Research Article

Stem Cell & Regenerative Medicine

Pulmonary Diseases and Adult Healing Cells: From Bench Top to Bedside

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ABSTRACT

Primitive healing cells, i.e., pluripotent stem cells and totipotent stem cells, have been isolated from the skeletal muscle and blood of adult mammals, including humans. The current study was undertaken to determine the location of these cells with respect to normal and regenerating lung parenchyma of the adult rat. Adult rats were euthanized following the guidelines of Mercer University's IACUC. The lungs were fixed, cryosectioned and stained with two antibodies diagnostic for primitive adult stem cells, i.e. SSEA-4 for pluripotent stem cells and CEA-CAM-1 for totipotent stem cells. In non-injured lung tissue SSEA-4 positive stem cells were located in areas of the smooth muscle within the parenchyma and bronchioles, whereas CEA-CAM-1 positive stem cells were located within the smooth muscle and visceral pleura. Both primitive stem cells were present in injured lung parenchyma undergoing repair. IRB-approved clinical studies are ongoing to address their functional significance in human clinical pulmonary injury and repair.

Keywords

Healing cells, Totipotent stem cells, Pluripotent stem cells, Endogenous stem cells, Adult stem cells, CEA-CAM-1, SSEA, Lung, Repair, Rat, Human, COPD, IPF, FEV_1.

Introduction

Each year in the United States, 349,000 people die from some form of pulmonary disease, represented by an age-adjusted mortality rate of 121.4 deaths per 100,000 populations [1]. Preceded only by cardiovascular disease and all types of cancer, pulmonary disease is the third leading cause of mortality for Americans, where it is responsible for one in every seven deaths [2]. While in recent years advances in surgical, minimally invasive, and chemotherapeutic treatment modalities have resulted in a decline in the death rates for both cardiovascular disease and cancer, mortality rates for lung disease continue to climb [3]. Not only is lung disease devastating to its victims, it also burdens the U.S. economy with $81.6 billion annually in direct healthcare costs and a total of $157.8 billion annually when the indirect expenses of patient care are considered [4].

Pulmonary disease constitutes a broad category of illness. Significant mortality and morbidity can be attributed to both infectious (pneumonia and tuberculosis) and neoplastic (small cell and squamous cell cancer) causes of lung disease. In contrast, chronic lung diseases are progressive in nature, leading to decreased lung function, which ultimately leads to oxygen starvation of peripheral tissues and death. They are particularly destructive and account for the highest mortality rates within the category of pulmonary disease.

The diagnosis for lung diseases is based on spirometry, a.k.a., lung function tests [5] and the saturation of oxygen within the arterial blood [5]. Spirometry measures the amount of airflow passing through the respiratory passages of the lungs during inspiration and expiration [6,7]. Two major components of spirometry are

measured to make the diagnosis for lung disease: 1) the greatest volume of air that can be breathed out in the first second of a breath, a.k.a., the forced expiratory volume in one second (FEV_1) and 2) the greatest volume of air that can be breathed out in a single large breath, a.k.a., the forced vital capacity (FVC) [8]. Normally, 75-80% of the FVC comes out in the first second [5]. A ratio of FEV_1/FVC of less than 80% of predicted value for age of the individual in someone with associated symptoms defines a person as having COPD [5,8]. A reduction in FVC with either a proportionate decrease in airflow or an increase in airflow for the observed FVC defines IPF [5]. An analysis of arterial blood for oxygen saturation is used to determine the need for long-term oxygen therapy. This therapy is recommended for those with an FEV_1 less than 35% of age-adjusted predicted value and/or those with a peripheral oxygen saturation of less than 92% (GOLD Staging System) Table 1 [9].

Severity	FEV_1 % of Predicted Value
GOLD-1 (Mild)	> 80
GOLD-2 (Moderate)	50 – 79
GOLD-3 (Severe)	30 – 49
GOLD-4 (Very Severe)	<30 or chronic respiratory failure

Table 1: GOLD Staging System.

Chronic Obstructive Pulmonary Disease (COPD) alone is the fourth leading cause of death in the United States, claiming 122,283 lives in 2003 [8,9]. The term COPD denotes slowly progressive and irreversible obstruction of the airways. Three pathological processes are involved in the etiology of COPD: chronic bronchitis, small airway obstruction involving the respiratory bronchioles and alveoli, and emphysema [8]. Chronic bronchitis involves the inflammation and eventual scarring of the lining of the bronchi that occurs when they are inflamed and/or infected. The inflammation of chronic bronchitis narrows these airways, reducing the flow of air during respiration. Chronic bronchitis also causes the deposition of thick mucus or phlegm, leading to chronic cough and the expectoration of mucus. Emphysema occurs when the tissues of the lungs are damaged, usually as a result of smoking. Emphysema is a progressive, chronic disease that leads to irreversible pulmonary damage that causes difficulty in breathing and shortness of breath. Emphysema leads to destruction of the alveoli, terminal bronchioles, and respiratory bronchioles.

