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Therapeutic Potential of Lung Epithelial Progenitor Cells Derived from Embryonic and Induced Pluripotent Stem Cells

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Abstract

Embryonic stem (ES) cells derived from preimplantation blastocysts and induced pluripotent stem (iPS) cells generated from somatic cell sources are pluripotent and capable of indefinite expansion in vitro. They provide a possible unlimited source of cells that could be differentiated into lung progenitor cells for potential clinical use in pulmonary regenerative medicine. Because of inherent difficulties in deriving endodermal cells from undifferentiated cell cultures, applications using lung epithelial cells derived from ES and iPS cells have lagged behind similar efforts devoted to other tissues, such as the heart and spinal cord. However, during the past several years, significant advances in culture, differentiation, and purification protocols, as well as in bioengineering methodologies, have fueled enthusiasm for the development of stem cell-based lung therapeutics. This article provides an overview of recent research achievements and discusses future technical challenges that must be met before the promise of stem cell applications for lung disease can be realized.

Keywords

bioengineering; COPD; cystic fibrosis; alpha-1-antitrypsin deficiency; surfactant protein deficiency

INTRODUCTION

Respiratory diseases are a major cause of mortality and morbidity worldwide, and current treatments offer no prospect of cure or disease reversal. Current therapies rely on management of symptoms or lung transplantation. Unfortunately, patients requiring lung

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replacement will often succumb to their disease owing to the scarcity of donated lung tissue suitable for transplantation. There is, therefore, a pressing need to develop novel therapeutic strategies to treat lung disease. Advances in stem cell research during the past decade have fueled a tremendous amount of excitement over the possibility of using adult or embryonic sources of stem cells to repair or regenerate damaged organs. The complex structure of the lung, the plethora of different cell types comprising the lung parenchyma, and the incomplete understanding of endogenous lung repair at a molecular and cellular level have made the development of stem cell therapeutics for pulmonary medicine appear rather daunting. Despite these challenges, significant progress has been achieved during the past several years in identifying early progenitor lung cells, developing procedures for the generation and purification of these progenitor cells from embryonic stem (ES) and induced pluripotent stem (iPS) cell sources, evaluating the therapeutic potential of these cells in animal models of lung disease, and their application in lung tissue regeneration using bioengineered three-dimensional matrices and scaffolding. This article reviews these recent developments in lung stem cell research and discusses future technical and scientific hurdles that must be cleared before stem cell therapeutics could be used for clinical treatment of acute, chronic, and inherited pulmonary diseases.

ENDOGENOUS LUNG EPITHELIAL PROGENITOR CELLS

The adult lung is a complex three-dimensional structure that is traditionally viewed as two major anatomically distinct regions—the conducting airways and the gas-exchanging airspaces, both of which are covered by a continuous layer of several types of endoderm-derived epithelial cells (Figure 1). Based on differences in epithelial cellular composition, the conducting airways can be further divided into two distinct regions: the proximal cartilaginous airway and the distal bronchioles. The proximal cartilaginous airways (trachea and bronchi) are lined mainly by ciliated and basal epithelial cells, as well as with lesser numbers of secretory goblet epithelial cells. Submucosal glands are also present in the cartilaginous airways. In contrast, the distal bronchioles (bronchioles, terminal bronchioles, and respiratory bronchioles) are lined predominantly by ciliated epithelial cells and by nonciliated columnar Clara cells. There is also a variety of less frequent cell types throughout the conducting airways, of which the neuroendocrine cells have attracted the most attention. The gas-exchanging airspaces (alveolar region) make up >99% of the surface area of the lung and are lined by a thin epithelium consisting of flattened squamous (type I) and cuboidal (type II) pneumocytes.

