin, laminin No significant differences between native and KO scaffolds except for no galactosylated proteins in a-gal KO scaffolds

Various antibiotics had been used in most of the decellularization protocols

compared to an SDS-based one [25]. Such discrepancies may be explained by the divergent ways of analysis, and/or decellularization procedures (dosing, exposure time) followed by different laboratories (summarized in Table 1).

Recently, a comprehensive study comparatively examined decellularized lung scaffolds derived from rat, pig, primate, and human by using the same protocol [26•]. The authors concluded that all scaffolds had the same mechanical properties as the native lungs, a finding consistent with what has been shown in mice where resistance and elasticity of mouse lungs were initially reduced during decellularization, but eventually returned to basal values [26•, 27]. Although the amount of collagen was almost the same in all species, human and primate lung scaffolds retained less glycosaminoglycans (GAGs), and human scaffolds had more elastin [26•]. Interestingly enough, cells seeded on mice lung scaffolds generated by different decellularization protocols behaved similarly regardless of the scaffolds' differences, indicating that optimal lung recellularization may not depend on the detergent being used for decellularization [14]. To avoid immune rejection in xenogeneic transplantation of pig-derived tissue into humans, pig lungs knocked out for 1,3 galactosyltransferase (a-gal)—a cell surface immunogenic protein—were decellularized and compared to wild-type (WT) pig lungs [16•]. Apart from the lack of the knocked-out protein in the ECM, the a-gal KO scaffolds were otherwise very similar to the WT lung both with regard to ECM protein composition and seeded cells' behavior [16•]. This further implied that the protein content of the lung scaffold ECM might not be as essential as it was hypothesized for successful regeneration. In one study, human lung tissue demonstrated significant ability to promote human endothelial cell proliferation compared to other species [26•], which indicated that cell-scaffold compatibility might be an important determinant of successful whole-lung recellularization compared to the method of decellularization. More work is still required to further understand the interaction between repopulated cells and lung decellularized scaffolds.

Optimizations of Decellularization for Regeneration

After the development of perfusion decellularization and the scaling up of this technique to large animal/human-size organs, many investigators have focused their efforts on further optimizing the resulting lung constructs with the aim to support its utility for clinical-scale bioengineering. The quality of the native lung before decellularization is certainly important. A study in mice lungs undergoing delayed necropsy or prolonged storage before decellularization demonstrated significant differences in histology and ECM components of the resulted scaffold [28]. However, freezing or thawing had no significant effect on the mechanical properties of decellularized mice lungs [22]. On a different note, a lower pH (pH = 8 compared to pH = 10–12) facilitated preservation of ECM proteins in

CHAPS-based decellularized lungs [29]. By modifying either the perfusion protocol or the type of bioreactor being used, the time required for decellularization of small and large animal lungs has been greatly diminished without impairing the quality of the created scaffold [5•, 20, 30].

After lung decellularization, there are several important aspects of the scaffold to examine to ensure its readiness for regeneration. Although the scaffolds are washed extensively with PBS, residual detergents may still have a detrimental effect on subsequent cell seeding. A methylene blue-based assay has been recently developed to measure the residual detergent. Several human cell lines utilized for lung recellularization were tested with this assay to illustrate their different thresholds for either SDS or SDC [31]. Terminal sterilization of lung scaffolds is quite challenging since the lung is an organ open to the air and hosts many microbes. Two major ways have been proposed for lung scaffold sterilization: gamma irradiation and peracetic acid (PAA) washouts. Irradiated mice lung scaffolds displayed increased lung resistance and elastance compared to non-irradiated ones [32]. Fused alveolar septa were evident in irradiated scaffolds, while PAA-treated lungs demonstrated a grossly appearance similar to the native or freshly decellularized lung, but with ECM depletion [28]. Recently, the use of supercritical carbon dioxide ($ScCO_2$) has been shown to successfully produce sterilized acellular lung matrices which are stable during long storage and have minimal ECM damage compared to PAA treatment [33]. It is essential to establish an effective and safe method of sterilization as the standard operation of lung scaffold decellularization for the potential clinical use in the future. Intact vasculature and airway are essential for the regeneration of the lung. Lung scaffolds can be generated after perfusion of detergents through the pulmonary artery or trachea. Perfusion via the artery has been determined to be a better approach since it better preserves airway structure, alveolar architecture, and ECM proteins [34, 35]. Perfusion from the vasculature with pressure or flow control resulted in different vasculature resistance [36], and perfusion with constant pressure has been reported to be more effective and reproducible when compared to constant flow [37]. By keeping the pressure on the trachea or pulmonary artery constant, a higher pulmonary artery flow could result in a lower vascular resistance [38]. In many of the published studies examining decellularized scaffolds, growing of the repopulated cells on the scaffold was the primary endpoint of successful decellularization, indicating that analysis of scaffold properties alone may not be sufficient to determine scaffold quality.

