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Future prospects for tissue engineered lung transplantation

Decellularization and recellularization-based whole lung regeneration

Tomoshi Tsuchiya[1,*], Amogh Sivarapatna[2], Kevin Rocco[2], Atsushi Nanashima[1], Takeshi Nagayasu[1], and Laura E Niklason[2]

[1]Division of Surgical Oncology; Department of Surgery; Nagasaki University Graduate School of Biomedical Sciences; Nagasaki, Japan;
[2]Departments of Anesthesia and Biomedical Engineering; Yale University; New Haven, CT USA

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The shortage of donor lungs for transplantation causes a significant number of patient deaths. The availability of laboratory engineered, functional organs would be a major advance in meeting the demand for organs for transplantation. The accumulation of information on biological scaffolds and an increased understanding of stem/progenitor cell behavior has led to the idea of generating transplantable organs by decellularizing an organ and recellularizing using appropriate cells. Recellularized solid organs can perform organ-specific functions for short periods of time, which indicates the potential for the clinical use of engineered solid organs in the future.

The present review provides an overview of progress and recent knowledge about decellularization and recellularization-based approaches for generating tissue engineered lungs. Methods to improve decellularization, maturation of recellularized lung, candidate species for transplantation and future prospects of lung bioengineering are also discussed.

Introduction

Lung transplantation is the last option for the treatment of terminal lung disorders such as chronic obstructive pulmonary disorder (COPD), which is the third leading cause of death in the United States.[1,2] More than 2000 lung transplants are performed annually in the United States.[3] Whereas lung transplantation is recognized as an established therapy that improves survival and provides an improved quality of life for transplant recipients,[4] there are several hurdles that make the transplantation of donor lungs a challenging endeavor.

One critical issue is the condition of the donor lungs. Donor lungs often suffer edema, atelectasis, or pneumonia originating from donor systemic problems or preoperative systemic control.[5] The long ischemic time also causes donor lung damage, which directly correlates with primary graft failure.[6] Another disadvantage of a lung transplant is the need for life-long administration of immunosuppression drugs in the recipients, which increases the risks of infections and cancer.[7] The biggest problem, however, is the acute shortage of transplantable organs. In 2011, there were 2280 patients waiting for lung transplantation in the US alone with the average waiting time of about 1 y.[8] The mortality for patients on the wait list is 15.7 per 100 wait-list years in the US.[8] In Japan, the situation is worse. The average waiting time in Japan is about 2 y and 9 mo and the waiting mortality is 43 per 100 wait-list years.[9] Thus, the demand of lungs greatly exceeds the supply. Further, a longer recipient waiting time for a suitable organ results in the deterioration of the patient's condition and increases operational risk. Given such unresolved problems, lung transplantation is seen as a necessary but currently imperfect treatment for lung disease.

In order to address the problem of organ shortage, several approaches for making transplantable lungs have been attempted, including: biomaterial improvement, 3-dimensional (3D) cell cultures, and ex vivo bioengineering.[10-12] Based on the progress of regenerative medicine and the development of recent stem cell advances, investigators have also begun to explore the idea of using decellularized native scaffolds for autologous cell recellularization to generate transplantable bioengineered lungs (**Fig. 1**).[13,14]

This review describes the recent developments in using decellularization and recellularization strategies for generating functional lungs for transplantation. We will begin by introducing the concepts of decellularization and recellularization followed by the current status of the cell replacement approach for ex vivo whole lung regeneration, including the use of stem/progenitor cells and studies of extracellular matrix (ECM) preservation. Finally, we address other important aspects of the methodology, such as protocols for organ maturation, the choice of candidate species, operation procedures and the prospect of clinical application of recellularized lung transplantation.

*Correspondence to: Tomoshi Tsuchiya; Email:tomoshi@nagasaki-u.ac.jp
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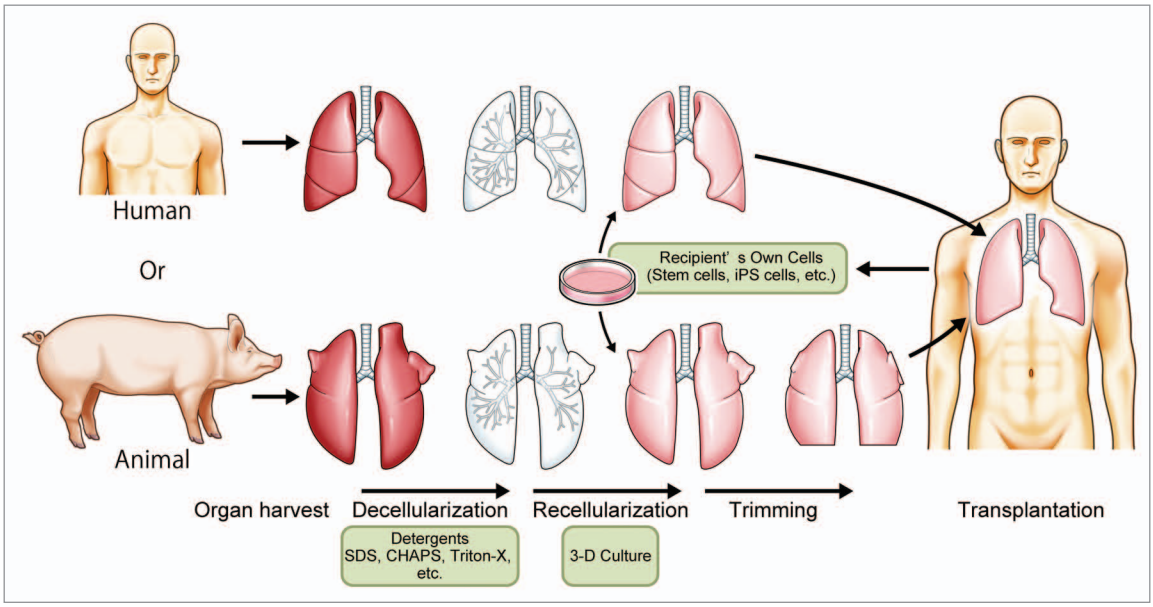

Figure 1. Schematic of decellularization and recellularization based whole lung regeneration for transplantation. Harvested human or animal lungs are decellularized by detergents such as SDS, CHAPS, or Triton-X. The decellularized lung is recellularized by the recipient's own stem/progenitor cells or iPSCs.