The emphysematous component has the most severe direct destructive effect on the pulmonary architecture, characterized by obliteration of both the respiratory bronchiolar and alveolar walls that are highly specialized for gas exchange [9]. Once lung tissue is irreversibly lost, the lungs lose their elasticity and, therefore, their intrinsic ability to obtain oxygen and eliminate carbon dioxide. Cigarette smoking is responsible for 80-90 % of the deaths that occur as a result of COPD, while the other causes include alpha-1-antitrypsin enzyme deficiency, air pollution, reactive airway disease, heredity, male gender, and increased age [10-12].

None of the existing medications for COPD have been shown to modify the long-term decline in pulmonary function that is the hallmark of the disease [9]. Nonetheless, pharmacologic treatment remains the mainstay of therapy for COPD. Therapy for COPD is directed towards relief of symptoms, prevention of complications, and minimization of side effects while disease progression still occurs [13]. Oxygen therapy, various inhalers and medications to improve vasodilation, antibiotics to combat the frequent lung infections, fluid replacement, and exercise rehabilitation programs, are the options available to patients with COPD [12,14]. Surgical therapies, including lung transplantation and lung volume reduction surgery (LVRS), show some promise but have their flaws as well [15]. Recently it has been shown that survivability, exercise tolerance, and health-related quality of life were all significantly greater in post-LVRS patients with severe upper-lobe emphysema and low exercise capacity compared to patients receiving medical therapy alone [15]. However, patients must undergo a strict selection process to determine if they are candidates for this procedure. Studies have found that patients with severe emphysema and more homogeneous patterns of airway destruction (i.e., not limited to the upper lobes) have a high associated mortality rate with LVRS [14,15]. Lung transplantation is an increasingly discussed therapeutic option, but its use remains limited primarily due to the widespread shortage of donor lungs and the stringent requirements for qualification for this therapy [16]. Thus, despite the positive aspects of these current and developing therapies, they remain only provisional and palliative treatments at best.

Interstitial pulmonary fibrosis (IPF) belongs to a large group of more than 200 lung diseases known as interstitial lung diseases (ILD), characterized by the involvement of the lung interstitium [17]. The cause(s) of these interstitial lung diseases are unknown (idiopathic) [17,18], however, it has been suggested that inflammation [19,20] within the lung stimulates fibrosis around the alveolar sacs [17,18,21,22]. IPF is manifested by the deposition of collagen [23] in the interstitium between the basement membrane of the capillaries and the adjacent basement membrane of the alveolar epithelium. This fibrosis within the alveolar sac interstitium [18-23] prevents the exchange of oxygen for carbon dioxide across the alveolar interstitial space. Fibrosis in interstitial lung diseases affects not only the interstitium, but also the air spaces, airways and blood vessels [7].

New therapeutic strategies continue to be developed for pulmonary diseases. Presently, much of the promise in treating chronic and incurable diseases of any organ system is centered on stem cell biology and the “holy grail” of regenerative medicine [24,25]. Despite the optimism and explosion of interest surrounding this new field, knowledge of the scientific basis for stem therapy for pulmonary diseases is still in its infancy [26].

Young et al. reported the characterization and isolation of adult pluripotent stem cells [27,28]. These putative healing cells were small in size (6-8 μm) and expressed stage-specific embryonic antigen-4 (SSEA-4) on their cell surfaces. A clone derived from a single cell revealed the presence of phenotypic expression

markers for 60 discrete cell types of ectodermal, mesodermal, and endodermal origin when exposed to general and specific induction agents. The clone maintained its differentiation capabilities in over 400 population doublings. Analyses were performed using an antibody-micro array-enzyme-linked immuno-culture assay [29]. Studies have shown that these cells may be useful in the treatment of myocardial infarction (MI), Parkinson's disease, vascular ischemia, and type-I diabetes mellitus [27,28,30-33].

A second population of putative healing cells was isolated and characterized from adult mammalian skeletal muscle and blood [34-36]. Like the SSEA-4 positive cells, these totipotent-like stem cells were cloned from a single cell. This novel cell population was small in size (<2 μm). Carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) was expressed on the surface of these cells. A clone revealed phenotypic expression markers for 65 discrete types of ectodermal, mesodermal, and endodermal origin when exposed to general and specific induction agents. The clone maintained its differentiation capabilities following more than 300 population doublings [37].

