# Chapter 18
# Acellular Lung Scaffolds in Lung Bioengineering

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## Introduction

Chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) are significantly increasing in prevalence and are predicted to be an increased major worldwide healthcare burden [1, 2]. There are currently no cures for these diseases and end-stage disease is associated with high mortality. While newly approved pharmaceutical interventions, such as Pirfenidone for IPF, have generated some excitement, it remains to be seen how effective these will be on a larger scale, and for what proportion of patients these treatments are suitable. For most patients, the only available treatment option at end-stage disease remains lung transplantation. However, there are not enough donor lungs to meet current transplantation needs and there are further complications associated with lung transplantation. Transplantation recipients require lifelong immunosuppression and the 5-year survival after lung transplantation remains approximately 50 % [1, 2]. Alternative options are therefore desperately needed for this patient population.

One active and promising area of research is the generation of pulmonary tissue using ex vivo methods. The basic concept is that a scaffold of either biologic or artificial origin could be seeded with an appropriate cell source to regenerate functional lung tissue for subsequent transplantation (Fig. 18.1). While both of these techniques are still in their relatively early stages, one of their purported benefits is that either biologic or synthetic scaffolds could be recellularized with autologous cells, thus minimizing the immunological complications which

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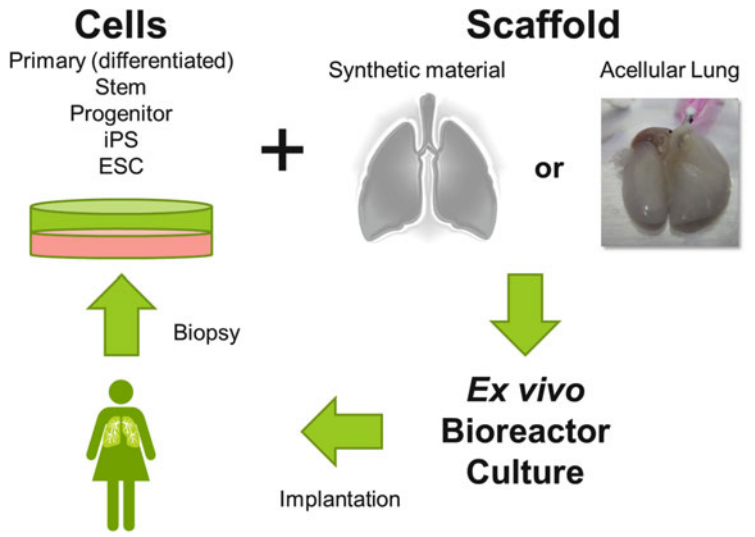


**Fig. 18.1** Schematic of ex vivo organ engineering. Autologous cells are obtained by a biopsy from the eventual transplant recipient and expanded in ex vivo culture. A scaffold, either synthetic or an acellular lung, is manufactured and repopulated ex vivo by the usage of a bioreactor to create a functional tissue suitable for re-implantation

typically accompany lung transplantation. While this approach is not yet feasible in lungs, similar approaches have already been used clinically in simpler tissues including trachea, skin, and bone [3, 4]. Lung tissue, however, is a considerably more structurally complex organ and consists of a variety of cell types which must be functionally recapitulated in vivo. Due to these intrinsic differences in complexity between tissues, progress has significantly lagged behind the advances made in other organs. Synthetic scaffolds made from biocompatible or natural polymers are one potential option. A number of different materials and manufacturing technologies have already been evaluated for lung and will be discussed in more detail in this chapter.

An exciting new and active area of research involves the use of acellular lung scaffolds derived from cadaveric or failed transplant lungs. Acellular tissue is generated by removing cells from the native organ while preserving the 3D macroarchitecture and the majority of the extracellular matrix (ECM) proteins [5–17]. Whole organ decellularization as a platform for organ regeneration was first described in the heart in 2008 [15] and beginning in 2010, several groups described similar techniques in lung [18–23]. The use of acellular lungs has since expanded beyond their usage in regenerative medicine and has become an incredibly powerful in vitro tool for studying cell–ECM interactions or the impact of diseased matrix on cellular behavior [24–29].

This chapter discusses the status of current areas of research investigating ex vivo regeneration of lung tissue, and includes a discussion of concepts learned from the literature on ex vivo tissue culture and organ preservation.

## Engineering a Scaffold

### *Designing and Manufacturing a Bioartificial Scaffold*

Proposed bioartificial scaffolds for lung engineering have been manufactured by various techniques and from a variety of different materials. An overview of the current approaches is given in Table 18.1. In general two different methods of scaffold generation can be distinguished: additive (layer-by-layer or unit-by-unit generation) and subtractive methods (generation by removing material to form the final scaffold). Additive techniques benefit from the possibility to generate scaffolds with interconnecting pores. Depending on the resolution needed, however, these techniques may lead to long fabrication times. Examples for additive techniques are rapid prototyping and 3D bioprinting [30]. Subtractive methods such as porogen forming techniques and sphere-templating have also shown promising initial results [31, 32] but are more limited regarding scaffold design. There are various other methods to produce scaffolds for tissue engineering purposes like solvent casting, particulate leaching, melt molding, or freeze drying. Synthetic materials used thus far are polymers like polyglycolic acid (PGA), poly-lactic-co-glycolic acid (PLGA), poly-L-lactic-acid (PLLA), polyurethane (PU), and polyvinyl (PV) in order to match the mechanical properties of lung tissue. Hydrogels made of collagen I, gelatin, Matrigel, alginate, fibrinogen–fibronectin–vitronectin combinations or PGA combined with Pluronic F-127 have been used as scaffolds as well [33, 34]. Further, synthetic scaffolds can be loaded with growth factors, ECM components (e.g., collagen or whole lung extracts) or peptide sequences known to facilitate cell attachment (e.g., RGD) [35].

The lung has a highly complex structure with varying structural composition and mechanical properties which are still unable to be completely recapitulated using synthetic approaches. While scaffolds fabricated via foaming techniques are structurally similar to peripheral lung tissue (especially the alveoli), they lack a vascular system and innervations. It is also difficult to tune the various mechanical properties needed throughout the lung for proper breathing motions. Additionally, the challenge of scaffold recellularization to create a fully functional organ has not yet been achieved. Thus, the use of the current methods exclusively may not solve the issue of whole lung replacement, but there are many areas for improvement which can still be explored.

### *Acellular Scaffolds*

Synthetic scaffolds could one day be accurately and precisely manufactured for the macro- and microarchitecture required for ex vivo lung bioengineering. However, the instructional cues which are needed on the scaffold for critical events such as initial cell attachment, potential cell-specific attachment cues, and differentiation

**Table 18.1** Artificial scaffolds used for Tissue Engineering of the lung

| Ref. | Scaffold | Study objective | In vitro/in vivo | Cell source seeding | Benefits/limitations |
|---|---|---|---|---|---|
| [83] | Polyglycolic acid and Pluronic F-127 hydrogel | Isolation and characterization of somatic lung progenitor cells and the promotion of alveolar tissue growth | In vitro, in vivo | Somatic lung progenitor cells | De novo generation of lung tissue, limited amount of cell source |
| [118] | PDLLA scaffolds (3D foam) | Suitability of scaffold for distal lung tissue engineering | In vitro | MLE-12 | No toxic effects, supports cell growth |
| [119] | Matrigel, synthetic poly-lactic-co-glycolic acid (PLGA) and poly-L-lactic-acid (PLLA) porous foams and nanofibrous matrices | Engineering of 3D pulmonary tissue constructs | In vitro | Murine embryonic day 18 fetal pulmonary cells | Limited amount of cell source; use of mixed populations |
| [120] | Collagen I gel | Effects of FGF10, FGF7, and FGF2 on murine fetal pulmonary cells to control epithelial cell behavior | In vitro | Fetal murine pulmonary cells | Addition of growth factors to influence cell proliferation and differentiation possible |
| [84] | Gelfoam sponge (collagen I) | Application for cell-based lung regeneration | In vivo | Fetal lung cells (d19) | No severe local inflammatory response, sponge degraded after several months |
| [31] | Patterned porous hydrogel from poly(2-hydroxyethyl methacrylate) (poly(HEMA)), Collagen I treated | Suitability of scaffold fabrication method to design scaffolds with different macro- and microstructure, cell attachment | In vitro | Mouse skeletal myoblast cells | Tailoring of scaffold macro- and microstructure possible |
| [85] | Matrigel plug combined with FGF2-loaded polyvinyl sponges | Generation of vascularized pulmonary tissue constructs | In vivo, subcutaneous | Fetal pulmonary cells (d17.5) | limited amount of cell source, induction of a host inflammatory response |
| [35] | Fibrinogen–fibronectin–vitronectin hydrogel | Ability of hydrogel to promote cell engraftment | In vivo | Primary ovine lung mesenchymal cells | Mechanical properties similar to lung, safe cell delivery, no atelectasis and scarring |

| | | | | | |
|---|---|---|---|---|---|
| [121] | Cylindrical-shaped bronchioles, fibroblasts embedded into collagen I with ASM cells on the outer surface, epithelial cells on the inner surface (lumen) | Development of a tissue-engineered bronchiole | In vitro | Human lung primary cells (fibroblasts, airway smooth muscle and epithelial cells) | Cell–cell interactions and airway remodeling events can be studied |
| [74] | Cellularized collagen matrix (Gelfoam) | Treatment of postpneumonectomy space | In vivo | BMSC | Cells only survive in early time points inside the scaffold (until d7) |
| [34] | Nanofibers coated with lung extracts | Effect of lung extract and modulus on cell differentiation | In vitro | Bone marrow cells | High throughput possible |
| [33] | Collagen-elastin hydrogels | Effect of elastin and cell seeding on hydrogel stiffness, Suitability as building blocks for lung engineering | In vitro | Lung fibroblasts | Adaption of mechanical properties to the lung tissue (alveolus) |
| [86] | Matrigel, decellularized rat lung slices | Potential of AF-MSCs to generate lung precursor cells | In vitro | AF-MSC | AF-MSC are a possible cell source for cell therapy, differentiation on lung matrix more effective, limited amount of cell source, lack of cell proliferation |
| [32] | Gelatin/microbubble-scaffolds | Vascularization of artificial scaffold for lung tissue engineering | In vitro, in vivo, in ovo, subcutaneous implantation in SCID mice | mPSC | Scaffolds promote angiogenesis and differentiation to alveolar pneumocytes |