The Concept of Decellularization and Clinical Usage

The ultimate goal of tissue engineering is to generate functional human tissue in vitro or ex vivo that has preserved organ architecture and is non-immunogenic to prevent organ rejection. Decellularized organs appear to be the ideal biomaterial for tissue engineering to achieve these two goals. Decellularized biological scaffolds preserve the 3D organ structure, while removing cells by treatment with detergent, resulting in an acellular and non-antigenic matrix, thus obviating the need for immunosuppression.[15] A method for decellularizing tissue, for example, would include extensive rinsing in hypotonic saline, treatment with dilute (0.1%) peracetic acid or incubation in Triton 100X and 0.1 M ammonium hydroxide and a terminal sterilization step with either ethylene oxide, gamma irradiation or E-beam irradiation.[16] Such methods have been shown to eliminate all intact cells (i.e., decellularize) and degrade any nucleic acid remnants to less than 200 base pairs with less than 50 ng of dsDNA per 1 mg dry weight of the extracellular matrix scaffold while leaving intact the constituent growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), bone morphogenic protein (BMP) and vascular endothelial growth factor (VEGF).[17,18]

ECM scaffolds are currently used for arterial grafts, heart valves, urinary tract reconstitution, skin reconstruction, dura mater grafts following intracranial surgery, and orthopedic applications. Some of the scaffolds are available commercially including: porcine heart valve (Hancock II®), bovine pericardium heart valve (PERIMOUNT Magna®), human heart valve (Synegraft®), human dermis (Alloderm®), porcine small intestinal scaffold (OaSIS®), and decellularized bone (Allograft c-ring®).

The Concept of Recellularization and Clinical Usage

The idea of recellularization of the acellular scaffold originally arose from the clinical usage of acellular matrices and studies of acellular organs.[19,20] In the field of cardiovascular surgery, for example, a glutaraldehyde treatment is used to eliminate immunogenicity, provide sterility and induce collagen cross-linking which increases tissue strength and flexibility in xenogeneic or allogeneic heart valves.[21] However, the degeneration of glutaraldehyde-fixed valves, with subsequent calcification and tissue failure, was proving to be a major disadvantage of these valvular prostheses.[22] Thus, the idea was conceived of covering the surface of porcine glutaraldehyde-fixed valve prostheses with autologous endothelial cells of the recipient in order to delay or eliminate valve degeneration and further reduce thromboembolic events.[23] As such, the concept of recellularization involves reseeding of cells into the matrix, where they can attach, migrate, proliferate and perform specialized functions as they would in native tissue so as to recapitulate organ function.

There is clinical and practical evidence that native cells can attach or migrate into a decellularized scaffold.[24,25] In animal studies, transplanted decellularized aortic valves or great vessel scaffolds were endothelialized and recellularized by the migration of recipient cells throughout the matrix in a time-dependent manner.[25,26] Several cases of these approaches have been successfully utilized in clinical trials including trachea, bladder, and heart valve.[27-30]

Lung Recellularization Studies Using Decellularized Scaffolds

Unlike simple organs such as skin, heart valve or bone, the lung has a complex 3D structure and therefore decellularized lung by itself cannot be used clinically. However, decellularized lung has been used as a scaffold in studies to elucidate the differentiation and behavior of stem/progenitor cells. Most recellularization studies have been performed using mouse lungs (**Table 1**), and the recellularization routes were limited to trachea or direct seeding on lung slices. The focus of most recellularizaion strategies has been on epithelial cells and few reports have described the complete endothelialization of decellularized lung scaffolds to date. Thus, the discussion described in the following sections will mainly focus on recellularization strategies using epithelial cells and stem/progenitor cell behavior on acellular lung scaffolds, with some discussion about endothelial cell re-seeding in the lung.

Epithelial cells

The alveolar epithelium consists of two major specialized epithelial cell types: the terminally differentiated squamous alveolar epithelial type I (AETI) cells, which constitute approximately 93% of the alveolar surface area, and the surfactant producing cuboidal alveolar epithelial type II (AETII) cells. While AETII cells only cover 7% of the alveolar surface area, they constitute 67% of the epithelial cell population within the alveoli, indicating their biochemical importance.[31]

In 1986, Jamson and colleagues succeeded in transforming AETII cells into AETI cells by directly seeding adult rat AETII cells onto 150 μm thick fragments of acellular human alveolar matrix.[32] Several groups have tried seeding fetal alveolar epithelial cells into decellularized lung via the trachea.[33-35] Price and colleagues demonstrated that the decellularized lung matrix bioreactor was capable of supporting the growth of fetal AETII cells.[33] Analysis of cryosections taken seven days after injection of fetal cells into lung matrices showed pro-SP-C, cytokeratin 18, and 40,6- diamidino-2-phenylindole (DAPI)-positive cells lining alveolar areas and the cells appeared to be attached to the matrix.[33] This indicates that the decellularized scaffold retains the components necessary to direct the differentiation of progenitor cells into cells appropriate for that organ. A proteomic analysis showed a significantly different composition for decellularized lung scaffold compared with liver scaffold.[35] Alveolar progenitor cells administrated to the liver failed to transcribe lung specific proteins of surfactant protein such as C (SP-C), an AETII cell marker, and aquaporin-5 (AQO-5), an AETI cell marker. Therefore, the ECM itself can affect alveolar epithelial cell behavior and differentiation depending on the organ from which it was derived, and thus is critical for controlling cell fate.

Endothelial cells

Like the epithelium, the endothelium exhibits significant functional heterogeneity. Proper function of the endothelium in the lung is critical for a variety of processes, including gas exchange and regulation of fluid and solute passage between blood and interstitial compartments (barrier function), supporting normal epithelial cell and progenitor cell behavior and function as well as non-respiratory metabolic processes.

Endothelial seeding in the vascular compartment of decellularized rat lung scaffolds showed uneven distribution and incomplete coverage using rat lung microvascular endothelial cells[13] and HUVECs.[14,34] For these reasons, thrombosis and ultimate failure of the organ occurred. Given their important role in the lung, the use of endothelial cells for recellularization needs to be examined more closely, including specific cell type, scale up potential and route of administration.

Embryonic stem cells (ESCs)

ESCs are derived from the inner cell mass of a blastocyst-stage embryo and can differentiate into any type of cell of ectodermal, endodermal or mesodermal origin.[36] For example, human ESCs can differentiate into AETII and bronchial epithelial cells.[37,38] Cortelia and colleagues immunohistologically confirmed that decellularized lung promoted differentiation of mouse ESCs into epithelial and endothelial lineages.[39] Jensen and colleagues seeded pre-differentiated murine ESCs with phenotypic characteristics of AETII cells into decellularized lungs and found that neovascularization developed in scaffolds that were subcutaneously implanted for 14 d.[40] Similarly, Longmire and colleagues demonstrated the step-wise derivation, purification, and culture of primordial lung and thyroid endodermal progenitors and purified Nkx2–1+ endodermal progenitors that differentiate into thyroid and lung lineages.[41] These reports demonstrate that the decellularized scaffold also supports the differentiation of ESCs into lung-specific lineages and that the scaffold can also be recellularized by ESC-derived lung epithelial cells.

Mesenchymal stem cells (MSCs)

MSCs are known to have a capacity for self-renewal and an ability to differentiate into cells of the mesenchymal lineage, including adipocytes and osteocytes.[42] MSCs can be derived from different tissues, including adipose and bone marrow, which is the main source of exogenous stem cells. Bone marrow-derived MSCs (BM-MSCs) are released into circulation upon inflammatory stimuli and facilitate recovery and repair in the inflammatory process.[43,44] A club cell secretary protein (CCSP) -expressing progenitor population in the bone marrow and its role in bronchial repair has also been reported.[45] Interestingly, an increase in bone marrow-derived endothelial cells within the lung capillary walls was observed during alveologenesis in mouse models of lung regeneration. This was achieved by treatment with granulocyte colony stimulating factor (GCSF),[46] hepatocyte growth factor (HGF)[47,48] or adrenomedullin.[49]

However, it is unknown whether MSCs can differentiate into lung epithelial cells in a decellularized scaffold. Daly and colleagues demonstrated that BM-MSCs administrated into the trachea initially target a region of the trachea, become established, and subsequently proliferate and migrate toward regions enriched in types I and IV collagen and laminin.[50] However, BM-MSCs predominantly express genes consistent with a mesenchymal and osteoblast phenotype suggesting that MSCs cannot differentiate into AETII cells in basal medium or small airway growth media (SAGM). Bonvillain and colleagues seeded adipose tissue derived MSCs onto decellularized lung[49]; while they attached and could

Table 1. Detergents and sources of cells for recellularization in the lung

Author	Article	Year	Species of the scaffold	Detergents used	Cell sources for re-seeding	Route of administration	Differentiated phenotype	Institution
Lwega-Mukasa et al.[32]	Exp Lung Res	1986	Human	0.1%Triton X-100, 2% SDS	Rat AETII cells	Direct seeding	AETI cells	Yale Univ.
Petersen et al.[13]	Science	2010	Rat (Fisher 344)	8mM CHAPS	A549 cells, HUVECs, Rat neonatal lung cells and RMECs	Trachea and pulmonary artery	N/A (Transplantable lung)	Yale Univ.
Ott et al.[14]	Nature Med	2010	Rat (Sprague Dawley)	0.1% SDS, 0.1%Triton X-100	A549 cells, rat fetal lung cells and HUVECs	Trachea and pulmonary artery	N/A (Transplantable lung)	Harvard Med School
Price et al.[33]	Tissue Eng Part A	2010	Mouse (C57BL/6)	0.1% Triton X-100, 2% SDC	Mouse fetal lung cells	Trachea	Epithelial (AETII cell-like)	Univ. of Minnesota
Cortiella et al.[39]	Tissue Eng Part A	2010	Rat (Sprague Dawley)	1% SDS	Mouse ESCs	Trachea	Epithelial (AETII cell-like)	Univ. of Texas Medical Branch
Song et al.[34]	Ann Thoracic Surg	2011	Rat (Sprague Dawley)	0.1% SDS, 0.1%Triton X-100	Rat fetal lung cells and HUVECs	Trachea and pulmonary artery	N/A (Transplantable lung)	Harvard Med School
Shamis et al.[35]	Tissue Eng Part C	2011	Rat (Lewis)	0.5% Triton X-100	Mouse AETII cells	Direct seeding	Epithelial (AETII cell-like)	Hebrew Univ.
Daly et al.[50]	Tissue Eng Part A	2011	Mouse (C57BL/6, BALB/C)	0.1% Triton X-100, 2% SDC	Mouse BM-MSCs and C10 epithelial cells	Trachea	Mesenchymal and osteoblast from BM-MSCs	Univ. of Vermont
Wallis et al.[51]	Tissue Eng Part C	2011	Mouse (BALB/C)	3 different protocols; (1) 0.1% Triton X-100, 2% SDC. (2) 0.1% SDS, 0.1%Triton X-100. (3) 8mMCHAPS.	Mouse BM-MSCs and C10 epithelial cells	Trachea	N/A (Distributed on scaffold)	Univ. of Vermont
Jensen et al.[40]	Tissue Eng Part C	2012	Mouse (C57BL/6)	0.1% Triton X-100, 2% SDC	Mouse ESCs	Trachea	Epithelial (AETII cell-like)	Univ. of Connecticut Health Center
Longmire et al.[41]	Cell stem cell	2012	Mouse (C57BL/6)	0.1% Triton X-100, 2% SDC	Mouse ESCs	Trachea	Epithelial	Boston Univ.
Bonvillain et al.[77]	Tissue Eng Part A	2012	Rhesus macaque	0.1% Triton X-100, 2% SDC	Rhesus BM-MSCs and AD-MSCs	Bronchioles	N/A (Distributed on scaffold)	Tulane Univ.
Booth et al.[78]	Am J Resp Crit Care	2012	Human	0.1% Triton X-100, 2% SDC	Human lung fibroblasts	Airway and vascular	N/A (Distributed on scaffold)	Univ. of Michigan
Sokocevic et al.[52]	Biomaterials	2013	Mouse (C57BL/6)	0.1% Triton X-100, 2% SDC	Mouse BM-MSCs and C10 epithelial cells	Trachea	N/A (Distributed on scaffold)	Univ. of Vermont

Triton X-100, Nonionic detergent used to solubilize proteins; mild non-denaturing detergent. SDC, Sodium deoxycholate; Water soluble ionic detergent used for disrupting and dissociated protein interaction. SDS, Sodium dodecyl sulfate; Anionic surfactant used for lysing cells and unraveling proteins. CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Non-denaturing zwitterionic detergent used to solubilize proteins. AETII; alveolar epithelial type II. AETI; alveolar epithelial type I. HUVECs; human umbilical vein endothelial cells. RMECs; Rat lung microvascular endothelial cells. ESCs; Embryonic stem cells. AECII; Alveolar epithelial cell. BM-MSCs; Bone marrow derived mesenchymal stem cells. AD-MSCs; Adipose derived mesenchymal stem cells. SP-B; surfactant protein B. iPSC; induced pluripotent stem cell

Table 1. Detergents and sources of cells for recellularization in the lung (continued)

Author	Article	Year	Species of the scaffold	Detergents used	Cell sources for re-seeding	Route of administration	Differentiated phenotype	Institution
Bonenfant et al.[53]	Biomaterials	2013	Mouse (C57BL/6)	0.1% Triton X-100, 2% SDC	Mouse BM-MSCs and C10 epithelial cells	Trachea	N/A (Distributed on scaffold)	Univ. of Vermont
Nichols et al.[79]	Tissue Eng Part A	2013	Human and Pig	2% SDS, 1% SDS	Mouse ESCs, human fetal lung cells, pig BM-MSCs and human AETII cells	Direct seeding	N/A (Distributed on scaffold)	Univ. of Texas Medical Branch
Nakayama et al.[80]	PLOS one	2013	Rhesus monkey	0.1% SDS, 0.01%Triton X-100	Human ESCs	Direct seeding	N/A (Upregulate lung associated gene expression)	Univ. California Davis
O'Neill et al.[81]	Ann Thoracic Surg	2013	Human and Pig	3 different protocols; (1) 1.8mM SDS. (2) 8mM CHAPS. (3) 3% Tween 20, 4% SDC.	Human small airway epithelial cells and AD-MSCs	Direct seeding	N/A (Distributed on scaffold)	Columba Univ.
Ghaedi et al.[56]	J Clin Invest	2013	Human and Rat (Fisher 344 or SD)	(1) 0.1% Triton X-100, 2% SDC for human lung. (2) 8mM CHAPS for rat lung.	Human AETII and iPSC-AETII cells	(1) Direct seeding for human lung. (2)Trachea for rat lung	AETI cells	Yale Univ.
Sun et al.[82]	Am J Physiol Lung Cell Mol Physiol	2013	Mouse	8mM CHAPS	Mouse fibroblast A9 cells	Direct seeding	N/A (Distributed on scaffold)	Yale Univ.
Elizabeth-Gilpin et al.[83]	J Heart Lung Transplant	2013	Rat, Human and Pig	For rat; 3 different protocols; .(1) 0.1% SDS, 1% Triton-X (2) 0.1% Triton X, 2% SDC2. (3) 8mM CHAPS. For human; 1% Triton-X	Human small airway epithelial cells, pulmonary epithelial cells and HUVECs	Direct seeding for slice. Via bronchus for human lung lobe.	N/A (Distributed on scaffold)	Harvard Med School

Triton X-100, Nonionic detergent used to solubilize proteins; mild non-denaturing detergent. SDC, Sodium deoxycholate; Water soluble ionic detergent used for disrupting and dissociated protein interaction. SDS, Sodium dodecyl sulfate; Anionic surfactant used for lysing cells and unraveling proteins. CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Non-denaturing zwitterionic detergent used to solubilize proteins. AETII; alveolar epithelial type II. AETI; alveolar epithelial type I. HUVECs; human umbilical vein endothelial cells. RMECs; Rat lung microvascular endothelial cells. ESCs; Embryonic stem cells. AECII; Alveolar epithelial cell. BM-MSCs; Bone marrow derived mesenchymal stem cells. AD-MSCs; Adipose derived mesenchymal stem cells. SP-B; surfactant protein B. iPSC; induced pluripotent stem cell

be maintained in vitro, there is no evidence that MSCs could differentiate into lung epithelial-specific lineages in decellularized lung.[51-53]

Induced pluripotent stems cells (iPSCs)

iPSCs have been generated from several somatic cell types by the forced expression of Oct3/4, Sox2, Klf4 and c-Myc (and recently Glis192), commonly known as the Yamanaka factors.[54,55] iPSCs, similar to ESCs, can differentiate into virtually any cell type in the body, but have the advantage that they do not require the destruction of an embryo. Additionally, a significant advantage of iPSCs is the ability to obtain autologous differentiated cells for clinical therapy since they are patient-specific.

