Tissue engineering for pulmonary diseases: Insights from the laboratory

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ABSTRACT

Recent advances in stem cell research and tissue engineering have opened new paradigms for future therapies towards many intractable diseases. Many tissue engineering approaches are also applied in the pulmonary research field. Several materials have been utilized as scaffolds to support lung tissue engineering to recapitulate the three-dimensional (3D) structure of the lung. Natural products and synthetic polymers are the two major components of the scaffold materials. Decellularization of allogeneic or xenogenic donor lungs is also utilized to obtain biological 3D matrix scaffolds. Decellularized lungs are recellularized with stem or progenitor cells. Cell sources are the key components for tissue engineering. The best cell source for tissue engineering is autologous cells obtained from patients because it does not induce an immunological response after transplantation. However, the stem/progenitor population in adult organs is generally small, and their capacity for proliferation or differentiation is limited. Knowledge about the endogenous stem/progenitor population in lung tissue has been expanded recently. Although the lung is the most challenging organ for tissue engineering because of its complex 3D structure and more than 40 different cell types, several breakthroughs in respiratory research have been made. These results give us a greater understanding of the possibilities and the limitations of tissue engineering for pulmonary diseases.

Key words: chronic obstructive pulmonary disease, progenitor cells, scaffold, stem cells, tissue engineering.

INTRODUCTION

The morbidity and mortality from pulmonary diseases are increasing worldwide because of an increase in the elderly population, high rates of smoking and exposure to air pollution. However, most of the clinical therapies for pulmonary diseases are mainly targeted towards improving the symptoms, and there are no therapies that can cure intractable lung diseases. Therefore, we need a new approach to treat pulmonary diseases.

Recent advances in stem cell research and tissue engineering, such as the establishment of inducible pluripotent stem (iPS) cells, open a new paradigm for future therapies of many intractable diseases. Bone marrow transplantations in leukaemia patients and skin grafts for burn injuries are already standard therapies. Recent reports using embryonic stem (ES) cells or iPS cells to treat severe diseases are promising future therapies. Some clinical trials using stem cells have already been started, although the results are controversial.

In contrast with these advances in other organs, stem cell research and tissue engineering in pulmonary diseases are limited. One reason is the complexity of the lung’s structure. The lung is a complex organ with a three-dimensional (3D) structure that is composed of over 40 cell types. Additionally, to mimic a functional lung, a perfect matching of gas and blood is needed. Therefore, the lung is the most challenging organ for tissue engineering.

The present review provides an overview of recent knowledge about tissue engineering approaches for pulmonary diseases.

TISSUE ENGINEERING

To reconstruct a tissue for clinical use, stem or progenitor cells are seeded on a biological scaffold with extracellular matrix (ECM) proteins, and these cells are expected to proliferate and differentiate into...
the proper cell populations. Organs with simple structures, such as the skin and trachea, are easily reconstructed by this strategy and utilized for clinical applications.

Because of the simple structures of the large airways in contrast with the distal lungs, several trials using tissue-engineered trachea or the main bronchus have already been performed.7-10 To engineer an artificial airway, a scaffold and stem/progenitor cells are required. The materials of the scaffold are important for proper cell adhesion, proliferation and differentiation. The transport of nutritional and growth factors is a required function for the scaffolds. There are two major scaffolds that are utilized for clinical trials: natural matrices and synthesized materials.10,11 Synthetic scaffolds are made of polymers, such as polypropylene or polyglycolic acid, or hydrogel. Decellularized natural matrices that were obtained from human donors are another source of material for the scaffold. The main cell sources for an engineered airway are allogeneic or autologous isolated chondrocytes and epithelial cells. Recently, mesenchymal stem cells (MSC) or iPS cells were experimentally used. In addition to the two key components, a scaffold and cells, a bioreactor for culturing the cells with the scaffold before transplantation is another matter in need of technical advancement.

In contrast with the large airways, the distal lungs are complicated organs with a 3D structure that is hard to reconstruct. Therefore, several different approaches were developed and tested to mimic the distal lungs (Fig. 1).

On-chip

The distal lung is a complex tissue that contains several hundred millions of alveoli with capillary networks for the contact of air with blood and is composed of many types of cells. Microdevices to mimic such conditions have been reported.13-18 Most of the devices are designed as a cell co-culture system that consists of monolayers of epithelial and endothelial cells on opposite sides of a permeable membrane. Polycarbonate or polydimethylsiloxane membranes that are coated with ECM proteins are used. Huh et al. developed a unique lung cell co-culture system that also mimics breathing movements to induce mechanical stress on the cells (Fig. 2).15 Lehmann et al. established a triple cell co-culture model that consisted of alveolar epithelial cells, macrophages and dendritic cells to mimic the immunological barrier function of the alveolar space.18 However, alveoli are constructed not only with epithelial and endothelial cells but also with mesenchymal cells, such as fibrocytes and pericytes; therefore, the development of a multicell co-culture system that includes these three cell types is needed in the future.

Co-culture systems in a dish or as an on-chip device would be useful for analysing cellular behaviour in lungs, and for drug discovery and screening.

ECM

Different ECM proteins induce different stimuli on the cells, and each organ has a distinct combination

Figure 1 Tissue engineering approaches to mimic the distal lungs (modified from Fujino et al.12).
of ECM proteins that are suitable for the organ-specific cells. For example, an ECM composition that is similar to the lung structure is a preferred and necessary substrate for the differentiation of respiratory epithelial cells from ES cells.

The ECM is one of the key components that provides the tissue structure and regulates cellular behaviour, including proliferation and proper differentiation. The lung ECM mainly contains collagen, elastin, laminin, fibronectin, entactin, proteoglycans and tenascin.

Collagen is composed of at least 19 different subtypes. The main subtypes that make up the lung’s structure are collagens I, III, IV and V. Types I, III and V are fibrillar collagens and are needed for structural components. Collagen IV is a nonfibrillar type and a key component of the basement membrane.

Elastin is important for the intrinsic recoil of the lung tissue. Elastic fibres are constructed with elastin and microfibrils, such as fibrillin. Elastic fibres are stable constructs; however, elastin can be degraded by elastases, such as metalloproteinases.

Laminin consists of at least 15 isoforms that result from various combinations of the α, β and γ chains. The prominent role of laminin is to provide an anchor for cells on the basement membrane. The major cell surface receptors for laminin are the integrins. Laminin is expressed not only on the basement membrane but also on alveolar epithelial cells. Laminin-5 is produced from alveolar type II epithelial (ATII) cells to facilitate migration to repair alveoli. Laminin-5 also plays a role in epithelial differentiation. Surfactant production was increased when ES-derived ATII cells were cultured on Laminin-5. Culturing of cells on laminin-coated dishes enhanced the efficiency of ATII differentiation from alveolar epithelial progenitor cells.

Fibronectin can affect cell adhesion, morphological changes, migration, proliferation and differentiation. The addition of soluble fibronectin enhances cell spreading and motility of wounded ATII cells in culture, which suggests an important role in alveolar repair. Other ECM proteins also induce proliferation and differentiation signals for lung cells.

The ECM proteins play a critical role in proper cell differentiation and function. However, an optimized combination or ratio of ECM proteins for lung tissue engineering has not been established yet. Further research in this field is necessary for the usage of an ECM in scaffolds or decellularized lungs.

**Scaffolds**

Recapitulating the 3D structure of a lung in a cell culture system has been attempted. Pedersen et al. demonstrated monolayered epithelial spheres using a free-floating suspension culture system. However, a scaffold is needed for use in lung regeneration therapy because cells need a scaffold to proliferate, and the elasticity provided by a scaffold is also needed for the proper function of the lung. Therefore, several materials have been utilized for scaffolds to support lung tissue engineering. Two major components of the scaffold materials are natural products and synthetic polymers. Single or mixed ECM proteins, such as collagen, Gelfoam (gelatin) and Matrigel (collagen, laminin and others), are used as natural scaffolds. Synthetic polymers are also used as a scaffold.

Culturing foetal rat lung cells in Gelfoam sponge leads to the development of alveolar-like structures and lined epithelial cells containing lamellar bodies. Andrade et al. injected Gelfoam sponges supplemented with foetal rat lung cells into rat lungs. The implanted Gelfoam showed porous structures similar to alveoli and was connected to pulmonary circulation. More than 100 days after the implantation, the Gelfoam sponge started to degrade and left behind a ‘lung tissue’-like structure. Interestingly, when Gelfoam sponges without foetal cells were implanted, endogenous lung cells migrated into the Gelfoam, but the sponge degraded rapidly and did not form an alveolar-like structure. These results suggest that both a scaffold and stem/progenitor cells are important for scaffold-based lung engineering.

Degradable synthetic polymers are also used for tissue engineering of the lung. Polylactic acid, polyglycolic acid and polylactic-co-glycolic acid are easily degraded by acid hydrolysis to lactic and glycolic acids. The degradation rate can be determined by the changing molecular weights of the polymers and the ratio of glycolic acid to lactic acid.
evaluation of the differentiation and tissue formation capacities of somatic lung progenitor cells that were isolated from adult sheep lungs. Progenitor cells were mixed with polyglycolic acid or Pluronic F-127, and implanted in the backs of nude mice or within the lungs of sheep. The implanted cells differentiated into several kinds of lung cells that expressed the Clara cell secretary protein or the surfactant protein C. The progenitor cells that were mixed with Pluronic F-127 showed a more alveolar-like structure.

Most previous reports used foetal lung cells to engineer lung tissues with scaffolds. As Andrade et al. reported, adult endogenous lung cells are not sufficient for tissue engineering. However, the use of foetal lung cells is a serious limitation of this strategy. Therefore, another sophisticated methodology that uses lung epithelial cells instead of foetal cells is needed. Franzdóttir et al. established a 3D co-culture system using lung epithelial cells that were cultured in an endothelial-rich stroma. This mimics lung development in which a small portion of the foetal digestive tract invades the vascular-rich stroma. Branching morphogenesis was also demonstrated in this elegant co-culture system (Fig. 3). Although developing a whole lung structure that includes pulmonary circulation is a distant problem, this is an interesting innovation for lung biology.

### Decellularized lung

New vascularization from the recipient’s vasculature is required for transplantation of ‘live’ engineered tissues. It can be easily achieved in tissues or organs with simple structures that only need an oxygen and nutrition supply. The earlier mentioned tissue-engineered trachea is a good example of a tissue that needs a blood supply for only oxygenation and nutrition. However, functional lungs require a perfect match of perfusion with ventilation, and therefore, engineering 3D-structured lungs is a difficult task.

Recently, decellularization of allogeneic or xenogenic donor organs, such as the heart, kidney and liver, were utilized to obtain biological 3D matrix scaffolds. Decellularized organs are then recellularized with progenitor cells, ES cells or iPS cells.

Decellularization is achieved by either a physical, an enzymatic or a chemical method. The method is selected to maximize the efficacy of cell removal with a minimum effect on the native ECM. Any remaining cell membranes or intracellular components can be immunogenic antigens, which could produce an inflammatory response or rejection. However, excessive destruction of the ECM causes the improper proliferation or differentiation of implanted progenitor cells.

Lung decellularization is achieved by perfusing both the airway and circulation with several detergents, such as sodium dodecyl sulfate, Triton X-100 or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The ECM proteins that remain differ according to the detergent used. Sodium dodecyl sulfate-based decellularization leads to more collagen loss from the lung construct and reduced mechanical strength compared with a CHAPS-based method. More than 50% of elastin and 95% of proteoglycans are lost with both methods.

The cell source for recellularization is another important issue for this technique. Petersen et al. used neonatal rat lung epithelial cells and microvascular lung endothelial cells to repopulate the acellular lungs. When the lungs were placed in a bioreactor with ventilation and circulation, the implanted epithelial cells differentiated into bronchial epithelial cells and surfactant-producing alveolar cells. The recellularized lungs can be transplanted in vivo and contribute to the gas exchange. Ott et al. also performed a similar experiment. They seeded human umbilical cord endothelial cells (HUVEC) in the pulmonary circulation of decellularized rat lungs and then implanted A549 (adenocarcinomic human alveolar basal epithelial) cells into the airway. Surprisingly, the implanted A549 cells differentiated into a multilayered squamous epithelium in the upper airways and single-layered alveolar type I and ATII epithelial cells in the distal lungs. Interestingly, vimentin-positive mesenchymal cells were also observed in the recellularized lung parenchyma, although it was not clear whether the origin was the A549 or human umbilical cord endothelial cells.

Decellularized lungs provide an opportunity for a direct connection to circulation. However, there are still many limitations remaining in this approach. First, the cell sources for the recellularization are not yet optimized. Most studies have utilized foetal lung cells or cancer cell lines, and they are not suitable for clinical use. Recent reports about protocols using MSC and iPS cells are promising. Second, it is not clear how long the lung matrix lasts or whether the original matrix is replaced by newly synthesized ECM proteins from the seeded cells. Original reports demonstrated that the function of implanted
recellularized lungs in vivo was limited to only a few hours to days because of lung oedema.\textsuperscript{42,43,46} Third, because allogeneic materials cannot be obtained clinically, the recipient’s response to the ECM proteins of the xenograft should be clarified, as the ECM of the xenograft may induce an inflammatory response.\textsuperscript{46}

**Xeno-embryo**

A different approach for tissue engineering is the use of a xeno-embryo as a biological reactor. Metanephroi that were derived from embryos and transplanted into the omentum demonstrated a normal kidney structure using the blood supply from the recipient’s arteries.\textsuperscript{47} Yokoo et al. injected human MSC into a rat embryo and performed a whole embryo culture. After 24 hours of culturing, kidney rudiments were isolated from the embryo and implanted into the omentum of an adult rat. A new kidney that was composed of human MSC developed in the omentum.\textsuperscript{48} This could be a potential strategy to engineer 3D-structured organs.

**CELL SOURCES FOR ENGINEERING LUNG TISSUE**

Cell sources are the critical issue for tissue engineering. The best cell source for tissue engineering is autologous cells obtained from the patients because they do not induce an immunological response after transplantation. However, stem/progenitor populations in adult organs are generally small, and the capacity for proliferation and differentiation is restricted. In addition, knowledge about the endogenous stem/progenitor population of the lung tissue is limited.

**Lung endogenous stem cells**

*Alveolar epithelial progenitor cells*

An alveolar type I epithelium injury is occurred in severe alveolar damage. ATII cells are believed to proliferate and subsequently differentiate to replace the injured type I cells (Fig. 4). During this process, a portion of the type II cell population becomes hyperplastic. Both of these events are frequently observed in a diseased or damaged lung, such as acute respiratory distress syndrome.

Regenerated and repaired alveoli were known to be composed of both bone marrow-derived cells and cells of a non-bone marrow origin based on experiments using mice, which bone marrow was reconstituted with green fluorescent protein-transgenic mice.\textsuperscript{49–52} This suggests that resident lung cells, including ATII cells\textsuperscript{53} and lung endogenous stem cells, contribute to alveogenesis. However, the potential role for lung endogenous stem cells to replace damaged ATII cells is not yet clear. Stem cell antigen-1 (Sca-1)-positive cells have been recently proposed to be endogenous stem cells of the lung in mice.\textsuperscript{54–57} It is known that a lung injury alone increased the number of cells with stem cell markers, such as Sca-1 and c-kit, within the lungs (Fig. 5).\textsuperscript{58} Most Sca-1+ cells were lung endogenous cells, while most of the c-kit+ cells were of bone marrow origin (Fig. 5), suggesting the importance of endogenous stem cells.

The response of supposed lung endogenous stem cells (Sca-1+/SP-C+/Clara cell secretary protein+/CD45− cells) and ATII cells was evaluated during the compensatory lung growth after a pneumonectomy in mice.\textsuperscript{53} Although the numbers of both Sca-1+ cells and ATII cells increased during the compensatory lung growth, the contribution of Sca-1+ cells was
0–25%, while the ATII cells were necessary for regrowth based on a cell kinetic model.

In spite of the increasing reports for stem cells in mice lungs, knowledge about lung endogenous stem cells in human is limited. There are two reasons for this limitation: (i) there is no specific marker for endogenous stem cells from human lungs; and (ii) the availability of human lung tissues is limited. Therefore, only a few reports of human lung endogenous stem cells are available now. One of the potential human lung stem cells is alveolar progenitor cells (AEPC) isolated from adult human lungs. AEPC has an epithelial phenotype with a MSC character. According to microarray analysis, AEPC shares many genes with ATII cells and mesenchymal stem cells, which suggests an overlapping phenotype with both the alveolar epithelium and the mesenchyme in these cells. The AEPC was present in lung fibrotic lesions and in some types of adenocarcinomas. The transitional phenotype between the epithelium and mesenchyme of AEPC suggests that these cells act as lung endogenous stem cells in tissue repair and carcinogenesis. Mesenchymal properties, such as anti-apoptotic activity and motility, may be beneficial for a functional epithelial progenitor to involve in alveolar repair. Therefore, AEPC could be a good candidate for the cell source for engineered lung.

MSC

MSC is one of the well-known populations of endogenous stem cells and present in many organs, such as bone marrow, skin and fat tissues. MSC is known to have a capacity for self-renewal and an ability to differentiate into cells of the mesenchymal lineage. Presence of MSC was also reported in lungs, and lung MSC can be isolated from neonate lungs and bronchoalveolar lavage fluid. MSC derived from surgical human lungs can be differentiated into aquaporin 5- and Clara cell secretory protein-expressing alveolar type I epithelial cells.

The role of MSC in lung regeneration or regrowth is not clear yet; however, the beneficial role of MSC on lung injuries has been extensively evaluated, and this advantage is mainly provided by immunomodulatory effect of MSC.

Although the potential roles of MSC in a cell therapy are well reported, capability of MSC as a cell source for tissue engineering is not clear.

c-Kit-positive cells

c-Kit is a transmembrane tyrosine kinase receptor, and its expression is detected in foetal tissue development, such as in lung. Binding of its ligand, a stem cell factor, promotes cell proliferation and differentiation. After birth, c-kit is persistently expressed in the germ cells, melanocytes and mast cells of the adult. Recently, some rare c-kit-expressing cell populations were reported as endogenous tissue stem/progenitor cells, such as in the central nervous system, heart and liver.

The main population of c-kit-positive cells in adult human lungs is composed of mast cells, but other cell types including lung cancer stem cells also express c-kit. Lindsey et al. identified quantitative trait loci of the mouse genome that were associated with the development of spontaneous airspace enlargement and identified c-kit as a key molecule.

Recently, Kajstura et al. isolated c-kit-positive, lineage-negative cells from adult human lungs and expanded them in culture. When this expanded cell population was injected in injured lungs of C57BL/6 mice, the cells differentiated not only into epithelial cells but also into mesenchymal and endothelial cells without rejection. It is not yet clear whether the naive c-kit-positive, lineage-negative cells within lungs have the same capacity as stem cells in situ. The stemness of the c-kit-positive cells may be acquired with the cell culture conditions. Although the presence and the characteristics of the c-kit-positive stem cells within human lung are still under discussion, the clonal capacity of the cells shows a potential usage in tissue engineering.
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**Exogenous stem cells**

**Bone marrow-derived cells**

Bone marrow is the main source of exogenous stem/progenitor cells. Bone marrow-derived stem/progenitor cells are released into the circulation upon inflammatory stimuli and that these cells facilitate the recovery form and repair of the inflammatory process. An increase in bone marrow-derived endothelial cells within the lung capillary walls was observed during alveologenesis in mouse models of lung regeneration by treatment with granulocyte colony stimulating factor, or adenomedullin, and then, the number of bone marrow-derived cells in the alveolar walls gradually decreases after treatment. A Clara cell secretory protein-expressing progenitor population in the bone marrow and its role in bronchial repair was also reported.

Because of the ease of obtaining bone marrow-derived cells, including MSC, these cells are a potential cell source for tissue engineering.

**ES cells**

ES cells are derived from the inner cell mass of a blastocyst-stage embryo and can differentiate into any type of cell. ATII cells and bronchial epithelial cells that were differentiated from human ES cells have been reported. However, none of the reports succeeded in differentiating ATII cells with a 100% efficacy, and therefore, the differentiated cells may be contaminated with some pluripotent cells. Differentiation into the endodermal lineage from ES cells is known to be harder than differentiation into the mesodermal or ectodermal lineage. Because lung epithelial cells originate from the endoderm, differentiating lung epithelial cells may be difficult and requires more technical advances for the culture conditions. In addition, although ES cells are truly pluripotent cells and can be a source for any lung cell, clinical applications are limited by ethical issues and the risk of teratoma formation.

**iPS cells**

iPS cells have been generated with the Yamanaka factors, Oct3/4, Sox2, Klf4 and c-Myc (recently Gli1) from several somatic cell types. iPS cells have a pluripotency similar to ES cells but do not require the destruction of an embryo. The most important advantage for tissue engineering is the ability to establish the patient’s own autologous stem cells. There are several reports about iPS cells that were generated from patients with pulmonary diseases, such as cystic fibrosis, α-1 antitrypsin deficiency-related emphysema and surfactant protein B deficiency.

Due to the limited knowledge about the endogenous progenitor cells of the lung and the proper methodology for differentiation, a tissue engineering approach for the lung that uses iPS cells has not been reported yet.

**Lung cancer stem cells**

Cancer stem cells get much attention recently. Lung cancer stem cells may be provided from the lung endogenous stem cells described earlier because the same stem cell markers, such as c-kit and CD133, are also expressed in lung cancer cells. Therefore, using endogenous stem cells isolated from a patient may require caution due to carcinogenesis. Further research is needed in this area.

The stem/progenitor cell population for treating lung diseases was reviewed well by Moodley et al., and the recent development of a novel cell isolation technique from human distal lung tissues should enhance our knowledge on the cell characteristics of the lungs for tissue engineering.

**TISSUE ENGINEERING FOR PULMONARY DISEASES**

Chronic obstructive pulmonary disease is becoming a leading cause of death worldwide. Emphysema is one of the characteristics of chronic obstructive pulmonary disease. To reverse the destruction of the lung structure, regenerative approaches have been extensively reported for small animal models. However, clinical applications have not yet been established.

Two main strategies for tissue engineering have been studied for the treatment of chronic obstructive pulmonary disease. The first method is the implantation of a scaffold with stem cells within alveolar spaces. The other strategy is the use of cell sheets to enhance lung regrowth after a volume reduction surgery.

Andrade et al. implanted Gelfoam sponges supplemented with foetal rat lung cells into adult rat lungs. The implanted sponges reached the alveolar spaces, and the cells inside formed an alveolar-like structure. Neovascularization was also observed in the sponge. The Gelfoam degraded several months after the implantation. Although a careful selection of the materials for the sponge and progenitor cells for the implant is needed for a clinical application, this is a potential approach for a tissue engineering treatment for an emphysematous lung.

Volume reduction surgery in patients with pulmonary emphysema increases the residual volume and improves the symptoms. However, because of high morbidity and mortality rates, this procedure is not commonly performed on patients. Shigemura et al. cultured adipose tissue-derived stromal cells on a polyglycolic acid felt sheet and then covered the cut edge of the remaining lung tissue after a lung-volume reduction surgery in rats. The alveolar and vascular regeneration was enhanced in the area covered by the sheet. Hepatocyte growth factor that
was secreted from the adipose tissue-derived stromal cells played a role in this accelerated lung regrowth after the surgery. This new strategy using a tissue engineering approach may improve the outcome of a volume reduction surgery for emphysema patients.

CONCLUSION

Regenerative medicine is a promising strategy for many diseases. Knowledge of and technological advances using ES and iPS cells are growing, and clinical applications will follow in the near future. In contrast with this progress in stem cell research, a tissue engineering approach in pulmonary diseases is limited and far from clinical use. Although many challenges have been overcome and several breakthroughs in respiratory research have been made, there are still obstacles; therefore, we need a new understanding of the possibilities and limitations of tissue engineering for pulmonary diseases. In the near future, new technologies will be developed for pulmonary medicine.

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