Respiratory Tissue Engineering: Current Status and Opportunities for the Future.

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Abstract

Currently, lung disease and major airway trauma constitute a major global healthcare burden with limited treatment options. Airway diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) have been identified as the fifth highest cause of mortality worldwide and are estimated to rise to fourth place by 2030. Alternate approaches and therapeutic modalities are urgently needed to improve clinical outcomes for chronic lung disease. This can be achieved through tissue engineering of the respiratory tract. Interest is growing in the use of airway tissue engineered constructs as both a research tool to further our understanding of airway pathology, validate new drugs and pave the way for novel drug therapies, and also as regenerative medical devices or as an alternative to transplant tissue. This review provides a concise summary of the field of respiratory tissue engineering to date. An initial overview of airway anatomy and physiology is given, followed by a description of the stem cell populations and signalling processes involved in parenchymal healing and tissue repair. We then focus on the different biomaterials and tissue engineered systems employed in upper and lower respiratory tract engineering, and give a final perspective of the opportunities and challenges facing the field of respiratory tissue engineering.

1. Introduction

Currently, lung disease and major airway trauma constitute a major global healthcare burden with limited treatment options. Airway diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) have been identified as the fifth highest cause of mortality worldwide and are estimated to rise to fourth place by 2030 (1). Additionally, tracheal, bronchial and other lung cancers are predicted to become the sixth leading cause of mortality by the same year. In spite of recent advances in the development of novel therapeutic agents and surgical interventions, treatment options for such conditions are primarily palliative, with restoration or replacement of damaged lung tissue ultimately required for success. Unfortunately, lung transplantation has several limitations of its own, with a 5 year mortality rate of approximately 50% post-transplantation in addition to the complications of lifelong immunosuppression (2). In cases of extensive tracheobronchial injury due to cancer, stenosis, infection or congenital abnormalities, allografts or prosthetic devices are indicated; unfortunately, allograft transplantation is also marred by the complications of immunosuppressive treatment, while artificial prosthesis is associated with numerous complications including device migration and dislodgement, material degradation and failure, tissue granulation and tracheal stenosis (3). Bacterial colonisation and chronic infection of the implantation site is a further life-threatening complication adding to the severity of the clinical case (4, 5). A new approach is therefore needed urgently to address this medical crisis.

Regarding drug therapy regimens for COPD and CF, the majority of medicines today principally alleviate symptoms of the failing airways and retard disease progression until transplantation is indicated. Current cell-based *in vitro* models of the respiratory tract employed in drug development of these medicines lack physiological relevance, hindering successful generation of new therapeutic agents. Common cell-based models, for example, consist of two-dimensional (2D) monolayers of a single tracheobronchial epithelial immortalised cell line cultured on a semi-permeable membrane insert at an air-liquid interface (ALI) to induce cell polarisation, differentiation and mucus

production. Although such models are useful as a tool for drug transport studies (6), they fall short in toxicity studies as they lack the physiological complexity of the airways (7, 8).

Respiratory tissue engineering is one such approach that has the potential to improve clinical outcomes for chronic lung disease through tissue regeneration or through improved *in vitro* modelling. In a landmark case in 2008, a 30 year old patient with end-stage bronchomalacia received a tissue-engineered tracheal segment from decellularised donor tissue that had been re-seeded with autologous chondrogenic mesenchymal stem cells (MSCs) and primary tracheal epithelial cells to become the first recipient of a tissue-engineered airway transplant (9). The success of this *ex vivo* engineered tracheobronchial tissue has provided impetus for exploration of more advanced tissue-engineered airway constructs in the laboratory that can be used as successful surgical medical devices, including methods to recellularise the entire lungs (10, 11). However, it is not the first example of a tissue engineered-airway construct. Indeed, procedures incorporating other acellular material and polymeric scaffolds have been reported since the turn of the millennium, while engineered three-dimensional (3D) *in vitro* models of the respiratory tract can be seen in the literature dating back to the 1970s (12). Recent advances in our understanding of lung stem cell biology have also identified potential pools of cell types that could be utilised for regenerative purposes in line with tissue engineered biomaterials (13).

This review serves to summarise the field of respiratory tissue engineering to date. An explanation of lung and airway anatomy and physiology is provided, including the stem cell populations involved in parenchymal healing and tissue repair and signalling factors modulating these processes. Finally, this review focuses on the different materials and tissue engineered systems employed in upper and lower respiratory tract engineering, with a final perspective of the opportunities and challenges facing the field of respiratory tissue engineering provided.

2. Anatomy and physiology of the airways

The lungs are the essential organs of the respiratory tract that enable animals to exchange oxygen and carbon dioxide with the environment to facilitate respiration within cells and tissue. The human respiratory tract contains nearly 50 different cell types along its hierarchical structure (14) in distinct proximal (conducting) and distal (respiratory) zones (8). The larger proximal airways include the trachea, bronchi and bronchioles of greater than 2mm in diameter; these regions are reinforced by cartilaginous rings to prevent collapse (Fig. 1; (15)). The distal airways, composed of non-cartilaginous conducting airways with an internal diameter of less than 2mm, include the bronchioles that terminate at the alveoli, the structural units responsible for gas exchange in the respiratory zone. The diverse range of cell types spread across the airways is primarily separated into these defined regions as a reflection of their functionality, and thus, from a tissue engineering perspective, it is crucial to consider the pertinent cells, extracellular matrix (ECM) composition and architecture required for successful *in vitro* mimicry of a particular airway section and for proximal or distal tissue regeneration *in vivo*.

2.1. Anatomy and physiology of the conducting region of the respiratory tract

Within the tracheobronchial region, a pseudostratified epithelial layer composed of three main cell types- ciliated epithelial cells, goblet cells and basal cells- is supported by the ECM of pulmonary interstitium and cartilage in a specific three-dimensional (3D) structure (16). These cells perform an essential role in innate host defence by providing a physical barrier and by producing mucus and serous secretions that allow the body to clear environmental toxins and infectious agents entering the conducting apparatus following inhalation. The respiratory epithelium operates as an interface between the host and its external environment (17, 18); in this regard, cells present along the walls of the tract have a barrier function, preventing the easy passage of potentially pathogenic substances. Mucus-producing cells assist in this defensive activity, trapping foreign bodies so that they can be removed by the ciliary action of the pseudostratified columnar epithelium. The presence

of a mucus layer also acts as a diffusional buffer to protect the cells from noxious gases that may be inhaled. In addition to mucus expression, the epithelium can secrete a host of other signalling molecules and inflammatory mediators to recruit immune cells that elicit an appropriate response to pathogen infection or invasion, including IL-1, IL-8 and leukotrienes (reported in (17)). Descending further down the proximal region through the branching bronchioles towards the alveoli, the pseudostratified epithelial layer is replaced by a simple cell monolayer composed of Clara cells (also known as club cells) and neuroendocrine cells with a concomitant reduction in the density of mucus-secreting cells and ciliated epithelia.

The fibro-cartilaginous ECM found below the epithelium hosts a range of cellular and non-cellular components. Smooth muscle cells, fibroblasts, chondrocytes, and inflammatory cells constitute the cellular content, mediating muscle contraction, matrix composition, and signalling processes. Non-cellular molecules such as collagen, elastin and various glycosaminoglycans (GAGs) modulate structural support and morphogenesis in tandem with growth factors and morphogens such as epidermal growth factor, bone morphogenetic proteins and fibroblast growth factors (19-22), which can dictate cell differentiation when engineering an *in vitro* tissue engineered tracheobronchial model. A continuous network of fibrillar collagen I and III supports the epithelium and airway smooth muscle (20),reinforced by a series of C-shaped cartilage rings located along the outside of the upper respiratory tract. This cartilage is hyaline in nature, composed predominantly of type II collagen and proteoglycans including aggregans, decorin, biglycan and fibromodulin (23, 24); it conveys increased structural integrity to the trachea and bronchi, preventing airway collapse and ensuring transit of inhaled air to the alveoli (19). The ECM architectural design in the conducting region allows for longitudinal flexibility but lateral rigidity (25), a combination that must be incorporated into any engineered construct that is implanted for tracheobronchial regeneration in order to preserve large airway patency and functionality.

2.2. Anatomy and physiology of the alveolar region of the respiratory tract

Exchange of oxygen and carbon dioxide that is essential for survival occurs in the alveolar region in the distal airways. The alveolar sacs themselves are populated by squamous type I pneumocytes to mediate gas exchange and surfactant-secreting cuboidal type II pneumocytes (26). Type I pneumocytes occupy approximately 93% of the surface area of the alveoli; this cell population has adapted to maximise rapid gas transfer to and from the bloodstream by adopting a thin squamoid shape and by residing at a close proximity to capillaries, reducing the gaseous diffusion barrier to approximately 1μm in thickness . The surfactant produced by type II cells is critical for reducing surface tension within the alveolar sacs from 70mN/m to 22-23mN/m, preventing their collapse during breathing (27, 28).

The ECM of the alveolar region complements the cellular functionality, consisting of thin fused capillary and alveolar epithelial basement membranes to maximise gas exchange between the respiratory tract and blood (20). This membrane is rich in fibronectin and the proteoglycan perlecan which can influence type II pneumocyte behaviour (29, 30). The ECM protein composition changes in the distal airways relative to the conducting region, with an increased elastin component providing more recoil in the airway walls in response to influx and efflux of air. Collagen, elastin and glycosaminoglycans provide the lower parenchymal region of the airways with a viscoelastic behaviour to withstand expansion and contraction during tidal ventilation (31, 32). As elastin fibres are stretched during tidal breathing, collagen fibres coiled around them uncrimp and reduce excessive strain on the elastin, preventing excessive stretch during alveolar expansion and ensuring elastic recoil following exhalation (33); again, from a tissue engineering perspective, incorporation of such ECM components and arrangements is paramount to maximise correct modelling of the 3D *in vivo* milieu for *in vitro* research applications.

3. Stem cell populations of the airways

In order to implement a successful tissue engineering-based approach to heal or replace physiological tissue, constructs should ideally include progenitor cell populations for *ex vivo* culture

or recruit them following implantation so that a regenerative response can be recapitulated and enhanced *in vivo*. Respiratory epithelial cells have a low rate of cell turnover but following injury to the surface of the tract, a rapid response is initiated to restore the epithelial barrier (34). Distinct groups of progenitors appear to marshal reparative processes in distinct regions of the lungs, while some evidence also points to the recruitment of a universal stem cell population that can assist in the repair along the entire respiratory tract (35).

Stem cells involved in airway development and repair processes can be broadly divided into two classifications: endogenous progenitor cells specific to an upper or lower respiratory region and exogenous stem cells that arise from other adult tissue or embryonic stem cells. Within the first set (Fig. 2), basal cells have been identified as the progenitors of the tracheal and bronchial epithelium (36, 37), a variant Clara cell subpopulation in the bronchus and bronchioles (38) and type II alveolar cells in the alveolar regions (39); the capacity to act as progenitors appears to be an additional role along with the basic cellular functions outlined earlier and evidence suggests that following injury to the lung, mechanisms are triggered to activate this regenerative capacity (40). A controversial multipotent lung stem cell has also been claimed to exist (35). Exogenous sources of stem cells that have been proposed for use include bone marrow-derived nonhaematopoietic or mesenchymal stem cells (41-43), embryonic stem cells (ESCs) (44-47), human amniotic fluid-derived stem cells (48) and induced pluripotent stem cells (49). Endogenous mesenchymal progenitors include fibroblast growth factor 10-releasing mesenchymal cells (reported in (50)), but knowledge of these cells is limited and the literature is focused on airway epithelial progenitors at present. All cell sources are of interest from a tissue-engineering perspective as whether the cell population desired is derived from a pool of stem cell progenitors, currently residing epithelia or a combination of both, the result is the same: regeneration of respiratory tissue that can perform adequate re-epithelialisation, and possible prevention of remodelling at the site that contributes to fibrosis, loss of lung function and potentially respiratory disease (51).

3.1. *Endogenous lung stem cell populations of the conducting region of the respiratory tract*

Basal cells have the capacity to act as multipotent progenitors of the tracheobronchial region of the respiratory tract. These cells occupy approximately 30% of the pseudostratified epithelium of the lung (52) and facilitate ciliated columnar epithelial attachment to the basement membrane, in addition to other potential roles in inflammatory processes and neurogenic signalling (53). Transgenic lineage labelling studies have demonstrated that following naphthalene and sulphur dioxide-induced injury to the airways, basal cells give rise to both ciliated cells and Clara cells in the subsequent reparative responses (36, 37). Interestingly, different ratios of ciliated cells: Clara cells are derived from the basal progenitors depending on the different type of insult to the upper respiratory tract, with more basal cells differentiating into Clara cells following their selective depletion with naphthalene. This group has since identified a molecular pathway by which basal cells preferentially differentiate into ciliated cells via IL-6 and JAK/STAT3 signalling (54). Clara cells in their own rite, as well as a subpopulation of naphthalene-resistant variant Clara cell, are capable of renewing the tracheobronchial and bronchiolar epithelium (38). From these studies, it is hypothesised that Clara cells provide a transiently amplifying population in response to injury while basal cells are longer-term progenitors of ciliated, secretory and Clara cell types. More recent work by Chen et al. that identified three pools of proximal conducting, distal conducting and alveolar progenitors mirrors this pattern of different progenitor responses to different forms of injury (55). In this study, it was shown that bleomycin administration selectively depleted epithelial cell types present within the distal lung cell fractions as well as reducing the colony-forming ability by remaining progenitor cells within each of the three progenitor pools. This suggested that the combined loss of epithelial cells and colony-forming ability in distal airways may account for the preferential effect of bleomycin on distal lung tissue remodelling. In response to the injury, preferential expansion of the subset of relatively undifferentiated distal progenitors occurred. Such variation in cellular response is important to consider for recapitulating fibrotic disease and tissue

remodelling using respiratory tissue engineered constructs, impacting on cell selection and the method of inducing airway damage.