Recently, Ghaedi and colleagues generated a homogeneous population of AETII and AETI cells from human iPSCs (iPSC-AETII) using step-wise derivation, purification, and culture expansion.[56] Since embryonic lung arises from definitive endoderm (DE), the first step requires saturating concentrations of activin A exposure for generating DE from iPSCs. For generating anterior foregut endoderm (AFE) from DE cells, NOGGIN and SB-431524 are then used, followed by the addition of a cocktail of trophic factors containing EGF/BMP4/Wnt3a/FGF10/KGF

for generating alveolar progenitors.[56] Interestingly, an air–liquid interface provided by a rotating bioreactor culture system was a potent inducer of AETI phenotype from iPSC-AETII cells.[57] These techniques provide a method for large-scale production of alveolar epithelium for tissue engineering, providing an important stepping stone toward the ultimate goal of ex vivo lung regeneration from autologous cells.

Stem/progenitor cells and mixed cell repopulation

Other candidate stem/progenitor cells have been used for recellularization. Alveolar progenitor cells (AEPCs) isolated from human lungs, c-kit-positive cells, and E-Cadherin and leucine-rich repeat-containing G-protein-coupled receptor 6 double positive (E-Cad/Lgr6$^+$) cells might have the potential for differentiation into lung specific lineages.[58,59] Fujino and colleagues demonstrated that AEPCs have an epithelial phenotype with an MSC character. According to microarray analysis, AEPCs share many genes with AETII cells and mesenchymal stem cells, which suggests an overlapping phenotype with both the alveolar epithelium and the mesenchyme in these cells.[58] Kajstura and colleagues isolated c-kit-positive cells from adult human lungs and when the cell population was injected into injured lungs of C57BL/6 mice, the cells differentiated not only into epithelial cells, but also into mesenchymal and endothelial cells without rejection.[59] Oeztuerk-Winder and colleagues identified E-Cad/Lgr6$^+$ cells as a distinct population of human alveolar stem cells. E-Cad/Lgr6$^+$ single-cell injection in the kidney capsule produce differentiated bronchioalveolar tissue, while retaining self-renewal.[60] These cells may potentially act as endogenous lung stem cells. However, the use of both cells for lung recellularization has not been reported yet.

In contrast to the stem/progenitor cell seeding studies, fetal or neonatal lung cells were used for re-seeding decellularized lung with the goal of achieving total organ regeneration rather than as a system for studying the behavior of stem/progenitor cells in the scaffold.[13,14] Because the lung has two divaricate systems of airway and vessels, regenerated lung was seeded with pulmonary epithelial cells from airways (via trachea) and vessel endothelial cells from the pulmonary artery in addition to cells derived from fetal or neonatal whole lung. In this system, the administrated mixture of lung cell types migrated to and attached to the appropriate sites and displayed remarkable hierarchical organization. Recent studies support this homing and engrafting phenomenon; endothelial progenitor cells (EPCs) utilize adhesion molecules for homing to sites of neovascularization similar to the leukocytes.[61]

Current Status of Recellularized Lung Transplantation

Two institutions in the US reported the first recellularized tissue engineered lung in 2010.[13,14] Using a bioreactor, they cultured pulmonary epithelium (whole cell suspension) and vascular endothelium in the acellular lung matrix. They then transplanted the engineered lungs into rats, which participated in gas exchange for 120 min in one case and 30 min in the other. Physiological treatment, including dry ventilation with positive end-expiratory pressure (PEEP), hyperosmolar perfusion before transplantation, conservative weaning and frequent suctioning through an endotracheal tube, could prolong the oxygenation for as long as 7 d at levels comparable to cadaveric lung transplants.[34]

Though demonstrating proof of concept, graft longevity and homeostasis are still limited. Three day air-ventilated lungs show greatly dilated airways with cell debris evident. Overall, it appears that ventilation with air in the bioreactor causes some destruction of the airway epithelium and dilation of peripheral airspaces.[62] This epithelial damage due to ventilation means that the recellularized lung has incomplete architecture. Technical hurdles still need to be addressed to optimally ventilate lungs in bioreactors to mimic physiological conditions.

The Role of ECM in the Lung

Transplantation studies of recellularized lung reveal that the alveolar structure of the bioengineered lung is fragile and the histological integrity of the recellularized lung seems to be determined by damage to the ECM at the thin barrier of the alveoli.[63] On the other hand, although cytokines and growth factors are present within ECM in very small quantities, they modulate cell proliferation and proper differentiation.[16,64] For this reason, investigators have focused on the ECM of the lung.

Collagen is the most abundant protein within the mammalian ECM. Greater than 90% of the dry weight of the ECM from most tissues and organs consists of collagen.[65] The main subtypes that make up the lung's structure are collagens I, III, IV, and V. Type I collagen is the major structural protein present in tissues. Type IV collagen forms a complex branch network and is largely present within the basement membrane of most vascular structures and within tissues that contain an epithelial cell component.[64] In nature, collagen is closely associated with glycosylated proteins, growth factors and other structural proteins such as elastin and laminin, which provide unique tissue properties.

The non-collagenous glycoproteins of fibronectin and laminin are both important ECM basement membrane proteins for cell adhesion.[64,66-68] They have important biological functions in the lung, including formation and maintenance of the vasculature.[69] Integrins are the major cell surface receptors for laminin.[70] The crucial role of the β-1 integrin chain in mediating hematopoietic stem cell interactions with fibronectin and laminin has been firmly established.[71]

Glycosaminoglycans (GAGs) are found on cell surfaces, within intracellular vesicles, and are incorporated into the ECM.[72] They bind growth factors and cytokines, help control macromolecular and cellular movement across the basal lamina, and contribute to the properties of the ECM by using negatively charged 'tails' to sequester water.[16] Because GAGS are intrinsically part of the cell surface, removal of cells and cell components will ultimately cause the depletion of cell-bound GAGs.

Elastic fibers are constructed with elastin and microfibrils, such as fibrillin. Elastin contributes to tissue elasticity, stretch, and the intrinsic tissue recoil property essential for breathing.[73] Additionally, elastic fibers provide the pulmonary vasculature

with resilience and are critical to the function of arteries.[74] Elastic fibers are highly stable constructs; however, elastin can be degraded by elastases, such as metalloproteinases.[16] The de novo synthesis of elastin is difficult to achieve because of low post-natal production and slow turnover. In addition, without sufficient elastin content, tissue degradation and calcification could occur upon implantation.[75] Therefore, it would be highly desirable to preserve elastin during decellularization.

The Effect of Decellularization on ECM and Cell Components

The challenge for any decellularization protocol is the effective removal of all cellular and nuclear material while retaining ECM structure and composition. Previous work has shown that detergent based decellularization can damage ECMs, with decreases in collagen, elastin, and GAG content of 0–30%, 60% and almost 90% compared with the native lung, respectively (**Fig. 2A**).[13,63] Histological and immnohistochemical studies also reveal extreme reduction of other matrix components including elastic fibers, fibronectin, and laminin (**Fig. 2B**).[63,76] Consequently, the preservation of the ECM during the decellularization process is critical for producing a suitable scaffold for recellularization and whole lung engineering.

Considerable effort has gone into determining the best protocols for decellularization.[69,76] The most commonly used detergents are Triton-X 100/sodium deoxycholate (Triton/SDC), sodium dodecyl sulfate (SDS), or 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (**Table 1**). There are few direct comparisons of various protocols utilizing different detergents, but Weiss et al. reported Triton/SDC is less disruptive to native ECM when compared with CHAPS and SDS approaches.[63,76] However, when cells were intratracheally inoculated into the various decellularized lungs, the results were comparable for initial binding and short-term (2 wk) proliferation of two different cell types, a stromal progenitor cell and a mouse lung epithelial cell line.[76]

Cellular and nuclear remnants from the decellularization process can elicit an immune response. However, the complete elimination of all cell membrane and nuclear materials is very difficult (**Fig. 2A**).[18] In one study of commercially available ECM scaffold materials, remnant DNA fragments were common, but do not appear to present significant risk, suggesting some amount of remnant nuclear material is acceptable.[84] Furthermore, proteomic analysis utilizing mass spectrometry has revealed a wide range of residual nuclear, intracellular and cytoskeletal proteins to be present in the lungs, despite reports of effective decellularization.[50-53] A recipient of such a material would be expected to mount an immune response to the foreign remnants in the ECM graft, yet it is unclear that such remnants have a negative effect on immunoreaction.[85] A preliminary study of BALB/c mice implanted with porcine-derived ECM of the small intestinal submucosa showed that all animals elicited a Th2-type response, which is correlated with transplant acceptance.[86] Interestingly, recipient cells produced inflammatory cytokines such as TNF-α and IL-1 and are decreased in the surrounding tissue, while anti-inflammatory cytokines such as IL-5 and IL-10 are increased. In a clinical trial, a transplanted recellularized trachea had remnant donor cellular elements in cartilaginous areas, yet it avoided rejection without the use of immunosuppressive drugs.[27] On the

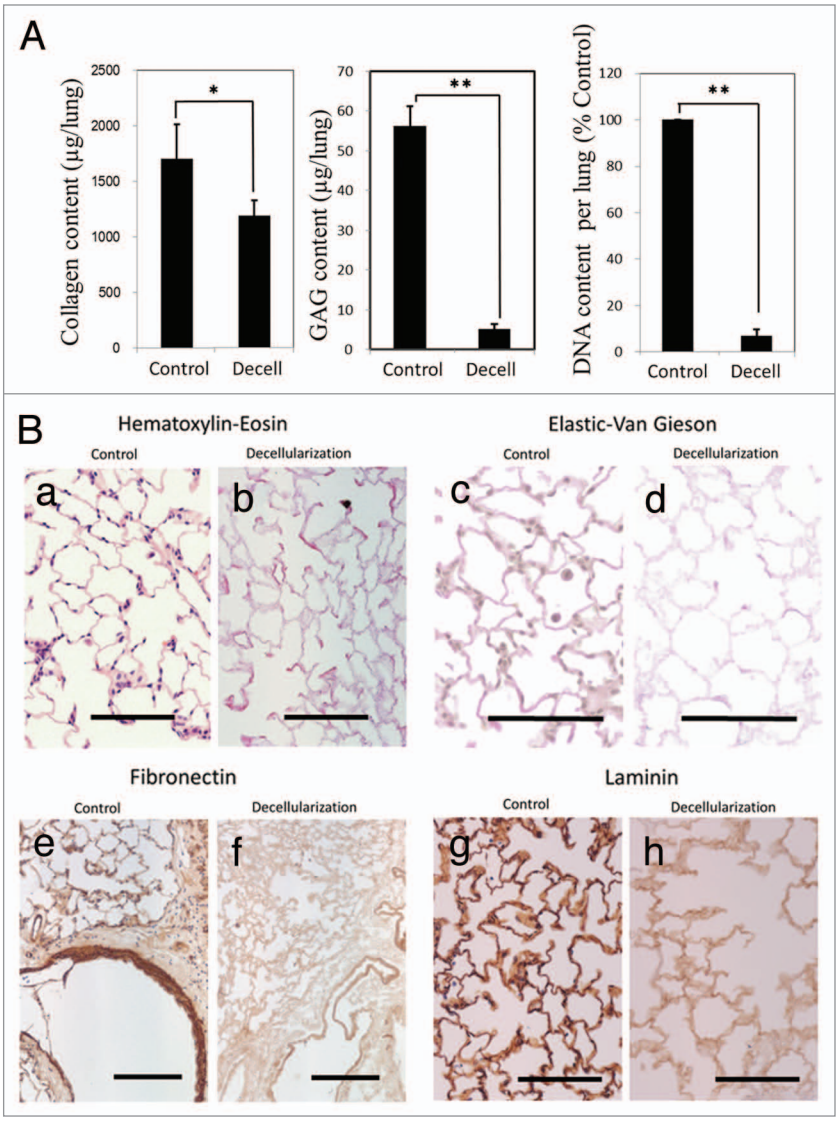

Figure 2. (**A**) Comparison of Collagen, GAG, and DNA content in untreated control lung vs. decellularized lung. Asterisk indicates a significant difference between the groups (*$P < 0.05$, **$P < 0.005$). Data are expressed as mean values ±SD, n = 5. (**B**) Histological and immunohistochemical comparison of the ECM in untreated control lung vs. decellularized lung. Scale bar = 100 µm

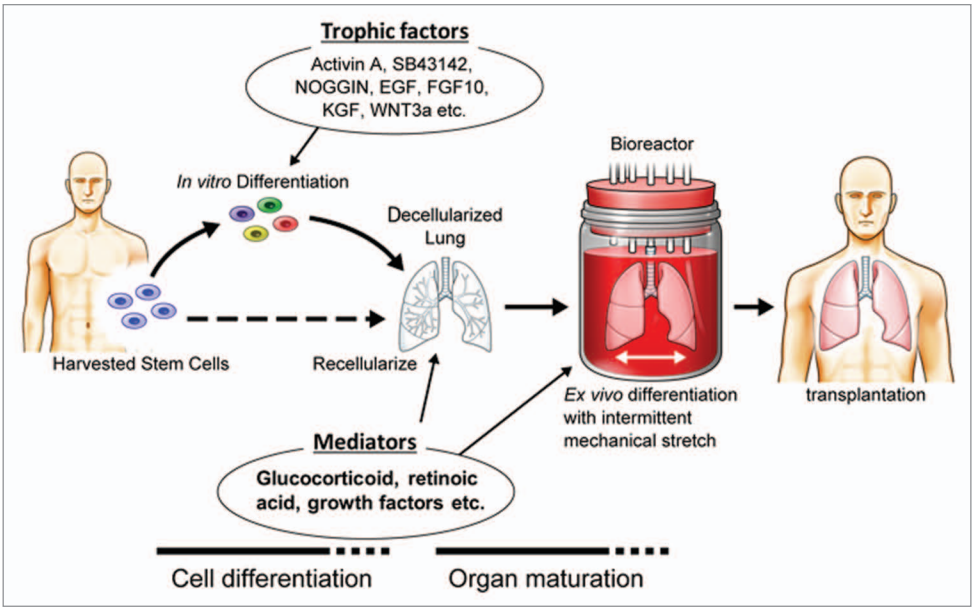

Figure 3. Possible approaches for recellularization and maturation of decellularized lung. Harvested stem cells can be matured and differentiated in vitro or left in the progenitor cell state using trophic factors and re-seeded into decellularized lung scaffolds. Mediators can then be used to support maturation, followed by bioreactor culture using intermittent mechanical stretch to simulate fetal breathing. Length and extent of organ maturation ex vivo can vary.

basis of results obtained with other engineered tissues, retained ECM elements and remnants of cell components might provide helpful signals to both graft and host cells as well as reduce the inflammatory response. It is also possible that a weak immunoreaction mainly induces a Th-2 type response, which might induce graft acceptance or activate remodeling processes.[86]

Methods for Improving Decellularized Matrix Integrity

Because detergent-based decellularization has been shown to cause some damage to lung barrier function, there is much interest in protocols that preserve or strengthen the ECM while still achieving complete decellularization. One approach is to vary the pH of the CHAPS-based methods. Our results suggest that lower pH solutions (less basic) might reduce ECM damage. Most notably, more neutral pH detergents decreased the loss of GAG and elastin content, but did not effectively remove all DNA (unpublished data). Another method is to coat the decellularized scaffold with either collagen or Matrigel via the trachea in order to enhance cell adhesion and preserve the mechanics of the scaffold.[40] This might repair the damage to the microarchitecture of the recellularized lung following decellularization, however there was no obvious improvement in lung architecture, cellular adhesion, or phenotypic expression of reseeded alveolar epithelial type II cells.[40]

The route by which the detergents are administered also affects lung microarchitecture integrity. Original decellularization methods utilized vascular perfusion of detergents but were found to cause barrier damage that resulted with blood in the airways upon implantation after reseeding.[11,12] Decellularization via the airway might be more effective than vasculature perfusion because total surface area of the respiratory field is extremely large (100 to 140 m^2) and more easily accessed.[87] Maghsoudlou et al. reported a method of intermittent breathing of detergent to produce an acellular scaffold with improved preservation of pulmonary microarchitecture, including basement membranes.[88] In a preliminary pathological and immunohistochemical study, however, airway-based decellularization decreased ECM retention, including proteoglycans, elastic fibers, fibronectin and laminin more than our perfusion-based method (unpublished data).

