

# Stem cells and cell therapy approaches in lung biology and diseases

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Cell-based therapies with embryonic or adult stem cells, including induced pluripotent stem cells, have emerged as potential novel approaches for several devastating and otherwise incurable lung diseases, including emphysema, pulmonary fibrosis, pulmonary hypertension, and the acute respiratory distress syndrome. Although initial studies suggested engraftment of exogenously administered stem cells in lung, this is now generally felt to be a rare occurrence of uncertain physiologic significance. However, more recent studies have demonstrated paracrine effects of administered cells, including stimulation of angiogenesis and modulation of local inflammatory and immune responses in mouse lung disease models. Based on these studies and on safety and initial efficacy data from trials of adult stem cells in other diseases, groundbreaking clinical trials of cell-based therapy have been initiated for pulmonary hypertension and for chronic obstructive pulmonary disease. In parallel, the identity and role of endogenous lung progenitor cells in development and in repair from injury and potential contribution as lung cancer stem cells continue to be elucidated. Most recently, novel bioengineering approaches have been applied to develop functional lung tissue *ex vivo*. Advances in each of these areas will be described in this review with particular reference to animal models. (Translational Research 2010;156:188–205)

**Abbreviations:** AEC = alveolar epithelial cell; ALI = acute lung injury; ARDS = acute respiratory distress syndrome; BASC = bronchioalveolar stem cell; CCSP = Clara cell secretory protein; CF = cystic fibrosis; CFTR = cystic fibrosis transmembrane conductance regulator; CLP = cecal ligation and puncture; COPD = chronic obstructive pulmonary disease; eNOS = endothelial nitric oxide synthetase; EPC = endothelial progenitor cell; ESC = embryonic stem cell; FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; GFP = green fluorescent protein; HSC = hematopoietic stem cell; IPF = idiopathic pulmonary fibrosis; KGF = keratinocyte growth factor; LPS = lipopolysaccharide; MCT = monocrotaline; MHC = major histocompatibility complex; MSC = mesenchymal stromal (stem) cell; PH = pulmonary hypertension; pro-SPC = pro-surfactant protein C; Sca-1 = stem cell antigen-1

**M**any lung diseases remain incurable and continue to have substantial morbidity and mortality. This includes chronic obstructive pulmonary disease (COPD), notably emphysema, the only major disease category increasing in prevalence

and one of the leading causes of death worldwide, as well as less common but equally devastating diseases such as pulmonary fibrosis, cystic fibrosis (CF), pulmonary hypertension (PH), and the acute respiratory distress syndrome (ARDS). Although symptomatic care

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for each of these conditions has improved, lung transplantation remains the only option for many patients. However, there is a severe shortage of donor lungs, and thus, many patients on waiting lists die prior to transplantation. Furthermore, lung transplantation is often a poor option with a 5-year mortality of approximately 50% and many problems associated with the required lifelong immunosuppression. New options are thus desperately needed.

Recent findings that embryonic stem cells and stem cells derived from adult tissues might be used by surgeons in repair and regeneration of injured or diseased tissues has stimulated extensive investigations into whether these approaches could be used for lung diseases. In parallel, there is increased understanding of the identity and roles of endogenous progenitor cells in the lungs, including possible roles as lung cancer stem cells. These are exciting and rapidly moving fields that hold promise for improved understanding of lung biology and as a potential therapeutic approach for lung diseases.

This review will highlight recent advances in understanding of endogenous lung progenitor cells followed by respective consideration of embryonic and adult-derived stem cells, with particular focus on advances in cell therapy approaches for specific lung diseases with adult stem cells (summarized in Table 1<sup>1-21</sup>).

## ENDOGENOUS LUNG STEM AND PROGENITOR CELLS

Undifferentiated multipotent endogenous tissue stem cells have been identified in nearly all tissues and are thought to contribute to tissue maintenance and repair. These are rare unspecialized cells that are often localized to specialized niches within each tissue and usually cycle infrequently. Importantly, they exhibit self-renewal capacity and can give rise to daughter progenitor or transit amplifying cells. Both stem and progenitor cells may give rise to the differentiated cells of the organ.<sup>22,23</sup>

As the lung is a complex organ composed of more than 40 different differentiated cell types (Fig 1), identification of endogenous progenitor cells has been challenging, and it is now clear that different progenitor cell populations are localized in different anatomic regions of the lung. In the alveoli, surfactant producing type 2 alveolar epithelial cells (AECs) are well recognized as progenitors for the type 1 AEC.<sup>23</sup> In the airways, different putative airway progenitor cell populations and hierarchies have been identified along the tracheobronchial tree in mouse models after selective ablation of epithelial cells by exposure to toxic chemicals or through cell-type-specific expression of cell-toxic genes in transgenic mice (Fig 2).<sup>22-26</sup> In the trachea, large airways, and large airway submucosal glands, a subpopulation of basal epithelial cells that express p63 and cytokeratins

5 and 14 have been implicated. In mice, the predominant epithelial cell of the smaller airways is the nonciliated Clara cell that exhibits characteristics of transit-amplifying cells after injury to ciliated airway epithelial cells. However, unlike transit-amplifying cells in tissues with higher rates of epithelial turnover, such as intestine, Clara cells exhibit a low proliferative frequency in the steady state, are broadly distributed throughout the bronchiolar epithelium, and contribute to the specialized tissue function. In more distal airways, cells resistant to the epithelial toxin naphthalene, which poisons the cytochrome P450 isozyme found in Clara cells and results in desquamation of the differentiated airway epithelial lining, termed toxin-resistant variant Clara cells have been identified as having progenitor functions and have been termed as bronchiolar stem cells (reviewed in Reference 25). Naphthalene-resistant cells are also located within discrete microenvironments within bronchioles that include the neuroepithelial body and bronchoalveolar duct junction (reviewed in References 22 and 25).

At the bronchoalveolar duct junction in mouse lungs, a population of naphthalene-resistant cells that stain for the Clara cell marker, Clara cell secretory protein (CCSP), and type 2 AEC marker pro-surfactant protein C (pro-SPC) have been described.<sup>27</sup> Although rare, these cells proliferate in response to naphthalene injury and during compensatory lung growth after unilateral pneumonectomy in mice.<sup>28</sup> Furthermore, when pro-SPC/CCSP dual-positive cells were isolated using methods developed for enrichment of type 2 AEC, some dual-labeled cells exhibited a unique cell-surface phenotype, Sca1<sup>pos</sup>/CD34<sup>pos</sup>/CD45<sup>neg</sup>/CD31<sup>neg</sup>. These cells were found to self-renew in culture and give rise to progeny expressing CCSP, pro-SPC, or the type 1 AEC marker aquaporin 5. As such, these cells have been termed bronchioalveolar stem cells (BASCs).<sup>27</sup> However, depending on the techniques used, there is some ambiguity in published results and the toxin-resistant Clara cells, BASCs, and other cells may represent different interpretations of the same cell population(s).<sup>22</sup> This highlights the need for specific markers of the different cell populations and rigorous methods of lineage tracing as well as further underscoring the importance of the *in vivo* microenvironment on cell behavior.<sup>29</sup> For example, expression of stem cell antigen-1 (Sca-1), once thought to be a feature of murine hematopoietic cells, has now been described as a marker for both putative bronchiolar progenitor cells as well as for fibroblastic progenitor cells in the lung.<sup>30-32</sup> Furthermore, recent data using chimeric wild-type–green fluorescent protein (GFP) chimeric mice, generated by implanting 8–16 cell GFP mouse embryo aggregates into pseudopregnant wild-type females, demonstrates that the bronchiolar progenitor cells do not seem