*Abbreviations*: *AF-MSC* amniotic fluid mesenchymal stem cells, *ASM* airway smooth muscle cells, *BMSC* bone marrow stem cells, *FGF* fibroblast growth factor, *HEMA* 2-hydroxyethyl methacrylate, *MLE-12* mouse epithelial cell line, *mPSC* mouse pulmonary stem/progenitor cells, *PDLLA* Poly-DL-lactic-acid, *PLGA* poly-lactic-co-glycolic acid, *PLLA* poly-L-lactic-acid, *SCID* severe combined immunodeficient

cues are not known. Furthermore, if these criteria were known, the lung scaffold would likely also need to be engineered with a material and manufacturing process selection which matched the mechanical and gas diffusion properties of native lung. This makes the engineering of a completely synthetic scaffold daunting. While synthetic materials could be engineered to include specific integrin binding sites to enhance cell adhesion (e.g., Arg-Gly-Asp (RGD) binding sites), it remains unknown what specific integrin binding sites need to be included and in what spatial arrangement they need to be. On the other hand, acellular scaffolds retain many of the native integrin binding sites in their correct spatial arrangement, and decellularization processes preserve the general organ architecture and ECM composition. Lung ECM has also long been known to provide instructional cues during prenatal development, postnatal tissue regeneration, remodeling responses following injury, and general tissue homeostasis [36–40]. Similarly, acellular scaffolds have been shown to have biologically inductive clues [21, 22, 27, 41, 42]. While hybrid materials, consisting of synthetic and acellular matrix components, are also an attractive possibility, these concepts are in their infancy. Hybrid materials could be utilized to enhance cell adhesion and biological activity while taking advantage of the ability to more precisely manufacture scaffolds or scaffold components with synthetic materials [21, 34, 35, 43, 44]. Differences between acellular and synthetic scaffold approaches are summarized in Table 18.2. Owing to the current advantages of acellular scaffolds, we will focus our discussion in the remainder of this chapter on their manufacture, assessment, and usage.

## Decellularization

### *Methods of Decellularization*

The derivation of a cell-free ECM is not a new concept. Lwebuga-Mukasa and colleagues first described the generation of acellular lung scaffolds in 1986 for the study of rat type II alveolar epithelial (AEII) cell behavior on a native basement membrane [45]. This technique was heavily explored in simple tissues in the 1990s and early 2000s [46–49] and has made strides into the clinic. Acellular biologic scaffolds have been created from a variety of different simple tissues, including skin, esophagus, and trachea [4]. Decellularization was first applied to complex tissues using whole organ perfusion decellularization in heart in 2008 [15]. Beginning in 2010, several groups described similar techniques in lung [18–23] and since this time, the field has grown rapidly.

The basic goal of any decellularization technique is to remove the endogenous cell population while retaining the macroarchitecture of the organ or the tissue, along with the ECM composition (Fig. 18.2). Maintenance of mechanical tissue properties is also thought to be critical in evaluating decellularization protocols. A variety of methods have been described to decellularize tissue. Most commonly, a

**Table 18.2** Comparison of biologic vs. synthetic scaffold approach for ex vivo bioengineering

| | Biologic (acellular) scaffold | | Synthetic scaffold | | Potential hybrid design | |
|---|---|---|---|---|---|---|
| Differentiation and engraftment cues | + | Retains native integrin binding sites | – | Lacks specific integrin binding sites (must be engineered into scaffolds) | + | Could be engineered with specific ECM components or engineered integrin sites |
| Immunogenicity | + | Antigen removal during decellularization | +/– | Unknown/variable depending on material | +/– | Unknown/variable depending on the artificial matrix material chosen |
| Manufacturability | + | Native architecture largely retained | – | Complex architecture possible | – | Complex architecture possible |
| | – | Large variability between donor scaffolds | + | Precise control possible (i.e., repeatability) | + | More ability than acellular to be controlled, but ECM incorporation introduces a degree of variability |
| Long-term storage | – | Degradation with long-term storage | + | Improved storage stability | +/– | Improved storage stability, but would likely loose biologic activity under long-term storage |

series or combination of detergents, solvents, acids/bases, and hypotonic or hypertonic solutions are used to remove the majority of cellular components. Alternative methods include physical methods such as freeze/thaw cycles and/or biological agents such as enzymatic treatment [48]. Methods of decellularization are comprehensively reviewed elsewhere [50]. In general, most protocols last from 1 to 7 days.

There are a variety of published reports on techniques for decellularizing mouse, rat, porcine nonhuman primate and human lungs (Tables 18.3 and 18.4). In the lung, maintenance of both large and small airways and vessels is critical, in addition to the more delicate structures such as alveolar, capillary, and lymphatic systems which can be damaged through the use of excessive pressure during decellularization [51]. Perfusion decellularization has been most commonly utilized for whole lung decellularization, but there have also been reports of excising segments or slices from native lung and decellularizing these smaller segments [28, 29, 52, 53]. Detergents are the most commonly utilized decellularization agents used in perfusion based lung decellularization. There are several studies which have directly compared differences between these methods, and endpoint comparisons included assessment of proteomic composition, the mechanical properties of the final acellular scaffold, and recellularization efficacy [23, 41, 52]. The most commonly utilized detergents for lung are either the ionic detergents sodium deoxycholate (SDC) and sodium dodecyl sulfate (SDS), which are often used in

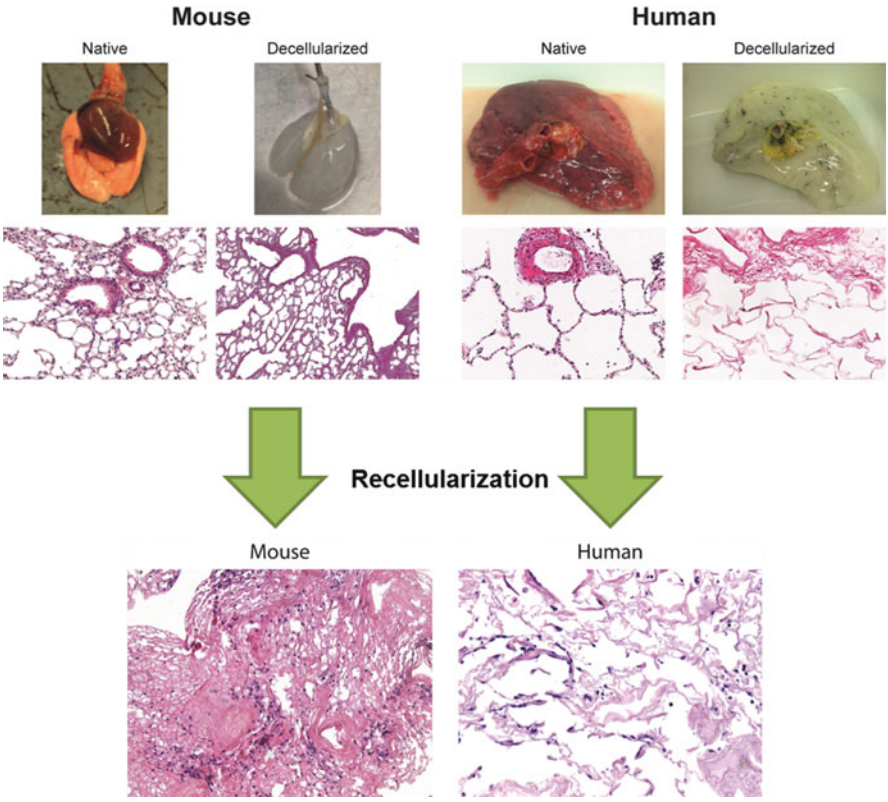


**Fig. 18.2** Overview of the decellularization and recellularization process. Representative images of native and decellularized lungs from mice and humans (*upper panel*) demonstrating loss of pigmentation following decellularization, whereby the lungs become translucent white in color. H&E staining reveals complete cellular removal and gross maintenance of histological architecture. Histological analysis following recellularization with murine alveolar epithelial cells (C10) (*left*) and human bronchial epithelial cells (HBE) (*right*) into acellular mouse and human lung slices. Cells can be seen to have attached to the acellular lungs after 1 day of slice culture

combination with the nonionic detergent Triton X-100 [18, 22–27, 29, 41, 54–58]. Zwitterionic detergents such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) have also been used [19, 52, 59, 60], but some reports demonstrate that these may be more damaging than ionic or nonionic detergents due to their efficiency in denaturing proteins [48]. Many protocols also incorporate additional rinses and incubations for the purpose of removing organic components which are difficult to remove with the other detergents. The most commonly utilized additional steps are the use of hypertonic solution for lysis of cells (e.g., 1 M NaCl), or DNase/RNase to clear residual DNA and RNA. While both vascular-only perfusion and a combination of vascular and airway perfusion have produced acellular scaffolds capable of supporting recellularization, there is no consensus on the best route of administration and removal of decellularization agents.

**Table 18.3** Compiled studies of ex vivo lung bioengineering using rodent and small primate decellularized whole lung scaffolds

| Ref. | Scaffold | Study objective | Method of decellularization | Length of decellularization process | Endpoint assessments |
|---|---|---|---|---|---|
| [122] | Alveolar basement membrane (calf, dog, rabbit, adult/newborn rat) | Basement membrane | Filtered distal lung homogenate, saline, 4 % Triton X-100 with protease inhibitors, $NaHCO_3$ rinse, distilled $H_2O$ rinse | 26–52 h depending on homogenate volume | Histology, IF, SEM, amino acid analysis, carbohydrate analysis |
| [45] | Acellular alveolar vs. amniotic basement membranes | Differentiation on different basement membranes | Distilled $H_2O$, 0.1 % Triton X-100, 2 % SDC, NaCl, pancreatic DNase Type 1S | >2 days | Cell attachment and morphology |
| [20] | Mouse (female C57/BL6) acellular lungs | Effect of matrix on spatial engraftment of E17 fetal lung homogenate | Airway and vascular perfusion: distilled $H_2O$, 0.1 % Triton X-100, 2 % SDC, 1 M NaCl, porcine pancreatic DNase | 3 days (approximately 63 h) | Histology, quantification of ECM proteins, IF, SEM, function with Flexivent, bioreactor with fetal type II cells |
| [19] | Rat acellular lungs (male Fischer 344) | Development of bioartificial lung for orthotopic transplantation | Vascular perfusion only (1–5 mL/min with less than 20 mmHg arterial pressure) CHAPS, NaCl, EDTA, PBS | 4 h | Histology, IF, DNA quantification assay, collagen assay, GAG assay, western blots, SEM, TEM, micro-CT imaging |
| [21] | Rat acellular lung (Sprague Dawley) | Comparison of matrices including decellularized rat lung in ability to support mESCs | Fast freeze/thaw cycles, 1 % SDS, DNase, RNase, PBS, Penicillin/Streptomycin, Amphotericin, DMEM | >6 weeks | Quantification of DNA, IH, confocal microscopy, flow cytometry, 2 photon microscopy, presence of SP-A |
| [18] | Rat acellular lung (Sprague Dawley) | Development of bioartificial lung for orthotopic transplantation | Vascular perfusion only: pulmonary artery pressure kept constant at 80 $cmH_2O$, heparinized PBS with 0.1 % SDS, deionized water, Triton X-100, and PBS with Penicillin, Streptomycin, Amphotericin B | 3 days (approximately 75 h) including incubation with antibiotics | Histology, morphology, mechanical function, fluoroscopy, gas exchange, transplantation, protein analysis |

(continued)

**Table 18.3** (continued)

| Ref. | Scaffold | Study objective | Method of decellularization | Length of decellularization process | Endpoint assessments |
|---|---|---|---|---|---|
| [58] | Rat acellular lung (Sprague Dawley) | Orthotopic transplantation | Vascular perfusion only: pulmonary artery pressure kept constant at 80 cm$H_2O$, heparinized PBS with 0.1 % SDS, deionized water, Triton X-100, and PBS with Penicillin, Streptomycin, Amphotericin B | 3 days (approximately 75 h) including incubation with antibiotics | Histology, IH, morphology, fluoroscopy, functional analysis, transplantation of seeded lungs with fetal pulmonary cells and Pulmonary artery and vein with endothelial cells |
| [123] | Rat acellular liver and lung (Lewis) | Cellular differentiation on 3D in vitro scaffold | Lung lobes cut into 300 μm thick, 0.5 % Triton X-100, 10 mM ammonia, mechanical disruption, PBS, distilled water | N/A | Histology, TEM, environmental scanning, PCR, IH, liquid chromatography with tandem mass spectrometry |
| [22] | Mouse acellular lung (C57BL/6; BALB/C) | Initial binding and recellularization of MSCs in acellular scaffold; directed seeding with integrin blocking | Same as [20] | 3 days (approximately 72 h) | Histology, IF, EM, dye perfusion to assess vascular continuity, mass spectrometry, western blot, lung mechanics with Flexivent, inoculation of bone-marrow-derived MSCs |
| [23] | Mouse acellular lung and lung slices (BALB/C) | Comparison of detergent-based decellularization protocols | Airway and vascular perfusion. 3 different protocols assessed: (1) [20]; (2) [18]; (3) [19] | 3 days (approximately 72 h) | IH, mass spectrometry, western blot, mechanics with Flexivent, gelatinase assay, DNase, RNase, comparative recellularization with MSCs and C10s |
| [55] | Normal rhesus macaque acellular lung | Initial binding and recellularization of MSCs in acellular scaffold | Airway and vascular perfusion: PBS, EDTA, Penicillin/Streptomycin at initial harvest: pulmonary artery: PBS + Heparin + Sodium Nitroprusside with pressures 25–30 mmHg; then trachea and vasculature: deionized $H_2O$ [20] | 2–3 days (approximately 48–72 h) | Histology, morphology, IH, western blot, genomic DNA, proteomics, seeding with bone marrow and adipose derived rhesus MSCs |

| | | | | | |
|---|---|---|---|---|---|
| [57] | Mouse acellular lung and lung slices (C57/BL6) | Seeding with and differentiation of mESCs-derived endodermal lung precursors | Same as [20] | 3 days (approximately 72 h) | Phenotypic assessment of mESCs and their capacity for differentiation |
| [56] | Mouse acellular lung (C57BL/6) | Comparison of timing of decellularization, pre-coating, and support of mESCs and their differentiation capacity into alveolar epithelial cells | Same as [20] | 1 vs. 3 days (approximately 24 h vs. 50 h) | Histology, morphology, EM, western blot, gelatinase assay, IF, mechanical properties with Flexivent, viability of differentiated mESCs seeded into the scaffold, subcutaneous implantation of scaffold |
| [60] | Rat acellular lung | Comparison of different detergent-based protocols | Two approaches: (1) [19]; (2) NaCl, EDTA, SDS | 4 h | Histology, collagen assay, elastin assay, GAG assay, DNA assay, mechanical testing with linear strips |
| [99] | Rat acellular lung | Creation of perfusable human lung cancer nodules | Same as [18] | 3 days (approximately 75 h) including incubation with antibiotics | Recellularization with human A549, H460, or H1299 and cultured with perfused, oxygenated media for 7–14 days |
| [54] | Mouse acellular lung and lung slices (C57BL/6) | Effect of time to necropsy, length of storage, and two different methods of sterilization | Same as [20] | 3 days (approximately 72 h) | Histology, IH, morphology, mass spectrometry, seeded lungs with MSCs and C10 epithelial cell line |
| [24] | Mouse acellular lung and lung slices (C57BL/6) | Effect of recipient age and injury on de- and recellularization | Same as [20] | 3 days (approximately 72 h) | Histology, IH, mass spectrometry, inoculation with MSCs and C10 epithelial cell line |
| [69] | Rat and mouse acellular lung slices | Engraftment and survival of fibroblasts through a β1-integrin and FAK-dependent pathway through ERK | Same as [19] | 1 Day | Histology, SEM, immunohistochemistry, DNA assay, recellularization with mouse A9 cells |

(continued)

**Table 18.3** (continued)

| Ref. | Scaffold | Study objective | Method of decellularization | Length of decellularization process | Endpoint assessments |
|---|---|---|---|---|---|
| [41] | Rat, porcine, human acellular lungs | Evaluation of the capacity of acellular lung scaffolds to support recellularization with lung progenitors derived from human induced pluripotent stem cells (iPSCs) | Same as [18] | 1 Day | Histology, IF, quantitative PCR, implantation of rat acellular scaffold recellulalarized with iPSC, blood gas analysis in orthotopic transplantation |
| [124] | Rat acellular lung | Comparison of different perfusions (manual, trachea + vasculature constant flow, vasculature only constant pressure) | Airway and vascular perfusion: PBS, 0.1 % Triton X-100, 2 % SDC, NaCl, Pancreatic DNase, $MgSO_4$, $CaCl_2$, PBS with Penicillin/Streptomycin and Amphotericin B | Up to 3 days | Histology, nucleic acid detection, IF, western blot, inoculation with C10 epithelial cell line |
| [86] | Rat acellular lung | potential of AF-MSCs to generate lung precursor cells in the decellularized lung | Same as [124] | Approximately 1 day | Viability assay (TUNEL staining), IF, quantitative PCR |
| [62] | Mouse acellular lung | Measurement of local stiffness by AFM | Same as [21] | >6 weeks | IH, IF, SEM, 2 photon microscopy AFM local stiffness measurement |
| [125] | Rat acellular lung | Influence of pH during decellularization on ECM | Same as [19] | 4 + 48 h after treatment | Histology, IH, DNA quantification assay, collagen assay, GAG assay, western blot, TEM, subcutaneous implantation |

*Abbreviations*: *A9* transformed subcutaneous murine fibroblasts, *AFM* atomic force microscopy, *C10* immortalized murine alveolar epithelial cells, *$CaCl_2$* calcium chloride, *CHAPS* 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, *$cmH_2O$* centimeters of water (pressure), *CT* computed tomography, *DMEM* Dulbecco's modified Eagle's medium, *DNase* deoxyribonuclease, *DNA* deoxyribonucleic acid, *E17* embryonic day 17, *EDTA* ethylenediaminetetraacetic acid, *FBS* fetal bovine serum, *GAG* glycosaminoglycan, *$H_2O$* water, *IF* immunofluorescence, *IH* immunohistochemistry, *mESCs* murine embryonic stem cells, *$MgSO_4$* magnesium sulfate, *mM* millimolar, *mmHg* millimeters of mercury, *MSC* mesenchymal stem cells, *NaCl* sodium chloride, *$NaHCO_3$* sodium biocarbonate, *PBS* phosphate buffered saline, *PCR* polymerase chain reaction, *RNase* ribonuclease, *SEM* scanning electron microscopy, *SP-A* surfactant protein A, *TEM* transmission electron microscopy

**Table 18.4** Summary of decellularization methods for human and porcine lungs

| Ref. | Species | Decellularization agents | Perfusion parameters | Instillation route | Days |
|---|---|---|---|---|---|
| [19] | Human | CHAPS, NaCl, and EDTA | Constant pressure (25 mmHg) | Airway and vasculature | 1 |
| [27] | Human/ IPF | Triton X-100, SDC, NaCl, DNase | Unspecified | Airway and vasculature | 3 |
| [126] | Human/ porcine | SDS, Triton X-100 | Constant pressure (30 $cmH_2O$) | Vascular | 4–7 |
| [52] | Human/ porcine | (a) SDS; (b) CHAPS; (c) Tween-20, SDC, peracetic acid | None—lung segments and agitation | N/A | 1 |
| [44] | Human/ porcine | Freeze/thaw; graded SDS perfusion | Varying flow rates (100–500 mL/h) | Airway and vasculature | 7 |
| [61] | Porcine | Triton X-100, SDC, NaCl | 12–25 mL/min (15 mmHg) | Airway and vasculature | 1 |
| [29] | Human/ IPF | SDS, Triton X-100, NaCl | None—thin lung slices | N/A | 2 |
| [26] | Human/ porcine | Triton X-100, SDC, NaCl, DNase Peracetic Acid | Constant flow rates (1 L, 2 L, 3 L/min) | Airway and vasculature | 3 |
| [25] | Human/ COPD | Triton X-100, SDC, NaCl, DNase Peracetic Acid | Constant flow rate 2 L/min | Airway and vasculature | 3 |
| [73] | Human/ rat | SDS, Triton X-100 | Constant pressure (50 $cmH_2O$) | Vascular | 4–7 |
| [41] | Human/ porcine/ rat | (a) SDS; (b) SDC; (c) CHAPS | Constant pressure (30 $cmH_2O$) | Vascular | 4–7 |

*Abbreviations*: *CHAPS* 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, *COPD* chronic obstructive pulmonary disease, *IPF* idiopathic pulmonary fibrosis, *NaCl* sodium chloride, *SDC* sodium deoxycholate, *SDS* sodium dodecyl sulfate

How differences in protocols and routes of administration for decellularization reagents might affect recellularization protocols or potential immunogenicity of implanted scaffolds is not yet known. There is currently no set of standards for demonstrating that a protocol has generated an optimal acellular scaffold. However, Crapo et al. proposed three minimal criteria: (1) <50 ng dsDNA per 1 mg ECM dry weight; (2) <200 bp DNA fragment length; (3) absence of visible nuclear content in histological sections by 4′,6-diamidino-2-phenylindole (DAPI) or hematoxylin-eosin (H&E) staining [50]. However, these are generic criteria for all acellular scaffolds and there are tissue and organ-specific requirements, such as preservation of mechanical properties that are likely important for lung. Furthermore, differences in retention of ECM components and mechanics have also been observed [23, 52] and these may be critical criteria in establishing lung-specific guidelines which must be met with the various protocols utilized in different laboratories.

## *Scaling Up Decellularization Protocols for the Clinic*

Scaling up decellularization protocols from rodent lungs to potential clinical sources (e.g., large animal xenogeneic sources: e.g., porcine or human scaffolds) presents a new set of further challenges. In addition to anatomical differences, there are practical differences in handling organs of this size and it is not a simple matter of scaling up volumes. While rodent and macaque lungs have been decellularized by hand, higher pressures and volumes must be utilized for sufficient inflation of perfusion pathways (e.g., vasculature, airways, etc.) in larger organs. This ensures that perfused solutions reach distal airspaces and capillary beds and that the ensuing cellular debris is cleared from the lungs. All of the published protocols to date for decellularizing whole large animal or human lungs utilize perfusion pumps to generate acellular scaffolds which can support recellularization [19, 20, 25–27, 41, 44], and a recent report demonstrates a potential automated scheme which minimizes many of the practical issues [61]. While not a model for clinical translation, human and porcine lung segments have also been decellularized using small segments in order to improve high throughput study [28, 29, 52, 53] (Table 18.4). There are a variety of techniques which have been reported for assessing the efficacy of the decellularization protocol as well as for characterizing the remaining scaffold. Most reports characterize scaffolds using histologic, immunofluorescent staining, and DNA detection/quantification (Fig. 18.2). We will next discuss these endpoint assessments.

## *Residual Extracellular Matrix and Other Proteins*

Owing to the importance of ECM components, retention of key ECM components is a critical parameter to assess as an endpoint when evaluating potential decellularization protocols. The precise combination of ECM proteins that must be retained to preserve the ability of the acellular scaffold to give organotypic cues for cellular differentiation and functional tissue level assembly remains unknown. The major structural and functional molecules in the ECM include proteins such as collagens, elastin, fibronectin, and laminins as well as a variety of glycoproteins including glycosaminoglycans (GAGs). Collagens are the chief structural components of the lung and are responsible for overall mechanical strength while elastin gives the lung its elastic properties of reversible distension and intrinsic recoil. GAGs help control macromolecular and cellular movement across the basal lamina and may also play a role in the mechanical integrity of the lung, although less is known about their exact role, matrix molecules are generally highly conserved proteins in eukaryotic organisms and therefore it is generally thought that these scaffolds will have minimal to no immune response if used in a xenogeneic context. This may theoretically explain the lack of an adverse immune response seen in xenotransplantation of other decellularized organs such as skin, trachea, and esophagus [4, 7, 11, 12].

There are a variety of techniques which have been used to evaluate ECM components, including histology, immunohistochemistry, western blotting, mass spectrometry-based proteomics, and component-specific assays such as Sircol Collagen Assay, Fastin elastase, etc. (Table 18.3). The majority of lung decellularization techniques result in significant loss of elastin and sulfated GAGs in all species studied thus far [18–20, 22–24, 54–56]. In head-to-head comparison studies of lung decellularization protocols, SDS and SDC have been found to retain more elastin as compared to CHAPS-based protocols [23, 60]. However, despite the differences in retention of ECM components, inoculated cells appear to behave similarly in the recellularization/repopulation assays currently used (including histological and immunofluorescence evaluation). Therefore, it remains unknown if there is an optimal decellularization protocol, and if so, which is best suited for translation to the clinic.

A recently emerging trend is the use of mass spectrometry proteomic analysis to help delineate differences between protein loss and retention in protocols or in scaffold source [22–26, 41]. This assessment has also been used to aid in the selection of optimal protocol parameters such as flow rates or pressures [26]. For example, proteomic analysis can help delineate the impact of changes in protocols during different steps, decellularization agents, or in decellularization parameters (e.g., flow rate, pressure, rinse volumes, etc.) by quantifying or semiquantitatively assessing which choices preferentially retain certain ECM components or minimize/maximize retention of cellular-associated proteins [25–27]. In addition to detecting ECM composition and residual proteins in acellular scaffolds, it has been used for distinguishing differences between decellularization methods or lung origin, including disease states or donor age [23–27, 41, 53, 55]. These assessments also yield critical information for those studying cell–ECM interactions as it can help delineate differences in the underlying matrix.

One particularly striking and consistent result amongst the various groups utilizing this analytical approach is the amount and breadth of non-ECM proteins detected in the scaffold following decellularization. In particular, cytoskeletal elements and cell-associated proteins appear to be retained in the scaffolds, while in general, lesser secreted proteins are detected. This suggests that transmembrane proteins and their associated cytoskeletal elements may remain anchored to the ECM with currently used decellularization protocols. The impact of these residual proteins on recellularization, including potential immunogenicity remains unknown. Furthermore, in the current reports, proteomic assessment has been limited in scope and generally only the most abundantly expressed proteins are reported.

## *Mechanical Assessments of Decellularized Scaffolds*

A variety of in vitro assessments have been utilized to assess the mechanical properties of acellular scaffolds. Investigators have explored both micro-[62, 63]

and macroscale [22, 23, 44, 64, 65] mechanical measurements of acellular lungs as well as force tension relationships in linear strips of decellularized lungs [52, 60]. While techniques such as atomic force microscopy (AFM) are useful in obtaining topographical information and initially assessing mechanical properties of the scaffolds [27, 62, 63, 65], these results have yet to be correlated to recellularization or functional performance. Traditional lung mechanics testing of acellular scaffolds has shown that in the absence of cells and surfactants, acellular scaffolds are stiffer than their naïve counterparts [22]. Introduction of exogenous surfactant into the acellular scaffolds can partially restore lung compliance [22]. This is an important finding and indicates that during recellularization strategies, serial measurements of lung mechanics could be used as a noninvasive and nondestructive means to assess functionality of the regenerating scaffold. For example, decreases in elastance could be used as a measurement of *de novo* surfactant production. However, as acellular lungs are often leaky following decellularization, interpreting results in this context can be challenging [44]. The importance and challenges of measuring mechanical properties in ex vivo bioengineering is discussed in more detail in the review by Suki [66].

## Recellularization

### *Recellularization of Acellular Scaffolds for Bioengineering New Lung*

The lung is a complex organ with a variety of different functions. These include gas exchange, immune system surveillance, and ciliary clearance of inhaled foreign objects. In order to accomplish all of these diverse functions, lung tissue utilizes a variety of different cell types, all of which uniquely contribute to some critical aspect of lung function [67]. Following a variety of acute injuries, such as infection or chemical insult, the lung has the capability to repair itself through activation of endogenous regeneration. The heterogeneous cell population of the lung is replenished by resident stem or progenitor cells, which differentiate into the various adult cell types [68]. Once implanted, it is thought that any ex vivo regeneration strategy must recapitulate these functions, whether it is through a completely biological strategy (i.e., functioning tissue) or some combined artificial and biological solution. It is therefore likely that lung tissue grown ex vivo require some minimal restoration of these subtypes so that it will function once transplanted.

While a variety of cell sources are being investigated for recellularizing acellular and artificial scaffolds, obtaining sufficient cell numbers with any source remains a significant open question. The ideal solution is thought to be the usage of an autologously derived source of cells to minimize post-transplantation immune complications which are a significant cause of morbidity in transplanted patients. One potential source is the use of fully differentiated primary adult cells. However,

these cells may not have sufficient replicative capacity to fully recellularize the organ, plus, normal repair and regeneration following normal lung injury (e.g., illness) may not be possible. Nevertheless, these sorts of repopulation studies may shed light on recellularization strategies using other cell types. It remains unknown if multiple cell types could be isolated from the eventual transplant recipient, grown to sufficient numbers ex vivo and then used in a recellularization approach to restore functionality. While it has been shown that a strategy such as integrin blocking can be used to direct initial cell engraftment of a single cell population [22, 69], scaling this clinically and further adding the complex challenge of uniquely directing the right cell population to a specific architectural location would be challenging. Alternatively, autologous endogenous lung progenitor cells from the various compartments could be utilized (e.g., distal and proximal epithelial progenitor cells, endothelial progenitor cells, etc.) along with stromal cells to recellularize acellular scaffolds. However, the same challenges of obtaining sufficient cell numbers for an initial seeding strategy and directing cells to their correct compartment remain. In both instances, it remains unknown if normal cells could be obtained from a patient with a preexisting lung disease or if isolated diseased cells could be gene-corrected prior to subsequent recellularization. Recent work indicates that the scaffold may more significantly contribute to phenotype than cell-origin. Fibrotic scaffolds were found to induce a pro-fibrotic profile, independent of whether normal or IPF-derived human fibroblasts were used in repopulation assays, whereas the normal lung scaffold did not induce a pro-fibrotic profile if either cell type was used [29]. An allogeneic cell source could also be used, but this re-introduces the potential for immune complications following transplantation. Furthermore, the identification of *bona fide* distal airway lung progenitor cells in the adult human lung remains controversial.

A potentially more appealing autologous approach is the use of induced pluripotent stem cells (iPS) which are derived from reprogramming somatic cells to a stem-cell-like state. While iPS cells avoid the ethical controversies surrounding the use of embryonic stem cells (ESCs)—stem cells derived from the inner blastocyst of in vitro fertilized embryos—iPS cells have been shown to retain epigenetic memory of their tissue origin and have been shown to form teratomas [70]. iPS cells are typically derived from dermal fibroblasts and thus, differentiating them into the various lung cell types has been challenging. However, despite this limitation, recent work has demonstrated that human iPS cells can be differentiated into cells expressing a distal pulmonary epithelial cell immunophenotype and seeded into acellular human lung scaffolds [71–73]. These results further encourage the use of this approach in moving towards the clinic.

Other potential approaches include the use of fetal homogenates or ESCs. As previously mentioned, ethical concerns remain for either of these approaches, as well as the potential for teratoma formation with ESCs. While initial studies have shown that ESCs can engraft in acellular murine lungs [21, 57], seeding into acellular lungs was not sufficient to induce differentiation. Optimized in vitro differentiation protocols must be used in conjunction with seeding and repopulation strategies. Significantly, ESC-derived murine Nkx2-1GFP+ progenitor cells were

able to recellularize acellular murine lungs and form alveolar structures, while in contrast, seeding with undifferentiated ESCs resulted in nonspecific cell masses in distal regions of acellular lungs. Fetal homogenates have the distinct advantage of containing all the necessary cell populations, and have been shown to have some capacity for self-assembly. These cells have been successfully used in the current rodent models of ex vivo regeneration and transplantation. However, in both instances, ethical concerns remain in obtaining these cells and the need for immunosuppressive drug treatment post-transplantation remains unknown. Tables 18.5 and 18.6 summarize recellularization approaches in animal and human models and the phenotype adopted by seeded cells.

## *Implantation of Recellularized Scaffolds*

Important proof of concept studies have shown that recellularized scaffolds can be implanted and participate in gas exchange for short time periods. Decellularized rat lungs re-endothelialized with human umbilical vein endothelial cells (HUVEC) and recellularized with fetal rat lung homogenates and A549 epithelial cells were transplanted into rats that had undergone previous pneumonectomy [18, 19]. While the ex vivo regenerated lungs were shown to contribute to gas exchange following transplantation, the transplants developed significant pulmonary edema and/or hemorrhage resulting in respiratory failure after several hours. In a subsequent study, survival for 14 days was achieved after implantation but lung function progressively declined and the histologic appearance of the graft at necropsy demonstrated significant atelectasis and indications of fibrotic-like alterations [58]. A third study also confirmed the feasibility of short-term survival (60 min) following orthotopic transplantation of a rat lung recellularized with iPS cells [73]. Transplanted grafts were perfused and partial pressure of carbon dioxide in the blood was maintained within normal limits over the observation period. However, blood gas measurements were taken from the left pulmonary vein and represent a mix of blood which had perfused both the left (bioartificial lung) and the naïve lung, and thus is likely not representative of active gas exchange in the transplanted lobe. Additionally, occasional alveolar hemorrhage was observed. Despite these limitations, these studies, nonetheless, provide proof of concept that acellular lungs can be recellularized, surgically implanted, and might minimally participate in gas exchange. However, they also demonstrate the significant challenges that remain in translating towards the clinic. A recellularized acellular lung needs to meet a number of functional requirements in order to be clinically transplantable: adequate gas exchange, waste transport, unidirectional mucociliary clearance, and the ability to maintain physiologic airway pressures and volumes. Thus far, there has been a compartmentalized approach to the respiratory system, separating regeneration of the trachea, vasculature, proximal airways, and distal lung. An animal model which accomplishes restoration of all of these functions has

**Table 18.5** Distribution and phenotype of cells seeded onto animal models of acellular scaffolds

| Ref. | Cells used for seeding | Scaffold | Route | Duration | Distribution | Final phenotype |
|---|---|---|---|---|---|---|
| [45] | ATII | Acellular alveolar vs. amniotic basement membranes | Direct seeding | 8 days | N/A | Alveolar matrices: ATI; amnionic membranes ATII |
| [21] | mESC | Rat (Sprague Dawley) acellular lung | Trachea | 21 days | Proximal-distal regiospecific CC10, proSP-C expression | Tracheobronchial: CC10, Ck18; distal lung: proSP-C, CD31, PDGFRα |
| [18] | HUVEC (DsRed) | Rat acellular lung | Pulmonary artery | 9 days | All vessels | Endothelial cells |
| | A549 | Rat acellular lung | Trachea | 9 days | Airways/alveoli | Airway/alveolar epithelium |
| | HUVEC (DsRed) | Rat acellular lung | Pulmonary artery | 9 days | Entire vasculature | Endothelial cells |
| | Rat fetal lung cells (GD19-20) | Rat acellular lung | Trachea | 9 days | Airways/alveoli | proSP-A, proSP-C, TTF-1/Nkx2.1 (ATII); T1α (ATI); Vimentin (fibroblast) |
| [19] | Neonatal (7d) lung epithelial cells (rat) | Rat acellular lungs (Fischer 344) | Trachea | 8 days | Alveolar, small airways | CCSP (Clara cell), proSP-C (ATII), Aqp5 (ATI), Ck14 (basal cell) |
| | Lung vascular endothelium (rat) | Rat acellular lungs (Fischer 344) | Pulmonary artery | 7 days | Microvascular | CD31 |
| [20] | Fetal lung (E17) | Mouse acellular lungs | Tracheal | 7 days | Alveolar | $Ck18^{+}$/proSP-$C^{+}$ (ATII); CD11b, Aqp5, CCSP, CD31, and vimentin |
| [22] | mBM-MSCs | Mouse acellular lung | Trachea | 28 days | Parenchymal > airway (squamous) | MSCs: no evidence for transdifferentiation |
| | C10—hATII (non-tumorigenic) | Mouse acellular lung | Trachea | 28 days | Parenchymal | N/A |

(continued)

**Table 18.5** (continued)

| Ref. | Cells used for seeding | Scaffold | Route | Duration | Distribution | Final phenotype |
|---|---|---|---|---|---|---|
| [58] | Rat fetal (GD17-20) pneumocytes | Rat acellular lung | Trachea | 14 days | Alveolar/distal bronchioles > trachea/ bronchi | CCSP (airways); TTF-1, proSP-C (alveolar) |
| | HUVEC | Athymic nude rat | Pulmonary artery | 14 days | Proximal to distal vasculature | $CD31^{+}$ |
| [123] | Mouse ATII (primary or P2) | Acellular lung microscaffold | Direct seeding | 22 days | Alveolar | proSP-C/SP-C (ATII-like, from primary or cultured ATII); Aqp5, PDPN (ATI); CCSP (primary) |
| [23] | mBM-MSCs | Mouse acellular lung | Trachea | 14 days | Alveolar | MSCs |
| | C10-hAECII (non-tumorigenic) | Mouse acellular lung | Trachea | 14 days | Large and small Airways | Squamous morphology |
| [55] | Rhesus BM-MSCs | Rhesus macaque | Secondary bronchus | 7 days | Alveolar septae, terminal bronchioles, respiratory bronchioles | MSCs phenotype |
| | Rhesus AD-MSCs | Rhesus macaque | Secondary bronchus | 7 days | Alveolar septae, terminal and respiratory bronchioles | MSCs phenotype |
| [56] | mESCs diff. to $TTF\text{-}1^{pos}/proSP\text{-}C^{pos}$ | Mouse acellular lung | Immersion | 14 Day | Alveolar | TTF-1/Nkx2.1, proSP-C (alveolar); PDFGRα (mesenchymal) |
| [57] | mESCs | Mouse acellular lung | Trachea | 10 days | Hypercellular sheets (alveolar) | Ciliated cells (airways); $T1\alpha^{neg}$ (alveoli) |
| | $Nkx2.1^{GFP}$ | Mouse acellular lung | Trachea | 10 days | Alveolar | Nkx2.1/T1α (alveoli) |
| [54] | Mouse BM-MSCs | Mouse acellular lung | Trachea | 28 days | Alveolar | N/A |
| | C10-mATII (non-tumorigenic) | Mouse acellular lung | Trachea | 28 days | Alveolar | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| [24] | mBM-MSCs | Mouse acellular lung | Trachea | 28 days | Alveolar | N/A |
| | C10-mATII (non-tumorigenic) | Mouse acellular lung | Trachea | 28 days | Alveolar | N/A |
| [69] | A9—murine immortalized fibroblasts | Rat and mouse acellular lung | Slices | 14 days | Alveolar | ProCol1α1 |
| [73] | Human iPS-derived epithelial progenitors and iPS-derived endothelium | Rat whole lung | Tracheal and pulmonary artery | 10 days of iPS/derived endothelial cell culture under perfusion, followed by iPS-derived epithelial progenitor cell installation | Alveolar and vascular | Epithelial: Nkx2.1, T1-$\alpha$, CCSP |
| | | | | | | Endothelial: CD31 |

*Abbreviations*: *A549* adenocarcinomic human alveolar epithelial cell line, *ATI/II* alveolar epithelial cell type I/II, *Aqp* aquaporin, *C10* human colorectal adenocarcinoma epithelial cell line, *CC10* Clara cell-specific protein, *CCSP* Clara cell secretory protein, *CD* cluster of differentiation, *Ck* cytokeratin, *E17* embryonic day 17, *h* human, *HUVEC* human umbilical vein endothelial cell, *iPS* induced pluripotent stem cell, *m* murine, *mBM-MSC* murine bone-marrow-derived mesenchymal stem cell, *mESC* murine embryonic stem cell, *MSC* mesenchymal stem cell, *Nkx2.1* NK2 homeobox 1, *P2* passage number 2, *PDGFR* platelet-derived growth factor receptor, *PDPN* podoplanin, *Col1α1* collagen type 1 α1, *SP-A/C* surfactant protein A/C, *TTF-1* thyroid transcription factor 1

**Table 18.6** Recellularization studies in porcine and human acellular scaffolds

| Ref. | Species | Lung origin | Recellularization technique | Cell types | Summary |
|---|---|---|---|---|---|
| [19] | Human | Normal | Segment incubated with cells | A549; endothelial progenitor cells | Human scaffolds support recellularization |
| [27] | Human | Normal/IPF | Thin slices | hLF | hLFs on IPF scaffolds increase α-SMA expression |
| [71] | Human | Normal | Thin slices | hiPS-ATII, ATII | hiPS-ATII cells adhered to acellular lung matrix and a subpopulation differentiated to ATI phenotype |
| [126] | Human/ porcine | Normal | Thin slices; single lobe recellularization SAEC and pressure-controlled perfusion | HUVEC, SAEC, PAEC | Cell survival for 3 days in an acellular perfused upper right lobe recellularized with PAECs |
| [52] | Human/ porcine | Normal | Thin slices | hMRC-5, hSAEC | Acellular and human porcine lungs supported cell viability |
| [42] | Human | Normal | Thin slices and rotating bioreactor | hiPS-ATII, ATII | Rotating bioreactor culture at ALI enhanced ATII to ATI differentiation |
| [127] | Human | Normal | Thin slices | hBM-MSCs, hAT-MSCs | Both MSC populations attached to the acellular lung matrix; culture on acellular lung matrices enhanced SPC, AQP5, and Caveolin-1 |
| [44] | Human/ porcine | Normal | Excised segments (~0.5 $cm^3$) injected with cells | mESC, hFLC, BMMSCs, hAEC | Porcine/human lungs supported cellular attachment; 1 % SDS protocol minimized T-cell activation |
| [29] | Human | Normal/IPF | Thin slices | Normal and IPF-derived hLF | ECM contributed more significantly to IPF phenotype rather than cell-origin |
| [97] | Human/ porcine | Normal | Physiologic instillation and thin slices | hMSC, hBE, CBF12, hLF | Normal acellular porcine and human lungs are nontoxic |
| [25] | Human | Normal/ COPD | Physiologic instillation and thin slices | hMSC, hBE, CBF12, hLF | COPD lungs do not support viability comparable to normal lungs |

*Abbreviations*: *α-SMA* alpha smooth muscle actin, *ALI* air–liquid-interface, *BMMSCs* porcine bone-marrow-derived mesenchymal stem cells, *CBF12* human endothelial progenitor cells, *COPD* chronic obstructive pulmonary fibrosis, *hAEC* human alveolar epithelial cells, *hAT-MSC* human adipose derived mesenchymal stem cells, *hBE* human bronchial epithelial cells, *hBM-MSC* human bone-marrow-derived mesenchymal stem cells, *hFLC* human fetal lung cells, *hiPS* human induced pluripotent cells, *hLF* human lung fibroblasts, *hMSC* human bone-marrow-derived mesenchymal stem cells, *HUVEC* human umbilical vein endothelial cells, *IPF* idiopathic pulmonary fibrosis, *mESC* murine embryonic stem cells, *PAEC* pulmonary alveolar epithelial cells, *SAEC* human small airway epithelial cells

not been achieved and it will likely be several years before this can be accomplished.

Most recellularized artificial scaffolds have been explored in a limited context and primarily in vitro (Table 18.1). The main research focus up until now has been on the structural development of candidate scaffold designs and materials for lung tissue engineering. There have also been studies investigating cell differentiation of progenitor cells to generate sufficient amounts of cells to repopulate the artificial/decellularized organ. Implantation of artificial scaffolds has only been performed in a few cases and was performed either subcutaneously [32] or into the pleural cavity following pneumonectomy [74]. The distal lung has been the predominant research focus to date. However, no candidate scaffolds have yet to include considerations for vascularization and therefore it remains unclear if these scaffolds could be viable once transplanted. Thus far, there have been no reports of an attempt to transplant a whole bioartificial lung.

## *Immunogenicity of Implanted Scaffolds*

The a priori assumption for clinical use of decellularized lung scaffolds is that acellular scaffolds will be nonimmunogenic because the cellular material has been removed, including cell-associated immunogens, such as Toll-like receptors (TLR) and enzymes associated with xenogeneic immunogenicity, such as (alpha1,3) galactose. However, some ECM and other proteins identified in the remaining decellularized scaffolds are known to be immunogenic [46, 75–77]. This issue has not yet been adequately studied or resolved. Further, cells inoculated into decellularized scaffolds secrete ECM and other proteins [22]. Thus, inoculated cells may considerably remodel the scaffold and generate their own basement membrane, shielding the denuded basement membrane, which can be immunogenic [78].

Some of these remaining proteins may also be beneficial with regard to their ability to induce an immune response. A growing body of literature suggests that decellularized scaffolds can polarize macrophages to the anti-inflammatory M2 phenotype, which is viewed as a more permissive, regenerative phenotype [4, 7, 46, 79–81]. Further, recent work in lung repair and regeneration has demonstrated the critical role that the immune system has in orchestrating normal repair and regeneration in adult lungs [82]. To date, with the exception of the use of fetal homogenates, no recellularization studies have included immune cells. Thus, it is unknown whether retention of these immunogenic components may actually be beneficial in a regeneration strategy. However, one study of interest demonstrated that co-culture of a recellularized acellular lung slice with CD206+ macrophages was found to increase fibroblast proliferation and prolong survival [28]. While this study was a model of disease, it demonstrates the critical role that one immune cell population can have on recellularization in the scaffolds through orchestrating paracrine signaling.

Some groups have implanted recellularized artificial scaffolds. Cortiella and colleagues used PGA and Pluronic F-127 hydrogels and showed that the foreign body response was reduced by usage of the latter [83]. A Gelfoam sponge recellularized with fetal lung cells induced no severe local immune response [84] while a Matrigel plug combined with FGF2-loaded polyvinyl sponge did [85]. This provides evidence that the scaffold material is in part responsible for triggering the immune reaction of the recipient, and that usage of natural matrices like collagen I, fibrinogen–fibronectin–vitronectin, and gelatin seem to be less inflammatory than PGA or polyvinyl [35, 74, 86].

## *Environmental Factors in Ex Vivo Lung Regeneration*

The majority of published work focuses on decellularization methods, lung origin (i.e., disease state or age), and cell sources. There have been limited investigations into the addition or supplementation of exogenous growth factors to scaffolds, and especially a lack of studies examining the role of environmental cues, such as mechanical stretch or oxygen control, in generating functional lung tissue. Despite the presumed importance that factors such as mechanical stimuli and oxygen tension will have in regeneration schemes, they have remained largely unexplored in acellular scaffolds. These critical factors are known to play roles in both embryonic development and post natal repair and regeneration [87–89].

Traditional in vitro cell culture is performed at 20 % oxygen, however, physiologic oxygen levels in individual cells vary depending on the tissue type, tissue density, and cell/tissue proximity to blood vessels [90]. It has long been known that hypoxia can mediate angiogenesis and that vascular endothelial growth factor (VEGF) expression is upregulated in hypoxia [91]. During embryonic development, the lung environment is hypoxic (1–5 % oxygen) [90] and lower oxygen tension levels have been shown to positively influence in vitro differentiation. Lowering oxygen tension to levels typically encountered by cells in the developing embryo has been shown to enhance in vitro differentiation of ESC and iPS cells to Nkx2-1$^{+}$ lung/thyroid progenitor cells [92]. Further studies of cellular differentiation in acellular scaffolds are needed to clarify the potential role of oxygen tension in an ex vivo regeneration strategy.

There is also a large and growing body of literature that delineates the importance of mechanical stimuli on embryonic lung development as well as in normal and diseased tissue repair and regeneration in vivo and ex vivo [88, 89]. Mechanical stretch is known to induce upregulation of surfactant protein C (SP-C) mRNA and protein expression in ATII cells, while shear stress on endothelial cells is critical for VEGF expression [93]. Several studies have examined the effect of mechanotransduction on fetal or adult lung cells in vitro; [93–96] but there is no available information on effects of stretch on development of lung epithelial tissue from embryonic or adult stem cells or from endogenous lung progenitor cells. We have observed upregulation of lung epithelial genes in murine bone-marrow-

derived mesenchymal stem cells seeded into acellular mouse lungs and ventilated (Wagner et al. unpublished data). In particular, we found that SP-C mRNA was significantly upregulated at physiologic tidal volumes; a result we also observed in human ATII cells ventilated in small segments of acellular human lung (Wagner et al. unpublished data) using an artificial pleural coating on excised acellular segments, permitting ventilation [97]. While perfusion parameters have not yet been studied in detail, cultivation of a recellularized human lobe was done under perfusion conditions [41] and a rotating bioreactor culture was found to have positive effects on iPS cells differentiating into distal lung epithelial cells [42].

In addition to utilizing a scaffold from a suitable source and using an optimized decellularization protocol, precise control of the mechanical and gaseous environment with bioreactor technologies (e.g., stimuli mimicking stretch from breathing and shear stress induced by blood flow or breathing) will be necessary for a successful regeneration scheme.

## Lessons Learned from Ex Vivo Organ and tissue Culture

Despite rigorous research efforts, it remains challenging to keep normal, healthy tissue slices and organ explants viable. Most in vitro studies (i.e., lung slices) are not kept longer than a few days while the difficulties in maintaining adequate tissue viability for more than a few hours for candidate donor lungs for transplantation is known to be extremely difficult and is a major limiting factor in maximizing the number of organs available for transplantation. In both of these research areas, tissue slices and organs are generally derived from healthy tissue sources, which are the end goals of an ex vivo strategy. Thus, the challenge of generating functional lung tissue ex vivo is even more daunting considering the challenges experienced in these fields.

Despite the fact that it is widely regarded that sophisticated bioreactor technologies will be needed for ex vivo lung tissue regeneration, there have been limited reports to date examining the effect of the various parameters which could be controlled in bioreactors (e.g., oxygen tension, mechanical ventilation, and vascular perfusion). Additional factors, such as optimal media formulation, have also been minimally explored in the current literature. However, several studies have strongly established the groundwork and the necessity of incorporating bioreactor technologies with ex vivo schemes to maintain or enhance phenotypes. Culture of hATII cells and hiPS-ATII cells in a rotating bioreactor at air–liquid-interface (ALI) was found to be beneficial in maintaining the phenotypic expression of distal epithelial lung cells [42]. In whole lungs or lobes, limited data is available on the viability of cells following recellularization. A single study demonstrated that human small airway epithelial cells (SAECs) instilled into a whole acellular human lung lobe could be maintained for 3 days with constant media perfusion [41]. However, a major limiting factor in both of these studies is that only short time points were analyzed and longer ex vivo schemes will likely be necessary for generating

functional lung tissue [30]. One resource which may be beneficial in guiding the development of optimal lung bioengineering strategies is the ex vivo organ and tissue culture literature describing practices and strategies utilized in those fields for optimizing and maintaining the viability of tissue and organs.

## *Acellular Lungs as Ex Vivo Models of Disease*

In addition to their potential use as scaffolds for tissue engineering, there has been rapid growth in the use of acellular lungs as ex vivo models which more closely recapitulate diseased in vivo environments. These experiments provide a new opportunity for insight into cell–ECM interactions capable of driving disease phenotypes. Human fibroblasts from normal human lungs seeded onto acellular scaffolds derived from fibrotic lungs were found to increase their alpha-SMA expression [27], and the ECM was found to contribute more significantly to IPF correlated gene expression changes in fibroblasts rather than cell-origin (i.e., from IPF or normal lungs) [29]. However, many cell-associated proteins, characteristic of pulmonary fibrosis (e.g., TGF-beta, Ctnnb1, etc.) are retained in decellularized mouse lungs following bleomycin injury [24]. In addition, ECM-associated proteins and matrikines (ECM derived peptides which are liberated by partial proteolysis of ECM macromolecules) are detectable by proteomic approaches following decellularization [22, 23, 25–27, 53, 54]. These proteins, in addition to the detected ECM components, may significantly contribute to the phenotypic changes observed by several groups in recellularization assays. In particular, observation of acquisition of a more fibrotic phenotype by normal fibroblasts in acellular human IPF lungs, may be attributed to these residual proteins, rather than the ECM components alone [27, 29].

Similarly, in acellular lungs derived from murine models of emphysema and from human patients with COPD, cells were unable to remain comparably viable as the same cells seeded into healthy acellular scaffolds [24, 25]. This suggests that either the matrix is impaired in COPD or that the residual protein composition is significantly altered as compared to normal acellular lungs. These studies generate exciting insight into the potential role of the matrix and matrix-associated proteins in driving disease phenotypes and provide proof of concept for use of acellular lungs as a novel platform for studying cell–matrix interactions.

A further novel use of acellular scaffolds in disease models has been utilized to study the role of macrophages in IPF using a Transwell culture setup of thin acellular lung slices recellularized in the Transwell insert, with macrophage co-culture [28]. Decellularized mouse lung slices seeded with murine fibroblasts were co-cultured with $CD206^+$ or $CD206^-$ macrophages from day 14 of murine lungs following bleomycin-induced lung injury (or in the absence of macrophages). $CD206^+$ macrophages were found to increase fibroblast proliferation and survival in the lung slices. However, there was no induction of α-SMA expression. Nonetheless, this study takes advantage of the ability to selectively study cells and cell

combinations in isolation using acellular lungs. Similarly, the human fibroblast cell line MRC5 was seeded onto slices of normal human decellularized lung slices and stimulated with rhCHI3L1, a prototypic-chitinase-like protein recently shown to be elevated in human IPF. The addition of rhCHI3L1 induced α-SMA expression in the MRC5 cells and they adopted a contractile phenotype, as assessed by histology [28].

In addition to repopulation assays, it has also been suggested that recellularized acellular scaffolds could also be used for studying infectious diseases [98] and used as models for cancer development [99]. Thus, studies to date have likely only begun to demonstrate the utility of acellular tissue as ex vivo models of disease which more closely recapitulate in vivo microenvironments than traditional in vitro setups.

## *Precision Cut Tissue Slices*

"Precision cut tissue slices" for ex vivo analysis have been used since the mid-nineteen eighties, when Smith et al. first reported on liver tissue that was sliced into 250 μm thin sections with low variation in thickness (<5 %) [100]. Highly delicate slices (thickness in general 25–300 μm) were fabricated with a device called a Krumdieck tissue slicer. This device overcame the variations in thickness previously seen due to manual cutting of tissue with a razorblade. This thickness also reduced the risk of malnutrition and lack of oxygenation for cells inside the tissue slice [101, 102]. Tissue slicers (Krumdieck or devices from Alabama Research and Development or Leica) use a core, drilled from the tissue that is to be sliced, and generate slices by cutting this core with a knife rotating perpendicular to the core axis. Another possibility for slice generation is the use of vibratomes (e.g., Leica, Zeiss), using a vibrating knife, thereby reducing mechanical impact to the tissue [103]. Several organs have been used to produce tissue slices including brain, heart, liver, kidney, and lung [102–108].

In general, the stiffness of most organ tissue is itself sufficient for slice generation. However, as lung tissue requires high elasticity for breathing movements and high surface area to volume for gas exchange, its density is low compared to other organs. Therefore, it needs to be filled with a supporting material in order to be sliced. One commonly used material to infiltrate the lungs is low melting agarose (used at 37 °C between 1 and 3 % w/v). After allowing the filled lungs to cool and the agarose to gel, the lung can be sliced. Slices have been used in diverse studies, some of which are listed in Table 18.7. Typical experimental durations have been reported in the range of 24–72 h [101, 102]. Using the current techniques, slice cultures seem to decrease in viability after 72 h and thus there is currently no possibility of long-term cultivation. Prolongation of this cultivation period would greatly expand the repertoire of studies which can currently be conducted using this technique. Some possible avenues of exploration of major interest would be the study of disease development or tissue regeneration.