Interaction Between Cells and Scaffolds

After decellularization and characterization of the lung scaffold, the next step is to repopulate it with cells and regenerate a functional lung construct with similar physiology with the native lung. Although many groups have recellularized lung scaffolds with various cell types, creating a completely regenerated lung that functionally resembles a native lung remains a big challenge. In the following paragraphs, the current knowledge on lung scaffold recellularization by using different cell types is presented.

Recellularization by Primary Cells

In the first several reports of rodent lung recellularization, human umbilical vein endothelial cells (HUVECs) or rat lung microvascular endothelial cells (RMECs) were used to regenerate the vasculature, while A549 or primary rat lung epithelial cells were used for airway regeneration [2, 3••]. Engraftments of cells and expression of correlated gene markers were detected on both vasculature and airway of the regenerated construct [2, 3••]. Other investigators tried to reseed mouse lung scaffolds with neonatal mouse lung cells and showed engraftment only in the airway [4]. Another group reported recellularization of mouse lung scaffolds with mouse bone marrow-derived mesenchymal stromal cells (MSCs) [13]. MSCs thrived in the parenchyma and airway, but no lung epithelial cell differentiation was observed [13, 14]. The observed viability of MSCs not only confirms scaffold biocompatibility but also shows its limited potential to control cell fate [6, 13, 14]. Interestingly, 2 weeks after inoculation, MSCs had a tendency to remain in the parenchyma while C10 epithelial cells preferred to line the airways [14]. In addition, C10 cells retained expression of several lung epithelial marker genes after 3 days of perfusion culture on a rat scaffold [37]. However, functional tests of mice lungs seeded with C10 cells have not yet been performed.

In an effort to increase proliferation of MSCs and C10 cells, decrease apoptosis, and avoid multilayered aggregates of MSCs, static culture systems were replaced by dynamic suspension cultures [39]. The static culture system in this experiment did not provide medium perfusion through the vasculature, which might be the reason for the less than ideal behavior of the seeded cells. Type II alveolar epithelial cells isolated from neonatal rat lungs were used to seed acellular rat lung, and cells persisted within the scaffold 7 days after inoculation. However, the expression of several rat type II cell marker genes significantly decreased while mesenchymal marker genes increased upon culture, which might be either because of the culture medium being used (DMEM + 10% FBS) which supports the proliferation of mesenchymal cells but not of epithelial cells or because of the epithelial-to-mesenchymal transition of the cells on the scaffold [40].

Porcine and human lung scaffold regeneration is more challenging than rodent lung regeneration, because of the requirement for large cell numbers, volumes of medium, and bioreactors. Pilot seeding experiments of acellular pig and

human lung slices with various primary and stem cells, including pig MSCs, human alveolar epithelial cells (AECs), human small airway epithelial cells (SAECs), and human primary lung cells have been done and cells were shown to attach and proliferate on the scaffolds [7•, 8]. The first attempt of seeding a decellularized human lung lobe was performed with 500 million human pulmonary airway epithelial cells from the trachea, and the lobe was cultured in the bioreactor for 4 days [8]. Cell attachment and engraftment on the matrix was observed with the cells having a flattened epithelial phenotype [8]. Another group used primary human lung epithelial and endothelial cells isolated and immortalized by virus to regenerate both adult and pediatric human lungs [41•]. Extensive regeneration of the acellular scaffold was observed after 20–25 days of culture and alveolar-capillary junction formation was evident [41•]. Using an advanced clinical-scale bioreactor for long-term human lung culture [42], a recently published study demonstrated the regeneration of human acellular lung by airway stem cells and endothelial cells isolated from a cadaveric human lung [43••]. Human basal cells expressing Krt5/P63 and human pulmonary endothelial cells expressing CD31 were infused into the lung from airway and vasculature, then cultured for 7 or 10 days while ventilation was initiated on day 3 [43••]. It should be noted that this study described an enhancement of basal cell proliferation by co-culturing them with pulmonary endothelial cells, which suggest a cross-talk among epithelial cells, endothelial cells, and matrix [43••].