The lung epithelium undergoes a slow but constant renewal under normal healthy conditions, with turnover thought to occur every 30 to 50 days (1). Unless the injury is too severe or chronic, the lung epithelium responds to damage by vigorously reestablishing an epithelial layer with normal structure and function. To date, most studies indicate that the damaged lung epithelium is repaired by resident lung progenitor cells serving as the source of the new epithelial cell population, with only a possible minor contribution coming from circulating or bone marrow–derived stem/progenitor cells. Moreover, current evidence suggests that these adult resident lung progenitor cells are not identical to the embryonic cells that form lung tissue during embryonic development (2). Instead, cell lineage studies and mouse injury models have suggested that these lung cells are made up of divergent

progenitor cells that reside in discrete microenvironmental niches located in particular areas of the respiratory tree. Despite the generally accepted notion that the adult lung epithelium is repaired by resident epithelial progenitor cells, the identity of these cells and the location and molecular and cellular composition of their niches within the respiratory tree remain ill defined. In the trachea, the basal cell and the so-called intermediate cell (located just above the basal cell) are thought to have the capacity to produce all the major epithelial cells in the trachea, including basal, ciliated, and goblet cells (3). Bromodeoxyuridine labeling in a mouse model of naphthalene-induced tracheal injury indicated the presence of a putative stem cell niche in the tracheal submucosal glands that could possibly harbor a subpopulation of basal progenitor cells to serve as the cell source for the production of basal or intermediate cells in the proximal cartilaginous airway (4). Clara cells have traditionally been accepted as progenitor cells of the bronchiolar airways (5, 6). Studies in mice in which Clara cells have been depleted by naphthalene injury suggest the presence of a subset of injury-resistant Clara cells that have the ability to regenerate the bronchiolar epithelium (7). These cells are located within discrete microenvironments within bronchioles that include neuroepithelial bodies and bronchioalveolar duct junctions. Other investigations have also provided evidence of a progenitor pool of Clara-like cells in the bronchioalveolar duct junctions of mice (8, 9). Interestingly, these cells, in addition to expressing Clara cell secretory protein (CCSP), were found to express surfactant protein C (SPC), which has been traditionally considered a protein restricted in expression to alveolar type II cells. These CCSP/SPC “double-positive” cells were proposed to act as progenitor cells for not only the bronchiolar epithelium but also the alveolar epithelium. However, a subsequent study reported that it was unlikely that these double-positive cells served as progenitor cells for the alveolus (10).

In the gas-exchanging region of the lung, alveolar type II epithelial (ATII) cells have been traditionally considered the progenitor cell of the alveolar epithelium, based on data published by numerous laboratories. Collectively, these studies have shown that ATII cells exhibit biological hallmarks of progenitor cells, including the ability to proliferate as well as differentiate into other cell types (ATI cells). Moreover, animal models of lung injury have shown that ATII cells are critical in repopulating the injured alveolus, not only by generating new ATII cells but also by differentiating into and replacing the thin, injury-prone ATI cells. It remains uncertain if all ATII cells or only a putative sub-population of ATII cells act as progenitor cells for the alveolar epithelium (11).

EXOGENOUS LUNG EPITHELIAL PROGENITOR CELLS

The repair capacity provided by endogenous lung epithelial progenitor cells is often insufficient. Inadequate, delayed, or impaired re-epithelialization of the injured lung is regarded as a key factor in the pathogenesis of several life-threatening pulmonary diseases, such as chronic obstructive pulmonary disease (COPD). Moreover, the natural repair capacity provided by endogenous epithelial progenitor cells appears to diminish with age. It is reasonable to propose that the repair process provided by the lung epithelial progenitor cells could be greatly enhanced or restored by the introduction of endogenous epithelial progenitor cells to debilitated lungs, thereby reversing the effects of injury, disease, or aging. Although this approach is promising in theory, it would be very difficult to obtain sufficient

numbers of primary lung epithelial progenitor cells that could be used effectively and routinely in most clinical settings. Recent investigations to resolve this problem have focused on generating lung epithelial progenitor cells from differentiated cultures of embryonic stem cells. Moreover, with the recent discovery that iPS cells can be created from dermal skin fibroblasts, investigations have begun to examine the possibility of generating lung progenitor epithelial cells from somatic cells. If these efforts succeed, patient-specific iPS cells could be obtained, thereby avoiding the immune rejection problems that might occur if heterologous sources of embryonic stem cells were employed. Moreover, iPS cells offer the possibility of generating gene-corrected, patient-specific lung progenitor cells from individuals with genetic diseases affecting the lung, including cystic fibrosis, alpha-1-antitrypsin deficiency, and surfactant protein deficiencies.