The current and future studies are focused on determining the location, identities, and inherent qualities of the healing cells in the lung and other organs in order to ascertain their functional significance with respect to repair and regeneration [38,39].

Materials and Methods

The use of animals in this study complied with the guidelines of Mercer University Institutional Animal Care and Use Committee and criteria of the National Research Council for the humane care of laboratory animals as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (National Academy Press, 1996). The use of adult humans reported herein complied with the guidelines of Mercer University School of Medicine and the Medical Center of Central Georgia Institutional Review Boards. All individuals signed consent forms before participating in this study.

Animal Study

Tissue Harvest

Postnatal Sprague-Dawley rats (n=10) were euthanized using inhalation of carbon dioxide. The anterior chest wall and abdomen were washed with Betadine solution and incised. Each adult rat lung was removed under aseptic conditions. The adult rat lungs were placed in 50 ml conical polypropylene tubes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) containing 40 ml of cold ELICA fixative, consisting of aqueous 0.4% v/v glutaraldehyde, 2% w/v paraformaldehyde, and 1% w/v D-glucose, pH 7.4, with an osmolarity 1.0 [34]. The adult rat lungs were allowed to remain in the fixative for one to 24 weeks at ambient temperature. After fixation, the adult rat lungs were stored at ambient temperature in Dulbecco's Phosphate Buffered Saline (PBS, Invitrogen, GIBCO, Grand Island, NY), pH 7.4. Pieces of lung tissue were removed from the buffer, placed into Tissue Tek OCT Compound 4583 (Miles Laboratory, Ames Division, Elkhart, IN) embedding medium and frozen at -20°C. The frozen pieces of lung tissue were cryostat sectioned on a Tissue Tek Cryostat II (GMI, Ramsey, MN) to a thickness of seven microns, placed on positively-charged microscope slides (Mercedes Medical, Sarasota, FL) and maintained at -20°C until stained immunocytochemically, following established procedures for ELICA analyses [29,34].

Immunocytochemistry

Tissue sections were incubated with 95% ethanol to remove the cryostat embedding medium and washed in running water for five minutes. They were incubated in 5.0% (w/v) sodium azide (Sigma, St. Louis, MO) in PBS for 60 minutes. The sections were washed in running water for five minutes and incubated with 30% hydrogen peroxide (Sigma) for 60 minutes to irreversibly inhibit endogenous peroxidases [29,34]. Tissue sections were rinsed with running water for five minutes and incubated for 60 minutes with a blocking agent (Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in PBS [34]. The blocking agent was removed. The sections were rinsed with running water for five minutes, and incubated with primary antibody for 60 minutes. The primary antibodies consisted of 0.005% (v/v) carcinoembryonic antigen cell adhesion molecule-1 (CEA-CAM-1) in PBS for blastomere-like stem cells [29,34-36]; 1 μg per ml stage-specific embryonic antigen-4 for epiblast-like stem cells (SSEA-4, Developmental Studies Hybridoma Bank, Iowa City, IA) in PBS [29,34-36]; and smooth muscle alpha-actin (IA4, Developmental Studies Hybridoma Bank) in PBS [29,34-36]. The primary antibody was removed. The sections were rinsed with running water for five minutes, and incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated anti-mouse IgG (H + L) affinity purified, rat adsorbed (BA-2001, Vector Laboratories) in PBS [29,34-36].

The secondary antibody was removed. The sections were rinsed with running water for five minutes, and then incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure Ultra, Curtin Matheson Scientific, Houston, TX) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vectastain ABC Reagent Kit, Vector Laboratories) in PBS. The avidin-HRP was removed. The sections were rinsed with running water for five minutes, and incubated with AEC substrate (Sigma) for 60 minutes. The AEC substrate was prepared as directed by the manufacturer (Sigma). The substrate solution was removed. The sections were rinsed with running water for 10 minutes and then cover-slipped with VectaMount (Vector Laboratories) [29,34-36].

Positive and negative controls were included to assure validity of the immunocytochemical staining [29,34-36]. The positive controls consisted of adult-derived totipotent stem cells (positive for CEA-CAM-1) [35], pluripotent stem cells (positive for SSEA-4) [34], and smooth muscle surrounding blood vessels within the tissue (positive for IA4) [34]. The negative controls consisted of the staining protocol with PBS alone (no antibodies or substrate), without primary antibodies (CEA-CAM-1, SSEA-4 or IA4), without secondary antibody (biotinylated anti-mouse IgG), without

avidin-HRP, and without substrate (AEC) [29,34].

Visual Analysis

Stained sections were visualized using a Nikon TMS phase contrast microscope with bright field microscopy at 40x, 100x, and 200x. Photographs were taken with a Nikon CoolPix 995 digital camera. Digital photographs were cropped using Adobe Photoshop 7.0.

Human Clinical Study

Healing Cell Harvest

Endogenous adult stem cells were isolated from blood from 1 female with diagnosed interstitial pulmonary fibrosis (IPF) and 1 female with diagnosed chronic obstructive pulmonary disease (COPD) as previously described [36]. In brief, adult human blood (n=2) was obtained by venipuncture following standard acceptable medical practice. The blood was collected using sterile procedures and placed in 10-ml EDTA hemovac tubes (Beckton-Dickinson), inverted several times to mix and then refrigerated at 4°C for 48 hours until further processing to isolate endogenous stem cells within the peripheral blood plasma fraction [35,36].

Stem Cell Isolation

After 48 hours of gravity separation, the blood had separated into a floating plasma fraction and a sedimented cellular fraction. The cellular fraction contained hematopoietic stem cells, red blood cells, white blood cells, and most mesodermal stem cells [35,36]. The plasma fraction was withdrawn using a sterile pipette, placed in a second sterile tube and refrigerated at 4°C.

Stem Cell Identification

Totipotent stem cells are Trypan blue positive very small spherical-shaped cells that are < 2.0 microns in size [35-39]. Transitional-totipotent stem cell/pluripotent stem cells display a peripheral rim that stains with Trypan blue and a central core that does not. They are >2.0 to <6.0 microns in size [35,36]. Pluripotent stem cells do not stain with Trypan blue and are 6.0 to 8.0 microns in size [27,32-34]. Germ layer lineage stem cells do not stain with Trypan blue and are > 8.0 to <20.0 microns in size [38,39].

Treatment

The healing cells for each individual were segregated into two populations: all the totipotent stem cells and one-half of the pluripotent stem cells were processed for nebulization (breathing) into the lungs to form endodermal-derived cells lining the conductive system of the lungs, i.e., respiratory epithelium and type-I and type-II pneumocytes. The other half of the pluripotent stem cells and all the mesodermal stem cells were processed for intravenous infusion as a systemic delivery system to the lungs to assist in the formation vasculature and stromal lung tissue. The forced expiratory volume in one second (FEV_1) was measured in each participant before treatment began and at one month following treatment.

Results

One aspect of the current study was to determine the location of primitive stem cells within the lung parenchyma and stroma of the adult rat. In normal non-regenerating lung tissue, CEA-CAM-positive and SSEA-4-positive cells are located in the smooth muscle wall of the blood vessels and structures within the bronchial tree, i.e., bronchi, bronchioles, and alveolar ducts (Figure 1).

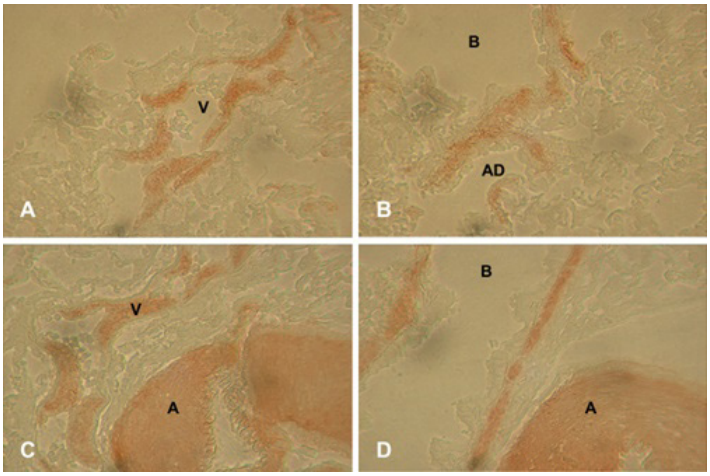

Figure 1: Normal non-regenerating lung tissue.
A: CEA-CAM-positive cells in tunica media of a vein, 200x mag.
B: CEA-CAM-positive cells within smooth muscle layer of alveolar ducts (AD) and bronchiole (B), 200x mag.
C: SSEA-4-positive cells in tunica media (smooth muscle layer) of an artery (A) and vein (V), 200x mag.
D: SSEA-4-positive cells in smooth muscle wall of a bronchiole (B) and an artery (A), 200x mag.