3.2. Endogenous lung stem cell populations of the alveolar region of the respiratory tract

In the alveolar region of the respiratory tract, type II alveolar epithelial cells are widely referred to as the progenitor for type I cells. It has been shown to be the case that in addition to their surfactant-producing function, these cells can give rise to type I pneumocytes both under homeostatic conditions and in response to bleomycin-induced injury (56, 57). Indeed, surfactant protein C^+ (SPC^+) type II cells have been recently confirmed as alveolar progenitors and as long-term stem cells in the adult lung (58). This new study builds on that of Rock and colleagues (57) by showing that in addition to their propensity to repopulate the alveolar region following bleomycin administration these cells have clonal properties, with long-term self-renewal described for over a year. Outside of the alveoli, a putative bronchioalveolar stem cell niche population has been proposed for both bronchiolar and alveolar regeneration in rodents (59), though at present, no corresponding cell type has been discovered in humans (60). More recently, a previously unidentified alveolar epithelial subpopulation expressing $\alpha4\beta6$ integrin and no surfactant activity has been identified as a possible unique distal progenitor stem cell (61). This study showed that following administration of bleomycin to induce a progenitor response to alveolar injury, the damaged epithelium in the murine alveoli were replaced primarily with cells derived from non-SPC-expressing progenitor cells present at the time of injury. While the results in this work point to the $\alpha4\beta6$ integrin-expressing subpopulation as this source, further lineage tracing data specific to this cell type is required for absolute confirmation. Finally, a $Krt5^+/p63^+$ pneumocyte-forming distal airway progenitor cell with has been reported after sublethal H1N1 virus administration (62); further investigation might yield yet another source for restoring the respiratory zone of the airways.

3.3. Other lung stem cell populations

In contrast to the multitude of progenitor cells described above that have regional restrictions, a report by Kajstura et al. has claimed to identify a multipotent lung stem cell with not only the potential to differentiate into any cell of epidermal origin, but also of mesodermal origin (35). This study assessed a c-kit^{+} population for the properties of clonality, self-renewal and engraftment through serial transplantation into mouse lungs. The ramifications of this paper could be revolutionary, not only for respiratory tissue engineering and regeneration but also for stem cell biology, as a multipotent cell capable of differentiating down two different germ lineages has never been discovered. As such, this work has been met with some consternation over the choice of controls, lack of lineage tracing and the choice of cellular markers (63); further investigation is warranted to confirm their identity and potential before their extensive interaction with tissue engineered constructs can be considered.

The role of extrapulmonary sources of lung stem cells in tissue repair to injury is one that is still inconclusive, but from a tissue engineering perspective, these cells hold potential for exploitation in *in vitro* or *in vivo* applications. Current evidence has identified bone marrow as a possible source of such cells. Repopulation of alveolar regions with bone marrow-derived stem cells has been reported from analysis of sex-mismatched transplant tissue in humans, whereby male-derived cells detected in lungs from female donors exhibited variable differentiation into type II cells within some samples (41). More recent work suggests that the source of these cells is non-haematopoietic in origin and may point to a primitive stem cell population residing in bone marrow (42), while surgeries using aortic allografts as tracheal replacements provide evidence of mesenchymal stem cell involvement (43). Embryonic stem cells (ESCs), popular for their pluripotency, have naturally been tested for induction into epithelial cells by various methods, with studies deriving alveolar epithelium from murine (45, 47) and human (44) ESCs in addition to proximal epithelial cells (46). At present, the ethical sensitivity of embryonic cells limits their widespread use in the clinical setting. Two alternative sources of pluripotent cells that can differentiate into lung cell epithelial cell lineages but have no ethical ambiguity are human amniotic fluid stem cells (hAFSCs) and induced pluripotent

stem cells (iPSCs) (48, 49). iPSCs were recently developed by transduction of 4 transcription factors and show great promise as a tool for tissue regeneration and as a means of creating disease-specific drug development tools (64, 65). However, this extrapulmonary stem cell source has current limitations that require resolution, including concerns of tumorigenesis (66), the method of transcription factor transfection and efficiency of iPSC production, and genotoxicity issues (67). Future studies will reveal their potential for respiratory tissue regenerative strategies, such as with the clinical trial investigating the derivation of iPSCs from the skin fibroblasts of patients with end-stage lung disease currently underway (68). hAFSCs have shown broad multilineage potential with derivation down six cell lineages across all three germ layers (69) and have been described to differentiate into alveolar epithelial cells (48), though further research to clearly characterise the stem cell and the degree of its "broad multipotency" are warranted before they are considered for use in lung regenerative trials (70).

While lung stem cell populations continue to be identified *in vivo* or developed *in vitro* (Table 1), the majority of endogenous progenitors have not been studied in isolation using three-dimensional (3D) culture on tissue engineered constructs. This might be largely due to the fact that most characterisation studies have been conducted using animal models with inherent differences between human and rodent cellular markers and anatomical distribution (71-73), in addition to a lack of standardisation of protocols for isolation of significant quantities of human progenitor cells. Optimisation of biomaterial composition, structure and mechanical properties for endogenous cell recruitment carried out *in vitro* with these cell types could pave the way for superior scaffold implant design for tissue regeneration or enhanced understanding of embryological processes. Most respiratory 3D studies to date have instead utilised primary tracheobronchial or alveolar epithelial cells which inadvertently in themselves are mixed cell populations containing the endogenous progenitors that might contribute to earlier proliferation observed on the constructs (74). These studies incorporate a wide range of acellular and polymeric, natural and synthetic components with different formulations and scaffold structure, which are discussed in Section 5. Like the endogenous

progenitors, extrapulmonary sources of stem cells such as ESCs and iPSCs are also difficult to obtain in large numbers, require careful *in vitro* maintenance in order to retain their stemness and also pose a risk of tumorigenesis *in vivo* (75). MSCs are an alternative cell source that is easier to obtain in larger numbers, less difficult to culture *in vitro* and has a more favourable safety profile. With improved standardisation of cell isolation and maintenance practice (reviewed in (76)), MSCs could be a universally successful therapeutic option for respiratory tissue engineering strategies.

4. Signalling factors in airway growth and development

Healing processes in the airways require complex coordination of a range of growth factors for processes such as proliferation, differentiation, and vascularisation of tissue to restore functionality (reported in (77)). In the airways, bone morphogenetic proteins (BMPs), notably BMP4, have been identified for their roles in lung development and airway healing following acute injury (78, 79). BMPs regulate a variety of proliferation, differentiation, angiogenesis, apoptosis, and regeneration across the body (80) and this has been exploited for tissue regeneration purposes; for example, recombinant BMP2 is available as a commercial bone graft substitute (81); investigations into their use for respiratory regeneration are currently limited.

Members of the fibroblast growth factor family have also been linked with respiratory epithelial proliferation and differentiation, as has hepatocyte growth factor (HGF) and epidermal growth factor (46, 82, 83). HGF, in particular, is under investigation as a possible therapeutic agent for the treatment of pulmonary fibrosis. This growth factor has been detected at higher levels in patients suffering from pulmonary fibrosis (84), and plays a role in the modulation of alveolar epithelial and endothelial apoptosis, as well as fibroblast differentiation into pro-fibrotic myofibroblasts (85). Increasing the levels of HGF in the respiratory tract, either by gene transfection or by intratracheal administration, reduced lung fibrosis in bleomycin-induced models of pulmonary fibrosis (86-89). Mechanisms of reparative action determined in these studies confirmed an anti-apoptotic effect on alveolar epithelial cells, reduction of pro-inflammatory molecules, a decrease in transforming growth

factor beta-1 (TGFβ1)-mediated epithelial-mesenchymal transition and reduction in collagen deposition. Indeed, recognition of HGF's role in regenerating respiratory tissue has instigated several clinical trials for the use of small molecule HGF-mimetics for the treatment of acute lung injury and pulmonary fibrosis (reviewed in (90)). Recent evidence suggests that HGF can be supplied to the damaged airways by bone marrow-derived MSCs (91), suggesting yet another benefit of the use of such stem cells for 3D respiratory tissue modelling *in vitro* or tissue regeneration.

A small molecule, all-*trans* retinoic acid, is a media supplement often added to primary airway epithelial cell culture (92), and has shown mixed potential for reversal of COPD-induced alveolar injury in human and animal trials (93-97). From a tissue engineering perspective, the inclusion of such a molecule within an *in vitro* 3D culture system could enhance maintenance of airway cell culture (98, 99). Further delineation of specific growth factors in the respiratory tract, their mechanisms of action, as well as their temporal release in morphogenesis, tissue development and responses to injury, could be incorporated into tissue modelling or regenerative medicine strategies for the airways in future.

5. Respiratory tissue engineering approaches: Biomaterial and cell sheet technologies

The majority of respiratory tissue engineering research has focused on the use of biomaterial scaffolds for 3D airway *in vitro* modelling or for the fabrication of regenerative implants. Scaffolds employed are typically composed of natural polymeric materials (e.g. collagen) or donor ECM (decellularised tissue), synthetic polymeric materials (e.g. poly-ε-caprolactone (PCL)) and composites of synthetic and natural materials (e.g. decorin-gelatin-PCL). All three categories have been employed in engineering both the conducting region and alveolar region of the airways in a variety of formulations, including hydrogels, porous polymeric sponges, and decellularised/ECM-based constructs. The choice of scaffold type has often reflected the tissue engineering application in mind, with most 3D *in vitro* modelling applications investigating hydrogel-based formulations and *ex vivo* culture and other regenerative approaches favouring the use of acellular ECM material and porous

polymeric scaffolds. Cell sheet tissue engineering has also been investigated as a “scaffold-free” means of repairing damaged lung tissue. Careful examination of the source material, biomaterial scaffold and potential application of the engineered construct is critical in order to enhance the field of respiratory tissue engineering.

5.1. Tissue engineering of the conducting region of the respiratory tract

Tissue-engineered constructs of the conducting region have been created and investigated for either developing an improved *in vitro* analogue of the tracheobronchial region for disease modelling and drug development, or for the manufacture of tubular or patch scaffolds to regenerate diseased or compromised airways to restore access to the lower regions of the lung for effective respiration. For the former application, hydrogel scaffolds have had widespread use (Table 2), while for the latter, decellularised (DC) tissue has risen to prominence since the clinical report of 2008 (9). Porous polymeric scaffolds have also been manufactured for this purpose (Table 3).

Hydrogels

Tracheobronchial cells cultured at an air-liquid interface (ALI) upon a type I collagen gel, either as a gel alone or as a set gel suspension containing fibroblasts, can enhance cellular proliferation and differentiation to yield a physiologically similar pseudostratified epithelia (100-103). The 3D multicellular environment can have a prominent influence on respiratory epithelial cells, including the composition and mechanical properties of the hydrogel. Pageau et al., for example, discovered that a collagen concentration range of 2mg/ml-3mg/ml was optimal for the co-culture model to prevent excessive fibroblast-induced contraction (103). This study also identified that the change of fibroblast cell type affected the epithelial cell phenotype and contractile properties. Additionally, other studies have shown that Matrigel®, a basement membrane analogue derived from mouse sarcoma cells (104), induced the formation of spheroid-like structures from human bronchial epithelial cells within the gel and displayed alveolar and pulmonary acinar characteristics when used

as the cellular substrate, similar to its effects on type 2 pneumocytes (105, 106). Further improvements have been made to hydrogel models by introducing physical strain and immunological components to better mimic the airways and understand inflammatory disease (107, 108).

It is worth noting that in the majority of upper airway co-culture studies involving collagen hydrogels (101, 103, 105, 107), the human foetal lung fibroblast IMR-90 cell line was used as the secondary cell for culture with airway epithelium (109). The origin of the fibroblast has been observed to modulate cell morphology and differentiation and should always warrant consideration for any airway tissue engineered system, as prominently demonstrated through the use of different primary sources of fibroblasts by Kobayashi and colleagues in culture with tracheal epithelial cells (110), as well as through the observed cancerous change in epithelial cells in the presence of lung cancer-associated fibroblasts (103). When taken into consideration with scaffold composition effects on cell signalling and behaviour, the importance of selection of the appropriate source of cells for successful *in vitro* tissue engineering applications is re-emphasised.

While collagen-based hydrogels are undoubtedly the most prominent choice of biomaterial for *in vitro* respiratory tissue engineering applications, other natural polymers and composites have been considered. Risbud and colleagues, for example, validated the immunocompatibility of a chitosan-gelatin hydrogel through a 7-day culture period with macrophages and found that the biocompatible material supported the growth of human primary respiratory epithelial cells (111). Cornelissen et al. directly compared fibrin hydrogels to those made from collagen in a bid to create a biomaterial that could be derived from autologous blood in a patient (112); fibrin was found to be non-inferior to collagen as a material for tracheal epithelial cell culture, highlighting its potential as a substrate for engineering the conducting region. Overall, hydrogel models of the conducting respiratory region have demonstrated efficacy as an ECM mimic of the proximal airways.

In spite of their widespread use as an ECM mimic of the upper respiratory tract, gel scaffolds suffer from one major disadvantage- their high water content (113). Due to this property, gel materials are viscoelastic in nature and are awkward to handle as a material; furthermore, they suffer from weak mechanical properties, requiring extended *ex vivo* culture periods and combinations with synthetic polymers to reduce scaffold collapse *in vivo* (114-117). Thus, their role in the design of tracheobronchial constructs for *in vivo* regeneration is quite limited and their use is generally restricted to *in vitro* airway reconstruction for disease modelling. That said, employing hydrogels as a cell delivery agent could be a promising regenerative approach for the airways in the future. In this role, the material might act to enhance delivery and retention of mesenchymal stem cells to the damaged tissue (118-120), providing an *in situ* reservoir of growth factor-secreting cells that can repair damaged respiratory tissue by paracrine action (121, 122).

Decellularised Tissue

As an alternative to hydrogel materials for regenerating the conducting airways, the use of decellularised (DC) tissue has increased significantly since the clinical report of Macchiarini et al. (9, 123). Donor tissue can have its antigenic components removed by various steps of detergent addition to leave a natural scaffold maintaining tissue architecture and embedded signalling factors (124), allowing for the re-seeding of the material with the recipient's cells; in the case above, autologous bronchial epithelial cells and bone-marrow derived MSCs differentiated into chondrocytes were seeded on the DC trachea and cultured in a dual-chamber bioreactor with oxygen exposure. Proof-of-concept with a cell-seeded tissue-engineered patch of porcine jejunal segment was performed and demonstrated that the presence of both cell types were necessary to prevent stenosis and infection in the graft (125, 126). More recent work has delivered success with intraoperative-based seeding and eliminating the use of a bioreactor through use of pharmacological cell boosting agents (127, 128).

DC trachea engineered as outlined above can still be subject to a lack of vascular supply, ultimately resulting in an avascular scaffold core unable to support viable tissue growth. To address this limitation, a case study by Delaere et al. adopted a different approach using untreated donor trachea with a "pre-vascularisation" period within the recipient patient's forearm to allow autologous vessel growth prior to implantation at the site of the tracheal defect which would become the first vascularised tracheal allotransplant (129). The patient received a course of immunosuppressive therapy to prevent rejection of the allogeneic tissue that was tapered to allow gradual replacement of the donor cells with patient cells. This procedure was very successful, with the initial process leaving a vascularised scaffold rich in patient-derived mucosa that integrated well into host trachea. No stents were required and the patient was discharged within one week of the tracheal transplant procedure. This procedure has since been performed in five other patients (130).

Another approach towards tracheal regeneration using donor tissue of note is the use of allogeneic aortas as a means to induce *in situ* tracheal regeneration in the body. Following on from pioneering studies with autologous and allogeneic aortic tissue in animals (131-133), Martinod and colleagues conducted three clinical cases to some degree of clinical success with stent insertion (134, 135). Further studies have been conducted with cryopreserved aorta as an alternative to fresh aorta (136, 137), though at the time of writing, these await human trials. While this procedure does not strictly involve a tissue engineered scaffold, the processes of guided healing and directed regeneration mirrors that which is sought with airway constructs and is important to note.

DC trachea is not without its drawbacks, however. Ultimately, the use of tissue-based scaffolds suffers from the same major limitation as transplantation- the requirement for donors. For widespread clinical application, mass-production of this type of material becomes an implausible challenge at present. Furthermore, donor tissue segments may be limited in their dimensions, which may not fit all recipients depending on the location and size of the injury. For acellular trachea, the process of decellularisation can weaken the tissue mechanical properties in spite of retention of

architecture, increasing the risk of graft collapse following implantation *in vivo* (138). The ideal method of decellularisation remains to be determined, with differences seen in structural integrity and ECM composition between the three most popular protocols (139). Tissue heterogeneity due to long-term storage prior to surgical implantation is another caveat that must be taken into account (140). Finally, there is the risk of disease transmission between donor and recipient. Thus, although they show great promise, acellular material is not the conclusive solution for tracheobronchial tissue regeneration just yet.

Porous polymeric scaffolds

Porous polymeric scaffolds hold the potential to address the supply and compatibility issues of donor tissue and to further improve the *in vitro* representation of the tissue architecture and composition of the conducting region, creating more fibrous structures that are reminiscent of the tissue architecture outlined in Section 2. These scaffolds are typically sponge-like materials that composed of either naturally-occurring polymers, synthetic polymers, or a composite of natural and synthetic material (141). Synthetic and semi-synthetic polymeric materials have been explored as biomaterials in tissue engineering because of their potential to produce constructs with more customisable biocompatible and biodegradable properties than some natural substrates, with manufacturing techniques such as freeze-drying, electrospinning and 3D printing involved in scaffold production.

As with hydrogels, collagen is a popular choice for porous polymeric scaffolds. Non-woven collagen scaffolds can sustain growth of human nasal epithelium in 3D *in vitro* culture (142); in the same study, Hyaff®, a hyaluronic acid derivative membrane compared to the collagen scaffold, was found to be non-adhesive for respiratory epithelial cells, a finding that has been disputed elsewhere (98, 143), with differences in primary cell isolation as the possible reason for differences in cell adherence. Other *in vitro* work by Pfenninger and colleagues has also validated the use of collagen for tracheal engineering through analysis of a collagen membrane (144). Concerning the use of

porous collagen scaffolds for *in vivo* regeneration of partial tracheal defects, collagen sponges coated with a type I collagen vitrigel can reproducibly induce *in vivo* formation of rat tracheal epithelium, as well as supporting tissue repair by *ex vivo* culture of epithelial cell and fibroblast co-culture prior to implantation (145-147). Vitrification of the hydrogel involves drying a "traditional" collagen hydrogel in order to form a more robust glass-like material that can be subsequently rehydrated and utilised for tissue engineering applications (148).

Porous natural materials are more robust than hydrogels in general but still suffer from weak mechanical properties; therefore, combination with synthetic materials can benefit from the synergy of natural cell-binding ligands and greater synthetic mechanical properties. Poly-ε-caprolactone (PCL) is an example of a polymer that can support respiratory cells with the advantage of being suitable for a range of fabrication methods and different cell types in conjunction with natural polymers. Electrospun mixtures of decorin, gelatin and PCL, for example, created a highly fibrillar network that supported primary human airway epithelial cells expressing markers and morphological features of a differentiated airway epithelium (149). Freeze-dried PCL-type II collagen scaffolds cultured with chondrocytes in a bioreactor were grafted into rabbits and maintained for a mean of 52 days (150). Certain combinations of natural materials can also reinforce the mechanical properties of one natural material alone, such as the incorporation of electrospun silk fibroin into a dense collagen tubular construct (151). In this study, the inclusion of the silk mesh not only provided increased mechanical strength, but also introduced a fibrous component that can mimic tracheobronchial architecture; such a scaffold holds great promise for future *ex vivo* bioreactor culture for large airway regenerative purposes.

Synthetic materials have been tested alone as the source material for tracheobronchial scaffolds for both *in vitro* modelling and *in vivo* regeneration applications. A novel approach of individually manufacturing electrospun layers of polyethylene terephthalate (PET) for culture of epithelial,

fibroblastic and dendritic cellular components of the scaffold before stacking them to create a combined immunocompetent “triculture” system with appropriate cell localisation has been designed (152). Initial findings have indicated that 3D co-culture with the fibroblasts has enhanced epithelial cell functionality, while immune responses following allergen exposure showed favourable dendritic cell migration; such a tissue engineering-inspired model could pave the way for pharmacological and toxicological screening of novel therapeutics for respiratory delivery. *In vivo* regeneration of a segmental tracheal defect has shown potential with pre-vascularisation of a chondrocyte-seeded polyglycolic acid-silicone construct in the sternohyoid muscle prior to implantation in the trachea (153), while a well-characterised electrospun PET-polyurethane scaffold pre-cultured with mesenchymal stem cells demonstrated cell attachment and expression of markers of epithelial differentiation in a rodent model (154). Furthermore, Jungebluth and colleagues have performed a proof-of-concept study in a 36 year old male with a polyhedral oligomericsilsesquioxane (POSS)-poly-(carbonate-urea)urethane (PCU) scaffold processed by an extrusion-phase inversion method (155). Computed tomography scans and virtual imaging of the patient’s airway were employed to match the construct dimensions to the site of implant to ultimately achieve the first *in vivo* implantation of a synthetic graft in man.

5.2. Overall, solid polymeric scaffolds can offer a balance between improved handling and mechanical properties and the ability for tailoring scaffolds to customisable sizes and shapes to match different airway dimensions, potentially surpassing the limitations of hydrogels and DC tissue encountered with tissue engineering of the conducting region of the respiratory tract. However, synthetic materials are not without their limitations. Firstly, these polymers can lack suitable ligands that are required for initial cell adherence and repopulation of constructs, as well as subsequent growth and differentiation. This drawback can be typically overcome through coating the material with ECM proteins to enhance cell attachment, such as with the integrin ligand RGD peptide (156). Of greater concern, perhaps, are issues with the biocompatibility and biodegradability of synthetic materials, where the presence of foreign material or its by-

products *in vivo* can induce damaging pro-inflammatory responses. Implantation of a synthetic scaffold can induce a foreign body reaction that encapsulates the biomaterial in a fibrotic capsule, rendering it useless for tissue regeneration (157), while for materials like poly(lactic-co-glycolic acid) (PLGA), acidic metabolic products can elicit tissue damage in its local environment in the body (158). Finally, prolonged or absent rates of material degradation in tandem with replacement with host tissue can be a challenge for implanted synthetic scaffolds; in the case of tissue engineering and the conducting region of the respiratory tract, however, non-degradable natural and synthetic implants appear to yield favourable clinical outcomes and thus might be of greater benefit in this application (123, 155).*Tissue engineering of the alveolar region of the respiratory tract*

The alveolar region is one of great interest for pharmaceutical drug delivery research and for chronic lung disease, for which a tissue engineering approach could be applied for improved 3D *in vitro* modelling. Firstly, it is in this area where an aerosolised therapeutic is absorbed into the bloodstream in order to elicit a systemic effect (159), and thus drug transport of novel agents can be adequately assessed with improved *in vitro* tools. Secondly, the pathophysiology of severe and incurable lung conditions like COPD and pulmonary fibrosis is linked with dysfunction in ECM structure and composition (29), a physiological component that a scaffold system can incorporate into a disease model. Furthermore, there is potential to further develop these constructs into functional units that can restore respiratory function in damaged alveolar segments or provide an alternative to lung transplantation. Alveolar tissue engineering has seen a similar trend in research to that of the conducting region, where earlier work focused on hydrogels with some porous solid scaffolds and more recent research has expanded on the use of acellular whole lungs to preserve alveolar architecture (Table 4).

Hydrogels

Hydrogel scaffolds have been investigated as an environment for forming 3D alveolar structures for distal airway modelling, where epithelial cells have been cultured within the gel matrix. One of the first studies reporting the use of a gel scaffold for the support of airway cell culture by Blau et al. described the culture of foetal rabbit type 2 pneumocytes with reconstituted basement membrane (Matrigel®) (160). When gels of a sufficient thickness were manufactured, the cells had sufficient space within the material to form spherical clusters around a central lumen, as well as polarise and differentiate to maintain their arrangement for up to 22 days. The propensity for type 2 alveolar cells to form these alveolar-like structures *in vitro* has been replicated with other species (161, 162) and with type I collagen gels (163), with expression of markers of differentiation such as pro-surfactant protein C and loss of cytokeratin 18 observed, suggesting transition from type II alveolar cells to flattened type I cells.

These studies have demonstrated the feasibility of reproducibly culturing and maintaining distal airway constructs in different research laboratories with great potential to enhance understanding of processes such as alveolar morphogenesis and progression of disease. In this regard, hydrogel materials can facilitate alveolar sac formation by suspending the embedded cells in such a 3D arrangement. However, their use for respiratory drug development applications is limited as this system has no means of exposing the apical side of the alveolar barrier to a drug formulation and conversely, no method of collecting transported drug on the basolateral side of the alveolar bundle to assess drug absorption through the airways and epithelial barrier permeability; such an assessment is a standard measure used in current two-dimensional airway *in vitro* models (6) that would have to be carried forward into 3D models. Additionally, encased alveolar units described above are infeasible for *in vivo* tissue regeneration or replacement as their successful integration with the surrounding airway branches is unlikely. *Ex vivo* culture of cells for regenerative purposes is a possibility, provided that a routine method of hydrogel digestion and viable cell isolation is present. With all matters considered, the utility of hydrogel scaffolds of the alveolar region lies principally in its role as a tool for basic research.

Porous polymeric scaffolds

Porous scaffold materials can remedy the regenerative limitations of hydrogel scaffolds by providing a structure in which the struts of the pores can provide a framework for alveolar cells to grow on and a hollow pore to develop in to the luminal airway on the apical side of the cells, with the correct tailoring of pore size and interconnectivity within the construct.

One of the earliest reports concerning airway tissue engineering described foetal rat lung cells cultured on a gelatin sponge matrix that developed into alveolar bundles with microvilli and lamellar bodies (12, 164); Since then, these early studies have been expanded on through foetal lung cell culture on the purified gelatin product Gelfoam® (165) and on other materials of a similar structure to achieve cell growth, ciliation and alveolar development, such as highly-porous lyophilised collagen-glycosaminoglycan (CG) scaffolds (166). Cell seeding on Gelfoam followed by insertion into healthy lung tissue in a rat model led to successful alveolar unit formation, vascularisation of scaffold segments and host remodelling of the construct into natural tissue. This promising study required pre-seeding with foetal lung cells- in their absence, the scaffolds integrated with surrounding tissue but failed to form alveoli. Should an appropriate human stem cell source be identified for human lungs, this scaffold system could develop into a regenerative procedure in cases of emphysema or other chronic lung disease affecting the lower respiratory tract. While the CG scaffolds were not implanted *in vivo*, foetal lung cells seeded not only formed alveolar structures and markers of alveolar surfactant production, but also deposited smooth muscle actin and elastin around the alveolar units, indicating enhanced functionality with potential for *in vivo* regeneration.

The use of synthetically manufactured porous sponges have not been reported as much as natural materials for alveolar regeneration, with the most prominent study conducted by Cortiella et al. using two synthetic polymers in different formulations (167). In this study, a polyglycolic acid (PGA) mesh and a Pluronic F127 (PF127) thermoresponsive gel were compared as suitable substrates for isolated somatic lung progenitor cells and found that in spite of favourable *in vitro* findings, cell-

seeded PGA mesh induced a foreign body reaction *in vivo*. Inclusion of PF127 with the PGA mesh led to the formation of alveolar-like structures, showing that alteration of the scaffold composition was critical in the success rate of the implant. Overall, porous scaffolds such as these can be manufactured to resemble alveolar architecture and provide a means of furthering alveolar regeneration through tissue engineering.

Decellularised (DC) tissue

The concept of DC whole lung has been the subject of recent interest since the publication of two studies outlining the preparation and transplantation of acellular rat lungs using primary epithelial and vascular cells and foetal airway cells (10, 11). Since then, parallel investigations have been conducted with murine (168, 169), primate (170), and more recently human tissue (171-173), as well as analysis with different stem cell populations such as bone marrow-derived MSCs (174). This emergent field could have great implications for transplant medicine, where the possibility of using acellular cadaveric donor tissue could reduce numbers of patients waiting to receive a transplant from viable sources (175). DC lung has also been utilised as a model for lung cancer (176), paving the way for improved disease modelling.

While these studies do indicate the possibility of developing an entire, intact DC human lung that can be repopulated and grafted into patients as an alternative for transplantation, this form of therapy is still far from clinic-worthy. Firstly, to seed an entire organ such as the lungs alone would require vast quantities of cell types, the exact selection of which progenitor or progenitors is currently uncertain. As with DC tracheal tissue, there is a degree of disparity between the effects of different DC protocols on the resultant acellular lung (177, 178), and particularly in the case of using lungs that are deemed unsuitable for transplantation, organ storage, donor age and disease status can adversely affect mechanical properties of the resultant scaffold (173, 179, 180). For now, a more plausible and beneficial clinical application for these whole-organ constructs could be as a sophisticated *in vitro* drug delivery platform that could provide information on drug deposition fate

in the lungs following pulmonary administration, a critical factor in effective delivery to the lungs (181), and systemic absorption, given that the vascular and airway systems remain intact and independent of each other in the DC organ. Furthermore, the use of damaged lung tissue from patients with COPD or other chronic respiratory illness could advance our understanding of the pathogenesis and progression of disease. Indeed, a recent comparison of fibroblast culture on decellularised lung tissue slices from donor tissue of normal patients and those with idiopathic pulmonary fibrosis revealed that in the presence of stiffer, fibrotic tissue, the cells adopted a myofibrolastic phenotype that was independent of transforming growth factor-β, suggesting that the mechanical properties of the lung tissue induced such a change (182). Additionally, prolonged fibroblast culture on DC lung tissue slices have been observed to develop fibrotic lung tissue *in vitro*, indicating that the use of an immortalised cell source or disruption of the tissue basement membrane could be exploited to develop a novel disease model (183). Use of such disease models for subsequent drug assessment could greatly boost the successful delivery of inhaled therapeutics to the diseased environment by improving *in vitro-in vivo* correlations in diseased status.

5.3. Cell sheet culture and respiratory tissue engineering

In a different approach to the abundance of biomaterials-based respiratory tissue engineering research that has developed in the field, cell sheet culture can develop intact cell layers for implantation without the requirement of a supporting scaffold. This method, pioneered by Okano and colleagues in 1993 (184), utilises cell culture plates coated with a thermoresponsive polymer that facilitates the removal of cell monolayers through reduction of the temperature from 37°C to below 32°C. In this manner, proteolytic cleavage of cell surface proteins and cell-cell contacts by trypsinisation is avoided, allowing cells to be transferred in a completely intact form, even with deposited ECM, for insertion into a site of injury. As well as offering this advantage, absence of the scaffold can reduce any potential inflammatory effects following implantation (185).

Cell sheet culture has been applied to both tracheobronchial and alveolar tissue engineering. For the upper airways, addition of an epithelial cell sheet to a pre-vascularised Dacron® PET graft implanted in a rabbit trachea induced the development of a mature pseudostratified columnar epithelium along the luminal side at 4 weeks that did not fully form in its absence (186). This indicates that the combination of such an approach with other biomaterial constructs could maximise re-epithelialisation of the tracheobronchial airway, an outcome critical for clinical success (4). Kanzaki et al. have also reported the sealing of intraoperative airway leaks in the lower lung tissue using skin fibroblast cell sheets (187, 188), and have recently developed a non-invasive method of delivering the cells via thoracoscopic surgery (189). Having considered these studies, we believe that there is an opportunity to combine both cell sheet and biomaterial scaffold-based respiratory tissue engineering. In addition to utilising cell sheets to repair possible defects and trauma in recellularised DC lungs, enhanced epithelialisation of tracheobronchial constructs and reduction of granulation at anastomoses (190) through the use of cell sheet sealing is possible.

6. Future perspectives of respiratory tissue engineering: Opportunities and challenges

Respiratory tissue engineering has advanced as a field significantly within the past decade and though this area of research is still quite nascent relative to other facets of tissue engineering such as skin, bone, and cartilage, it has the capacity to become the platform to advance treatment of chronic airway disease and adequately address the growing clinical need for novel treatments for these conditions. This can be achieved through two pathways- (i) utilisation of the constructs as a research tool for translational medicine to further our understanding of airway physiology and pathology, to validate new drugs and excipients and ultimately to pave the way for novel drug therapies; and/or (ii) utilisation of respiratory tissue engineered constructs as a regenerative medical device or as an alternative to transplant tissue. These applications have been alluded to throughout this review and are summarised here as the opportunities and challenges facing the future of respiratory tissue engineering.

6.1. In vitro respiratory drug development and disease models

One avenue for respiratory tissue engineering research to pursue is the use of scaffolds as a means of enhancing *in vitro* disease models and the drug development process. As mentioned earlier, current cell-based *in vitro* models of the respiratory tract consist of 2D monolayers of primary tracheobronchial epithelial cells or an immortalised cell line cultured on a semi-permeable membrane insert at an air-liquid interface (ALI) to induce cell polarisation, differentiation and mucus production (6). ALI culture has played a significant role towards the *in vitro* recapitulation of the *in vivo* environment, presenting the cells with an apical side resembling the lumen of the respiratory tract and a basolateral side to represent vascular supply of nutrients (191), with increased expression of cilia in primary cells and differences in barrier and mucus-secreting properties of cell lines observed (192, 193). However, the absence of an extracellular component with co-cultured cells in a 3D environment can result in an over-simplification of the airway barrier, rendering this drug development tool lacking in physiological relevance. Inadequate data obtained from this system can increase the risk of drug candidate failure due to poor *in vitro-in vivo* correlation between the apparent pharmacokinetic and pharmacodynamic characteristics of the compound, culminating in great expense and time lost that delay the development process of new medicines. While the use of *in vivo* animal models can provide more toxicological information that might not be currently attainable with *in vitro* models, some animal species used in preclinical testing are not always suitable as *in* vivo models for human respiratory drug research because of structural and physiological differences between species (71-73). Therefore, combined with the drive to implement the reduction, refinement and replacement of animal models in research (194, 195), more sophisticated models based on 3D human normal and diseased tissue are required to provide *in vitro* models that improve predictive validity of drug compounds in humans and thus increase the number of successfully formulated therapeutics. Successful development of novel drugs that can actually target the pathophysiological source of the disease more directly will greatly improve treatment options for sufferers of chronic airway disease; for example, the recent revolutionary drug used to

treat cystic fibrosis, ivacaftor, was developed in line with improved *in vitro* culture using primary bronchial epithelial cells (196).

Much of respiratory tissue engineering research can be linked to studies of three-dimensional (3D) airway modelling as an *in vitro* tool for basic research and drug development, which has seen a transition from single cell type culture on inserts towards co-culture and the inclusion of scaffold material (Fig. 3). Recognising the effects of mesenchymal cells on epithelial proliferation and differentiation from earlier work (197), Le Visage and colleagues expanded on epithelial cell insert culture by investigating the use of a tracheobronchial co-culture model in which human bronchial epithelial cells and human MSCs were cultured in monolayers on opposite sides of a Transwell® insert, akin to Fig. 3(b) (198). The presence of hMSCs altered the temporal pattern of mucin secretion by the epithelial monolayer, but the authors noted a prominent limitation in their model that when the MSCs were induced towards a chondrogenic lineage, they detached from the insert before any discernible effects on the bronchial epithelium could be observed, negating the co-culture system. It was concluded that the inclusion of a biomaterial substrate might be more favourable in the future in order to provide a scaffold for MSC differentiation. In spite of this study, a triple co-culture system of the same style compromising alveolar epithelial cells, macrophages and dendritic cells has shown promise for studying immunological responses to inhaled particulates (199, 200), but there is an overall consensus in the literature that introducing an ECM analogue into the co-culture environment, often through the use of a biomaterial scaffold, could enhance cell culture, cell-cell signalling and functionality(Fig. 3(c)). Accordingly, models in which the epithelium is cultured at an ALI over a scaffold substrate embedded with co-cultured cells are the subject of much interest and are even available now as commercial 3D research products, such as the MatTek EpiAirway-FT® technology (191). Use of airway tissue engineering provides the opportunity to develop long-lasting, reproducible, primary co-culture models for improved drug development and understanding of respiratory disease.

6.2. Respiratory regenerative medicine

Considering the success of the human tracheal transplant cases (9, 129, 155, 201), it could be presumed that the prospect of a tissue engineered tracheal replacement device and possibly even a whole-lung construct is not far off the horizon; unfortunately, there is still much work to be done. Of the 14 patients who have received a tracheal DC allotransplant or synthetic scaffold, half have been reported to have died (190), although the scaffolds themselves may not be directly responsible for the deaths. The need for airway stents is another issue that has not yet been solved. Additional *in vitro* studies have been recommended to further elucidate the mechanisms of scaffold integration, healing and cartilage production prior to wide-scale clinical trials (202). Of course, it cannot be denied that the reported cases have shown excellent promise and proof of concept that such constructs can be translated to the clinic where they can affect change. For acellular tissue, the principal challenges lie in standardising donor tissue selection and long-term storage, decellularisation processes and, perhaps the most challenging aspect, the appropriate selection of cell sources for *ex vivo* recellularisation and restoration of functionality prior to implantation.

DC tissue is not the only avenue forward for respiratory regenerative medicine. Natural polymeric and synthetic scaffolds offer potential to address the shortcomings of the need for donor tissue and their production is more often easier to reproduce, customise and scale-up for clinical production. Use of natural materials appropriately strengthened through crosslinking and advanced manufacturing techniques like electrospinning and 3D printing offer many advantages. These natural or semi-synthetic composite structures can act as an “off-the-shelf” device that is available on immediate demand in the clinic. The challenge lies in assessing whether these constructs can operate as cell-free implants, given the nature of the airways being an open interface that requires an epithelial barrier (4), or whether an *ex vivo* pre-seeded system would be better. The use of growth factor and gene-enriched scaffolds seen in other areas of tissue engineering (203, 204) could also provide new opportunities for using these scaffolds as regenerative implants.

7. Conclusions

In conclusion, the field of respiratory tissue engineering has progressed significantly from the early experiments of Douglas et al. (12, 164), and holds great potential for the future of clinical therapies for chronic airway disease and respiratory drug development. The airways represent a complex system between the host and its environment, leading to challenges in effective *in vitro* representation or surgical treatment of the organ. As further knowledge accrues on airway and lung stem cells, signalling factors are revealed and further advances are made in biomaterial development, amalgamation of pulmonary stem cell biology research and tissue engineering can bring with it the development of *in vitro* systems and regenerative implants to address the significant burden of lung disease and major airway trauma.

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References

1. Mathers, C.D., and Loncar, D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med **3**, e442, 2006.

2. Sueblinvong, V., and Weiss, D.J. Stem cells and cell therapy approaches in lung biology and diseases. Transl Res **156**, 188, 2010.

3. Walles, T. Tracheobronchial bio-engineering: biotechnology fulfilling unmet medical needs. Adv Drug Deliv Rev **63**, 367, 2011.

4. Grillo, H.C. Tracheal replacement: a critical review. Ann Thorac Surg **73**, 1995, 2002.

5. Steger, V., Hampel, M., Trick, I., Muller, M., and Walles, T. Clinical tracheal replacement: transplantation, bioprostheses and artificial grafts. Exp Rev Med Devices **5**, 605, 2008.

6. Forbes, B., and Ehrhardt, C. Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm **60**, 193, 2005.

7. Berube, K., Prytherch, Z., Job, C., and Hughes, T. Human primary bronchial lung cell constructs: the new respiratory models. Toxicology **278**, 311, 2010.

8. Klein, S.G., Hennen, J., Serchi, T., Blomeke, B., and Gutleb, A.C. Potential of coculture in vitro models to study inflammatory and sensitizing effects of particles on the lung. Toxicol In Vitro **25**, 1516, 2011.

9. Macchiarini, P., Jungebluth, P., Go, T., Asnaghi, M.A., Rees, L.E., Cogan, T.A., Dodson, A., Martorell, J., Bellini, S., Parnigotto, P.P., Dickinson, S.C., Hollander, A.P., Mantero, S., Conconi, M.T., and Birchall, M.A. Clinical transplantation of a tissue-engineered airway. Lancet **372**, 2023, 2008.

10. Ott, H.C., Clippinger, B., Conrad, C., Schuetz, C., Pomerantseva, I., Ikonomou, L., Kotton, D., and Vacanti, J.P. Regeneration and orthotopic transplantation of a bioartificial lung. Nat Med **16**, 927, 2010.

11. Petersen, T.H., Calle, E.A., Zhao, L., Lee, E.J., Gui, L., Raredon, M.B., Gavrilov, K., Yi, T., Zhuang, Z.W., Breuer, C., Herzog, E., and Niklason, L.E. Tissue-engineered lungs for in vivo implantation. Science **329**, 538, 2010.

12. Douglas, W.H., Moorman, G.W., and Teel, R.W. The formation of histotypic structures from monodisperse fetal rat lung cells cultured on a three-dimensional substrate. In Vitro **12**, 373, 1976.

13. Hogan, B.L., Barkauskas, C.E., Chapman, H.A., Epstein, J.A., Jain, R., Hsia, C.C., Niklason, L., Calle, E., Le, A., Randell, S.H., Rock, J., Snitow, M., Krummel, M., Stripp, B.R., Vu, T., White, E.S., Whitsett, J.A., and Morrisey, E.E. Repair and regeneration of the respiratory system: Complexity, plasticity, and mechanisms of lung stem cell function. Cell Stem Cell **15**, 123, 2014.

14. Breeze, R.G., and Wheeldon, E.B. The cells of the pulmonary airways. Am Rev Respir Dis **116**, 705, 1977.

15. Kleinstreuer, C., Zhang, Z., and Donohue, J.F. Targeted drug-aerosol delivery in the human respiratory system. Ann Rev Biomed Eng **10**, 195, 2008.

16. Huang, S., Wiszniewski L., and Constant S. The use of in vitro 3D cell models in drug development for respiratory diseases. In:Kapetanovic, I. M., ed. Drug Discovery and Development-Present and Future. Croatia: InTech; 2011, pp. 169-190.

17. Knight, D.A., and Holgate, S.T. The airway epithelium: structural and functional properties in health and disease. Respirology **8**, 432, 2003.

18. Tam, A., Wadsworth, S., Dorscheid, D., Man, S.F., and Sin, D.D. The airway epithelium: more than just a structural barrier. Ther Adv Respir Dis **5**, 255, 2011.

19. McGowan, S.E. Extracellular matrix and the regulation of lung development and repair. FASEB J **6**, 2895, 1992.

20. Dunsmore, S.E., and Rannels, D.E. Extracellular matrix biology in the lung. Am J Physiol **270**, L3, 1996.

21. Weaver, M., Dunn, N.R., and Hogan, B.L. Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. Development **127**, 2695, 2000.

22. Papakonstantinou, E., and Karakiulakis, G. The 'sweet' and 'bitter' involvement of glycosaminoglycans in lung diseases: pharmacotherapeutic relevance. Br J Pharmacol **157**, 1111, 2009.

23. Drake, R.L., Vogl, W., Mitchell, A.W.M., and Gray, H. Gray's anatomy for students. Philadelphia: Elsevier/Churchill Levingstone; 2005.

24. Temenoff, J.S., and Mikos, A.G. Review: tissue engineering for regeneration of articular cartilage. Biomaterials **21**, 431, 2000.

25. Belsey, R. Resection and reconstruction of the intrathoracic trachea. Br J Surg **38**, 200, 1950.

26. Crapo, J.D., Barry, B.E., Gehr, P., Bachofen, M., and Weibel, E.R. Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis **126**, 332, 1982.

27. Rooney, S.A., Young, S.L., and Mendelson, C.R. Molecular and cellular processing of lung surfactant. FASEB J **8**, 957, 1994.

28. Lopez-Rodriguez, E., and Perez-Gil, J. Structure-function relationships in pulmonary surfactant membranes: from biophysics to therapy. Biochim Biophys Acta **1838**, 1568, 2014.

29. Dunsmore, S.E. Treatment of COPD: a matrix perspective. Int J Chron Obstruct Pulmon Dis **3**, 113, 2008.

30. von der Mark, K., and Park, J. Engineering biocompatible implant surfaces: Part II: Cellular recognition of biomaterial surfaces: Lessons from cell–matrix interactions. Progress in Materials Science **58**, 327, 2013.

31. Suki, B., Ito, S., Stamenovic, D., Lutchen, K.R., and Ingenito, E.P. Biomechanics of the lung parenchyma: critical roles of collagen and mechanical forces. J Appl Physiol **98**, 1892, 2005.

32. Suki, B., and Bates, J.H. Lung tissue mechanics as an emergent phenomenon. J Appl Physiol **110**, 1111, 2011.

33. Abraham, T., Hirota, J.A., Wadsworth, S., and Knight, D.A. Minimally invasive multiphoton and harmonic generation imaging of extracellular matrix structures in lung airway and related diseases. Pulm Pharmacol Ther **24**, 487, 2011.

34. Crosby, L.M., and Waters, C.M. Epithelial repair mechanisms in the lung. Am J Physiol Lung Cell Mol Physiol **298**, L715, 2010.

35. Kajstura, J., Rota, M., Hall, S.R., Hosoda, T., D'Amario, D., Sanada, F., Zheng, H., Ogorek, B., Rondon-Clavo, C., Ferreira-Martins, J., Matsuda, A., Arranto, C., Goichberg, P., Giordano, G., Haley, K.J., Bardelli, S., Rayatzadeh, H., Liu, X., Quaini, F., Liao, R., Leri, A., Perrella, M.A., Loscalzo, J., and Anversa, P. Evidence for human lung stem cells. New Engl J Med **364**, 1795, 2011.

36. Rock, J.R., Onaitis, M.W., Rawlins, E.L., Lu, Y., Clark, C.P., Xue, Y., Randell, S.H., and Hogan, B.L. Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc Natl Acad Sci USA **106**, 12771, 2009.

37. Hong, K.U., Reynolds, S.D., Watkins, S., Fuchs, E., and Stripp, B.R. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. Am J Pathol **164**, 577, 2004.

38. Rawlins, E.L., Okubo, T., Xue, Y., Brass, D.M., Auten, R.L., Hasegawa, H., Wang, F., and Hogan, B.L. The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. Cell Stem Cell **4**, 525, 2009.

39. Uhal, B.D. Cell cycle kinetics in the alveolar epithelium. Am J Physiol **272**, L1031, 1997.

40. Rock, J.R., and Hogan, B.L. Epithelial progenitor cells in lung development, maintenance, repair, and disease. Annu Rev Cell Dev Biol **27**, 493, 2011.

41. Albera, C., Polak, J.M., Janes, S., Griffiths, M.J., Alison, M.R., Wright, N.A., Navaratnarasah, S., Poulsom, R., Jeffery, R., Fisher, C., Burke, M., and Bishop, A.E. Repopulation of human pulmonary epithelium by bone marrow cells: a potential means to promote repair. Tissue Eng **11**, 1115, 2005.

42. Kassmer, S.H., Bruscia, E.M., Zhang, P.X., and Krause, D.S. Nonhematopoietic cells are the primary source of bone marrow-derived lung epithelial cells. Stem Cells **30**, 491, 2012.

43. Seguin, A., Baccari, S., Holder-Espinasse, M., Bruneval, P., Carpentier, A., Taylor, D.A., and Martinod, E. Tracheal regeneration: Evidence of bone marrow mesenchymal stem cell involvement. J Thorac Cardiovasc Surg **145**, 1297, 2013.

44. Samadikuchaksaraei, A., Cohen, S., Isaac, K., Rippon, H.J., Polak, J.M., Bielby, R.C., and Bishop, A.E. Derivation of distal airway epithelium from human embryonic stem cells. Tissue Eng **12**, 867, 2006.

45. Siti-Ismail, N., Samadikuchaksaraei, A., Bishop, A.E., Polak, J.M., and Mantalaris, A. Development of a novel three-dimensional, automatable and integrated bioprocess for the differentiation of embryonic stem cells into pulmonary alveolar cells in a rotating vessel bioreactor system. Tissue Eng Part C Methods **18**, 263, 2012.

46. Wong, A.P., Bear, C.E., Chin, S., Pasceri, P., Thompson, T.O., Huan, L.J., Ratjen, F., Ellis, J., and Rossant, J. Directed differentiation of human pluripotent stem cells into mature airway epithelia expressing functional CFTR protein. Nat Biotechnol **30**, 876, 2012.

47. Sun, H., Quan, Y., Yan, Q., Peng, X., Mao, Z., Wetsel, R.A., and Wang, D. Isolation and characterization of alveolar epithelial type II cells derived from mouse embryonic stem cells. Tissue Eng Part C Methods **20**, 464, 2014.

48. Carraro, G., Perin, L., Sedrakyan, S., Giuliani, S., Tiozzo, C., Lee, J., Turcatel, G., De Langhe, S.P., Driscoll, B., Bellusci, S., Minoo, P., Atala, A., De Filippo, R.E., and Warburton, D. Human amniotic fluid stem cells can integrate and differentiate into epithelial lung lineages. Stem Cells **26**, 2902, 2008.

49. Otsuki, K., Imaizumi, M., Nomoto, Y., Wada, I., Miyake, M., Sugino, T., and Omori, K. Potential for respiratory epithelium regeneration from induced pluripotent stem cells. Annals Otol Rhinol Laryngol **122**, 25, 2013.

50. Warburton, D., El-Hashash, A., Carraro, G., Tiozzo, C., Sala, F., Rogers, O., Langhe, S.D., Kemp, P.J., Riccardi, D., Torday, J., Bellusci, S., Shi, W., Lubkin, S.R., and Jesudason, E. Lung organogenesis. Curr Top Dev Biol **90**, 73, 2010.

51. Beers, M.F., and Morrisey, E.E. The three R's of lung health and disease: repair, remodeling, and regeneration. J Clin Invest **121**, 2065, 2011.

52. Mercer, R.R., Russell, M.L., Roggli, V.L., and Crapo, J.D. Cell number and distribution in human and rat airways. Am J Respir Cell Mol Biol **10**, 613, 1994.

53. Evans, M.J., Van Winkle, L.S., Fanucchi, M.V., and Plopper, C.G. Cellular and molecular characteristics of basal cells in airway epithelium. Exp Lung Res **27**, 401, 2001.

54. Tadokoro, T., Wang, Y., Barak, L.S., Bai, Y., Randell, S.H., and Hogan, B.L. IL-6/STAT3 promotes regeneration of airway ciliated cells from basal stem cells. Proc Natl Acad Sci USA **111**, E3641, 2014.

55. Chen, H., Matsumoto, K., Brockway, B.L., Rackley, C.R., Liang, J., Lee, J.H., Jiang, D., Noble, P.W., Randell, S.H., Kim, C.F., and Stripp, B.R. Airway epithelial progenitors are region specific and show differential responses to bleomycin-induced lung injury. Stem Cells **30**, 1948, 2012.

56. Adamson, I.Y., and Bowden, D.H. Derivation of type 1 epithelium from type 2 cells in the developing rat lung. Lab Invest **32**, 736, 1975.

57. Rock, J.R., Barkauskas, C.E., Cronce, M.J., Xue, Y., Harris, J.R., Liang, J., Noble, P.W., and Hogan, B.L. Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. Proc Natl Acad Sci USA **108**, E1475, 2011.

58. Barkauskas, C.E., Cronce, M.J., Rackley, C.R., Bowie, E.J., Keene, D.R., Stripp, B.R., Randell, S.H., Noble, P.W., and Hogan, B.L. Type 2 alveolar cells are stem cells in adult lung. J Clin Invest **123**, 3025, 2013.

59. Kim, C.F., Jackson, E.L., Woolfenden, A.E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R.T., and Jacks, T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell **121**, 823, 2005.

60. Rock, J., and Konigshoff, M. Endogenous lung regeneration: potential and limitations. Am J Respir Crit Care Med **186**, 1213, 2012.

61. Chapman, H.A., Li, X., Alexander, J.P., Brumwell, A., Lorizio, W., Tan, K., Sonnenberg, A., Wei, Y., and Vu, T.H. Integrin alpha6beta4 identifies an adult distal lung epithelial population with regenerative potential in mice. J Clin Invest **121**, 2855, 2011.

62. Kumar, P.A., Hu, Y., Yamamoto, Y., Hoe, N.B., Wei, T.S., Mu, D., Sun, Y., Joo, L.S., Dagher, R., Zielonka, E.M., Wang, de Y., Lim, B., Chow, V.T., Crum, C.P., Xian, W., and McKeon, F. Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. Cell **147**, 525, 2011.

63. Moodley, Y. Evidence for human lung stem cells. New Engl J Med **365**, 464; author reply 465, 2011.

64. Takahashi, K., and Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell **126**, 663, 2006.

65. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell **131**, 861, 2007.

66. Harding, J., and Mirochnitchenko, O. Preclinical studies for induced pluripotent stem cell-based therapeutics. J Biol Chem **289**, 4585, 2014.

67. Hong, S.G., Dunbar, C.E., and Winkler, T. Assessing the risks of genotoxicity in the therapeutic development of induced pluripotent stem cells. Mol Ther **21**, 272, 2013.

68. Mayo Clinic. A phase I study of iPS cell generation from patients with COPD. In: clinicaltrials.gov [Internet].Bethesda (MD): National Library of Medicine (US). 2013- [cited 2014 August 15]. Available from: http://clinicaltrials.gov/ct2/show/NCT01860898. Identifier: NCT01860898.

69. De Coppi, P., Bartsch, G., Jr., Siddiqui, M.M., Xu, T., Santos, C.C., Perin, L., Mostoslavsky, G., Serre, A.C., Snyder, E.Y., Yoo, J.J., Furth, M.E., Soker, S., and Atala, A. Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol **25**, 100, 2007.

70. Siegel, N., Rosner, M., Hanneder, M., Valli, A., and Hengstschlager, M. Stem cells in amniotic fluid as new tools to study human genetic diseases. Stem Cell Rev **3**, 256, 2007.

71. Cryan, S.A., Sivadas, N., and Garcia-Contreras, L. In vivo animal models for drug delivery across the lung mucosal barrier. Adv Drug Deliv Rev **59**, 1133, 2007.

72. Zosky, G.R., and Sly, P.D. Animal models of asthma. Clin Exp Allergy **37**, 973, 2007.

73. Liu, X., and Engelhardt, J.F. The glandular stem/progenitor cell niche in airway development and repair. Proc Am Thorac Soc **5**, 682, 2008.

74. Pohl, C., Hermanns, M.I., Uboldi, C., Bock, M., Fuchs, S., Dei-Anang, J., Mayer, E., Kehe, K., Kummer, W., and Kirkpatrick, C.J. Barrier functions and paracellular integrity in human cell culture models of the proximal respiratory unit. Eur J Pharm Biopharm **72**, 339, 2009.

75. Knoepfler, P.S. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells **27**, 1050, 2009.

76. Bara, J.J., Richards, R.G., Alini, M., and Stoddart, M.J. Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic. Stem Cells **32**, 1713, 2014.

77. Kirkpatrick, C.J., Fuchs, S., and Unger, R.E. Co-culture systems for vascularization — Learning from nature. Adv Drug Deliv Rev **63**, 291, 2011.

78. Warburton, D., and Bellusci, S. The molecular genetics of lung morphogenesis and injury repair. Paediatr Respir Rev **5 Suppl A**, S283, 2004.

79. Masterson, J.C., Molloy, E.L., Gilbert, J.L., McCormack, N., Adams, A., and O'Dea, S. Bone morphogenetic protein signalling in airway epithelial cells during regeneration. Cell Signal **23**, 398, 2011.

80. Abe, E. Function of BMPs and BMP antagonists in adult bone. Ann NY Acad Sci **1068**, 41, 2006.

81. McKay, B. Local sustained delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2). Abstract presented at the Annual International Conference of the IEEE: Engineering in Medicine and Biology Society, Minneapolis, MN, 2009, pp 236.

82. Ware, L.B., and Matthay, M.A. Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair. Am J Physiol Lung Cell Mol Physiol **282**, L924, 2002.

83. Takeyama, K., Dabbagh, K., Lee, H.M., Agusti, C., Lausier, J.A., Ueki, I.F., Grattan, K.M., and Nadel, J.A. Epidermal growth factor system regulates mucin production in airways. Proc Natl Acad Sci USA **96**, 3081, 1999.

84. Yamanouchi, H., Fujita, J., Yoshinouchi, T., Hojo, S., Kamei, T., Yamadori, I., Ohtsuki, Y., Ueda, N., and Takahara, J. Measurement of hepatocyte growth factor in serum and bronchoalveolar lavage fluid in patients with pulmonary fibrosis. Respir Med **92**, 273, 1998.

85. Crestani, B., Marchand-Adam, S., Quesnel, C., Plantier, L., Borensztajn, K., Marchal, J., Mailleux, A., Soler, P., and Dehoux, M. Hepatocyte growth factor and lung fibrosis. Proc Am Thorac Soc **9**, 158, 2012.

86. Dohi, M., Hasegawa, T., Yamamoto, K., and Marshall, B.C. Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. Am J Respir Crit Care Med **162**, 2302, 2000.

87. Watanabe, M., Ebina, M., Orson, F.M., Nakamura, A., Kubota, K., Koinuma, D., Akiyama, K., Maemondo, M., Okouchi, S., Tahara, M., Matsumoto, K., Nakamura, T., and Nukiwa, T. Hepatocyte growth factor gene transfer to alveolar septa for effective suppression of lung fibrosis. Mol Ther **12**, 58, 2005.

88. Gazdhar, A., Fachinger, P., van Leer, C., Pierog, J., Gugger, M., Friis, R., Schmid, R.A., and Geiser, T. Gene transfer of hepatocyte growth factor by electroporation reduces bleomycin-induced lung fibrosis. Am J Physiol Lung Cell Mol Physiol **292**, L529, 2007.

89. Gazdhar, A., Temuri, A., Knudsen, L., Gugger, M., Schmid, R.A., Ochs, M., and Geiser, T. Targeted gene transfer of hepatocyte growth factor to alveolar type II epithelial cells reduces lung fibrosis in rats. Hum Gene Ther **24**, 105, 2013.

90. Chakraborty, S., Chopra, P., Hak, A., Dastidar, S.G., and Ray, A. Hepatocyte growth factor is an attractive target for the treatment of pulmonary fibrosis. Expert Opinion Investig Drugs **22**, 499, 2013.

91. Gazdhar, A., Susuri, N., Hostettler, K., Gugger, M., Knudsen, L., Roth, M., Ochs, M., and Geiser, T. HGF expressing stem cells in usual interstitial pneumonia originate from the bone marrow and are antifibrotic. PloS One **8**, e65453, 2013.

92. Gray, T.E., Guzman, K., Davis, C.W., Abdullah, L.H., and Nettesheim, P. Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. Am J Respir Cell Mol Biol **14**, 104, 1996.

93. Mao, J.T., Goldin, J.G., Dermand, J., Ibrahim, G., Brown, M.S., Emerick, A., McNitt-Gray, M.F., Gjertson, D.W., Estrada, F., Tashkin, D.P., and Roth, M.D. A pilot study of all-trans-retinoic acid for the treatment of human emphysema. Am J Respir Crit Care Med **165**, 718, 2002.

94. Lucey, E.C., Goldstein, R.H., Breuer, R., Rexer, B.N., Ong, D.E., and Snider, G.L. Retinoic acid does not affect alveolar septation in adult FVB mice with elastase-induced emphysema. Respiration **70**, 200, 2003.

95. March, T.H., Cossey, P.Y., Esparza, D.C., Dix, K.J., McDonald, J.D., and Bowen, L.E. Inhalation administration of all-trans-retinoic acid for treatment of elastase-induced pulmonary emphysema in Fischer 344 rats. Exp Lung Res **30**, 383, 2004.

96. Ishizawa, K., Kubo, H., Yamada, M., Kobayashi, S., Numasaki, M., Ueda, S., Suzuki, T., and Sasaki, H. Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema. FEBS Lett **556**, 249, 2004.

97. Roth, M.D., Connett, J.E., D'Armiento, J.M., Foronjy, R.F., Friedman, P.J., Goldin, J.G., Louis, T.A., Mao, J.T., Muindi, J.R., O'Connor, G.T., Ramsdell, J.W., Ries, A.L., Scharf, S.M., Schluger, N.W., Sciurba, F.C., Skeans, M.A., Walter, R.E., Wendt, C.H., and Wise, R.A. Feasibility of retinoids for the treatment of emphysema study. Chest **130**, 1334, 2006.

98. Huang, T.W., Cheng, P.W., Chan, Y.H., Yeh, T.H., Young, Y.H., and Young, T.H. Regulation of ciliary differentiation of human respiratory epithelial cells by the receptor for hyaluronan-mediated motility on hyaluronan-based biomaterials. Biomaterials **31**, 6701, 2010.

99. Huang, T.W., Chan, Y.H., Su, H.W., Chou, Y.S., and Young, T.H. Increased mucociliary differentiation and aquaporins formation of respiratory epithelial cells on retinoic acid-loaded hyaluronan-derivative membranes. Acta Biomater **9**, 6783, 2013.

100. Paquette, J.S., Tremblay, P., Bernier, V., Auger, F.A., Laviolette, M., Germain, L., Boutet, M., Boulet, L.P., and Goulet, F. Production of tissue-engineered three-dimensional human bronchial models. In Vitro Cell Dev Biol Anim **39**, 213, 2003.

101. Vaughan, M.B., Ramirez, R.D., Wright, W.E., Minna, J.D., and Shay, J.W. A three-dimensional model of differentiation of immortalized human bronchial epithelial cells. Differentiation **74**, 141, 2006.

102. Wang, Y., Wong, L.B., and Mao, H. Creation of a long-lifespan ciliated epithelial tissue structure using a 3D collagen scaffold. Biomaterials **31**, 848, 2010.

103. Pageau, S.C., Sazonova, O.V., Wong, J.Y., Soto, A.M., and Sonnenschein, C. The effect of stromal components on the modulation of the phenotype of human bronchial epithelial cells in 3D culture. Biomaterials **32**, 7169, 2011.

104. Hughes, C.S., Postovit, L.M., and Lajoie, G.A. Matrigel: a complex protein mixture required for optimal growth of cell culture. Proteomics **10**, 1886, 2010.

105. Delgado, O., Kaisani, A.A., Spinola, M., Xie, X.J., Batten, K.G., Minna, J.D., Wright, W.E., and Shay, J.W. Multipotent capacity of immortalized human bronchial epithelial cells. PloS One **6**, e22023, 2011.

106. Wu, X., Peters-Hall, J.R., Bose, S., Pena, M.T., and Rose, M.C. Human bronchial epithelial cells differentiate to 3D glandular acini on basement membrane matrix. Am J Respir Cell Mol Biol **44**, 914, 2011.

107. Choe, M.M., Sporn, P.H., and Swartz, M.A. An in vitro airway wall model of remodeling. Am J Physiol Lung Cell Mol Physiol **285**, L427, 2003.

108. Darveau, M.E., Jacques, E., Rouabhia, M., Hamid, Q., and Chakir, J. Increased T-cell survival by structural bronchial cells derived from asthmatic subjects cultured in an engineered human mucosa. J Allergy Clin Immunol **121**, 692, 2008.

109. Nichols, W.W., Murphy, D.G., Cristofalo, V.J., Toji, L.H., Greene, A.E., and Dwight, S.A. Characterization of a new human diploid cell strain, IMR-90. Science **196**, 60, 1977.

110. Kobayashi, K., Suzuki, T., Nomoto, Y., Tada, Y., Miyake, M., Hazama, A., Nakamura, T., and Omori, K. Potential of heterotopic fibroblasts as autologous transplanted cells for tracheal epithelial regeneration. Tissue Eng **13**, 2175, 2007.

111. Risbud, M., Endres, M., Ringe, J., Bhonde, R., and Sittinger, M. Biocompatible hydrogel supports the growth of respiratory epithelial cells: possibilities in tracheal tissue engineering. J Biomed Mater Res **56**, 120, 2001.

112. Cornelissen, C.G., Dietrich, M., Kruger, S., Spillner, J., Schmitz-Rode, T., and Jockenhoevel, S. Fibrin gel as alternative scaffold for respiratory tissue engineering. Ann Biomed Eng **40**, 679, 2012.

113. Brown, R.A. In the beginning there were soft collagen-cell gels: towards better 3D connective tissue models? Exp Cell Res **319**, 2460, 2013.

114. Naito, H., Tojo, T., Kimura, M., Dohi, Y., Zimmermann, W.H., Eschenhagen, T., and Taniguchi, S. Engineering bioartificial tracheal tissue using hybrid fibroblast-mesenchymal stem cell cultures in collagen hydrogels. Interact Cardiovasc Thorac Surg **12**, 156, 2011.

115. Hong, H.J., Lee, J.S., Choi, J.W., Min, B.H., Lee, H.B., and Kim, C.H. Transplantation of autologous chondrocytes seeded on a fibrin/hyaluronan composite gel into tracheal cartilage defects in rabbits: preliminary results. Artif Organs **36**, 998, 2012.

116. Hong, H.J., Chang, J.W., Park, J.K., Choi, J.W., Kim, Y.S., Shin, Y.S., Kim, C.H., and Choi, E.C. Tracheal reconstruction using chondrocytes seeded on a poly(l-lactic-co-glycolic acid)-fibrin/hyaluronan. J Biomed Mater Res A **102**, 4142, 2014.

117. Chang, J.W., Park, S.A., Park, J.K., Choi, J.W., Kim, Y.S., Shin, Y.S., and Kim, C.H. Tissue-Engineered Tracheal Reconstruction Using Three-Dimensionally Printed Artificial Tracheal Graft: Preliminary Report. Artif Organs **38**, E95, 2014.

118. Salinas, C.N., and Anseth, K.S. Mesenchymal stem cells for craniofacial tissue regeneration: designing hydrogel delivery vehicles. J Dent Res **88**, 681, 2009.

119. Gao, J., Liu, R., Wu, J., Liu, Z., Li, J., Zhou, J., Hao, T., Wang, Y., Du, Z., Duan, C., and Wang, C. The use of chitosan based hydrogel for enhancing the therapeutic benefits of adipose-derived MSCs for acute kidney injury. Biomaterials **33**, 3673, 2012.

120. Mathieu, E., Lamirault, G., Toquet, C., Lhommet, P., Rederstorff, E., Sourice, S., Biteau, K., Hulin, P., Forest, V., Weiss, P., Guicheux, J., and Lemarchand, P. Intramyocardial delivery of mesenchymal stem cell-seeded hydrogel preserves cardiac function and attenuates ventricular remodeling after myocardial infarction. PloS One **7**, e51991, 2012.

121. Curley, G.F., Scott, J.A., and Laffey, J.G. Therapeutic potential and mechanisms of action of Mesenchymal Stromal Cells for Acute Respiratory Distress Syndrome. Curr Stem Cell Res Ther **9**, 319, 2014.

122. Antunes, M.A., Laffey, J.G., Pelosi, P., and Rocco, P.R. Mesenchymal stem cell trials for pulmonary diseases. J Cell Biochem **115**, 1023, 2014.

123. Gonfiotti, A., Jaus, M.O., Barale, D., Baiguera, S., Comin, C., Lavorini, F., Fontana, G., Sibila, O., Rombola, G., Jungebluth, P., and Macchiarini, P. The first tissue-engineered airway transplantation: 5-year follow-up results. Lancet **383**, 238, 2014.

124. Crapo, P.M., Gilbert, T.W., and Badylak, S.F. An overview of tissue and whole organ decellularization processes. Biomaterials **32**, 3233, 2011.

125. Macchiarini, P., Walles, T., Biancosino, C., and Mertsching, H. First human transplantation of a bioengineered airway tissue. J Thorac Cardiovasc Surg **128**, 638, 2004.

126. Go, T., Jungebluth, P., Baiguero, S., Asnaghi, A., Martorell, J., Ostertag, H., Mantero, S., Birchall, M., Bader, A., and Macchiarini, P. Both epithelial cells and mesenchymal stem cell–derived chondrocytes contribute to the survival of tissue-engineered airway transplants in pigs. J Thorac Cardiovasc Surg **139**, 437, 2010.

127. Jungebluth, P., Bader, A., Baiguera, S., Moller, S., Jaus, M., Lim, M.L., Fried, K., Kjartansdottir, K.R., Go, T., Nave, H., Harringer, W., Lundin, V., Teixeira, A.I., and Macchiarini, P. The concept of in vivo airway tissue engineering. Biomaterials **33**, 4319, 2012.

128. Jungebluth, P., Moll, G., Baiguera, S., and Macchiarini, P. Tissue-engineered airway: a regenerative solution. Clin Pharmacol Ther **91**, 81, 2012.

129. Delaere, P., Vranckx, J., Verleden, G., De Leyn, P., and Van Raemdonck, D. Tracheal allotransplantation after withdrawal of immunosuppressive therapy. N Engl J Med **362**, 138, 2010.

130. Delaere, P.R., Vranckx, J.J., and Den Hondt, M. Tracheal allograft after withdrawal of immunosuppressive therapy. N Engl J Med **370**, 1568, 2014.

131. Martinod, E., Zegdi, R., Zakine, G., Aupecle, B., Fornes, P., D'Audiffret, A., Chachques, J.C., Azorin, J., and Carpentier, A. A novel approach to tracheal replacement: the use of an aortic graft. J Thorac Cardiovasc Surg **122**, 197, 2001.

132. Martinod, E., Seguin, A., Pfeuty, K., Fornes, P., Kambouchner, M., Azorin, J.F., and Carpentier, A.F. Long-term evaluation of the replacement of the trachea with an autologous aortic graft. Ann Thorac Surg **75**, 1572, 2003.

133. Martinod, E., Seguin, A., Holder-Espinasse, M., Kambouchner, M., Duterque-Coquillaud, M., Azorin, J.F., and Carpentier, A.F. Tracheal regeneration following tracheal replacement with an allogenic aorta. Ann Thorac Surg **79**, 942, 2005.

134. Azorin, J.F., Bertin, F., Martinod, E., and Laskar, M. Tracheal replacement with an aortic autograft. Eur J Cardiothorac Surg **29**, 261, 2006.

135. Wurtz, A., Porte, H., Conti, M., Desbordes, J., Copin, M.C., Azorin, J., Martinod, E., and Marquette, C.H. Tracheal replacement with aortic allografts. N Engl J Med **355**, 1938, 2006.

136. Seguin, A., Radu, D., Holder-Espinasse, M., Bruneval, P., Fialaire-Legendre, A., Duterque-Coquillaud, M., Carpentier, A., and Martinod, E. Tracheal replacement with cryopreserved, decellularized, or glutaraldehyde-treated aortic allografts. Ann Thorac Surg **87**, 861, 2009.

137. Makris, D., Holder-Espinasse, M., Wurtz, A., Seguin, A., Hubert, T., Jaillard, S., Copin, M.C., Jashari, R., Duterque-Coquillaud, M., Martinod, E., and Marquette, C.H. Tracheal replacement with cryopreserved allogenic aorta. Chest **137**, 60, 2010.

138. Partington, L., Mordan, N.J., Mason, C., Knowles, J.C., Kim, H.W., Lowdell, M.W., Birchall, M.A., and Wall, I.B. Biochemical changes caused by decellularization may compromise mechanical integrity of tracheal scaffolds. Acta Biomater **9**, 5251, 2013.

139. Haykal, S., Soleas, J.P., Salna, M., Hofer, S.O., and Waddell, T.K. Evaluation of the structural integrity and extracellular matrix components of tracheal allografts following cyclical decellularization techniques: comparison of three protocols. Tissue Eng Part C Methods **18**, 614, 2012.

140. Baiguera, S., Del Gaudio, C., Jaus, M.O., Polizzi, L., Gonfiotti, A., Comin, C.E., Bianco, A., Ribatti, D., Taylor, D.A., and Macchiarini, P. Long-term changes to in vitro preserved bioengineered human trachea and their implications for decellularized tissues. Biomaterials **33**, 3662, 2012.

141. Lyons, F., Partap, S., and O'Brien, F.J. Part 1: scaffolds and surfaces. Technol Health Care **16**, 305, 2008.

142. Ziegelaar, B.W., Aigner, J., Staudenmaier, R., Lempart, K., Mack, B., Happ, T., Sittinger, M., Endres, M., Naumann, A., Kastenbauer, E., and Rotter, N. The characterisation of human respiratory epithelial cells cultured on resorbable scaffolds: first steps towards a tissue engineered tracheal replacement. Biomaterials **23**, 1425, 2002.

143. Huang, T.W., Chan, Y.H., Cheng, P.W., Young, Y.H., Lou, P.J., and Young, T.H. Increased mucociliary differentiation of human respiratory epithelial cells on hyaluronan-derivative membranes. Acta Biomater **6**, 1191, 2010.

144. Pfenninger, C., Leinhase, I., Endres, M., Rotter, N., Loch, A., Ringe, J., and Sittinger, M. Tracheal remodeling: comparison of different composite cultures consisting of human respiratory epithelial cells and human chondrocytes. In Vitro Cellular Dev Biol Anim **43**, 28, 2007.

145. Nomoto, Y., Kobayashi, K., Tada, Y., Wada, I., Nakamura, T., and Omori, K. Effect of fibroblasts on epithelial regeneration on the surface of a bioengineered trachea. Ann Otol Rhinol Laryngol **117**, 59, 2008.

146. Kobayashi, K., Suzuki, T., Nomoto, Y., Tada, Y., Miyake, M., Hazama, A., Wada, I., Nakamura, T., and Omori, K. A tissue-engineered trachea derived from a framed collagen scaffold, gingival fibroblasts and adipose-derived stem cells. Biomaterials **31**, 4855, 2010.

147. Tani, A., Tada, Y., Takezawa, T., Imaizumi, M., Nomoto, Y., Nakamura, T., and Omori, K. Regeneration of tracheal epithelium using a collagen vitrigel-sponge scaffold containing basic fibroblast growth factor. Ann Otol Rhinol Laryngol **121**, 261, 2012.

148. Takezawa, T., Ozaki, K., Nitani, A., Takabayashi, C., and Shimo-Oka, T. Collagen vitrigel: a novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. Cell Transplant **13**, 463, 2004.

149. Hinderer, S., Schesny, M., Bayrak, A., Ibold, B., Hampel, M., Walles, T., Stock, U.A., Seifert, M., and Schenke-Layland, K. Engineering of fibrillar decorin matrices for a tissue-engineered trachea. Biomaterials **33**, 5259, 2012.

150. Lin, C.H., Hsu, S.H., Huang, C.E., Cheng, W.T., and Su, J.M. A scaffold-bioreactor system for a tissue-engineered trachea. Biomaterials **30**, 4117, 2009.

151. Ghezzi, C.E., Marelli, B., Donelli, I., Alessandrino, A., Freddi, G., and Nazhat, S.N. The role of physiological mechanical cues on mesenchymal stem cell differentiation in an airway tract-like dense collagen-silk fibroin construct. Biomaterials **35**, 6236, 2014.

152. Harrington, H., Cato, P., Salazar, F., Wilkinson, M., Knox, A., Haycock, J.W., Rose, F., Aylott, J.W., and Ghaemmaghami, A.M. Immunocompetent 3D model of human upper airway for disease modeling and in vitro drug evaluation. Mol Pharm **11**, 2082, 2014.

153. Luo, X., Liu, Y., Zhang, Z., Tao, R., He, A., Yin, Z., Li, D., Zhang, W., Liu, W., Cao, Y., and Zhou, G. Long-term functional reconstruction of segmental tracheal defect by pedicled tissue-engineered trachea in rabbits. Biomaterials **34**, 3336, 2013.

154. Ajalloueian, F., Lim, M.L., Lemon, G., Haag, J.C., Gustafsson, Y., Sjoqvist, S., Beltran-Rodriguez, A., Del Gaudio, C., Baiguera, S., Bianco, A., Jungebluth, P., and Macchiarini, P. Biomechanical and

biocompatibility characteristics of electrospun polymeric tracheal scaffolds. Biomaterials **35**, 5307, 2014.

155. Jungebluth, P., Alici, E., Baiguera, S., Le Blanc, K., Blomberg, P., Bozoky, B., Crowley, C., Einarsson, O., Grinnemo, K.H., Gudbjartsson, T., Le Guyader, S., Henriksson, G., Hermanson, O., Juto, J.E., Leidner, B., Lilja, T., Liska, J., Luedde, T., Lundin, V., Moll, G., Nilsson, B., Roderburg, C., Stromblad, S., Sutlu, T., Teixeira, A.I., Watz, E., Seifalian, A., and Macchiarini, P. Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study. Lancet **378**, 1997, 2011.

156. Hersel, U., Dahmen, C., and Kessler, H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials **24**, 4385, 2003.

157. Bryers, J.D., Giachelli, C.M., and Ratner, B.D. Engineering biomaterials to integrate and heal: The biocompatibility paradigm shifts. Biotechnol Bioeng **109**, 1898, 2012.

158. Sung, H.J., Meredith, C., Johnson, C., and Galis, Z.S. The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. Biomaterials **25**, 5735, 2004.

159. Labiris, N.R., and Dolovich, M.B. Pulmonary drug delivery. Part I: physiological factors affecting therapeutic effectiveness of aerosolized medications. Br J Clin Pharmacol **56**, 588, 2003.

160. Blau, H., Guzowski, D.E., Siddiqi, Z.A., Scarpelli, E.M., and Bienkowski, R.S. Fetal type 2 pneumocytes form alveolar-like structures and maintain long-term differentiation on extracellular matrix. J Cell Physiol **136**, 203, 1988.

161. Mondrinos, M.J., Koutzaki, S., Jiwanmall, E., Li, M., Dechadarevian, J.P., Lelkes, P.I., and Finck, C.M. Engineering three-dimensional pulmonary tissue constructs. Tissue Eng **12**, 717, 2006.

162. Mondrinos, M.J., Koutzaki, S., Lelkes, P.I., and Finck, C.M. A tissue-engineered model of fetal distal lung tissue. Am J Physiol Lung Cell Mol Physiol **293**, L639, 2007.

163. Sugihara, H., Toda, S., Miyabara, S., Fujiyama, C., and Yonemitsu, N. Reconstruction of alveolus-like structure from alveolar type II epithelial cells in three-dimensional collagen gel matrix culture. Am J Pathol **142**, 783, 1993.

164. Douglas, W.H., and Teel, R.W. An organotypic in vitro model system for studying pulmonary surfactant production by type II alveolar pneumonocytes. Am Rev Respir Dis **113**, 17, 1976.

165. Andrade, C.F., Wong, A.P., Waddell, T.K., Keshavjee, S., and Liu, M. Cell-based tissue engineering for lung regeneration. Am J Physiol Lung Cell Mol Physiol **292**, L510, 2007.

166. Chen, P., Marsilio, E., Goldstein, R.H., Yannas, I.V., and Spector, M. Formation of lung alveolar-like structures in collagen-glycosaminoglycan scaffolds in vitro. Tissue Eng **11**, 1436, 2005.

167. Cortiella, J., Nichols, J.E., Kojima, K., Bonassar, L.J., Dargon, P., Roy, A.K., Vacant, M.P., Niles, J.A., and Vacanti, C.A. Tissue-engineered lung: an in vivo and in vitro comparison of polyglycolic acid and pluronic F-127 hydrogel/somatic lung progenitor cell constructs to support tissue growth. Tissue Eng **12**, 1213, 2006.

168. Price, A.P., England, K.A., Matson, A.M., Blazar, B.R., and Panoskaltsis-Mortari, A. Development of a decellularized lung bioreactor system for bioengineering the lung: the matrix reloaded. Tissue Eng Part A **16**, 2581, 2010.

169. Cortiella, J., Niles, J., Cantu, A., Brettler, A., Pham, A., Vargas, G., Winston, S., Wang, J., Walls, S., and Nichols, J.E. Influence of acellular natural lung matrix on murine embryonic stem cell differentiation and tissue formation. Tissue Eng Part A **16**, 2565, 2010.

170. Bonvillain, R.W., Danchuk, S., Sullivan, D.E., Betancourt, A.M., Semon, J.A., Eagle, M.E., Mayeux, J.P., Gregory, A.N., Wang, G., Townley, I.K., Borg, Z.D., Weiss, D.J., and Bunnell, B.A. A nonhuman primate model of lung regeneration: detergent-mediated decellularization and initial in vitro recellularization with mesenchymal stem cells. Tissue Eng Part A **18**, 2437, 2012.

171. Nichols, J.E., Niles, J., Riddle, M., Vargas, G., Schilagard, T., Ma, L., Edward, K., La Francesca, S., Sakamoto, J., Vega, S., Ogadegbe, M., Mlcak, R., Deyo, D., Woodson, L., McQuitty, C., Lick, S., Beckles, D., Melo, E., and Cortiella, J. Production and assessment of decellularized pig and human lung scaffolds. Tissue Eng Part A **19**, 2045, 2013.

172. Gilpin, S.E., Guyette, J.P., Gonzalez, G., Ren, X., Asara, J.M., Mathisen, D.J., Vacanti, J.P., and Ott, H.C. Perfusion decellularization of human and porcine lungs: Bringing the matrix to clinical scale. Journal Heart Lung Transplant **33**, 298, 2014.

173. Wagner, D.E., Bonenfant, N.R., Parsons, C.S., Sokocevic, D., Brooks, E.M., Borg, Z.D., Lathrop, M.J., Wallis, J.D., Daly, A.B., Lam, Y.W., Deng, B., Desarno, M.J., Ashikaga, T., Loi, R., and Weiss, D.J. Comparative decellularization and recellularization of normal versus emphysematous human lungs. Biomaterials **35**, 3281, 2014.

174. Daly, A.B., Wallis, J.M., Borg, Z.D., Bonvillain, R.W., Deng, B., Ballif, B.A., Jaworski, D.M., Allen, G.B., and Weiss, D.J. Initial binding and recellularization of decellularized mouse lung scaffolds with bone marrow-derived mesenchymal stromal cells. Tissue Eng Part A **18**, 1, 2012.

175. Nichols, J.E., Niles, J.A., Vega, S.P., and Cortiella, J. Novel in vitro respiratory models to study lung development, physiology, pathology and toxicology. Stem Cell Res Ther **4 Suppl 1**, S7, 2013.

176. Mishra, D.K., Thrall, M.J., Baird, B.N., Ott, H.C., Blackmon, S.H., Kurie, J.M., and Kim, M.P. Human lung cancer cells grown on acellular rat lung matrix create perfusable tumor nodules. Ann Thorac Surg **93**, 1075, 2012.

177. Petersen, T.H., Calle, E.A., Colehour, M.B., and Niklason, L.E. Matrix composition and mechanics of decellularized lung scaffolds. Cells Tissues Organs **195**, 222, 2012.

178. Wallis, J.M., Borg, Z.D., Daly, A.B., Deng, B., Ballif, B.A., Allen, G.B., Jaworski, D.M., and Weiss, D.J. Comparative assessment of detergent-based protocols for mouse lung de-cellularization and re-cellularization. Tissue Eng Part C Methods **18**, 420, 2012.

179. Bonenfant, N.R., Sokocevic, D., Wagner, D.E., Borg, Z.D., Lathrop, M.J., Lam, Y.W., Deng, B., Desarno, M.J., Ashikaga, T., Loi, R., and Weiss, D.J. The effects of storage and sterilization on de-cellularized and re-cellularized whole lung. Biomaterials **34**, 3231, 2013.

180. Sokocevic, D., Bonenfant, N.R., Wagner, D.E., Borg, Z.D., Lathrop, M.J., Lam, Y.W., Deng, B., Desarno, M.J., Ashikaga, T., Loi, R., Hoffman, A.M., and Weiss, D.J. The effect of age and

emphysematous and fibrotic injury on the re-cellularization of de-cellularized lungs. Biomaterials **34**, 3256, 2013.

181. Carvalho, T.C., Peters, J.I., and Williams, R.O., 3rd. Influence of particle size on regional lung deposition--what evidence is there? Int J Pharm **406**, 1, 2011.

182. Booth, A.J., Hadley, R., Cornett, A.M., Dreffs, A.A., Matthes, S.A., Tsui, J.L., Weiss, K., Horowitz, J.C., Fiore, V.F., Barker, T.H., Moore, B.B., Martinez, F.J., Niklason, L.E., and White, E.S. Acellular normal and fibrotic human lung matrices as a culture system for in vitro investigation. Am J Respir Crit Care Med **186**, 866, 2012.

183. Sun, H., Calle, E., Chen, X., Mathur, A., Zhu, Y., Mendez, J., Zhao, L., Niklason, L., Peng, X., Peng, H., and Herzog, E.L. Fibroblast engraftment in the decellularized mouse lung occurs via a beta1-integrin-dependent, FAK-dependent pathway that is mediated by ERK and opposed by AKT. Am J Physiol Lung Cell Mol Physiol **306**, L463, 2014.

184. Okano, T., Yamada, N., Sakai, H., and Sakurai, Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). J Biomed Mater Res **27**, 1243, 1993.

185. Yang, J., Yamato, M., Kohno, C., Nishimoto, A., Sekine, H., Fukai, F., and Okano, T. Cell sheet engineering: recreating tissues without biodegradable scaffolds. Biomaterials **26**, 6415, 2005.

186. Kanzaki, M., Yamato, M., Hatakeyama, H., Kohno, C., Yang, J., Umemoto, T., Kikuchi, A., Okano, T., and Onuki, T. Tissue engineered epithelial cell sheets for the creation of a bioartificial trachea. Tissue Eng **12**, 1275, 2006.

187. Kanzaki, M., Yamato, M., Yang, J., Sekine, H., Kohno, C., Takagi, R., Hatakeyama, H., Isaka, T., Okano, T., and Onuki, T. Dynamic sealing of lung air leaks by the transplantation of tissue engineered cell sheets. Biomaterials **28**, 4294, 2007.

188. Kanzaki, M., Yamato, M., Takagi, R., Kikkawa, T., Isaka, T., Okano, T., and Onuki, T. Controlled collagen crosslinking process in tissue-engineered fibroblast sheets for preventing scar contracture on the surface of lungs. J Tissue Eng Regen Med **7**, 383, 2013.

189. Maeda, M., Yamato, M., Kanzaki, M., Iseki, H., and Okano, T. Thoracoscopic cell sheet transplantation with a novel device. J Tissue Eng Regen Med **3**, 255, 2009.

190. Delaere, P.R., and Van Raemdonck, D. The trachea: the first tissue-engineered organ? J Thorac Cardiovasc Surg **147**, 1128, 2014.

191. Berube, K., Pitt, A., Hayden, P., Prytherch, Z., and Job, C. Filter-well technology for advanced three-dimensional cell culture: perspectives for respiratory research. Altern Lab Anim **38 Suppl 1**, 49, 2010.

192. de Jong, P.M., van Sterkenburg, M.A., Hesseling, S.C., Kempenaar, J.A., Mulder, A.A., Mommaas, A.M., Dijkman, J.H., and Ponec, M. Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface. Am J Respir Cell Mol Biol **10**, 271, 1994.

193. Grainger, C.I., Greenwell, L.L., Lockley, D.J., Martin, G.P., and Forbes, B. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. Pharm Res **23**, 1482, 2006.

194. The Principles of Humane Experimental Technique. University of Michigan: Methuen & Co., 1959.

195. Wells, D.J. Animal welfare and the 3Rs in European biomedical research. Ann NY Acad Sci **1245**, 14, 2011.

196. Van Goor, F., Hadida, S., Grootenhuis, P.D., Burton, B., Cao, D., Neuberger, T., Turnbull, A., Singh, A., Joubran, J., Hazlewood, A., Zhou, J., McCartney, J., Arumugam, V., Decker, C., Yang, J., Young, C., Olson, E.R., Wine, J.J., Frizzell, R.A., Ashlock, M., and Negulescu, P. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. Proc Natl Acad Sci USA **106**, 18825, 2009.

197. Goto, Y., Noguchi, Y., Nomura, A., Sakamoto, T., Ishii, Y., Bitoh, S., Picton, C., Fujita, Y., Watanabe, T., Hasegawa, S., and Uchida, Y. In vitro reconstitution of the tracheal epithelium. Am J Respir Cell Mol Biol **20**, 312, 1999.

198. Le Visage, C., Dunham, B., Flint, P., and Leong, K.W. Coculture of mesenchymal stem cells and respiratory epithelial cells to engineer a human composite respiratory mucosa. Tissue Eng **10**, 1426, 2004.

199. Rothen-Rutishauser, B.M., Kiama, S.G., and Gehr, P. A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol **32**, 281, 2005.

200. Herzog, F., Clift, M.J., Piccapietra, F., Behra, R., Schmid, O., Petri-Fink, A., and Rothen-Rutishauser, B. Exposure of silver-nanoparticles and silver-ions to lung cells in vitro at the air-liquid interface. Part Fibre Toxicol **10**, 11, 2013.

201. Elliott, M.J., De Coppi, P., Speggiorin, S., Roebuck, D., Butler, C.R., Samuel, E., Crowley, C., McLaren, C., Fierens, A., Vondrys, D., Cochrane, L., Jephson, C., Janes, S., Beaumont, N.J., Cogan, T., Bader, A., Seifalian, A.M., Hsuan, J.J., Lowdell, M.W., and Birchall, M.A. Stem-cell-based, tissue engineered tracheal replacement in a child: a 2-year follow-up study. Lancet **380**, 994, 2012.

202. Vogel, G. Trachea transplants test the limits. Science **340**, 266, 2013.

203. Vo, T.N., Kasper, F.K., and Mikos, A.G. Strategies for controlled delivery of growth factors and cells for bone regeneration. Adv Drug Deliv Rev **64**, 1292, 2012.

204. Tierney, E.G., Duffy, G.P., Cryan, S.A., Curtin, C.M., andO'Brien, F.J. Non-viral gene-activated matrices-next generation constructs for bone repair. Organogenesis **9**, 22, 2013.