**Table 1.** Cell therapy with MSCs in experimental lung disease models

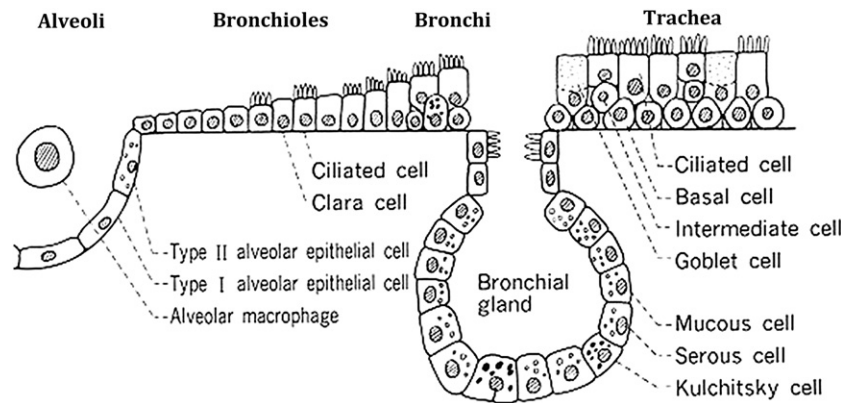
Animal model	Outcome	Potential mechanism	Reference
Bleomycin IT	Reduce fibrosis Reduce inflammation	IL-1RA Suppression of NO metabolite, proinflammatory and angiogenic cytokines Increase of MMP2 activity and reduction of Timp2	1,2, 3–6
<i>E coli</i> LPS IP	Reduce inflammation Improve mortality	Direct cell–cell contact and release of cytokines	7,8
LPS IT	Reduce inflammation	Angiotensin-1	9,10
Hyperoxia-induced lung injury	Prevent arrested alveolar and vascular growth	Paracrine-mediated mechanism	11–13
Cecal ligation and puncture-induced sepsis and acute lung injury	Improve mortality	Prostaglandin E(2)-induced IL-10 production	15
Papain-induced emphysema	Amelioration of emphysema	Reduce apoptosis by upregulation of Bcl-2 and Bax gene	14
Ovalbumin-induced asthma	Reduced airway hyperresponsiveness to methacholine Reduced airway eosinophilia	Inhibit Th2 phenotype development through paracrine effect	16
CFTR-KO mice with Naphthalene-induced injury	Detection of CFTR by PCR Restored CFTR function by patch clamp	Engraftment of airway epithelial cells	17
2% polidocanol-induced airway injury	Airway epithelium	Early engraftment Paracrine effect	18
MCT-induced PAH (Rat)	Improved RV pressure, RVH and RV function	ECM remodeling Secretion of VEGF	19–21

*Abbreviations:* IT, intratracheal; IP, intraperitoneal; LPS, lipopolysaccharide; ECM, extracellular matrix; RV, right ventricle; RVH, right ventricle hypertrophy; PAH, pulmonary artery hypertension; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; NO, nitric oxide; MMP2, matrix metalloproteinase 2; Timp2, tissue inhibitor of metalloproteinases 2.

to play a role in normal airway epithelial homeostasis and turnover but do participate in the setting of lung injury.<sup>33</sup>

Less information is available concerning potential progenitor cell populations contributing to other cells populations such as interstitial, smooth muscle, or endothelial cells in the lung.<sup>31,34</sup> Recently, several groups have identified what seem to be resident mesenchymal stromal (stem) cells in mouse lung as well as in both neonatal and adult human lungs.<sup>35–37</sup> At present, it is unclear whether these cells may participate in structural repair of lung tissue or may play a role in immune surveillance and immunomodulation. In addition to the role of endogenous lung stem and progenitor cells in repair from lung injury, increasing information suggests that mature differentiated lung cells may transdifferentiate and change phenotype. Best described for epithelial–mesenchymal transition, recent investigations describe a wider range of reversible phenotypes in epithelial and mucus cells.<sup>38–43</sup> These mechanisms may also play significant roles in injury or repair from injury. Thus, overall, although progress is being made in clarifying the identity and role of airway progenitor cells in mice, the role(s) of these cell populations remains unclear. Moreover, little corresponding data as yet exist in other animal models or in human lungs.

**Endogenous lung progenitor cells in human lung diseases.** Although it is attractive to speculate that failure of normal endogenous airway stem cell reparative function may contribute to chronic acquired lung diseases such as emphysema, evidence for this remains sketchy at present. More suggestive information is available for the genetic lung disease, cystic fibrosis (CF). Airway epithelium in CF patients contains primitive cuboidal cells that express primitive cell markers, including thyroid transcription factor and cytokeratin 7.<sup>44</sup> Neuroepithelial cells also express the cystic fibrosis transmembrane conductance regulator protein (CFTR), the defective protein in patients with CF that seems to play a role in neuropeptide secretion.<sup>45,46</sup> CFTR  $-/-$  mice also contain fewer pulmonary neuroendocrine cells during embryonic development but increased numbers of these cells postnatally.<sup>47</sup> This suggests that endogenous airway progenitor cell pathways in CF lungs may be altered, but this has not been extensively investigated. Recently, another population of putative airway progenitor cells expressing CCSP, SCA-1, stage-specific embryonic antigen 1, and the embryonic stem cell marker Oct-4 have been identified in neonatal mice.<sup>48,49</sup> These cells were able to form epithelial colonies and differentiate into both type 1 and type 2 alveolar epithelial cells. Interestingly, these cells were



**Fig 1.** Schematic showing different cell types in the lung. Figure courtesy of Barry Stripp, PhD, Duke University, North Carolina, USA.