In contrast, in lung parenchyma and stroma undergoing repair/regeneration, SSEA-4 positive and CEA-CAM-1-positive cells were located in the lung parenchyma surrounding bronchioles, alveolar ducts, and alveolar sacs (Figure 2A-D and Figure 3A,B). Positive controls (i.e., smooth muscle alpha-actin staining of smooth muscle within the lung vasculature, Figure 3C) and negative controls (absence of staining in the absence of primary antibody, secondary antibody, tertiary probe, substrate, or DPBS only, Figure 3D) yielded appropriate results.

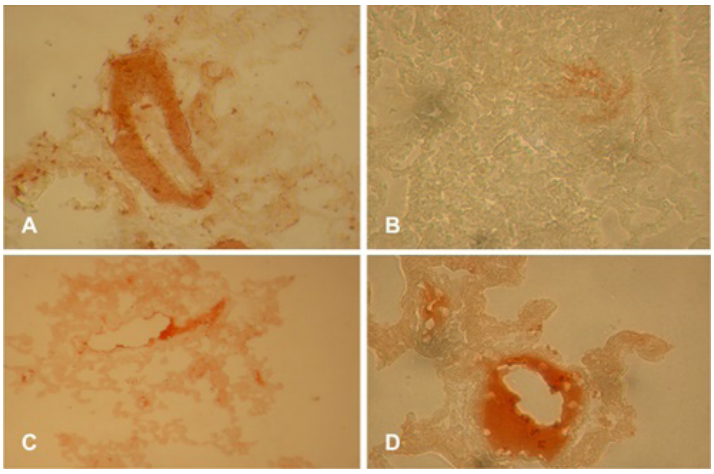

Figure 2: In regenerating lung tissue, CEA-CAM-positive cells (A,B) and SSEA-4-positive cells (C,D) are located amongst the tissues.
A: Regenerating lung tissue – CEA-CAM-positive cells, 100x mag.

B: Regenerating alveolar ducts – CEA-CAM-positive cells, 200x mag.
C: Regenerating bronchiole – SSEA-4-positive cells, 40x mag.
D: Regenerating alveolar duct – SSEA-4-positive cells, 200x mag.

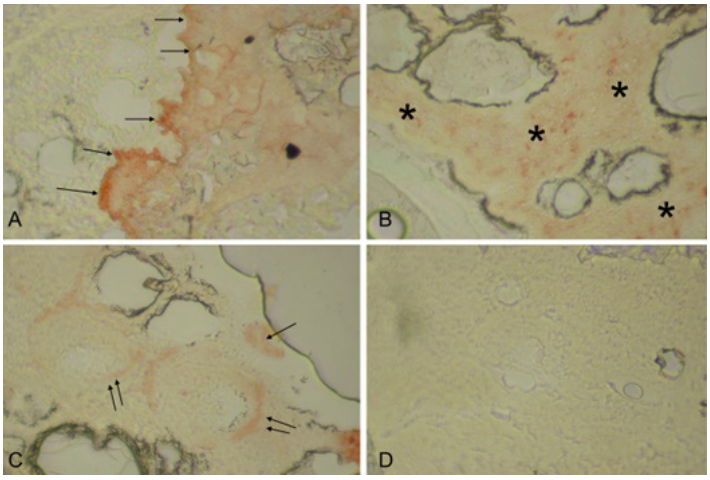

Figure 3: Regenerating lung tissue.
A: SSEA-positive staining along periphery of bronchopulmonary segment (arrows), 40x mag.
B: CEA-CAM-1-positive staining of cells within lung stroma (asterisks), 40x mag.
C: IA4-positive (smooth muscle alpha-actin) staining of cells within walls of bronchioles (double arrows) and alveolar duct (single arrow), 40x mag.
D: Negative procedural control demonstrating the absence of any staining within lung stroma or parenchyma, 40x mag.

Human Safety Study

The second aspect of the study was to ascertain the safety and efficacy for nebulized totipotent stem cells and pluripotent stem cells and transfused pluripotent stem cells and mesodermal stem cells to affect the FEV_1 values in these two individuals. One month after their first treatment, the volunteer with IPF demonstrated an increased FEV_1 from 14% to 27%, a 93% increase in their FEV_1. One month after their first treatment, the volunteer with COPD demonstrated an increased FEV_1 from 30% to 46%, a 53% increase in their FEV_1 (Figure 4).

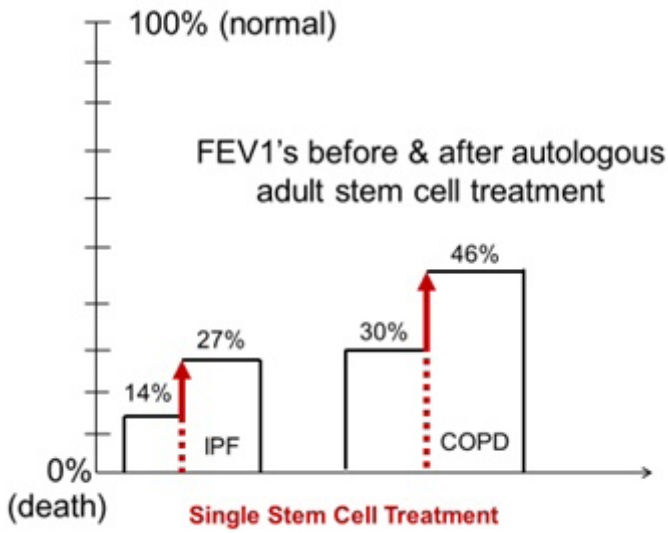

Figure 4: Human Clinical Model. Two human volunteers were studied. One volunteer had interstitial pulmonary fibrosis (IPF) with an initial forced expiratory volume in one second (FEV_1) of 14%. The second volunteer had chronic obstructive pulmonary disease (COPD) with an initial FEV_1 of 30%. Both volunteers were treated with their own endogenous adult totipotent stem cells, pluripotent stem cells, and mesodermal stem cells. The totipotent stem cells and half of the pluripotent stem cells were nebulized (breathed in). The other half of the pluripotent stem cells and mesodermal stem cells were given by intravenous infusion. One month after the first treatment, the volunteer with IPF increased their FEV_1 from 14% to 27%, a 93% increase in their FEV_1 from their initial starting value. One month after the first treatment, the volunteer with COPD increased their FEV_1 from 30% to 46%, a 53% increase in their FEV_1 from their initial starting value.

Discussion

One of the major functions of the lungs is to maintain 92-100% oxygen saturation in the arterial blood, which is necessary for normal metabolic functioning of the tissues [6]. To accomplish this, the lungs are composed of airways, glands and gas exchange units [40]. The airways (bronchi, bronchioles, alveolar ducts) allow the passage of air from the trachea to the gas exchange units [7]. These airways also assist in adjusting the air to body temperature as well as providing an environment for the removal of particulate material in the incoming air [7]. The glands secrete mucus, a sticky substance, which lines the airways, traps particulate material and assists in its removal from the lungs. The gas exchange units (alveolar sacs) are a composite of thin-walled vessels (capillaries) abutted against a single layer of epithelial cells (alveolar cells) [6,7]. Both structures are separated by an interstitial space (interstitium) containing their respective basement membranes [7]. This particular architecture allows for the rapid exchange of oxygen in the air with carbon dioxide in the blood, thereby maintaining the high level of oxygen saturation for the metabolic demands of the body tissues [6,7].

Diseases of the lungs affect one or more of these entities. One set of diseases, collective known as chronic obstructive pulmonary disease or COPD, affect both the airways and the glands [5,41-43]. Normally, the diameter of the airways decrease in size from the trachea to the alveolar sacs to limit the amount of dead space the air has to travel within the lungs before gas exchange can occur [7,42]. Contaminants in the incoming air cause a change in the airway architecture of the lung. This is reflected by an increase in bronchiole diameters, affectively increasing the amount of dead space through which the air must travel to reach the alveolar sacs [5,8,44]. There is also a concomitant decrease in alveolar duct diameters, effectively trapping the air in the lungs and preventing its reaching the alveolar sacs [45,46]. This results in a feeling of shortness of breath on exertion, initially, and then in advanced stages, even at rest [5,8,44]. The contaminants also stimulate an increased mucus production which can physically block airway passages and produce a chronic cough [5,8,47]. Together, these changes within the lung "obstruct" the airflow to the alveolar sacs limiting the amount of gas exchange that can occur.

While there is no known cure for either COPD or IPF, their symptoms are treatable, which can delay the progression of the

respective diseases. Potential treatments include improvement of air quality, limiting exposure from known causes, medication to adjust airway diameters, medication to limit inflammation, medication to decrease mucus secretion, long-term oxygen therapy, lung volume reduction surgery, and/or lung transplantation [1-5,9-14,18-25,41-47].