Pluripotent Stem Cells-Derived Cells

Published data on regeneration of acellular lung scaffold with differentiated cells derived from pluripotent stem cells—including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)—are scarce as opposed to regeneration of acellular lung scaffold with primary cells. Epithelial and endothelial cells are the most essential cells required for the regeneration of a decellularized lung scaffold. Mouse ESCs predifferentiated into type II alveolar epithelial-like cells expressing TTF-1 and Pro-SPC were seeded into whole mouse lung acellular scaffolds. Culture for 24–48 h without perfusion led to the attachment of these mouse ESCs to the scaffold while maintaining their main characteristics [5•]. Interestingly, after implanted subcutaneously for 14 days, proximal airway cells found in the recellularized lung constructs indicated potential transdifferentiation of the mouse ESC-derived type 2 cells to airway cells in vivo [5•]. Undifferentiated mouse ESCs were seeded on pig or human acellular lung to study the attachment and viability of the cells [7•]. To enhance the seeding of mouse ESCs, decellularized rat lungs were pre-treated with media conditioned by A549 human lung adenocarcinoma cells which were found to contain laminin [44]. A more than twofold increase of seeding efficiency and a more uniform distribution of the cells were observed in the recellularized lungs [44]. However, data on undifferentiated mouse ESC engraftment might not apply to the regeneration of decellularized lung scaffolds with later stage cells, since no lung-specific gene expression was observed in the ESC-seeded lungs.

Patient-specific iPSCs could become a clinically relevant resource for generation of personalized lung endothelial and epithelial cells. Efficient protocols for differentiation of endothelial cells from human ESCs/iPSCs have been developed and optimized over the past several years [45–47]. Generation of human lung and airway progenitors from ESCs/iPSCs was initially reported in 2012 [48, 49], and subsequently, protocols to generate proximal or distal airway epithelial cells have been developed [50•, 51–52]. However, differentiation of these cells is time-consuming (1–2 months), labor-intensive, and relatively inefficient, making it difficult to get enough lung airway epithelial cells for regeneration. In one report, TTF-1 expressing lung progenitor cells were introduced into decellularized mouse lung scaffolds and then the scaffolds were cut into slices and cultured [48]. Further differentiation of the progenitors was observed after 10 days of culture both proximally and distally inside the airway of the scaffold [48]. In 2013, a group reported the regeneration of rat and human lung using type 1 and type 2 alveolar epithelial cells derived from human iPS cells [50•]. In another study, after seeding both rat and human lungs with human iPS cells-derived lung progenitors, an increase of TTF-1 expression in seeded cells was observed [53]. The regenerated lungs were then orthotopically transplanted and the blood gases were analyzed [53]. Future studies focusing on lung epithelium regeneration should address the difficulty of low cell coverage of the airway and improve methods to demonstrate biological function of the regenerated lung.

While much work remains to be done in iPSC-derived epithelium, significant progress has been made in the required vasculature regeneration using iPSCs-derived endothelial cells. Over 75% endothelial coverage was achieved in a rat lung scaffold by seeding endothelial and perivascular cells derived from human iPS cells. The regenerated vasculature had an intact barrier function proved by the perfusion of microbeads and dextran [21••]. However, upscaling of this technique to a human acellular lung lobe showed lower levels of cell coverage, which might be due to the insufficient number of cells seeded in the scaffold [21••]. Another group performed lung regeneration of a rat scaffold only with rat primary endothelial cells [54]. Optimization of the seeding technique, cell and tissue culture, and assays to analyze the function of the regenerated vasculature remain an important area of research in lung vascular engineering. Results of the studies attempting seeding of various cells on different lung scaffolds are summarized in Table 2.

To more efficiently analyze the interaction between different cells and lung scaffolds in humans and pigs under different conditions, small sections of the acellular matrix can be used

Table 2 Seeding of lung scaffold with various cells

Reference	Scaffold	Cells	Morphology	Function
Ott et al. 2010 [2]	Rat lung	HUVECs A549 cell or rat FLCs	Engraftment on vasculature and airway. Cells regenerated the scaffold with both endothelial and epithelial cells, which expressed marker genes. Tumor-like tissue formed by A549 cells.	In vitro: gas exchange In vivo: gas exchange after orthotropic transplantation up to 6 h
Peterson et al. 2010 [3••]	Rat lung	RMECs Rat neonatal lung cells	Engraftment on vasculature and airway Cells regenerated the scaffold with both endothelial and epithelial cells, which expressed marker genes. Tight junctions in the endothelium were observed.	In vitro: lung compliance tests In vivo: gas exchange after orthotopic transplantation
Price et al. 2010 [4]	Mouse lung	Mouse fetal lung cells	Engraftment on airway. Cells regenerated the scaffold epithelial cells, which expressed marker genes.	None
Daly et al. 2012 [13]	Mouse lung	Mouse MSCs Mouse C10 cells	MSCs repopulate most in parenchymal regions also in large and small airways. No lung-specific marker genes expressed in MSCs despite a transient expression of TTF-1.	None
Wallis et al. 2012 [14]	Mouse lung	Mouse MSCs Mouse C10 cells	C10 cells lined in the airway while MSCs remained in the parenchyme after 14 days culture. No lung-specific marker genes expressed in MSCs.	None
Jensen et al. 2012 [5•]	Mouse lung	Mouse ESCs-derived type II alveolar epithelial cells	Cells attached to the scaffold while retained the expression of type II cell markers after 24–48 h culture in vitro. Cells proliferated and expressed type II cell markers as well as proximal airway cell markers after 14 days implantation in vivo. Nevascularization of the implanted scaffolds.	None
Bonvillain et al. 2012 [6]	Rhesus macaque lung	Rhesus macaque MSCs and ASCs	MSCs and ASCs adhered, elongated, and proliferated on the scaffold.	None
Longmire et al. 2012 [48]	Mouse lungs	Lung epithelial progenitor cells derived from mouse iPSCs	Cells attached on lung scaffold and proliferated. After cutting into pieces and cultured for 10 days, T1a expressing epithelial cells and cilia cells were observed.	None
Girard et al. 2013 [37]	Rat lungs	GFP expressing mouse C10 cells	Cells attached on lung pieces and proliferated after 3 days of culture. Expressions of lung epithelial marker genes were detected on the seeded scaffold.	None
Nichols et al. 2013 [7•]	Pig and human lungs	Murine ESCs, human FLCs, pig MSCs, human AECs	Cells attached on lung pieces and proliferated after 7 days of culture.	None
Ghaedi et al. 2013 [50•]	Rat and human lungs	Type 2 and type 1 alveolar epithelial cells derived from human iPSCs	Cells attached on both lung slices and whole rat lung scaffold and proliferated after 7 days of culture. Cells showed typical alveolar epithelial morphology and retained the expression of marker genes.	None
Gilpin et al. 2014 [8]	Rat and human lungs	HUVECs, human SAECs, human PAECs	Cells attached on rat and human lung slices and proliferated after 5 days of culture. Staining of integrins demonstrated interaction between cells and matrix components. On the human lobe, cells attached and engrafted on the matrix while having a flattened epithelial phenotype.	None
Gilpin et al. 2014 [53]	Rat and human lungs	Lung epithelial progenitor cells derived from human iPSCs	Cells attached on both lung slices and whole rat lung scaffold and proliferated after 5 days of culture. TTF-1 expression increased after seeding.	In vivo: blood gas analysis after orthotropic transplantation
Lecht et al. 2014 [44]	Rat lung	Mouse ESCs	Cells seeded on scaffold pre-treated with A549 cells' condition medium showed increased seeding efficiency and more uniform distribution.	None
Calle et al. 2015 [40]	Rat lung	Type II alveolar epithelial cells isolated from neonatal rat lungs	Cells attached and proliferated on the scaffold after 7 days of culture. Epithelial cells decreased while mesenchymal cells increased upon culture.	None
Crabbe et al. 2015 [39]	Mouse lung	Mouse MSCs Mouse C10 cells	Comparing to previous static culture system, suspension bioreactor for culture of the lung after seeding increased cell proliferation and decreased apoptosis after 28 days culture.	None
Ren et al. 2015 [21••]	Rat and human lung	HUVEC and hMSCs		

Table 2 (continued)