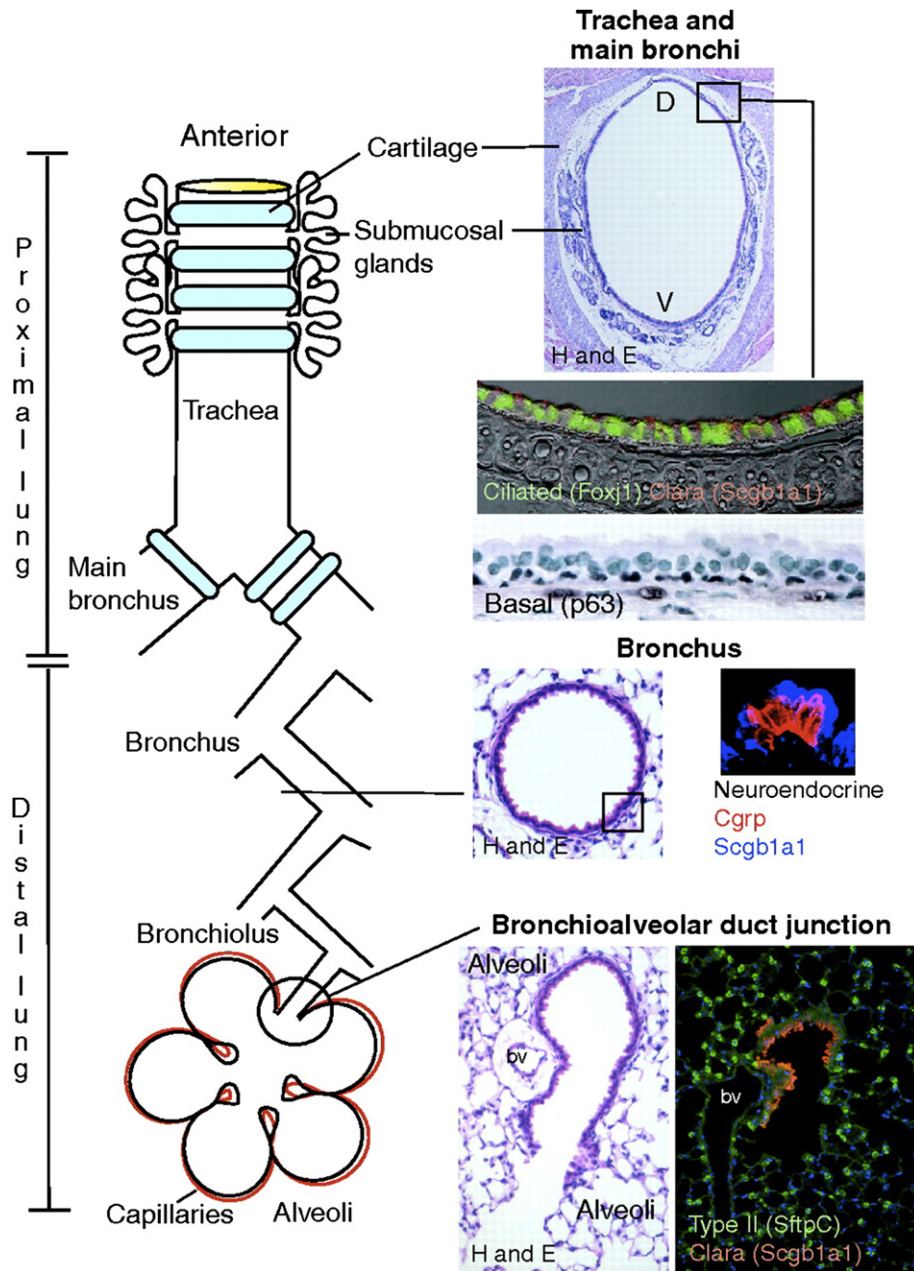
susceptible to infection with the severe acute respiratory syndrome virus raising the possibility that endogenous lung progenitor cells may be specific disease targets. Comparably, the basal epithelial cells of the trachea and upper airways seem more susceptible to infection with the common cold rhinovirus.<sup>50</sup> Endogenous progenitor cells may also be attractive candidates for targeting with gene transfer vectors that provide sustained expression. Variant Clara cells seem to be specifically targeted by recombinant adeno-associated vectors in adult mice.<sup>51</sup> The possibility remains that other endogenous stem or progenitor populations exist, and there is much room for additional information on regulatory mechanisms and pathways as have been elucidated in other epithelial progenitor cell populations.<sup>52</sup>

**Lung cancer stem cells.** There is intense current interest in the connections between endogenous stem or progenitor cells and cancer stem cells. Cancer stem cells have been defined in transplantation assays as the critical cells from tumors that are capable of propagating disease and are hypothesized to be the cells that maintain tumor progression and disease resistance.<sup>53,54</sup> Best described in breast and other solid cancer, increasing evidence suggests there may be multiple lung cancer stem cells.<sup>55-57</sup> Given the heterogeneity of lung cancers, this may not be surprising. BASCs have been suggested to have a role in development of lung cancer in mice, but their role in tumor maintenance has not been established, nor has the human correlation been demonstrated. Side population CD45-Hoechst-effluxing cells have been identified in several human lung cancer cell lines and exhibit tumorigenic properties when subcutaneously implanted into NOD SCID mice.<sup>58</sup> Side population cells have also been identified in clinical lung cancer specimens.<sup>58</sup> Several recent reports implicate CD133+ cells as both conferring resistance to chemotherapy and having tumor initiating properties for

lung cancers.<sup>59-62</sup> Recent studies have begun elucidating cell signaling and gene expression pathways including Wnt, hedgehog, c-kit, Akt, and others that may play roles in transformation of endogenous progenitor cells into lung cancer cells.<sup>60,61,63-68</sup> Despite growing data, additional work is needed to clarify the connections between endogenous lung progenitor cells and their potential roles as lung cancer stem cells and to determine their potential role as therapeutic targets.

#### EMBRYONIC STEM CELLS (ESCs) AND LUNG REPAIR

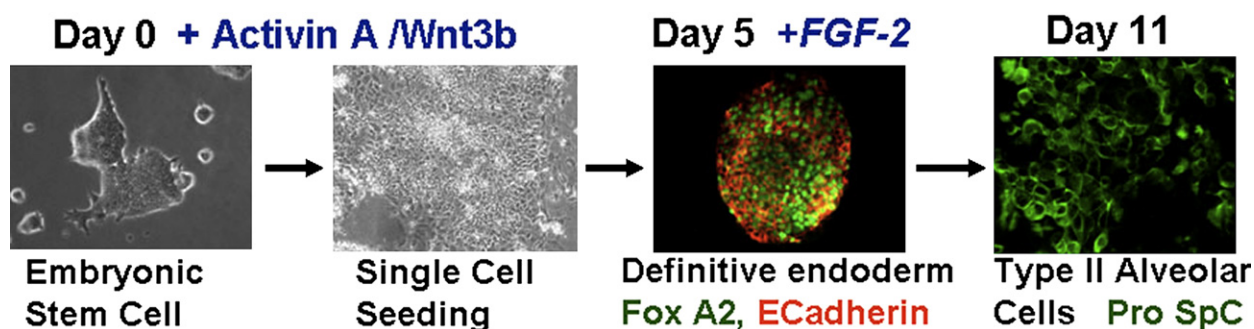
Progress using ESCs for lung regeneration or repair has been slower to develop. In studies during the past approximate 5 years, several laboratories have demonstrated that both mouse and human ESCs can be induced in culture to acquire phenotypic markers of type 2 AEC, including expression of surfactant proteins and lamellar bodies, and even form pseudoglandular structures.<sup>69-72</sup> In general, this occurred at a low level unless the ESCs were genetically engineered to select for surfactant protein-expressing cells.<sup>73</sup> More recent protocols and manipulation of cell signaling pathways guiding embryologic lung development and development of definitive endoderm have yielded more robust *in vitro* derivation of cells with phenotypic characteristics of type 2 alveolar epithelial cells from murine ESCs (Fig 3).<sup>74</sup> Derivation of airway epithelial cells from ESCs has proven even more elusive although development of cells with phenotypic markers of airway epithelial cells has been demonstrated after culture of the ESCs under air-liquid interface conditions.<sup>75,76</sup> Moreover, there are still only limited available studies of effects of ESC administration to lung *in vivo*. Endotracheal administration of mESC-derived type 2 AEC to fetal mice resulted in survival of the cells in the lungs and maintenance of pro-SPC expression during a 24-hour period.<sup>74</sup> Whether the cells truly engrafted or had



**Fig 2.** Schematic showing various types of lung endogenous progenitor cells located in different anatomic regions of the lung. CGRP = calcitonin gene-related peptide, Scgb1a1 = secretoglobulin, family 1A (also known as uteroglobulin), SftpC = surfactant protein C, D = dorsal, and V = ventral. Figure reproduced with permission from Rawlins and Hogan.<sup>24</sup> (Color version of figure is available online.)

functional significance was not determined. Most recently, transduction of one of the approved human ESC lines developed at the University of Wisconsin (line H9.2) with an SPC-promoter-driven neomycin expression cassette was used to develop a >99% pure population of ESC-derived cells with phenotypic characteristics of type 2 AEC, including surfactant expression and presence of lamellar bodies (hES-ATII).<sup>77</sup> Intratracheal administration of the hES-ATII cells 1 or

2 days after induction of acute lung injury following intratracheal bleomycin administration to immunocompromised SCID mice resulted in a substantial number of hES-ATII cells seeming to have engrafted in lung with persistent SPC expression up to 9 days later. Up to 20% of the total SPC-expressing cells seemed to be of hES-ATII origin. A small number of hES-ATII cells also seemed to have differentiated into type 1 AEC *in vivo*. No apparent engraftment was observed if



**Fig 3.** ESCs can be effectively manipulated *in vitro* to differentiate into type 2 alveolar epithelial cells using the lung development cell signaling pathway to guide ESCs differentiation. FGF-2 = fibroblast growth factor-2 and pro-Spc = pro-surfactant protein C. Figure courtesy of Christine Finck, MD, University of Connecticut. (Color version of figure is available online.)

hES-ATII cells were administered to naïve uninjured mice or if a control monocyte population was administered. In parallel, bleomycin-induced lung injury was significantly reduced in mice receiving hES-ATII cells but not monocytes or vehicle control. Reduced injury was measured by both qualitative and quantitative measures, including functional measurements of tidal volumes and arterial oxygen saturations. Whether the observed amelioration of lung injury resulted from structural engraftment of the administered cells or reflected a previously unsuspected paracrine effect of the hES-derived cells is not yet clear. Nonetheless, these results have several ramifications, including the study and potential use of ESCs in genetic lung diseases as human deltaF508 embryonic stem cell lines have been established in England and Belgium.<sup>78,79</sup> These cells exhibit normal morphology and protein expression compared with other hES cell lines but have not been studied in detail. With the new loosening of restrictions on study of human ESCs in the United States, it is anticipated that there will be further rapid advances in study of ESCs for lung injury and repair.

#### ADULT STEM CELLS AND LUNG REPAIR

**Structural engraftment of lung tissues by circulating or exogenously administered stem or progenitor cells.** It was initially speculated that adult bone-marrow-derived cells including hematopoietic stem cells (HSCs), mesenchymal stromal (stem) cells (MSCs), endothelial progenitor cells (EPCs), circulating fibrocytes, and other populations could structurally engraft as mature differentiated airway and alveolar epithelial cells or as vascular endothelial or interstitial lung cells. As such, a sizeable literature has developed over the past approximate 10 years predominantly based on studies in mice using techniques that evaluated histologic demonstration of tagged donor-derived marrow cells in recipient lungs after systemic or more intratracheal administration of

tagged donor marrow cells. Experimental approaches have included use of donor marrow cells from transgenic GFP expressing mice or use of sex-mismatched transplantation (ie, male donor cells into female recipients) and subsequent identification of donor cells using fluorescent *in situ* hybridization to detect the Y chromosome (reviewed in Reference 80). Lethal irradiation and myeloablation of the recipient mouse bone marrow prior to cell administration was frequently but not always used. Notably, prior lung injury, including radiation-induced lung injury resulting from myeloablative total body irradiation, was usually required to observe engraftment although lung injury did not always result in apparent increase of engraftment.<sup>81-83</sup>

However, it has become apparent that engraftment of exogenously administered adult stem cells as airway and alveolar epithelial cells is generally a rare occurrence of uncertain physiologic significance.<sup>84,85</sup> Several technical issues contributed to misinterpretation of results in the earlier reports including inadequate microscopic techniques in which donor-derived cells superimposed on resident airway or alveolar epithelial cells were not effectively discriminated. Furthermore, a variety of leukocytes, notably airway and alveolar macrophages, reside in the lung. Many of the early reports did not use antibodies directed against CD45 or other leukocyte markers to exclude the possibility that cells of donor origin detected in airway or alveolar epithelium were donor-derived leukocytes rather than epithelial cells. Other tools, such as detection of GFP by direct fluorescence as a marker of donor-derived marrow cells, can be subject to error in the presence of autofluorescent cells resident in the lung, notably alveolar macrophages.<sup>86</sup> Furthermore, fusion of marrow-derived cells with resident organ cells, rather than phenotypic conversion of the marrow cells, has been demonstrated in several organs, notably liver and skeletal muscle, but also in lung.<sup>87-89</sup> The extent of fusion in lung after

sex-mismatched transplantation may be underestimated as the Y chromosome may be lost from the heterokaryon cells resulting from fusion of donor-derived marrow cells with type 2 alveolar epithelial cells in mouse lungs.<sup>89</sup> A smaller body of literature in human bone marrow and lung transplantation that had also demonstrated apparent chimerism in lungs of the transplant recipients has also been called into question for the same reasons (reviewed in Reference 80).<sup>90-97</sup>

Nonetheless, recent studies with more rigorous techniques continue to demonstrate that engraftment of airway and alveolar epithelium, as well as of pulmonary vascular endothelium and of lung interstitium, with adult bone marrow or cord blood-derived stem cells, although rare, can occur under certain conditions, usually after previous perturbation through induction of lung injury.<sup>17,98-106</sup> Interestingly, several recent reports suggest that chronic or progressive lung injury may result in more substantial engraftment of type 2 AECs and of interstitial and pulmonary vascular cells with donor-derived cells in mouse or rat models.<sup>101</sup> However, not all chronic lung injury models resulted in substantial epithelial engraftment.<sup>107</sup> Notably, engraftment of pulmonary vascular endothelium and stimulation of neo-angiogenesis by exogenously administered EPCs has fostered recent clinical trials for treatment of pulmonary hypertension (as will be discussed further).<sup>108-110</sup> However, there are many variables still left to be explored that may increase epithelial, interstitial, or pulmonary vascular engraftment with circulating or donor-derived cells. For example, the types of marrow-derived, cord-blood-derived, or fully differentiated nonpulmonary cells that might engraft as lung epithelium, interstitium, or pulmonary vasculature remain to be fully explored. In addition to existing studies of HSCs, MSCs (of bone marrow and cord blood origin), EPCs, and fibrocytes, the possibility remains that there may be other cell populations that could be recruited to the lung or localized to the lung after systemic or other routes of administration.<sup>111,112</sup> A population of circulating bone-marrow-derived CD45+/CXCR4+/cytokeratin+ cells has been described to participate in re-epithelialization of denuded tracheal xenografts.<sup>113</sup> Most recently, a population of CCSP-expressing marrow cells has been suggested to engraft as airway epithelium.<sup>114</sup> Other sources of stem or progenitor cells, such as umbilical cord blood or adipose tissues, also have not yet been extensively characterized for ability to engraft as lung tissue.<sup>115-117</sup> The effect of age of either donor cells or of recipients is also less well explored, although one report demonstrated that transplantation of whole marrow into 1-day-old mouse pups, using a variety of conditioning regimens, did not increase the number of bone-marrow-derived cells over that observed after total marrow administration to adult mice.<sup>105</sup> Furthermore,

the route of administration of donor-derived cells is less well characterized as most studies have investigated engraftment after systemic administration of donor cells. Direct intratracheal administration of MSCs may enhance retention of donor-derived cells in the lung and possible epithelial engraftment.<sup>8,114</sup> However, this area is controversial and remains under-explored.

Furthermore, the ability to structurally engraft in adult lung may not solely be a property of stem or progenitor cells. For example, intratracheal administration of isolated type 2 alveolar epithelial cells results in rare engraftment in areas of injured lung after bleomycin administration to rats.<sup>118</sup> Notably, bleomycin-injured rats that received the type 2 AEC had less histologic injury and decreased hydroxyproline content. Comparably, intratracheal administration of neonatal mouse lung fibroblasts resulted in apparent alveolar and interstitial engraftment and engraftment was higher in areas of elastase-induced lung injury.<sup>119</sup> Systemically administered skin fibroblasts, transduced *ex vivo* to express angiopoietin-1, protected against lung injury produced by intratracheal endotoxin administration in rats.<sup>120</sup> These results suggest that lung injuries might be amenable to cell therapy approaches using a variety of cell types other than stem or progenitor cells.

The mechanisms by which circulating or systemically administered stem or progenitor cells might be recruited to lung remain poorly understood. After systemic (ie, venous) administration, many cells initially localize in lungs as the first major capillary bed encountered. Lung injury can result in increased localization and/or retention of marrow-derived cells in lung.<sup>87-89</sup> The range and identity of chemotactic soluble mediators released by injured lung cells and the role of upregulation of adhesion molecules with which circulating cells might interact remains poorly understood, but several recognized pathways including the SDF-1/CXCR4 axis and CD44 seem to be involved (reviewed in Reference 80).<sup>121-123</sup> Recipient immune responses also play significant yet poorly characterized roles in retention of cells in lung.<sup>89</sup> Commonly used approaches of sex-mismatched transplantation or cell administration may also result in clearance of cells.<sup>89</sup> The timing of cell administration after lung injury can also influence recruitment and phenotypic conversion. Systemic administration of MSCs 4 hours after lung irradiation resulted in apparent engraftment of cells as epithelial and vascular endothelial cells.<sup>124</sup> However, MSCs administered at later time points seemed to engraft as interstitial cells and to participate in the development of fibrosis.<sup>124,125</sup> As with engraftment, several factors including age of donor or recipient, type of cell administered, and route of administration might affect recruitment to lung.

Once lodged in or recruited to the lung, the mechanisms by which stem or progenitor cells might be induced to acquire phenotype of lung epithelial, interstitial, or vascular endothelial cells remain poorly understood. *In vitro* studies continue to demonstrate that soluble factors released from lung epithelial cells or from injured lung homogenates can induce expression of lung epithelial markers in several types of marrow-derived cells, possibly through activation of  $\beta$ -catenin signaling pathways.<sup>7,126,127</sup> One novel mechanism of inducing phenotypic change might involve release of membrane-derived microvesicles, a recently appreciated means of intercellular communication that involves horizontal transfer of mRNA and proteins between cells.<sup>128,129</sup>

**Immunomodulation of lung diseases by mesenchymal stem cells.** MSCs were first described in 1968 as an adherent, clonogenic, nonphagocytic, and fibroblastic-like population of bone marrow cells.<sup>130</sup> Since then they have been isolated from many other tissues including adipose tissues, cord blood, placenta, and amniotic fluid.<sup>59</sup> MSCs are multipotent and can differentiate into cells of mesodermal, endodermal, and ectodermal lineages.<sup>80</sup> Furthermore, these cells also have the ability to regulate hematopoietic cells and to secrete multiple regulatory molecules such as growth factors and anti-inflammatory cytokines that can modulate immune responses.<sup>131</sup> The mechanisms of MSC actions on inflammatory and immune cells are not well understood but likely involve both secretion of soluble mediators as well as cell–cell contact (Fig 4). Although the frequency of MSCs in the adult bone marrow is low (less than 0.1%); once isolated, MSCs can be expanded *ex vivo*, which makes it possible to manufacture these cells for potential therapeutic purposes. Furthermore, MSCs can easily be transduced or genetically manipulated to deliver or to secrete selected disease-modifying molecules. Moreover, MSCs also seem to be relatively immunoprivileged because of their low constitutive expression of major histocompatibility complex (MHC) type 1 and lack of constitutive expression of MHC type 2 and the costimulatory molecules CD80, CD86, and CD40.<sup>132</sup> This allows administration of allogeneic MSCs without significant host responses. Overall, these properties of MSCs make them an attractive therapeutic tool as a vector for treatment delivery or as immunomodulatory agents. As such, MSCs have now been used in clinical trials for inflammatory and autoimmune diseases such as graft versus host and Crohn's diseases as well as in acute myocardial infarction.<sup>133</sup> As will be discussed, immunomodulation and amelioration of lung injury has now been demonstrated in a variety of mouse models and suggests the most direct translational route toward clinical trials of cell therapies in lung diseases. As such, a trial of allogeneic MSCs in patients with

moderate–severe chronic obstructive pulmonary disease has been started in the United States.

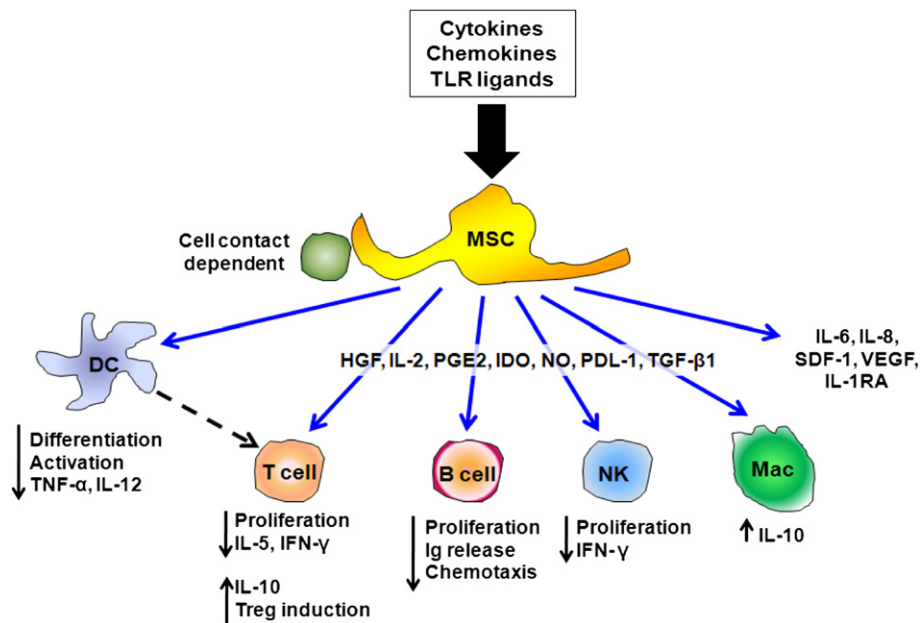
### STEM CELL AND CELL THERAPY APPROACHES FOR SPECIFIC LUNG DISEASES

**Acute lung injury (ALI).** A growing number of studies have demonstrated compelling data on the beneficial effects of MSCs in mouse models of ALI including acute bacterial pneumonia, administration of endotoxin or bleomycin, hyperoxia exposure, and cecal ligation and puncture (CLP)-induced sepsis.<sup>7-13,15,134,135</sup> Other available animal models of ALI have been used to evaluate for pathogenesis and pathophysiology of lung injury, such as acid-induced acute lung injury<sup>135,136</sup> and ventilator-induced lung injury.<sup>137</sup> However, none of these has yet been tested for therapeutic efficacy of MSC.

In the endotoxin injury model, mice receive *Escherichia coli* lipopolysaccharide (LPS) either via intraperitoneally or intratracheally followed by MSC administration systemically or intratracheally at 1 hour or 4 hours after injury, respectively. The LPS administration results in acute lung inflammation that begins several hours peaks between 24 and 48 hours and resolves after that.<sup>134</sup> Both systemic and intrapulmonary administration of bone-marrow-derived MSCs have been demonstrated to result in improved mortality, improvement of alveolar fluid clearance, and attenuation of inflammation and lung injury despite minimal, if any, engraftment of MSCs in the lung. Several paracrine mechanisms have been proposed for the MSC effects in this model including release of anti-inflammatory mediators such as IL-10, angiopoietin-1, and keratinocyte growth factor (KGF). Moreover, MSCs engineered to further express angiopoietin-1 potentiated the protective effect of MSCs.<sup>116</sup> The MSCs themselves may be influenced by the specific inflammatory environment in the injured lung. For example, endotoxin can act on toll-like receptor 4 (TLR-4) expressed by the MSCs resulting in increased production of cyclooxygenase-2 and subsequent increased production of the anti-inflammatory agent prostaglandin E2 by the MSCs.<sup>15</sup> Similar anti-inflammatory effects of MSCs have been found in endotoxin-treated isolated perfused human lungs.<sup>138</sup> Another interesting study performed by Zhao et al showed that the protection of lung injury prevented endothelial leakage by strengthening of the endothelial adherent junction barrier through activation of RhoGTPase Cdc42.<sup>139</sup> Altogether, these studies demonstrate the potent immunomodulatory effect of MSCs in mouse and isolated human lung models of acute lung injury and provide important preclinical data for potential clinical trials of allogeneic MSCs in ALI and ARDS.

**Fibrotic lung diseases.** Idiopathic pulmonary fibrosis (IPF) is a devastating chronic progressive pulmonary disease with high morbidity and mortality, and no





**Fig 4.** Schematic illustrating the range of *in vitro* immune-modulating effects described for MSCs. DC = dendritic cell; HGF = hepatocyte growth factor; IDO = indoleamine 2,3-dioxygenase; IFN- $\gamma$  = interferon  $\gamma$ ; Ig = immunoglobulin; IL = interleukin; IL-1RA = interleukin-1 receptor antagonist; Mac = macrophage; NK = natural killer; PGE2 = prostaglandin E-2; SDF-1 = stem-cell-derived factor 1; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ ; TGF- $\beta$ 1 = transforming growth factor-  $\beta$ 1; TLR = toll-like receptor; and VEGF = vascular endothelial growth factor. (Color version of figure is available online.)

effective treatment for this disease has been identified.<sup>140</sup> IPF is now considered to be caused by pro-inflammatory and noninflammatory pathways that lead to chronic epithelial injury. This triggers cellular apoptosis and a dysregulated immune response characterized by, among other things, elicitation of Th2 cytokines,<sup>141-143</sup> which promotes the expression of pro-fibrotic factors like transforming growth factor beta.<sup>144</sup> Other fibrotic lung diseases are a consequence of underlying auto-immune and inflammatory conditions through as yet unclear mechanisms. Several animal models of fibrotic lung diseases have been developed to study the pathophysiology and pathogenesis of human fibrotic lung diseases.<sup>145</sup> The best characterized model is the bleomycin model of pulmonary fibrosis. The drug was initially used as an anti-neoplastic agent; however, its use was limited by pulmonary toxicity and development of pulmonary fibrosis.<sup>145</sup> It can be administered via intraperitoneal, intravenous, subcutaneous, or intratracheal routes.<sup>145</sup> Other fibrotic lung disease models that have been characterized are fluorescein-isothiocyanate-induced fibrosis,<sup>146</sup> irradiation-induced fibrosis,<sup>147</sup> silica,<sup>148</sup> asbestos-induced fibrosis,<sup>149</sup> various transgenic mice including T-bet deficient mice,<sup>150</sup> and viral models of chronic fibrosis.<sup>151</sup>

The murine model that has been most extensively evaluated for the protective or therapeutic effects of MSCs in fibrotic lung disease is intratracheal adminis-

tration of bleomycin. The advantage of this model is that it requires only a single dose to result in injury and fibrosis, with fibrosis starting around day 14 and maximal responses around days 21–28.<sup>145</sup> However, resolution of fibrosis can occur in mice after day 21.<sup>145</sup> Systemic administration of MSCs 4 hours after bleomycin administration was demonstrated to reduce inflammation, fibrosis formation, and mortality at 14 days after injury, in part mediated by the IL-1 $\alpha$  receptor antagonist released by the MSCs.<sup>1,2</sup> However, the protective effects of MSCs were abrogated when MSCs were given at day 7 after injury.<sup>1</sup> In another study using asbestos-induced pulmonary fibrosis in mice, after total body irradiation and bone marrow transplantation, the chimeric mice were protected from development of lung injury and fibrosis as compared with nonchimeric mice.<sup>107</sup> However, the protective effect of transplantation is most likely from changes occurred after irradiation and bone marrow transplantation. Another study demonstrated a reduction in collagen deposition in bleomycin-induced lung fibrosis in mice after administration of MSC or lineage-negative HSCs engineered to overexpress KGF.<sup>152</sup>

In contrast, another bone-marrow-derived stem cell population, circulating fibrocytes, have been implicated in the pathogenesis of several lung diseases in both mouse and clinical models including pulmonary fibrosis, pulmonary hypertension, and the subepithelial

fibrosis that can develop in severe asthma, as well as in clinical bronchiolitis obliterans in lung and bone marrow transplant patients.<sup>153-156</sup> Further levels of circulating fibrocytes may be an indicator of worse prognosis in both asthma and IPF.<sup>157-158</sup> As such, based on current available data in the mouse models, cell-based therapy approaches may be less effective in established fibrotic lung diseases. However, studies using cell therapy as preventive modality for development of fibrosis (ie, after irradiation-induced lung injury) are needed. For example, administering marrow-derived cells early after radiation-induced lung injury in mice was protective, whereas administering the cells later resulted in the cells contributing to the radiation-induced damage.<sup>124</sup> One potential therapeutic avenue for fibrotic lung diseases is to inhibit the fibrocytes. Alternatively, these cells might be excellent vectors to deliver therapeutic agents directly at the site of fibrosis (ie, fibroblastic foci).

**Chronic obstructive pulmonary disease (COPD).** COPD is currently the fourth leading cause of death in worldwide.<sup>159</sup> Approximately 20% of patients with COPD present with emphysema that is characterized by destruction of terminal bronchioles and alveolar walls causing enlargement of air spaces. Patients generally die from progressive respiratory failure, and despite advances in the symptomatic treatment of emphysema, no cure remains.

Available experimental mouse models of emphysema include transgenic and knockout models and exposure to environmental agents such as tobacco smoke or intratracheal administration of degradative enzymes such as elastase.<sup>160,161</sup> Several studies have evaluated the role of stem cells on remodeling of the lung in mouse emphysema models using elastase-treated and transgenic (Tight-skin or Tsk) mice.<sup>161-163</sup> These studies used transplantation of wild-type mouse bone marrow cells into emphysematous mice and demonstrated that there were decreased emphysematous structural changes in chimeric mice. In particular, a study in Tsk mice that had already developed emphysematous changes at the time of transplantation demonstrated a reversal of these changes such that the lungs resembled those of age-controlled normal mice with nonsignificant engraftment of donor-derived cells in chimeric mice lungs.<sup>87</sup> Another study in rats was done to specifically evaluate the role of bone marrow stromal cells in irradiation and papain-induced emphysema.<sup>14</sup> In this study, plastic adherent bone marrow cells (bone-marrow-derived stromal cells) were systemically administered immediately after irradiation (7.5 Gy total body irradiation) and intratracheal instillation of 8% papain (0.05 mL/100 g body weight). Lungs were harvested

after 28 days and demonstrated a reduction in air-space enlargement, as quantified by mean linear intercept, and apoptotic cells in the bone-marrow-derived stromal cells treated group as compared with the controlled irradiated, papain-treated group.<sup>14</sup> Minimal engraftment by donor-derived cells was observed. These data suggest that bone-marrow-derived stromal cells can protect against progression of emphysema. The potential mechanism is likely through paracrine effects resulting in decreased alveolar apoptosis after injury. However, there is much yet to be learned about anti-inflammatory effects of bone-marrow-derived and other stem cell populations in models of COPD.