From Embryonic Stem Cells

Embryonic stem (ES) cells are undifferentiated, pluripotent cells (12), isolated from the inner cell mass of preimplantation blastocyst-stage embryos (5 to 8 days postconception in humans). Under well-defined culture conditions, ES cells can be maintained indefinitely as rapidly proliferating undifferentiated cells. In their undifferentiated state, they maintain the ability to give rise to cells of all three embryonic germ layers (ectodermal, mesodermal, and endodermal). The potential clinical use of ES cell-derived progenitor cells to regenerate or repair damaged tissue has fueled a tremendous amount of excitement and research activity in numerous medical disciplines. However, despite the plethora of documented research efforts devoted to other tissues, such as the heart and spinal cord, research publications examining the use of ES cells for pulmonary regenerative medicine have remained scarce. Although there are numerous political and financial issues that partly explain the lack of ES cell research in pulmonary medicine, the lack of progress is also due to the complex structural and cellular composition of the lung and the limited information regarding the identity of lung progenitor cells. Another factor that has slowed research efforts is the finding that endodermal cells, such as lung epithelial cells, are much more difficult to generate from undifferentiated ES cell cultures than are mesodermal and ectodermal cells (13). During embryonic development, the endoderm is the last of the three germ layers to form, and its cellular specification process is complex. As a consequence, the development of differentiation and purification procedures for ES cell-derived lung epithelial cells, compared to cardiomyocytes and neuronal cells, has proven to be especially difficult.

ATII cells are arguably the best-characterized cell type in the lung. Moreover, their progenitor properties are well documented and the potential high impact of their clinical applications well accepted. It is easy to understand why the first report in 2002 (14), as well as subsequent investigations by other laboratories, focused almost entirely on the generation of ATII cells from ES cell cultures (15–17). The generation of ATII cells from differentiated ES cell cultures has continued to be problematic, with the percentage of ATII cells in the differentiated cultures remaining a minority of the overall cell content. More robust generation of ATII cells from ES cell cultures has been achieved by the use of conditioned media from a human ATII tumor cell line (A549) in combination with compounds known to drive endodermal cell development (18); however, these procedures still have not achieved a level of purity that would be of clinical use. Moreover, these mixed cell cultures may

contain undifferentiated pluripotent cells, which could develop into teratomas after transplantation.

To circumvent the inherent difficulties in generating a pure culture of ES cell–derived ATII cells, our laboratory recently developed a culture and genetic selection procedure that reliably yields an essentially pure population of human ATII cells by generating stable transfected human embryonic stem (hES) cell lines containing a single copy of a SPC-promoter-driven neomycin-resistant gene cassette (19). Without having to form embryoid bodies, these hES cell lines can be differentiated and cultured in the presence of neomycin to produce pure preparations of hES cell–derived ATII cells with biological and phenotypic characteristics of ATII cells, including expression of surfactant proteins A, B, and C, presence of lamellar bodies, and the ability to proliferate and differentiate into ATI cells (Figure 2).

Failure to efficiently repopulate the alveolar epithelium after injury is a major reason for the development of pulmonary fibrosis. Alveolar epithelial cell damage results in denuded epithelial basement membranes, release of inflammatory and chemoattractant molecules (which facilitate the migration of fibroblasts into the alveolar space), and development of intra-alveolar fibrosis (20). When transplanted (via oropharynx intubation) into the lungs of SCID mice subjected to bleomycin-induced acute lung injury, a significant number of hES-ATII cells appeared to physically engraft into the alveolar epithelial basement membranes denuded by bleomycin injury. By the end of the study (10 days post injury), several of these engrafted cells expressed ATI phenotypic cell markers, suggesting that they had differentiated or were in the process of differentiating into ATI cells (21). Transplantation of the hES-ATII cells almost completely reversed or prevented acute lung injury in the bleomycin-treated mice when transplanted 1 or 2 days following injury, as demonstrated by recovery of body weight and arterial blood oxygen saturation, decreased collagen deposition, and increased survival (21). Although the data from this study argued that engraftment and differentiation of the transplanted hES-ATII cells played a major role in repairing or preventing tissue damage caused by bleomycin, it was unlikely that these events accounted for all the therapeutic benefits provided by the hES-ATII cells. For example, some injured lung alveolar epithelium regions appeared healthy after hES-ATII cell transplantation despite having no observable engrafted hES-ATII cells. Similar studies with bone marrow–derived mesenchymal stem cells (MSCs) have shown some reduction of mortality and lung injury despite minimal, if any, engraftment of MSCs in the lung (22–24). Several paracrine effects due to the release of anti-inflammatory mediators such as IL-10, angiopoietin-1, and keratinocyte growth factor from MSCs are thought to explain the benefit provided to the injured mouse lungs by MSCs (25). Further investigation will be required to determine if hES-ATII cells also provide similar paracrine repair/protection to injured lung epithelium.