Reference	Scaffold	Cells	Morphology	Function
		Endothelial cells and perivascular cells derived from human iPSCs	Cells regenerated rat and human vasculature on acellular scaffold. On rat scaffold, the continuity and integrity of the vasculature were greatly increased. Tight junction of endothelial cells was observed.	In vitro: barrier function analysis, platelet adhesion assay In vivo: vasculature perfusibility after orthotropic transplantation
Stabler et al. 2016 [54]	Rat lungs	RMECs	Cells regenerated rat vasculature on acellular scaffold. The continuity and integrity of the vasculature were greatly increased.	In vitro: 3D adhesion assay, barrier function analysis
Nichols et al. 2016 [41•]	Pediatric and adult human lung	Pulmonary airway, tracheal/bronchial, and vascular cells isolated from human lung and immortalized.	Cells attached and proliferated on the scaffold after 20 days of culture. Airway and vascular tissue formation was seen in some of the regenerated lungs. Extensive regeneration with epithelial cells in the pediatric lungs was observed. Alveolar-capillary junction formation was observed.	In vitro: lung compliance test
Platz et al. 2016 [16•]	Native ad a-gal KO pig lung	Human airway epithelial cells, vascular endothelial cells, fibroblasts, MSCs	Cells attached and proliferated on both types of scaffolds after 28 days of culture.	None
Gilpin et al. 2016 [43••]	Rat and human lungs	Pulmonary endothelial cells and lung basal cells isolated from cadaveric human lungs	Cells attached and proliferated on both rat and human scaffold while retained the expression of marker genes after 7 days culture. Extensive regeneration with organized architecture was observed.	In vitro: gas exchange

for in vitro culture [55, 56•]. In a recently published paper, seeding of mouse iPS-derived definitive endoderm cells onto 350-μm-thick pieces of rat lung showed that heparan sulfate proteoglycans were essential for promoting lung lineage differentiation of the endoderm cells into ciliated, club, and basal epithelial cells [57••]. In the future, seeding of patient-specific iPS-derived cells onto small pieces of human lung scaffold might be a useful platform for drug screening of ECM-related diseases ex vivo.

Ventilation and perfusion enabling gas exchange between circulating blood in the vasculature and inhaled air in the airway are the most basic biological functions of the lung. During ex vivo experiments, perfusion through the vasculature while ventilating through the airway of the regenerated lung allows investigators to assess the blood gases of the venous effluent. In a few studies, gas exchange was evident in the regenerated lung based on pO_2 and pCO_2 [2, 3••, 43••]. However, in the majority of pertinent studies, gas exchange was not documented. Following an orthotopic transplantation of recellularized rat lung with HUVECs and rat fetal lung cells, the transplanted lungs were able to provide oxygenation for days after transplantation in the recipient [2, 58]. However, in all of these pilot transplantation experiments, only half of the lung was removed from the recipient and replaced by a regenerated lung, indicating the insufficiency of the regenerated lung to fully substitute the physical function of the whole native lung.

To date, there is more research focusing on the regeneration of lung by primary cells than by iPSC-derived cells, which may be because of the complexity, long time course, cost, and low efficiency of the iPSC differentiation. The experience from primary cells could be used to facilitate the iPSC-related regeneration and eventually generate patient-specific lungs for transplantation (Table 3).

Bioengineered Lung and Lung Pathologies

It is well known that ECM plays an important role in maintaining the structure and physiological function of organs. ECM is a dynamic entity with continuous degradation and re-synthesis. The alteration of ECM is implicated in many respiratory diseases including asthma and pulmonary fibrosis [59]. Collagens, elastins, laminins, and others are all matrikines known to be affected in lung diseases [60] and are also the major ECM components after decellularization [8]. Regeneration on decellularized matrices renders us able to study the relationship of ECM with lung diseases and to seek for potential clinical solutions.