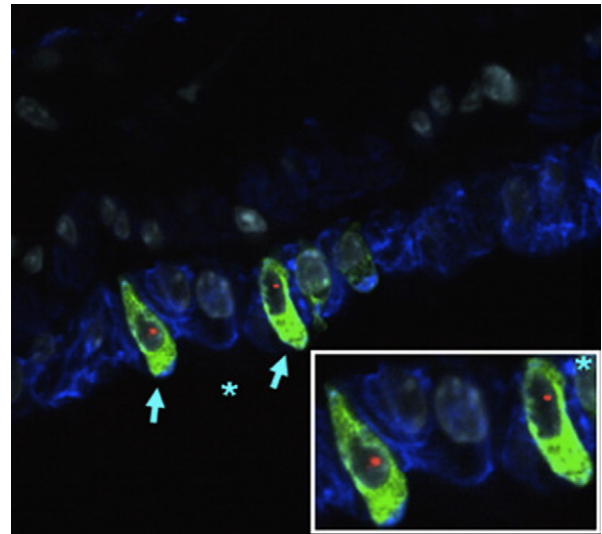
In a phase I, double-blind, placebo-controlled trial of PROCHYMAL™ (*ex vivo* cultured adult human mesenchymal stem cells) conducted by Osiris Therapeutics Inc. (Columbia, Md) in patients with acute myocardial infarction, an improvement in both forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) was noted in treated patients.<sup>164</sup> Although the mechanisms of improvement in pulmonary function in this patient population are not yet well understood, these observations have stimulated a multicenter, double-blind, placebo-controlled phase II trial of PROCHYMAL™ (Osiris Therapeutics) for patients with moderate–severe COPD (FEV1/FVC < 0.70, 30% ≤ FEV1 ≤ 70%), which was initiated in May 2008. The primary goal of the trial is to determine the safety of MSC infusions in patients with lung disease. The secondary goal is initial estimation of the potential efficacy of MSCs for decreasing the chronic inflammation associated with COPD, thus improving both pulmonary function and quality of life. The trial has recruited 62 patients in 6 participating U.S. sites. In the 6-month interim data analysis, no infusion toxicities or significant adverse events were reported. Notably, a significant decrease in the circulating inflammatory marker C-reactive protein, which is commonly increased in COPD patients, was noted in treated patients as was a trend towards improvement in quality of life indicators.<sup>165</sup> The trial ends in the fall of 2010, and further results will be forthcoming.

**Cystic fibrosis.** CF is an autosomal recessive disease caused by mutations in the gene encoding for the CF transmembrane CFTR resulting in abnormal ion transport across the respiratory epithelium leading to CF lung disease. The initial cell therapy approach for CF in mouse models was based on the concept of replacing defective airway epithelial cells with wild-type donor cells containing the correct CFTR gene. However, only rare engraftment (<0.025% of total airway epithelial cells) of airway and alveolar epithelium with adult bone-marrow- or cord-blood-derived stem cells with corresponding CFTR protein expression has been found

(Fig 5).<sup>17,80,104,105</sup> This was despite using naphthalene to denude the airway epithelium and conceivably enhance donor cell engraftment. In one study, intestinal engraftment was also assessed, and despite being rare, functional CFTR activity was detected by potential difference measurements in the rectal mucosa of recipient mice. This observation remains unexplained, as it is difficult to reconcile the rare engrafted cell observed with a physiologic change in potential difference. Transplant of whole marrow into 1-day-old CFTR knockout mice also did not increase the number of donor origin cells engrafted as either respiratory or intestinal epithelium.<sup>104</sup> These studies suggest that systemic administration of adult bone-marrow- or cord-blood-derived progenitor cells do not result in significant structural engraftment as CFTR-expressing cells as either airway or intestinal epithelium in mouse models. Although not yet investigated in CF mouse models, several recent studies have also found only low levels of airway epithelial engraftment after direct airway administration of donor marrow-derived cells in mice.<sup>8,103</sup> Overall these observations suggest that, unless significant advances are made in understanding the mechanisms by which cells are recruited to airway epithelium and induced to undergo phenotypic conversion to functional airway epithelial cells, correction of CF lung disease by structural engraftment of adult marrow- or cord-blood-derived stem cells is not likely at present. In contrast, as CF lung disease is characterized by chronic neutrophilic airway inflammation and high expression of multiple proinflammatory cytokines,<sup>166</sup> a potential therapeutic role of MSCs to abate inflammation in CF lung disease is suggested. However, there is no available information concerning the immunomodulatory effects of MSC in CF lungs. This would seem a promising area for study.

**Asthma.** Asthma is characterized by reversible air flow obstruction, hyper-responsiveness of the airway to smooth muscle agonists, and airway inflammation. In most cases of asthma, the patients remain symptomatically controlled by available medications including corticosteroids and bronchodilators. However, approximately 5% of asthmatics are refractory to conventional treatment and suffer substantial morbidity and mortality. The ability of MSCs to modulate immune systems has led to increasing interest in using MSCs as a potential therapeutic modality for severe refractory asthma. Several laboratories have now demonstrated that MSCs can ameliorate allergic airways inflammation in mice.<sup>16,167-169</sup> However the optimal dose, route of administration, timing of administration, and longevity of MSCs effects are not yet known.

**Pulmonary vascular diseases.** Pulmonary hypertension (PH) is a disease characterized by pulmonary vascular



**Fig 5.** CFTR expressed cells can be detected in female CFTR knockout mouse lungs after transplantation with male GFP stromal marrow cells. Donor-derived (Y chromosome, red), CFTR-positive (green), and cytokeratin-positive (blue) cells are indicated by *light blue arrows* in airway walls of lungs assessed 1 week after transplantation. Inset is a higher power view of the area marked by asterisk. Original magnification:  $\times 1000$ . Figure reproduced with permission from Loi et al.<sup>17</sup> (Color version of figure is available online.)

remodeling resulting in pulmonary artery obstruction that, in turn, leads to elevation of pulmonary arterial pressure, right-heart dysfunction, and ultimately, progressive respiratory failure. MSCs-based therapy had been explored as a potential therapeutic modality in this devastating disease.