From Induced Pluripotent Stem Cells

In 2006, Takahashi & Yamanaka made the seminal discovery that four transcription factors (Klf4, Sox2, Oct4, and c-Myc), when introduced into mouse fibroblasts through retroviral transduction, led to the formation of clones of cells with pluripotent properties (26). Just one

year later, human pluripotent cells were generated by either the same set of transcriptional factors or another set of transcriptional factors that did not include the oncogenic transgene c-Myc (27, 28). These reprogrammed pluripotent cells are now commonly referred to as induced pluripotent stem cells or iPS cells. Although there have been some concerns that human iPS cells are not as robust or pluripotent as hES cells (29), they do appear similar to hES cells morphologically, and they have been shown to differentiate into cell types of all three germ layers in vitro as well as form teratomas in immune-deficient mice in vivo (30). Across the globe, a frenzy of iPS cell research has occurred since publication of these seminal studies.

The primary interest and enthusiasm has been focused on the potential use of iPS cells in drug screening, modeling human diseases, and patient-specific therapies. Initially, iPS cells were made from somatic cells by retroviral or lentiviral transduction of the required transcription factors. Transgenes are inserted randomly by this method and may therefore disrupt normal gene expression. As a consequence, strenuous efforts by numerous laboratories in the past three years have focused on perfecting iPS technologies so that these pluripotent cells can be produced virus free and without insertional mutagenesis. Recently, iPS cells have been generated by transduction of the reprogramming factors by plasmid vectors and removable transposons as well as direct delivery of recombinant proteins encoded by the transcription factor transgenes (31–36). Other investigations have been initiated with the hope that iPS cells can be generated in the near future by incubation of somatic cells with low-molecular-weight compounds identified by high-throughput drug screens.

Proof-of-principle studies in rodents have made a strong case for the future clinical use of iPS cell–derived progenitor cells in the treatment of diabetes and Parkinson’s disease (37, 38). Currently, there are no published reports of preclinical or clinical use of iPS cells in pulmonary medicine. It is tempting to speculate that iPS cell–derived lung progenitor cell applications will prove equally promising in animal models of lung injury and disease. The treatment of genetic disorders has been demonstrated using mouse iPS cell lines in which the sickle hemoglobin allele was replaced by the normal allele by standard gene-targeting techniques (39). These cells when transplanted into affected mice resulted in restoration of normal functional hemoglobin, red blood cell counts, weight, and breathing rate (39). It is anticipated that gene-corrected iPS cell–derived lung progenitor cells will prove equally powerful and provide an attractive alternative to gene therapy for patients with inherited mutations that cause lung disease, including cystic fibrosis, alpha-1-antitrypsin deficiency, and surfactant protein deficiencies. Very recently, Kotton and colleagues reported the generation of iPS cells from dermal or liver fibroblasts from patients with cystic fibrosis and alpha-1-antitrypsin deficiency (40). In addition, our laboratory, in collaboration with Cole and colleagues at Washington University School of Medicine at St. Louis, have generated iPS cells from lung and dermal fibroblasts from neonatal infants with surfactant protein B (SPB) deficiency. Before gene-corrected iPS cells derived from patients with cystic fibrosis, alpha-1-antitrypsin, and SPB deficiency can be used clinically, it will be imperative to identify the correct lung progenitor cells for each disease and to be able to purify these progenitors sufficiently for transplantation studies.