Maturation of Recellularized Lung

In order to make a more complete and functional engineered lung, a variety of physiological and external factors are necessary (**Fig. 3**). The stepwise differentiation method of iPSCs gives us much information on generating definitive endoderm (DE), anterior foregut endoderm (AFE) and subsequently, a homogeneous population of human AETII and AETI cells from stem cells.[56] The process of organogenesis during fetal development also gives us insight into what is necessary for lung maturation.[89,90] During early gestation, glucocorticoids have several effects. They stimulate cell maturation and differentiation, while inhibiting DNA synthesis. They also stimulate antioxidant enzymes, increase phospholipid synthesis, and regulate pulmonary liquid metabolism, which produce surfactant associated.[91] Lung organogenesis is also controlled by mediators including Nkx2–1 and growth factors, such as PDGFs, FGFs (FGF 10/7/2) and insulin-like growth factors (IGFs).[92] Retinoic acid accelerates

fetal lung branching, leading to the development of the alveolar tree.[93] Therefore, treatment with combinations of growth factors or hormones, with appropriate timing, might facilitate maturation and establishment of the epithelial and endothelial interactions during recellularization.

Fetal breathing movements (FBMs) play an important role in regulating fetal lung growth and maturation.[92,94] During gestation, FBMs are detected at embryonic day (E) 14.5 in the mouse and at 10 wk gestation in human embryos.[92] FBMs cause lung cells to function biochemically as well as mechanically. Clinical reports and laboratory experiments have revealed that the absence of FBMs in the embryo leads to such conditions as pulmonary hypoplasia and akinesia.

In vitro, intermittent mechanical stretch (IMS) has been used to simulate FBMs.[95] In the 3D- cell culture model, IMS stimulated DNA synthesis and cell division of fetal lung fibroblasts and epithelial cells.[95,96] Accordingly, in rat models, IMS increases SP-C gene expression[97] and stimulates the secretion of lung surfactant lipids from AETII cells.[98,99] Furthermore, mechanical stretching has a significant impact on the synthesis and secretion of several of these ECM molecules in 3D culture or fetal rat lung cells including type I and type IV collagens, fibronectin, proteoglycans, and GAGs.[100-102] At the same time, IMS does not affect gene expression and activities of several matrix metalloproteinases (MMPs).[102] Because the ECM structure depends on a balance between ECM synthesis and its destruction by proteolytic enzymes, mechanical stretching might only affect synthesis resulting in ECM maturation.

Candidate Species for Decellularized Scaffolds and Transplantation

In order to generate enough transplantable lungs using recellularization approaches, the selection of a donor source for generating decellularized scaffolds is an important consideration. Given the issue of immunogenicity, human organs are the most favorable candidates. However, structural lung diseases such as emphysema or pulmonary fibrosis are often present in donor organs from older people. Using immunohistochemistry and mass spectrometry, it has been shown that age, lung condition, and the cell type used for recellularization may significantly impact the usefulness of decellularized whole lungs for ex vivo lung tissue regeneration.[52] Therefore, the ability to use donated human lungs might be limited because of the necessity of harvesting young and healthy lungs.

As a potential solution to the limited availability of healthy human lung tissue, animals may offer an ample supply of lungs. Among candidate species, concordant animals such as baboons would be ideal but are not viable candidates due to ethical concerns, limited populations, and the possibility of an unknown zoonosis. Among discordant animals, porcine lung might be suitable due to similarity in organ shape and size, availability in large numbers, and affordability. In addition, there is already extensive clinical experience using porcine organs and tissues. Components of the ECM may differ between human and pig, but that has not yet been demonstrated to be a problem. Many components of the ECM, such as collagen, are highly conserved across species.[65] This high degree of sequence homology is one reason that xenogeneic ECM can function as a suitable scaffold in mammalian recipients.

It has been shown that ECM derived from porcine tissues contains small amounts of the α-galactosyl epitope (gal epitope), which causes a hyperacute type of rejection via a complement response.[103] However, whereas the gal epitope has been shown to be present in the acellular matrix scaffold material,[104] most preclinical studies using acellular matrix scaffolds have failed to show convincing evidence of an adverse immunologic response and tissue remodeling.[105-107] Additionally, pigs have now been created with genetic modifications to reduce xenogenic rejection in humans.[108-110] For example, the alpha1,3-galactosyltransferase gene-knockout pig has been engineered to ensure more complete immunogenicity-deleted lungs.[111] The development of these gene-knockout animals may solve the potential danger of tissue rejection and coagulopathy in xenotransplantation between pigs and mammals.[112]

The operational procedures of engineered lung transplantation will be the final major issue. The standard technique of lung transplantation includes anastomosing the main bronchus, the pulmonary vein, and the pulmonary artery trunk. Until ECM components can be better preserved during decellularization, engineered lung remains at a mechanical disadvantage and surgical techniques must be developed to prevent tissue damage and rupture of the anastomoses. When xenogeneic engineered lungs are used, tissue trimming might also be needed (**Fig. 3**). For example, the porcine right lung has a specific tracheal bronchus with cranial lobe,[113] which will be sacrificed in the right lung transplantation. The shape of the lung is different between the species due to the difference in the thoracic cavity. Further animal transplantation studies focusing on pig lung into primate recipients are necessary to establish an optimized surgical technique for xenogeneic engineered lungs.[114]

Conclusion

To address the shortage of organs for lung transplantation, many approaches have been tried. Significant progress has been made using cellular therapies for lung regeneration, including recent advances using iPSCs that can be differentiated into lung epithelium. While still in the early stages, these patient specific cells can be derived in unlimited quantities and don't pose a risk of rejection in a transplant setting. Decellularized lung, which can maintain a near intact ECM and the complex microarchitecture of the lung, has been shown to be an ideal scaffold for reseeding with cells. Thus, recellularization based lung regeneration may prove to be an effective clinical therapy for future treatment of lung diseases.

However, there are still many issues that remain to be resolved before such bioengineered lungs become available for clinical use. Issues still to be resolved include: the ideal decellularization method, the ideal duration for whole lung recellularization

and the minimum number and types of necessary cells. Many of these issues are currently being addressed through research to address the current clinical problems in lung transplantation. The success of these approaches for lung bioengineering will result in substantial benefits for the field of transplantation medicine.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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