Several rodent models of PH are available, but the most commonly used involve administration of monocrotaline (MCT) or exposure to chronic hypoxia. Systemic or intratracheal MSC administration can attenuate MCT-induced increase in pulmonary artery pressure and improve pulmonary vascular resistance.<sup>19,20</sup> The protective effects of MSCs in this model occurred even when cells were administered systemically 7 days after MCT administration.<sup>21</sup> MSCs express several growth factors including vascular endothelial growth factors that promote neovascularization, and this might be one of the mechanisms by which MSCs protect against development of PH. Furthermore, EPCs can be transduced to express pro-angiogenic factors such as endothelial nitric oxide synthetase (eNOS) or inhibitors of smooth muscle cell proliferation such as calcitonin gene related peptide, which further improve right ventricle function.<sup>21</sup> Several studies in mice and dogs have demonstrated a role for exogenously administered EPCs in vasculogenesis and vascular repair in experimental models of pulmonary hypertension.<sup>108,170-172</sup> EPCs can also preferentially localize to areas of injured lung after systemic

administration and may also have paracrine effects to decrease inflammation.<sup>173</sup> As such, two pilot trials of autologous EPC administration for primary pulmonary hypertension conducted at Zhejiang University, Hangzhou, China, in both adult and pediatric patients demonstrated an increased 6-minute walk capacity and improved hemodynamic variables, including mean pulmonary artery pressure, pulmonary vascular resistance, and cardiac output, 12 weeks after systemic administration of autologous EPCs with conventional therapy compared with patients receiving conventional therapy alone.<sup>110,174</sup> Importantly, no adverse effects of EPC administration were noted, although long-term follow-up is pending. A therapeutic trial of administration of autologous EPCs transduced to express eNOS for patients with pulmonary hypertension, the Pulmonary Hypertension and Cell Therapy (PHaCeT) trial has been initiated at St. Michael's Hospital in Toronto, Ontario, Canada, and the Jewish General Hospital in Montreal, Quebec, Canada. As of March 2010, the PHaCeT trial has completed enrollment of the first 2 dose-panels with 3 patients receiving a total of 7 million early growth EPCs transfected to overexpress human eNOS in panel 1 and three more patients receiving 23 million cells in panel 2 (information courtesy of Duncan Stewart, MD, FRCPC). The cell delivery procedure was well tolerated, and there were no safety concerns. Notably, the first 6 patients showed a remarkable reduction in total pulmonary vascular resistance during the course of the 3-day delivery period, which might represent the effect of increased nitric oxide (NO) release by the engineered EPCs within the pulmonary microcirculation. The trial's Data Safety Monitoring Board has approved moving to panel 3, which calls for a total of 50 million cells, again in 3 divided doses over 3 days. Completion of the third dose panel will be followed by enrollment of an additional 3 patients at the highest tolerated cell dose, which should provide sufficient support to move forward with the design of a randomized controlled trial that can assess potential efficacy of this cell therapy approach in pulmonary artery hypertension.

**Lung cancer.** In addition to the possibility of endogenous lung progenitor cells functioning as cancer stem cells, bone-marrow-derived or circulating MSCs, EPCs, and fibrocytes may each contribute to development of primary and metastatic lung and other malignancies in mice, in part by providing a supportive stroma for the cancers and/or by participating in tumor vascularization.<sup>175-179</sup> In contrast, MSCs and EPCs have been demonstrated to home to areas of tumor development and EPCs and MSCs, as well as HSCs, engineered to release anti-tumor or pro-apoptotic agents, have been used to suppress tumor growth in mouse tumor models.<sup>180-186</sup> Cell-based therapies may thus be power-

ful new tools for lung cancer. In parallel, increased numbers of circulating EPCs also portended worse survival among those with non-small-cell lung cancer. Thus, circulating stem or progenitor cells may serve as useful biomarkers for lung cancer response to therapies.

**Lung tissue bioengineering.** Most recently, focus has been on exploration of 3-dimensional culture systems and bioengineering approaches to generate functional lung tissue *ex vivo* and *in vivo*.<sup>187,188</sup> These approaches have been increasingly successfully used in regeneration of other tissues including skin, vasculature, cartilage, and bone. Notably, MSCs isolated from amniotic fluid, umbilical cord blood, adipose tissues, or bone marrow can be seeded on biodegradable polyglycolic acid or other biosynthetic scaffolds and can generate tracheal cartilage for use in repair of congenital tracheal defects, as well as tendon tissue for use in congenital diaphragmatic defects.<sup>189,190</sup> Studies in animal models and a recent clinical investigation suggest safety and efficacy, and clinical trials in neonates with congenital tracheal or diaphragmatic defects are planned.<sup>189-191</sup> Most recently these approaches have resulted in successful clinical use of a bioengineered airway.<sup>192</sup>

The complex 3-dimensional architecture of the lung makes engineering functional lung parenchyma *ex vivo* a more difficult task. Studies both *in vitro* and *in vivo* using mixed fetal lung cells cultured in a 3-dimensional glycosaminoglycan or other type of scaffolds resulted in formation of alveolar-like structures in the scaffold.<sup>188,193</sup> Furthermore, stimulation of murine fetal lung cells in polymer scaffolds with different isoforms of fibroblast growth factor resulted in different patterns of development demonstrating the power of 3-dimensional culture systems to evaluate lung development and repair.<sup>194</sup> Fetal rat lung cells cultured in a biodegradable gelatin sponge, and subsequently injected into normal rat lungs, induced formation of branching, sacculated epithelial structures reminiscent of lung parenchymal architecture.<sup>193</sup> Mixed fetal murine epithelial cells admixed with Matrigel and injected subcutaneously into the abdominal wall of adult mice demonstrated cells that expressed pro-surfactant protein C (pro-SPC) after 1 week.<sup>195</sup> These studies demonstrate the potential of *in vivo* lung tissue generation using mixed populations of fetal lung cells. However, using fetal lung cells is not a practical approach, and lung tissue engineering with stem or progenitor cells is a more practical option.

However, there are few studies as yet evaluating whether stem or progenitor cells isolated from adult bone marrow, cord blood, or other sources can also comparably form airway or alveolar-like structures when cultivated in a 3-dimensional matrix or other scaffolding material and whether stem or progenitor cells

cultured in such fashion can be used for functional lung regeneration *in vivo*. A population of cells described as adult lung somatic progenitor cells isolated from adult sheep lungs cultured in synthetic polymer constructs resulted in expression of airway and alveolar epithelial markers by the cells.<sup>196</sup> Structures resembling lung airways and parenchyma developed when impregnated constructs were implanted subcutaneously in nude mice or inserted into the wound cavity after wedge lung resection in sheep. Adipose-derived MSCs, cultured *ex vivo* in sheets of polyglycolic acid and then applied to wound edges after lung volume reduction surgery in rats, accelerated alveolar and vascular regeneration.<sup>197</sup> Successful engineering of a portion of a bronchus and subsequent transplantation into a patient with bronchial stenosis has also been reported.<sup>198</sup> In this particular study, autologous bone-marrow-derived MSCs were cultured and differentiated into chondroblasts prior to seeding onto a de-cellularized allograft section of bronchus along with the patient's epithelial cells. By applying appropriate growth factors and physical force, a functional bronchus resulted and was used in transplantation. These studies demonstrate that MSCs and other stem cell populations can be used in *ex vivo* tissue bioengineering and herald a potential new era for lung transplantation.

## CONCLUSION

A continuing rapid accumulation of data in both animal models and in clinical trials suggests that cell-based therapies using adult tissue-derived stem/progenitor cells may be potential therapeutic strategies for lung repair and remodeling after injury and as a source of cells for *ex vivo* lung tissue engineering. In parallel, further understanding of the role of endogenous lung progenitor cells will provide insight into mechanisms of lung development and repair after injury and may provide novel therapeutic strategies. It is hoped new research programs will provide further understanding of mechanisms of repair of lung injury and provide a sound scientific basis for therapeutic use of stem and cell therapies in lung diseases.

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