Table 3 The comparison of lung regeneration by primary cells and iPSC-derived cells

	Isolated primary lung endothelial and epithelial cells[a]	Endothelial and epithelial cells derived from pluripotent stem cells[b]
Expandability	Low	High in pluripotent status Moderate to low after differentiation
Cost	Moderate	High
Compatibility for patients	Low	High
Cell survival on scaffold	Good	Moderate
Regeneration outcome	Good	Moderate
Potential tumorigenicity risk	Low	To be determined
Potential clinical application	Low	High

[a] Refers to primary cells isolated from human donor organs

[b] Refers to cells derived from patient-specific iPS cells

Decellularization of Abnormal Lungs to Analyze ECMs

The proteomic analysis of human lungs revealed significant differences in the ECM composition, as well as the structure and stiffness of the matrix between normal and fibrotic lungs [55]. Increased stiffness was also observed in decellularized mice with bleomycin-induced fibrosis [61]. The fibrotic scaffold could induce myofibroblast differentiation of seeded fibroblasts, indicating that the ECM affected the identity of the cells [55]. Another study demonstrated alterations of structure and ECM components in mouse lung scaffold related to age, and emphysematous and fibrotic injury [62]. Interestingly, MSCs and C10 mouse lung epithelial cells showed no difference in growth on aged and injured decellularized lungs, while C10s failed to survive after 2 weeks on the emphysematous scaffold when seeded through the airway [62]. This finding implies that scaffolds obtained from decellularization of lungs from individuals with different disease states may significantly affect cell growth and differentiation. A comparative analysis of normal and chronic obstructive pulmonary disease (COPD) human lungs uncovered the incapability of the diseased scaffold to support the proliferation of seeded human bronchial epithelial cells, endothelial progenitor cells, mesenchymal stem cells, and lung fibroblasts for more than a week [63••]. The authors hypothesized that apart from the ECM itself, the 3D architecture may also be important since cell attachment and proliferation were the same on solubilized ECM of normal and diseased lungs [63••], and the lung architecture can be severely affected by COPD. Decellularized-aged mouse lungs contained less structural proteins and seeded human bronchial epithelial cells and lung fibroblasts expressed less laminins on scaffolds derived from old donors compared to scaffolds derived from young donors [64]. In a study focusing on Marfan syndrome (MFS)—a common connective tissue disorder—decellularized mice lungs were compliant and had an emphysematous-like pattern in both early and late stages of the disease [65]. These data indicate that decellularization of normal or diseased human lung lead to scaffolds with different ECM composition, which may provide insight to the mechanism of various lung pathologies and suggest innovative treatment options targeting the ECM level.

Recently, pilot studies have reported on decellularized lung scaffolds derived from patients with interstitial lung disease. Analysis of human lung scaffold from patients with systemic sclerosis-related interstitial lung disease demonstrated abnormal lung architecture and ECM composition [66]. Lung scaffolds were seeded with fibrocytes, which is a cell type known to produce collagen and accumulate in patients with systemic sclerosis. A netrin-1-dependent regulatory pathway of the ECM was identified by antibody-mediated netrin-1 neutralization; this could potentially be a novel therapeutic target of the disease [66]. Another study showed that when seeding decellularized lungs of monocrotaline-induced pulmonary hypertensive rats, rat adipose-derived stem cells underwent more apoptosis [67]. Importantly, narrowed vasculature was observed by micro-CT in native lung as well as in decellularized scaffold [67], indicating that the scaffold might be a good model for disease analysis as it preserves the main characteristics of certain diseases. A comparison of scaffolds produced from the lungs of smokers vs. non-smokers demonstrated that primary lung cells proliferated significantly better in the setting of non-smokers' lungs [41•].

Decellularized lung scaffold will also be a tool to investigate the mechanism of other lung diseases. Cystic fibrosis is a disease caused by mutation of the CFTR gene and majorly affects the function of the lung. In 2012, human iPS cells generated from cystic fibrosis patients were differentiated into multipotent lung progenitors [49]. In a subsequent experiment, the CFTR mutation in iPS cells was targeted and corrected resulting in restoration of function in iPS-derived epithelial cells [68]. It can be hypothesized that new developments in cell biology and lung decellularization will provide us with new weapons to battle certain lung diseases. High-throughput deep sequencing of genetic or epigenetic modifications could also help to expand our knowledge of the mechanism of lung disease.

Disease Modeling with Bioengineered Lungs

Apart from investigating the decellularization of diseased lung and analyzing patient's specific cells of the matrix, another important area of research benefiting from lung bioengineering is the study of cell behavior by seeding cells from tissues of patients with certain lung diseases on the scaffolds. Human lung cancer cells seeded on decellularized rat lungs form tumor nodules on the scaffold with a similar pattern to human tumors. Specifically, human lung cancer cells produced matrix metalloproteinases (MMP) seen in human cancer patients, indicating the ex vivo culture system of cancer cells may be a more relevant model of studying diseased cells from patients than 2D culture [69, 70•]. This system was reported as a 4D tumor model since intact vasculature and airways of the lung scaffold allowed the nodule to be perfused by media, which could not be performed in 3D cultured nodules in Matrigel. Comparison of the gene expression profile of 2D, 3D, and 4D cultured human lung cancer cells by microarray analysis showed that 4D culture could potentially better predict poor survival in patients [71]. This model has been used to analyze the effect of human lung fibroblasts on metastasis and to study circulating cancer cells (CTCs) by collecting them in the perfusion media from the bioreactor [72–74]. With this model, nanoliposome-packaged microRNAs were shown to inhibit tumor growth and decrease metastasis of the tumors [75]. Moreover, a newly developed non-invasive method allows the real-time monitoring of cancer cell viability within decellularized lung scaffolds, which allows the evaluation of responses to various treatment protocols [76, 77•].

The hope is that maturation of 4D lung models will facilitate research on accurate disease modeling, especially with the development of new drugs (e.g., antineoplastics), validation of personalized medicine, and further characterization of lung disease mechanics. Cancer cells isolated from biopsy of patients could be expanded in vitro then infused in the decellularized scaffold for personalized drug testing. A recent study showed different cytotoxic effect on cancer cells growing on decellularized lung scaffold after treatment of cisplatin or erlotinib [77•]. However, discrepancy of drug activities has been frequently observed between 2D and 3D cell culture models. Whether or not 4D lung model will succeed in simulating the in vivo response to drugs is something that still needs to be determined. In the meantime, culturing a recellularized lung in bioreactor is costly and time-consuming, making high-throughput drug screening almost impossible with 4D models. Therefore, 4D lung models may initially serve as a higher level validation of cancer drugs for candidates emerged from screening by 2D or 3D models.

Since lung is one of the most common sites of metastatic colonization of cancer cells originating from other organs, this model has been also used to study the metastatic potential of other cancers such as breast cancer ex vivo [78]. Although this new culture model seems promising for the evaluation of cancer cells and for drug selection, the lack of normal lung tissue on the scaffold might be a barrier when researchers need to further mimic the behavior of cancer ex vivo. Thus, a recellularized lung with continuous vasculature and viable epithelial and mesenchymal cells could serve as an even better model to analyze cancer cell proliferation and metastasis. In most of the published studies, an established human lung cancer cell line (i.e., A549) was used; it remains unclear how much these cell lines are able to mimic the highly diverse lung cancer cells growing in a human cancer patient.

As soon as the current technical problems will be surpassed, 4D lung model may provide a platform for accurate disease modeling and drug screening. Commercial use and widespread adoption of 4D lung models may require a lengthy maturation process which will cost hundreds of millions of dollars in research. Once this technique will be established and accepted by NIH, pharmaceutical companies, and health care providers, it will hasten the progress of biological research as well as the development of new therapies. For NIH, many programs related to small molecule screening, drug toxicity, and mechanism of disease could benefit from this new model since it provides a distinct and effective method to investigate such experimental questions. For pharmaceutical companies, new drugs could be tested on 4D models prior starting clinical trials. For medical associates and their patients, a new method of highly personalized testing will allow patients to get more effective treatment while avoiding significant side effects.

Conclusions

The field of lung bioengineering has to overcome many more hurdles to re-create a regenerated lung capable of sustained high-level function. Optimization of epithelial and endothelial generation and engraftment will be required to improve the structural integrity of the lung and to enable gas exchange during physiologic ventilation and perfusion. Orthotopic transplantation will enable us to evaluate the in vivo function of the lung, while standardized in vitro assays will help us to assess specific parameters and modes of failure of regenerated lungs under well-defined conditions. In addition to the long-term goal of organ replacement, acellular lung scaffolds provide us with a unique and currently underexplored tool in analyzing/modeling lung diseases, such as cancer, fibrosis, emphysema, and COPD. An increased understanding of the biology of lung repair, regeneration, and development will be

complementary in supporting our engineering efforts to create bioartificial lungs.

Compliance with Ethical Standards

Conflict of Interest Tong Wu and Konstantinos P. Economopoulos declare that they have no conflict of interest. Dr. Harald C. Ott is founder and shareholder of IVIVA Medical Inc. This relationship did not affect the content of this article. In addition, Dr. Ott has a patent US 8470520 with royalties paid, and a patent US9005885 issued.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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