BIOENGINEERING OF LUNG EPITHELIUM

While intratracheal delivery of hES- or iPS cell-derived lung epithelial cells holds considerable promise as a therapy, the transplantation of engineered lung tissue is also an attractive prospect. To make this possible, appropriate scaffolding must be found that can support formation of tissue architecture and epithelial cell function. To this end, several groups have explored the use of natural and synthetic three-dimensional matrices to support fetal and adult pulmonary cells (41–45). A variety of scaffolds are capable of supporting tissue growth, differentiation of CCSP⁺ and SPC⁺ cells, and formation of distal airway-like structures. When implanted in vivo, these grafts show gradual resorption of the matrix and vascularization with contribution from recipient endothelial cells. It remains to be demonstrated whether these engineered tissues can communicate with recipient airways and significantly participate in gas exchange.

A major milestone in lung tissue engineering was achieved in 2008 with the transplantation of an engineered main stem bronchus in a patient with bronchomalacia secondary to tuberculosis (46). A segment of a cadaveric trachea was decellularized and seeded with the patient's bronchial epithelial cells internally and MSC-derived chondrocytes externally.¹ Within four days postoperatively, the luminal surface of the graft was indistinguishable from adjacent tissue by bronchoscopy. The engineered tissue restored the patient's lung function and appears to have provided a lasting cure.

Although a great technical feat, engineering a trachea is intrinsically much simpler than engineering a lung. In particular, the fine structure of the distal airways is liable to be damaged during decellularization. Despite this, whole lung decellularization has recently been reported. Cortiella et al. first examined the ability of the devitalized lung to act as a matrix supporting lung tissue differentiation from mouse ES cells (48). Their decellularization protocol resulted in the complete removal of cellular material and most DNA while retaining the basic architecture of the lungs from trachea to alveolus. Devitalized lung appeared superior to other simple matrices and two-dimensional culture in terms of cell survival and differentiation into epithelial and endothelial tissues. Furthermore, evidence of site-specific differentiation was observed, with cyst-like areas of proSPC⁺ cells in the distal airways and CCSP⁺ cells in the trachea and transitional airways. At 21 days of culture, surfactant protein A was detectable, indicating maturity of the epithelial compartment. Most recently, Petersen et al. demonstrated that devitalized lung scaffolds could be reseeded with neonatal rat lung cells to generate functional tissue-engineered lungs (49). A biomimetic bioreactor was designed in which the airways were ventilated under negative pressure and the pulmonary artery was perfused at physiologic pressure with culture medium. Introducing epithelial and endothelial cells into their respective compartments resulted in robust repopulation with appropriate regional distributions of various lung cell types. Although proSPB⁺ and proSPC⁺ cells were identified, mature SPB was not detected. Upon orthotopic left lung transplantation in rats, the engineered lungs

¹Of note, external and internal seeding of the trachea may have not been required. In canine transplantation studies, unseeded portions of the main stem bronchus were replaced with polypropylene meshes coated in collagen with no external or internal seeding; at three months the transplanted meshes were internally coated with squamous or ciliated columnar epithelium (47).

were shown to participate in P2/CO₂ exchange in vivo by blood gas analysis with elevated PO₂ and decreased PCO₂ in the left pulmonary vein relative to the pulmonary artery. Further improvements in the technique are needed before translation into human therapy, however. The integrity of the alveolar-capillary barrier was not complete, as evidenced in the appearance of red blood cells in airspaces following transplantation. Additionally, some clotting was noticed, likely due to exposure of collagen in basement membranes to the circulation.

Together, these decellularization experiments confirm the importance of the extra-cellular matrix in providing regional molecular cues for the differentiation and maintenance of the lung epithelium. Further understanding and identification of these specific molecular cues will facilitate the directed differentiation and culture of lung epithelial cells.