**Table 18.7** Studies applying slice cultivations

| Study type | Ref. |
|---|---|
| Pulmonary physiology | [108, 128–134] |
| Pharmacology | [128, 135–139] |
| Pathogenesis | [140, 141] |
| Toxicity | [107, 142] |
| Cellular effects of mechanical stretch | [108, 129, 134, 143] |
| Cytokine release | [142] |
| Viral infection and gene transfer | [144, 145] |
| Viral exacerbations | [146, 147] |

## *Ex Vivo Maintenance of Explanted Organs for Transplantation*

The shortage of donor organs is a major limiting factor in the treatment options for end-stage lung disease patients. It is further currently impossible to fully mimic all the diverse lung functions in a sustainable and practical manner (i.e., portable) with manmade technical devices. In addition to traditional allogeneic transplantation, one alternative approach could be to use intact xenogeneic organs to restore the function of complex organs as has been done with liver and kidney (Butler and McAnulty refs).

In the lung, progress has been slower. Cypel and colleagues investigated whether ventilation of explanted lungs for up to 4 h with subsequent inflation to full capacity and storage in 4 °C Perfadex solution could improve transplantation outcomes [109]. Although not reaching statistical significance, the incidence of primary graft dysfunction 72 h after transplantation was lower in the ex vivo perfusion group (15 %) compared to the control group (30 %, $p = 0.11$). This ex vivo perfusion at 37 °C (normothermic) and storage at 4 °C has been found to have no drawbacks on transplantation outcome compared to normal donor lungs and therefore seems to be very promising. Still there is no consensus about which solution is the best to use.

The US Food and Drug Administration (FDA) recently approved the "XVIVO Perfusion System with STEEN Solution" (XVIVO Perfusion Inc. Englewood, Colorado, USA) which has been shown to increase the time for evaluation of the functional suitability of a donor organ for transplantation. Donor lungs are kept at body temperature while flushing the vasculature up to 4 h with a sterile solution (STEEN Solution). STEEN solution is a normal oncotic pressure solution containing human serum albumin, dextran, and a low $K^+$ concentration. This solution is designed to prevent edema formation, thrombogenesis, and vascular spasm under normothermic conditions. Lungs remain ventilated during the evaluation period and cells are thus maintained in more physiologic oxygen levels. Waste products are removed by flushing of the vasculature. With this technique, a proportion of organs once regarded as nonideal can become suitable for

transplantation with similar rejection and 12-month survival rates compared to optimal donor organs.

For basic and translational research efforts, these studies are of major importance because they elucidate critical parameters, including perfusate content, temperature, and perfusion rates and pressures that need to be controlled and optimized for long-term cultivation of organs. It is likely that many of these parameters will also be critical to control in ex vivo organ culture techniques, regardless of whether it is simply for longer ex vivo culture for basic science or for preservation/maintenance for candidate transplant organs or tissue engineering schemes.

## Discussion and Outlook

While the prospect of utilizing acellular lung scaffolds clinically may still seem like science fiction to many, the progress made in the last few years has rapidly indicated that this may be a viable option in the not so distant future. The difficulties encountered in maintaining ex vivo viability of freshly explanted healthy organs highlight many of the challenges which the ex vivo regeneration field faces, in addition to those unique to the field. Ex vivo *w*hole organ cultures experience decreases in viability, selective survival of specific cell types, and loss of phenotypic expression over time with current techniques. Many of these same problems may plague ex vivo bioengineering strategies. Even in very thin tissue slice models of naïve tissue, where lack of nutrition and oxygenation is theoretically not of major concern, cells can only maintain their functionality, proliferative capacity, and viability for short periods of time (up to 72 h). This is exacerbated in cultivating whole organs, such as lung, where the need for proper control of medium oxygenation, osmolarity, pH, ventilation, and tissue perfusion in three dimensions is required to keep the tissue viable for long-term cultivation. This likely reflects the combination of a number of factors at play, whose importance we may not currently be fully aware of.

Chiefly among these may be media formulation. Currently, media formulations which have been optimized for two-dimensional (2D) cultivation of homogenous cell populations are utilized in cultivation of both precision cut tissue slices and recellularized acellular tissue slices. However, the media composition needed for whole organ cultivation needs to be optimized for multiple cell types and it remains unclear if the media formulations which are viewed as optimal in 2D are even optimal in that setup. Furthermore, stem and progenitor cells should sustain their capability to differentiate and replenish damaged or absent cell compartments and using a media which pushes these populations into a differentiated state may not be desirable. Therefore, the media formulation used should somehow be able to serve multiple roles simultaneously. To achieve this, different cell types and stem cells initially seeded into acellular scaffolds in an undifferentiated state may require the timed sequential addition of different growth factors, nutrients, and amino acids to regulate signaling pathways involved in cellular proliferation and differentiation.

Means of surveying and controlling the cultivation conditions and media formulation are needed. The knowledge from bioprocess engineering may help to fill the knowledge gap in the needs of whole organ cultivation and ex vivo bioengineering of lung. To date, no study has been conducted addressing the composition of organ-specific cultivation medium supporting long-term cultivation and cellular maintenance in recellularizing lung scaffolds.

Additionally, currently used cultivation conditions for either ex vivo naïve tissue or recellularized acellular scaffolds do not even remotely resemble the in vivo environment. These environments lack proper mechanical (stretch) and environmental stimuli (contact to certain media/air). For example in the lung, it has been shown that isolated ATII cells in tissue culture lose SP-C expression over time and transdifferentiate into alveolar epithelial type I (ATI) cells [110, 111]. A similar decrease in SP-C expression was observed when we cultured naïve murine and human lung tissue slices for 7 days in submerged culture (Uhl et al. unpublished data). As it is known that mechanical stimulation induces SP-C expression in ATII cells [112–114], this suggests, that ventilation of whole organ cultures or stretching of lung slices may be necessary to retain ATII cells in their progenitor state. On the other hand, nonphysiologic ventilation may cause alveolar epithelial cell damage. In a healthy organ the tolerance of cells to mechanical stimuli may be different to that in disease. Further, we know that the mechanics of the acellular lung are dramatically different than naïve lung [22], even despite administration of exogenous surfactant. This indicates the importance of maintaining precise control of the environmental parameters during the whole regenerative scheme.

Reseeding of decellularized matrices has currently been limited to only a few different cell types and often times in monoculture. Each additional cell type adds complexity, making interpretation of results utilizing homogenates or multiple cell types challenging. Usage of stem and progenitor cells (e.g., embryonic stem cells, mesenchymal stem cells, or iPS cells) is appealing for recellularization strategies as these cells can potentially differentiate into the multitude of cell types needed in a specific area of the scaffold. The potential for this approach was demonstrated with the use of ESC-derived murine Nkx2-1$^{GFP+}$ in acellular lungs. These cells repopulated distal airspaces and a subpopulation differentiated into Nkx2-1$^{GFP-}$ and acquired a morphology characteristic of ATI cells and expressed the phenotypic ATI marker podoplanin (T1α) [57]. While encouraging, the necessity of regenerating the multitude of cell types in the lung remains a challenge.

A clinical translation scheme of recellularization of decellularized organs will require precise process control. Metrics for assessing successful decellularization need to be established and a consistent decellularization scheme should be utilized. During recellularization phases, the initial seeding may be accomplished by attachment followed by migration and/or proliferation. We have observed that during this initial seeding phase, physiologic ventilation and perfusion are not feasible until cells have adequately adhered to the scaffold, and in fact, inclusion of these stimuli may even be detrimental. The initial properties and composition of the organ are not comparable to the in vivo situation. For example, in decellularized lungs, there is a lack of surfactant in the alveoli prior to recellularization, and this dramatically

effects mechanical properties [77]. It has also been shown that there is a loss of ECM components, such as elastin, following perfusion decellularization using most protocols [115]. The effect of the loss of these ECM components on initial engraftment and subsequent recellularization and regeneration remain unknown, but may be critical to the success of a regenerative scheme. The importance of preserving the native integrin binding sites in recellularization schemes has already been demonstrated and cells can be directed to certain ECM binding sites through integrin blocking [22]. It has been shown that fibroblasts seeded into acellular mouse lungs utilize a $ß_1$-integrin-dependent pathway and thus preservation of these integrin epitopes seems to be critical. As an accessory technology, a collagen I and Matrigel solution has been used as a pretreatment to coat the decellularized lungs via the trachea before cell seeding to enhance engraftment [56]. Alternatively, cells have also been injected in a hydrogel (Pluronic-F127) for recellularization [21, 44]. Addressing the question of how the matrix should be prepared before inoculation might be an extremely important aspect not yet explored in detail.

There are still major hurdles to overcome for ex vivo engineering. Using state-of-the-art ex vivo preservation techniques, freshly explanted organs, such as kidney and liver, can only maintain viability and function for 5 days [116, 117]. Perfusion at physiologic flow rates is needed in conjunction with the appropriate perfusates tailored in their chemical composition for lungs. This will also be essential for ex vivo recellularization strategies using acellular or synthetic scaffolds and unfortunately, these approaches are not yet mature enough for use. As cells first need to be distributed by migration and likely undergo differentiation inside the matrix, optimal media composition and environmental stimuli will be crucial for ex vivo bioengineering strategies. In order to control for and adapt these stimuli to the regenerating organ during the cultivation period, a range of ancillary technologies need to be integrated and developed into existing bioreactor technologies (e.g., sensors, pumps, and analytic and process control systems). While the road to translating acellular scaffolds into the clinic is long, steady progress has been made in this relatively young field and it has a promising future.

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