Currently, the treatment for COPD patients is very limited. At the onset of disease, there is minimal shortness of breath, but as the disease progresses, a patient's quality of life diminishes and supplemental oxygen and/or mechanical respiratory assistance may be required. An American Lung Association survey revealed that half of all COPD patients (51%) say their condition limits their ability to work, limits them in normal physical exertion (70%), household chores (56%), social activities (53%), sleeping (50%) and family activities (46%) [48]. None of the existing medications for COPD have shown the ability to modify the long-term decline in pulmonary function that is the hallmark of this disease. Therefore, the goal of pharmacotherapy for COPD is to provide relief of symptoms and prevent complications and/or progression of the disease with a minimum of side effects. Bronchodilator medications are available as inhaled aerosol sprays or oral medications, antibiotics, oxygen therapy, and systemic glucocorticosteroids are all contemporary treatment options [4,6,7,12,19,21,25,43]. Also available is pulmonary rehabilitation, a preventive health-care program provided by a team of health professionals to help people cope physically, psychologically, and socially with COPD. Finally, lung transplantation is being performed in increasing numbers and may be an option for some people who suffer from severe emphysema. Lung volume reduction surgery (LVRS) has shown promise and is being performed with increasing frequency. However, there is still no treatment option for COPD patients that will effect a cure for their disease allowing them to replace the destroyed tissue with new functional pulmonary tissue [3,10,16].

There is no known cure for pulmonary fibrosis diseases, such as interstitial pulmonary fibrosis. The clinical presentations for IPF include insidious onset, progression over several years, infrequent acute attacks, and failure to respond to immunosuppressive therapies [21,25]. This invariably leads to death of the individual due to oxygen starvation of their tissues [19,24,48]. The goals of treatment for IPF are to essentially reduce the symptoms, slow disease progression, prevent acute attacks, and try to prolong survival. It is essential that preventive care and symptom-based treatments should start with initial diagnosis of patient. Proposed symptom-based therapies include antibiotics, antioxidants, and corticosteroids. Lung transplantation is a potential therapy that has been shown to reduce the risk of death by 75%, however, five-year survival rates after transplant are estimated at 50%. Palliative care focuses on improving comfort and reducing symptoms of patients rather than treating the disease. This may include use of chronic opioids for cough and severe dyspnea. Oxygen therapy may benefit hypoxemia patient, although it has not been shown to improve survival [19,21,25,].

The current study demonstrates the presence of both SSEA-4 positive cells and CEA-CAM-1 positive cells within the smooth muscle layers of the bronchial tree and vasculature in the adult rat lung. The presence of these primitive stem cells reveals that the rat lung possesses a pool of cells that have the vast potentials for regenerating lung tissue following injury. Young et al. demonstrated the remarkable capabilities of these cells in other model systems [13-15, 17-19, 22]. The SSEA-4 positive stem cells, termed pluripotent stem cells, were capable of differentiating into any somatic cell type found in the body [15]. The CEA-CAM-1-positive stem cells, termed totipotent stem cells, also have the capability of differentiating into any somatic cell type, but display an additional capacity to differentiate to form germ cells [23]. These results suggest that the totipotent stem cells are even more primitive than the pluripotent stem cells. The demonstration of the presence of both these types of healing cells in areas of the lung actively undergoing repair and restoration suggests that repair of lung tissues *in vivo* by stem-cells may occur naturally in the rat lung.

The implications of these findings for lung tissue regeneration and repair are profound. Researchers in recent years have employed several different strategies for pulmonary tissue regeneration with only limited success. The highly complex embryological origins, histoarchitecture, and physiology of the lung account for the majority of these difficulties [6,7,49]. Furthermore, the study of the normal *in vivo* process of lung injury and repair/regeneration is hindered by the slow steady state turnover of pulmonary cells, making it difficult to observe stem cell migration to sites of injury and initiation of repair by them [7,49]. Finally, the fact that such a large number of structurally and functionally distinct cell types must differentiate from a common precursor into such complex histoarchitectural structures as alveoli, respiratory and conducting airways, and pulmonary vasculature and lymphatics makes any attempt to recreate such a process seem utterly impossible [7,49].

Some studies have attempted to show that bone marrow derived cells play a role in pulmonary repair [26,50,51]. In 2007, Spees et al. demonstrated engraftment of bone marrow derived cells within alveoli in rat models of asbestos-induced lung fibrosis [52]. While these cells differentiated into several different cell types, including some phenotypically similar to type II pneumocytes, their overall function was primarily profibrotic, with no ability to repair or regenerate functional pulmonary tissue [26]. Several other studies have attempted similar explanations for lung regeneration and repair, stressing migration of bone marrow-derived cells to sites of pulmonary injury, but the evidence for true repair resulting from this mechanism is limited [26].

Several other cell types have been used to attempt lung regeneration as well, with only minimal success. Type II pneumocytes have been transplanted into rat models of bleomycin-induced pulmonary fibrosis with only modest reductions in collagen deposition and fibrosis [53]. In rat models having undergone LVRS, adipose stromal cells that generate hepatocyte growth factor (HGF, a potent angiogenic factor) have been implanted to attempt to stimulate lung regeneration and angiogenesis [26,54]. This approach

significantly accelerated alveolar and vascular regeneration within the remaining lung, but these promising results only persisted for slightly more than one month [54]. Perhaps even more significantly, fetal rat lung cells applied to a scaffold material and implanted into adult rats were recently shown to form "alveolar-like units" that eventually became connected to the pulmonary circulation [26]. Although these structures only persisted for slightly more than one month, they did demonstrate regeneration of structures somewhat resembling normal healthy alveoli [26].

The current attempts at lung tissue regeneration using several different types of cells as "precursors" are exciting [26], but obviously underscore the need for new techniques using cell types with a more powerful and durable capacity for differentiation. Now that the characterization of adult-derived healing cells has been achieved in the lung tissue of the adult rat, the cells must be further evaluated for their functional significance and capacity for cell and tissue regeneration.

An alternative to pharmacological treatment involves the use of endogenous stem cells. Young et al. noted the presence of endogenous healing cells within the skeletal muscle and blood of adult mammals, including humans [34-36]. These endogenous healing cells consisted of totipotent stem cells, pluripotent stem cells, and germ layer lineage stem cells. In a pre-clinical animal model of Parkinson disease, genomically-labeled healing cells were shown to regenerate dopaminergic neurons and neural networks lost due to the action of a neurotoxin injected into the substantia nigra of the midbrain. Pyramidal neurons, interneurons, glial cells, and blood-filled capillaries were regenerated in the cerebral cortex that was previously lost due to their traumatic injury during the injection process. The pre-clinical animal study was followed by a human clinical trial for the safety and efficacy of a single treatment of autologous healing cells for Parkinson disease. Ten participants started the trial, with eight participants completing the trial. The participants were assayed for ten parameters, including Hoen-Yahr (H-Y) scoring for quality of life recorded before treatment and at 1, 2, 7, and 14 months after treatment. A single treatment with autologous healing cells resulted in 100% of participants demonstrating a better quality of life (H-Y score) at one and two months following treatment than before treatment. The seven-month follow-up showed that 25% reverted and began to decline (but at a H-Y score higher than before their stem cell transplants), 50% remained stable, and 25% continued to improve. At the 14-month follow-up, the results were the same for the same participants. Thus 25% continued to decline, 50% remained stable, and 25% continued to improve. These results suggested that it was safe for participants to receive their own healing cells and that 75% of the participants benefited from a single healing cell treatment for Parkinson disease [31,32,36].

Based on the basic science data presented herein, we proposed the hypothesis that nebulization concomitant with intravenous infusion would have a positive influence on the FEV_1 of patients with IPF and/or COPD.

Therefore, to determine if autologous healing cells could be a beneficial treatment for pulmonary diseases, a human volunteer with interstitial pulmonary fibrosis (IPF) and a second volunteer with chronic obstructive pulmonary disease (COPD) underwent individualized safety and efficacy studies. Autologous (self) healing cells from each individual were processed in a manner similar to that used in the Parkinson human clinical trial [31,32,36], with the following difference. The healing cells for each individual were segregated into two populations: all the totipotent stem cells and one-half of the pluripotent stem cells were processed for nebulization (breathing) into the lungs to hypothetically form endodermal-derived cells lining the conductive system of the lungs, i.e., respiratory epithelium and type-I and type-II pneumocytes. The other half of the pluripotent stem cells and all the mesodermal stem cells were processed for intravenous infusion as a systemic delivery system to the lungs to assist in the hypothetical formation vasculature and stromal lung tissue. The forced expiratory volume in one second (FEV_1) was measured in each participant before treatment began and at one month following treatment. As shown in Figure 4, one month after their treatment, the volunteer with IPF increased their FEV_1 by 93%, compared to the pre-treatment control value. The volunteer with COPD showed a 53% increase in their FEV_1.

The IPF and COPD participants in this short study are still alive six years after being treated. While a sample size of n=1 for each disease is anecdotal at best, it does suggest that it is safe for participants to receive their own endogenous healing cells as a treatment modality for their respective disease. While a single treatment with autologous healing cells did not cure these patients, it did increase their pulmonary function and presumably gave them a better quality of life. An increase of 93% for the IPF patient and an increase of 53% for the COPD patient suggests that treatment with these healing cells is efficacious with respect to an increase in quality of life. This research has exciting implications for patients suffering from COPD or any of the other chronic lung diseases. Continued studies in the form of IRB-approved clinical trials will assess the ability of endogenous healing cells to improve the quality of life in a larger number of participants with pulmonary diseases.

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