CONCLUSION

The lung is a complex organ composed of numerous different cell types and thus does not lend itself easily to stem cell-based regenerative therapeutics. For example, it was a great disappointment to the field when it was concluded from rodent models of lung injury that adult bone marrow-derived stem cells do not engraft or differentiate into lung epithelium to any significant degree. However, these and other studies revealed that intrapulmonary administration of bone marrow-derived MSCs, via putative paracrine mechanisms, attenuated inflammation and lung injury despite minimal if any engraftment of MSCs in the lung. These findings have fostered a multicenter double-blind placebo-controlled clinical trial using MSCs for patients with moderate to severe COPD. To date, no infusional toxicities or significant adverse events have been observed. A significant decrease in the blood inflammatory marker C-reactive protein and improvement in quality-of-life indicators have been observed in COPD patients treated with MSCs (50). Therefore, MSCs via their anti-inflammatory paracrine effects may provide a beneficial therapeutic for the treatment of COPD.

Because of their robust pluripotent nature, hES and iPS cells may exhibit more therapeutic potential for gene delivery and the repair and regeneration of injured lung epithelium than adult bone marrow-derived stem cells. However, the direct administration of hES or iPS cells could pose a risk of teratoma formation, especially to patients who are immunocompromised. Therefore, as in the case of the recently initiated clinical trials of hES-derived neural progenitor cells for spinal cord injuries, the first clinical applications of human pluripotent cells in pulmonary medicine are likely to be performed by using hES- or iPS cell-derived lung progenitor cells in transplantation studies. In this regard, more research on the identification and characterization of endogenous stem/progenitor lung cells is warranted. In addition, elucidation of potential surface protein markers that could be exploited for the purification (by flow or magnetic beads) of lung progenitor cells will be important.

Preclinical experiments in mouse models of acute lung injury support the transplantation of gene-corrected iPS cell-derived lung epithelial progenitor cells as an attractive alternative to virus-based gene therapy for inherited single-gene mutations causing lung disease, such as

surfactant protein deficiencies, cystic fibrosis, and alpha-1-antitrypsin deficiency. In this regard, investigations into the self-renewing “stem-like” properties of putative lung progenitor cells are critical. For example, gene-corrected transplanted lung progenitor cells without self-renewal capacity will, provide only a short-term therapeutic benefit, not a long-term cure.

Initial experiments in bioengineering of the lung have been informative and indicate that the tracheal epithelium may be very amenable to regeneration by stem cell therapeutic approaches. Moreover, bioengineering investigations using devitalized lung tissue have shown promise for at least partial recovery of lung structure and function by seeding with embryonic or fetal cells. These findings support the possible use of patient-derived iPS cells for lung regenerative medical applications in the future.

In short, despite numerous challenges, the work of numerous laboratories has led to significant therapeutic and scientific advances in stem cell applications to lung regenerative medicine during the past several years. These studies have thus far been life saving in at least one example, and it is hoped that with continued effort, other stem cell applications will provide therapies that will ease the morbidity and mortality suffered globally due to lung disease.

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Glossary

ES cell	embryonic stem cell
iPS cell	induced pluripotent stem cell
CCSP	Clara cell secretory protein
SPC	surfactant protein C
ATII cell	alveolar type II epithelial cell
COPD	chronic obstructive pulmonary disease
hES cell	human embryonic stem cell
MSCs	mesenchymal stem cells

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Figure 1. The three main lung epithelial regions and the major epithelial cells comprising each area. *Left panel:* a, bronchi of the proximal cartilaginous airway; b, distal bronchioles; c, gas-exchanging airspace (alveolar region). *Right panel:* a, major cell types of the bronchial epithelium; b, major cell types of the bronchiolar epithelium; c, major cell types of the alveolar epithelium.

Figure 2.

Summary of the procedure for differentiation of human embryonic stem (hES) cells into alveolar epithelial type II (ATII) and type I (ATI) cells in vitro. Stable hES cell lines carrying a single-copy SPC-promoter-neomycin-resistant transgene spontaneously differentiate in differentiation medium in 15 days to a culture containing ~13% hES-ATII cells. When genetically selected for 3 days (day 18) by the addition of G418, >99% of surviving cultured cells are hES-ATII cells. On removal of G418, the hES-ATII cells differentiate into hES-ATI cells after 8 days (day 26) of culture in DMEM containing 10% FBS. Please see References 19 and 21 for experimental details. Abbreviations: SPC, surfactant protein C; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum.