Mauricio Rojas *Editor*

Stem Cells in the Respiratory System

Stem Cell Biology and Regenerative Medicine

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Mauricio Rojas
Editor

Stem Cells in the Respiratory System

Editor
Mauricio Rojas
Department of Medicine
Emory University
Division of Pulmonary, Allergy and Critical
Care Medicine
Michael St. 615
30322 Atlanta, Georgia
USA
mrojas@emory.edu

Department of Medicine
University of Pittsburgh
Division of Pulmonary, Allergy and Critical
Care Medicine
15213 Pittsburgh, PA

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Preface

Lungs are one of the most complex organs; mature lung is composed of at least 40 morphologically differentiated cell lineages with distinct functions. The proximal airways contain mucous, ciliated, basal, Clara, and pulmonary neuroendocrine cells, whereas the distal airways contain mainly ciliated cells and nonciliated Clara cells. Alveolar units are almost entirely composed of distinct type I and type II alveolar epithelial cells, directly exposed to the exterior and with the entire blood passing through to be oxygenated. These particular factors make the lung a susceptible organ, a target for multiple types of internal and/or external injury. The mechanisms of lung repair are complex and, depending on the type of cell affected, the repair process might have different characteristics.

Because of their multipotentiality, stem cells are considered as a novel and important alternative cell-based therapy in lung injury. To name a cell as a stem cell, it must meet two strict criteria: extended self-renewal capacity and multilineage differentiation. Progenitor cells have some but not limitless self-renewal capacity and restricted lineage differentiation potential. The most completely characterized adult stem cell is the hematopoietic stem cell, which can differentiate into all blood cells, including lymphoid, myeloid, platelet, and red blood cell lineages.

Today, the concept of plasticity and transdifferentiation of stem cells and, in particular, adult mesenchymal stem cells has engendered significant controversy regarding their use as a therapeutic agent. The benefit of stem cell therapy has been undoubtedly observed, however apparently independently of a lasting cell engraftment and differentiation. The protective effect with bone marrow cell therapy has been explained more recently by a paracrine secretion of anti-inflammatory factors that enhances the recovery from diverse acute and chronic injuries.

Lately, there has been increasing interest in local or endogenous stem cells in the lung. There is experimental evidence that the airway epithelium likely turns over every 30–50 days. Thus, resident local cells can mediate reestablishment of the airway epithelium with normal structure and function unless an injury is too severe, extensive, or chronic. Although there may be some contribution from circulating stem/progenitor cells, most evidence supports the concept that local stem/progenitor cells are the main source of new cells with the potential to differentiate into all cell types in the normal epithelium.

Taken together, these observations suggest that the process of lung repair is a very dynamic and well-coordinated set of events. In this process, external cells, preferentially bone-marrow-derived mesenchymal stem cells, are recruited into the lung after injury to downmodulate inflammatory responses. This phase of the repair will mediate a diminution of the severity of the wound, and will create an appropriate milieu for local progenitor cells and potentially some recruited bone-marrow-derived stem/progenitor cells, to regenerate the normal lung epithelium and parenchyma and restore the lung function.

In this book, the authors discuss the potential role of different types of stem cells, in the context of physiological stress and lung injury. In Chap. 1, Susan Reynolds reviews the lung structure and function and their correlation with endogenous lung stem cells. Daniel Weiss reviews in Chap. 2 the different sources of adult mesenchymal stem cells, as well as the controversial issue of cell differentiation into alveolar epithelial cells and the implications for future cell therapies in the lung. Recruitment of nonhematopoietic cells into the injured lung has not been well documented. In Chap. 3, Ellen Burnham explores the implications of mobilization and recruitment of progenitor cells, endothelial cells, and epithelial cells. In Chap. 4, Robert Strieter explains the role of another type of bone-marrow-derived progenitor cell, the fibrocytes. These cells have been implicated in pulmonary fibrosis, but as discussed by Strieter, these cells have unique properties that make them an indispensable element in the process of lung repair. An additional important factor that can determine the magnitude of cell recruitment and can have implications on the fate of the recruited cells is the type of extracellular matrix to which stem cells are exposed. In Chap. 5, Jesse Roman presents an extended review of the different proteins that form the extracellular matrix and how each of them can induce the differentiation of stem cells into fibroblasts and myofibroblasts. A novel concept for the mobilization of stem/progenitor cells is the effect of physical activity. In Chap. 6, Partick Wahl describes in detail the effect that exercise can have on the recruitment and homing of these cells into the different organs. Finally, we dedicate two chapters to discuss some clinical applications of mesenchymal stem cells. First, in Chap. 7, Micheal Matthay discusses the role of stem cells in acute lung injury and repair, and, finally, in Chap. 8, we present a complete review of the use of mesenchymal stem cells in animal models of lung diseases. These studies support the translation of mesenchymal-stem-cell-based therapy for acute lung injury, pulmonary hypertension, cystic fibrosis, and lung transplant.

The objective of this book is to review the most relevant and recent concepts for the use of local, endogenous, or exogenous progenitor/stem cells in the prevention and repair of the lung after injury. This is a very dynamic field, currently in constant evolution. The authors presenting their work here are indisputable leaders in their field, making this book an exciting collection of reviews by an outstanding group of investigators.

Atlanta, GA, USA Mauricio Rojas

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Contributors

Shama Ahmad Department of Pediatrics, National Jewish Health, Denver, CO 80206, USA, ahmads@njhealth.org

Wilhelm Bloch Institute of Cardiovascular Research and Sport Medicine, German Sport University, Cologne, Germany; The German Research Center of Elite Sport, Cologne, Germany, w.bloch@dshs-koeln.de

Heather M. Brechbuhl Department of Pediatrics, National Jewish Health, Denver, CO 80206, USA

Kenneth L. Brigham Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Center for Translational Research in the Lung, McKelvey Center for Lung Transplantation, Emory University, Atlanta, GA 30322, USA, kbrigha@emory.edu

Ellen L. Burnham Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver School of Medicine, Aurora, CO 80045, USA, ellen.burnham@ucdenver.edu

Carter Co Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Center for Translational Research in the Lung, McKelvey Center for Lung Transplantation, Emory University, Atlanta, GA 30322, USA, cco@emory.edu

Moumita Ghosh Division of Cell Biology, Department of Pediatrics, National Jewish Health, Denver, CO 80206, USA

Naveen Gupta Cardiovascular Research Institute, University of California, San Francisco, CA 94143, USA, naveen.gupta@ucsf.edu

Smita Iyer Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Center for Translational Research in the Lung, McKelvey Center for Lung Transplantation, Emory University, Atlanta, GA 30322, USA, siyer3@emory.edu

Ellen C. Keeley Division of Cardiology, Department of Medicine, University of Virginia, Charlottesville, VA, USA

Jae W. Lee Departments of Medicine and Anesthesiology, University of California, San Francisco, CA 94143, USA, leejw@anesthesia.ucsf.edu

Susan Majka Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver School of Medicine, Aurora, CO 80045, USA; Division of Cardiology, Charles C. Gates Regenerative Medicine and Stem Cell Biology Program, University of Colorado Denver, Aurora, CO 80045, USA, susanmajka@mac.com

Michael A. Matthay Departments of Medicine and Anesthesiology, University of California, San Francisco, CA 94143, USA; Cardiovascular Research Institute, University of California, San Francisco, CA 94143, USA, michael.matthay@ucsf.edu

Borna Mehrad Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Virginia, Charlottesville, VA, USA

Marc Moss Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver School of Medicine, Aurora, CO 80045, USA, marc.moss@ucdenver.edu

Susan D. Reynolds Department of Pediatrics, Stem Cells and Developmental Biology, National Jewish Health, Denver, CO 80206, USA, reynoldss@njhealth.org

Mauricio Rojas Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Center for Translational Research in the Lung, McKelvey Center for Lung Transplantation, Emory University, Atlanta, GA 30322, USA, mrojas@emory.edu

Jesse Roman Department of Medicine, University of Louisville Health Sciences Center, Louisville, KY, USA, j.roman@louisville.edu

Robert M. Strieter Divisions of Pulmonary and Critical Care Medicine, Department of Medicine, University of Virginia, Charlottesville, VA, USA, strieter@virginia.edu

Viranuj Sueblinvong Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA, vsuebli@emory.edu

Patrick Wahl Institute of Cardiovascular Research and Sport Medicine, German Sport University, Cologne, Germany; Institute of Training Science and Sport Informatics, Cologne, Germany; The German Research Center of Elite Sport, Cologne, Germany

Daniel J. Weiss Division of Pulmonary and Critical Care, Vermont Lung Center, University of Vermont College of Medicine, Burlington, VT 05405, USA, dweiss@uvm.edu

Carl W. White Department of Pediatrics, National Jewish Health, Denver, CO 80206, USA

Chapter 1
Stem and Progenitor Cells of the Airway Epithelium

Susan D. Reynolds, Moumita Ghosh, Heather M. Brechbuhl, Shama Ahmad, and Carl W. White

1 Introduction

1.1 Tissue-Specific Stem Cells

A tissue-specific stem cell is defined as a cell that self-renews and has a differentiation potential equivalent to the cellular diversity of its resident tissue [1]; thus, proliferation and differentiation are the two parameters that are most commonly used to identify a tissue-specific stem cell. Still, these are relative terms rather than hard and fast definitions. For instance, a tissue-specific stem cell has a greater mitotic potential than other progenitor cells. It is thought that the stem cell spreads its allotted number of cell divisions over a long period, potentially the lifespan of the animal. In a similar vein, the tissue-specific stem cell has a greater differentiation potential than other progenitor cells. In a diverse tissue such as the hematopoietic system, differences in differentiation potential are easily discerned. However, in a simple tissue such as the airway epithelium, a single differentiated cell type may exist. Thus, the differentiation potential of the tissue-specific stem cell could be equivalent to that of a simple progenitor cell. The nuanced definition of potential, be it proliferation or differentiation, makes definitive identification of tissue-specific stem cells a difficult goal.

1.2 Lung-Epithelial-Tissue-Specific Stem Cells

Lung epithelial cells that fit the definition of a tissue-specific stem cell have been identified by their resistance to various chemical injuries and by their sequestration

S.D. Reynolds (✉)
Division of Cell Biology, Department of Pediatrics, National Jewish Health, Denver, CO 80206, USA
e-mail: reynoldss@njhealth.org

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in specialized microenvironments [2]. However, the standard definition cannot distinguish between a lung-tissue-specific stem cell and another progenitor cell type, the facultative progenitor (see below). Difficulty distinguishing lung facultative progenitor cells from lung-tissue-specific stem cells is a consequence of cell lifespan, which is long, and cellular diversity, which is low.

1.3 Facultative Progenitor Cell

Facultative progenitor cells fulfill essential cellular and biochemical functions in their quiescent state; however, facultative progenitor cells retain the ability to alter cellular structure and mitotic status in response to cellular damage. These injury-induced changes can be quite dramatic and result in loss of differentiation markers, biochemical functions, and a 10–20-fold increase in mitotic activity. Proliferation of facultative progenitor cells and regionally appropriate differentiation of their daughter cells results in maintenance of the facultative progenitor cell pool (self-renewal) and restoration of terminally differentiated cells. The abundance and broad distribution of facultative progenitor cells make them a critical component of epithelial defense against environmental challenge.

1.4 Lung Facultative Progenitor Cells

The major differences between lung facultative progenitor cells and the tissue-specific stem cells is differentiation status and abundance. Lung facultative progenitor cells are responsible for secretion, absorption, metabolism, immunomodulation, mucocillary clearance, and barrier maintenance. These cells fall into two major categories, basal cells and secretory cells. Secretory cells are further subdivided into several subclasses: Clara-like, Clara, alveolar type 2 cells. The various facultative progenitor cell types inhabit specific compartments of the normal lung airway: the tracheobronchial (basal and Clara-like cells), bronchiolar (Clara cells), and alveolar (alveolar type 2 cells) epithelia. Clara-like and Clara cells are the progenitors for terminally differentiated cells, ciliated cells. Alveolar type 2 cells are the progenitor for terminally differentiated alveolar type 1 cells. The presence of this vast reparative reservoir distinguishes the lung epithelium from tissues such as the intestine which are maintained exclusively through proliferation and differentiation of the tissue-specific stem cell.

1.5 Questions Relevant to Lung Stem Cells

The structural and functional diversity of the lung epithelium begs several questions regarding tissue-specific stem cells and their attributes. First, should the definition of a stem cell, as presented above, be refined to reflect specifics of the lung epithelium? Second, given the nuances of the lung epithelium, what attributes should a

lung stem cell exhibit? Third, given the species-specific differences in airway structure, do findings in mice relate directly to the human lung and visa versa? These questions shape the existing lung stem and progenitor cell literature as well as ongoing research. By keeping these questions in mind, the reader will be able to critically evaluate the data presented below.

2 Conducting Airway Structure and Function

2.1 Functional Domains

The conducting airway is a set of tubular structures that decrease in caliber from proximal to distal. For the purposes of this chapter, only the region extending from the trachea (proximal) through the terminal bronchiole (distal) will be discussed. Between these extremes are the bronchial, and bronchiolar regions.

2.2 Tracheobronchial Domain

The proximal portion of the conducting airway epithelium is termed the "tracheobronchial epithelium." This region contains two functionally distinct epithelia, the submucosal glands and the surface epithelium. The submucosal glands consist of acini that are linked by ducts to the lumen of the trachea (mouse) and to the trachea, bronchi, and bronchioles of the human airway [3]. The submucosal glands are further specialized into mucus and serous domains that secrete biochemically distinct proteins [4].

The tracheobronchial surface epithelium is pseudostratified, with each cell being in contact with the basement membrane. Basal cells are typically located adjacent to the basement membrane and contact it through hemidesmosomes. Basal cells have limited exposure to the lumen under normal conditions. Secretory and ciliated cells are the other major cell types in this region. They are linked to each other and to basal cells through desmosomes. Gap junctions serve as portals for movement of small molecules between secretory and ciliated cells. The human trachea, bronchi, and the first six generations of the bronchiolar epithelium are supported by cartilage. The synonymous region of the mouse airway is the trachea and bronchi.

2.3 Bronchiolar and Terminal Bronchiolar Domain

The distal portion of the conducting airway is termed the "bronchiolar epithelium." This region is a simple columnar or cuboidal epithelium. Secretory and ciliated cells are the main cellular constituents. Minor cell types include pulmonary neuroendocrine cells (PNECs). In the human bronchiole, basal cells are a rare cell type.

However, basal cells are not detected in the lower airways of mice. As a consequence of these anatomical distinctions, the mouse bronchiolar epithelium is most similar to the terminal bronchiolar epithelium of the human airway.

2.4 Origin of Airway Domains

Embryology studies in mice established that all airway epithelial cells, with the possible exception of neuroepithelial cells, are derived from the foregut endoderm [5]. In mice, airway and alveolar progenitors are specified very early in development, between embryonic days 2 and 4. Interestingly, this specification occurs prior to identification of the lung anlagen [6]. The trachea and esophagus begin to separate on embryonic day 9.5 [7, 8]. The process of airway tube formation is termed "branching morphogenesis." It is completed by the pseudoglandular stage of lung development [9].

Continuous labeling studies in hamsters supported the conclusion that neuroepithelial bodies (NEBs) serve as mitotic centers that promote airway segment lengthening [10]. NEBs are structurally similar to carotid bodies and are composed of PNECs. Neural peptides secreted by PNECs are epithelial mitogens [11]. These analyses indicated a central role for NEBs in airway segmentation and the establishment of unique secretory cell pools [12]. However, normal prenatal lung development in NEB-deficient mice suggested that this structure may serve as a marker for an as yet undefined signaling center [13]. Additional studies are needed to determine the functional significance of this secretory cell–NEB association.

Submucosal glands are formed in the postnatal period. These structures were not tagged in mice using a surfactant protein C promoter regulated system even when recombination was induced from embryonic day 0.5 through postnatal day 7 [6]. These data may indicate that mouse submucosal glands are derived from a different set of progenitor cells than those that form the surface epithelium. However, a cautious interpretation of these data is warranted considering that lineage tracing studies strongly support a lineage relationship between bronchial and glandular lineages in the mouse and human [3, 14, 15].

2.5 Birth Date of Airway Epithelial Cells

Lineage tracing analysis demonstrated that cells "born" during lung development persist into adulthood [16]; thus, two populations of epithelial cells may exist in the adult airway, those "born" during lung development and those resulting from proliferation and differentiation in air-breathing postnatal animals. Functional maturation of epithelial cells, particularly airway secretory cells, may be modulated by Wnt signaling during prenatal lung development [17]. The functional significance of "embryological" and adult cells and their impact on lung injury, repair, and susceptibility to chronic lung disease are under investigation.

3 Conducting Airway Progenitor Cell Types

3.1 Tracheobronchial Epithelium

Cellular mechanisms regulating replacement of terminally differentiated ciliated cells were the focus of early injury-repair studies. These studies demonstrated that the tracheobronchial epithelium is populated by two progenitor cell pools, the aforementioned basal cell and a specialized secretory cell, the Clara-like cell [12]. Histological and pulse–chase analysis of tracheobronchial repair after NO_2 or ozone exposure identified the Clara-like cell as the progenitor for ciliated cells [18].

3.2 Basal Cells

The basal cell was identified as a supportive cell type that anchored the epithelium to the basement membrane [19]. Consequently, the basal cell has been referred to as a "reserve" cell in the literature [19]. However, recent studies indicate that basal cells proliferate actively in the steady-state mouse trachea and bronchi. These cells increase their mitotic rate dramatically in response to Clara cell depletion [20].

3.2.1 Basal Cells – Surface Epithelium

Basal cells are distinguished from other epithelial cell types by their pyramidal shape and by their distinct keratin expression profile. In the steady state, basal cells express primarily keratins 5 and 14. Basal cells are distributed throughout the human airway. They are abundant in the trachea and the first six generations of the respiratory track. This region is pseudostratified and is supported by cartilage. Basal cells are also found in the bronchiolar epithelium of the human lung. In this region, the epithelium is columnar and basal cells are rare. In rodent lungs, basal cells are located primarily in the trachea and bronchi. Rare basal cells, one cell per high-powered field, are found in the mouse bronchial epithelium. As a consequence of these species-specific differences in airway structure, care must be taken to ensure that similar regions are compared.

3.2.2 Basal Cells – Submucosal Glands

Basal cells are also located in the glandular epithelium. Here, basal cells are found along the basement membrane of the gland ducts and the acini. These cells are thought to be contractile and as a consequence are referred to as "myoepithelial cells." In humans and mice, these cells express keratins 5 and 14. The mitotic index in the glandular epithelium is very low in the adult and may reflect the fact that this region is relatively protected from the environmental exposures that drive proliferation in the surface epithelium.

3.2.3 Basal Cells – Plural Membrane

The plural membrane is a final location of lung basal cells. This region is characterized by low cell density and basal cells are a rare cell type. Consequently, little is known of basal cell function within the visceral lining of the lung. These cells have been lineage-traced using the Wilm's tumor 1 gene (Wt1) promoter and several recombination substrates [21]. Interestingly, these cells served as progenitors for mesenchymal cells within the vascular walls. These studies raise the possibility that markers associated with basal cells of the epithelium are utilized, potentially in a different functional role, in cells of the plural membrane. These cells have not been characterized in the context of lung injury and repair.

3.3 Secretory Cells

Airway secretory cells are a specialized cell type that were first defined morphologically as a nonciliated cell. Subsequent ultrastructural analysis identified abundant rough endoplasmic reticulum and secretory granules as unique subcellular organelles [22]. Such cells secrete proteins into the luminal space, however, they are also a source of antioxidant compounds. Genetic alterations to airway secretory cells are associated with direct changes in cell function [23] as well as alterations to adjacent ciliated cells and to more distant inflammatory cells such as the alveoalar macrophage [24, 25].

3.3.1 Clara-Like and Clara Cells

Clara cells are defined structurally as nonciliated cells (reviewed in [26, 12]). The Clara cell is a multifunctional cell type that has been studied for nearly a century. These cells were originally described as cuboidal, nonciliated cells in human and rabbit terminal bronchioles. They contain a basally situated nucleus, an apical dome that extends variable distances into the airway lumen, and discrete, oval densely staining granules. These cells constitute approximately 50% of cells in the bronchial and bronchiolar epithelium and 70% of cells in the terminal bronchiolar epithelium. Their shape varies from columnar to cuboidal along the proximal to distal axis.

Ultrastructural and morphometric analysis by Plopper and colleagues provided insights into Clara cell function, and led to ongoing studies demonstrating critical roles in barrier maintenance, secretion, and metabolism [22]. Multispecies comparisons demonstrated that Clara cell structure varies among species and along the proximal to distal axis of the airway epithelium. Despite this heterogeneity, studies employing oxidant gas exposure and pulse–chase strategies indicated that most if not all rabbit [27] or rat [28, 29] Clara-like cells have the ability to proliferate in response to injury. The ultrastructural differences between proximal and distal airway secretory cells led to the designation of upper airway secretory cells as Clara-like cells [12].

3.3.2 Secretory Cell Molecular Markers

All mouse airway secretory cells, from the trachea to the terminal bronchioles, express a low molecular weight protein, Clara cell secretory protein (CCSP). Thus, CCSP expression in the mouse is synonymous with the Clara-like and Clara cell types. However, human proximal airway secretory cells are more readily recognized by expression of mucins such as Muc5Ac. In adult human airways, expression of CCSP is restricted to the terminal bronchioles. These "differences" in expression of CCSP in the adult human and the mouse have led to the conclusion that human airways do not have a constitutive population of non-mucus-secreting secretory cells. However, studies in mice suggest that mucus cells are derived from CCSP-expressing cells through a metaplastic transition [30, 31]. These mucus cells may be postmitotic, although there is controversy regarding this point. These studies suggest that the human airway does have secretory cells that are functionally similar, if not molecularly identical, to the CCSP-positive mouse Clara-like and Clara cells. However, the lineage relationship has not been evaluated in the human.

3.3.3 Secretory Cells – Cellular Specialization

Biochemical specialization of the airway is recognized by the establishment of molecularly distinct airway secretory cell types. These specialized cells are established during the middle stage of lung development, between embryonic days 12 and 14, in the mouse. Subdivision of the human conducting airway epithelium begins during the second trimester in human lung, and the earliest secretory cells are positioned within the luminal aspect of NEBs [32]. These spatially-restricted secretory cells are CCSP-positive. In the early postnatal period, secretory cell specialization can be identified by regionally specific expression of secretory protein messenger RNAs [33].

3.3.4 Secretory Cells – Submucosal Gland

Secretory cells are also located in the submucosal glands. As indicated above, the glands form in the postnatal period, suggesting a distinct molecular plan for this region. Glandular secretory cells of the adult human and mouse do not express CCSP. Rather they express a distinct repertoire of host defense proteins [34]. Molecular analysis of mechanisms regulating submucosal gland development revealed a complex role for the Wnt–β-catenin signaling pathway in bud formation and elongation [35, 36]. These studies demonstrated a clear role for β-catenin-dependent gene expression. However, the DNA binding cofactors for β-catenin, Lef1 and TCF4, were differentially regulated as a function of gland development. Gene deletion studies suggest a compensatory role for TCF family members in implementation of Wnt ligand signaling. These cofactors may be part of a positive–negative regulatory circuit that is regulated by the Wnt ligand, Wnt 3a [14].

3.4 Bronchiolar Epithelium

The bronchiolar epithelium contains two progenitor cell pools, Clara cells and PNECs.

3.4.1 Clara Cells

Clara cells are the most prevalent progenitor cells within the distal airways. These cells respond to ciliated cell injury by alterations in their differentiated functions and proliferation [29]. These changes are described further in Sect. 4. As indicated for Clara-like cells, Clara cells of the mouse are most readily recognized by expression of CCSP. These cells also express other secreted proteins, including SCGB3A2 [33] and enzymes involved in phase I and II metabolism [37].

A unique characteristic of mouse Clara cells has been exploited to evaluate the stem cell hypothesis. In this species, Clara cells express the monooxygenase cytochrome P450 2F2 [38]. This enzyme metabolized the xenobiotic agent naphthalene to a cytotoxic epoxide. Under conditions where the epoxide cannot be detoxified, Clara cells die via necrosis. This cellular toxicity initiates within 6 h of parenteral exposure and dead and dying cells slough between 24 and 48 h [39]. Similar methods cannot be used to evaluate stem cells in the human or cultures of human cells, as this species does not express cytochrome P450 2F2 in the secretory cell population. However, alternative agents that exploit the unique phase I and II metabolism of human secretory cells may exist and could be used to test the stem cell hypothesis in vitro.

3.4.2 Pulmonary Neuroendocrine Cells

PNECs are found as isolated cells or in clusters termed “neuroepithelial bodies” (NEBs). Human and mouse PNECs are recognized by dense core granules on transmission electron micrographs or by expression of neural peptides, such as calcitonin gene related peptide and chromogranin A, on paraffin sections. PNECs proliferate in response to various forms of epithelial injury in humans and mice. This results in an increase in the number of NEBs (hypertrophy) and in an increased number of cells per NEB (hyperplasia) [40]. Proliferation of PNECs is limited to one or two cycles as indicated by retention of ^{3}H-thymine deoxyribose by PNECs after naphthalene injury [41, 42].

Dual immunofluorescence analysis suggested a lineage relationship between PNECs and Clara cells [42]; however, formal lineage tracing has not been used to critically test this point in the adult mouse. Chimera studies and lineage tracing in utero suggest that PNECs are a distinct lineage [43]. Several studies identified the NEB as a potential stem cell microenvironment [2]. Interestingly, NEB structure changes with injury [44]. Alterations in cellular and cell–basement membrane interactions were observed but functional consequences were not investigated. Owing to the paucity of data regarding progenitor cell activity of PNECs, these cells will not be discussed further.

4 Facultative Progenitor Cell Pools

4.1 Basal Cells

Steady-state basal cells exhibit two molecular phenotypes, keratin 5+/14– and keratin 5+/14+. These two subsets were 80 and 20% of the steady-state basal cell population, respectively. Although the steady-state mitotic index of the trachea is low, about 10% using Ki67 as a mitotic marker, the basal cell subsets comprised approximately half of all mitotic cells in the mouse trachea (Cole et al. in press). Lineage tracing indicated that these steady-state basal cells were responsible for maintenance of the basal cell population. Contribution to the secretory and ciliated cell pools was not detected over a 40-day window, suggesting that the basal and secretory/ciliated lineages were distinct (Ghosh, M & Reynolds, S.D. unpublished).

4.1.1 Steady-State and Reparative Basal Cells

Basal cell phenotype and function varied in the context of secretory cell injury. Naphthalene-mediated depletion of the tracheal Clara-like cell pool initiated an epithelial repair process that was driven by the abundant and broadly distributed basal cell population. These progenitor cells were uniformly keratin 14 positive and were derived from the keratin 5+/14– and keratin 5+/14+ basal cell pools (Cole, in press). Increased keratin 14 gene expression was responsible for this altered molecular phenotype. The keratin 14+ basal cell population represented at least 80% of mitotic cells on recovery days 3 and 6. These cells were highly proliferative, with approximately 40% of cells cycling at a given time point. The unbiased distribution of keratin 14+ reparative cells along the proximal–distal axis and parallel restitution of the secretory cell population indicated that epithelial repair was mediated primarily by a broadly distributed population of basal cell progenitors rather than through activation of a proximally restricted tissue-specific stem cell.

4.2 Clara-Like and Clara Cells

Clara-like and Clara cells respond to ciliated cell depletion through dedifferentiation and proliferation. Pulse–chase studies in combination with ultrastructural analysis demonstrated that the initial event was a morphological change [28, 29, 18] (Fig. 1.1). Loss of secretory granules and endoplasmic reticulum resulted in the generation of a transient cell type, the type A cell. This cell entered the cell cycle. One of the two daughter cells redifferentiated through a type B intermediate to restore the Clara cell population. The other daughter differentiated into a nascent ciliated cell. Clara cells located throughout the bronchiolar epithelium had the capacity to undergo these morphological alterations and to proliferate. Mechanisms regulating differentiation of daughter cells have not been delineated. However, the fact that

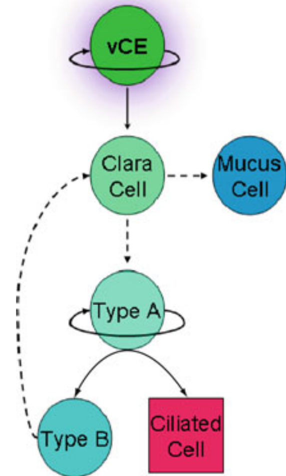

Fig. 1.1 The bronchiolar stem cell hierarchy. See the text for details

cellular representation varies in the bronchial and bronchiolar epithelium suggests that regionally specific signals regulate cell fate decisions.

4.2.1 Phenotypic Plasticity Is a Hallmark of Clara-Like and Clara Cells

Individual Clara cells refine their phenotype in response to alterations in the lung milieu, microenvironmental influences specific to trophic units, and exposure to environmental agents, including ozone, pathogens and their by-products, and chemotherapeutic agents. In response to injury, reparative Clara cells express surfactant protein B, potentially to maintain patency of the small airways during repair [45]. Analysis of the response of mouse Clara cells to allergic inflammation or the Th2 cytokine, interleukin-13 [46], suggest a lineage relationship between Clara cells and mucus cells [30, 31]. Pulse-labeling studies showed that mucus metaplasia of Clara cells generates a terminally differentiated cell that can no longer enter the cell cycle. Thus, metaplasia to a mucus-producing cell may provide critical protection of the airways but also lead to loss of reparative potential in chronic lung disease.

5 Evidence in Support of Lung Stem Cells

5.1 Classic Stem Cell Methods

A clear understanding of the assays used to identify a tissue-specific stem cell is critical to the interpretation of the studies that attempt to identify lung stem cells. Label retention has been used as a functional measure of stem-cell-like behavior

in multiple epithelial systems and is particularly well suited to those in which differentiated cell turnover is continuous, such as the gut, cornea, and interfollicular epidermis [47, 48, 49]. In contrast with these systems, the conducting airway epithelium is relatively quiescent in the steady state. Lineage tracing studies done in steady-state animals demonstrated persistence of airway epithelial cells over a period of months [16]; thus, label retention in the steady-state pulmonary epithelium was a measure of cellular lifespan and not necessarily an indication of stem cell character. Analysis of the steady-state epithelium also indicated that the majority of persistent cells were a self-renewing cell type [16] and suggested that stem cells are not active in the normal airway [50].

5.2 Injury and Lung Stem Cell Analysis

A distinction between analyses of classic tissue-specific stem cell hierarchies and those thought to maintain the lung epithelium is the need to impose injury on the system. In the lung, differentiated cells are long-lived and consequently the mitotic index is low [51, 41, 52, 42]. Exposures that primarily target terminally differentiated cell types (ciliated cells or alveolar type 1 cells) are repaired by the facultative progenitor cells [18, 29, 53]. These types of exposure do not activate putative stem cells [42]. As a consequence of this high level of quiescence, various forms of injury have been used to deplete the facultative progenitor cell and "activate" putative stem cells.

Numerous injury models have been used to deplete the facultative progenitor cell populations. Cell isolation and culture on plastic, Transwell plates, or in tracheal xenografts has been used to evaluate human lung stem cells [54, 55, 15, 56]. Inhaled acid or detergent has been used to deplete both tracheobronchial facultative progenitor cells, the Clara-like cell and the basal cell, in mice [57, 16]. Parenteral naphthalene exposure and genetic sensitization of these cell types to the antiviral drug ganciclovir have been used to reduce or eliminate the Clara-like cell and Clara cell populations [41, 58, 45].

The importance of "selective injury models" for advancement of the lung stem cell field is indicated by the paucity of knowledge regarding alveolar tissue-specific stem cells. Alveolar-biased injuries have been used to study reparative processes in this compartment (bleomycin, transforming growth factor β overexpression, butylated hydroxytoluene, hyperoxia, lipopolysaccharide). However, these injuries tend to be severe and lead to fibrosis rather than epithelial repair. Further, these injuries compromise multiple tissue types within the alveolar unit (mesenchyme and/or endothelial cells), confounding the interpretation of results (see below). In the absence of genetic methods or agents to deplete the alveolar type 2 progenitor cell pool, identification of alveolar tissue-specific stem cells has been limited to identification of cells that express stem-cell-associated markers such as hTERT [59, 60, 61].

5.3 Injury as a Confounding Variable

Different injury models provoke distinct reparative processes. For instance, injuries that deplete all surface epithelial progenitor pools result in a spatially restricted reparative process involving cells derived from uninjured compartments such as the submucosal glands [57]. The subsequent repair process was initiated from protected environments, including the gland ducts in the proximal trachea and rare cells residing in the hypercellular intercartilaginous regions. These studies suggested that the tracheobronchial stem cell was sequestered within a protective microenvironment located in the gland duct junction and the intercartilaginous zone.

Similar to the acid and detergent models, the naphthalene model resulted in depletion of tracheobronchial Clara-like and ciliated cells. Although the glandular epithelium was intact, lineage-tagged cells located in gland ducts did not contribute to repair of the surface epithelium (Ghosh, M & Reynolds, S.D. unpublished data). These data indicated that basal cells located within the gland duct junction are not obligate progenitors for repair of the surface epithelium. In contrast with other injury models, the uniform reparative process reported for the naphthalene model indicates that restoration of secretory and ciliated cells following naphthalene injury was mediated by a population of basal cells that is limited to the surface epithelium. These data suggested that injury is a critical parameter determinant of which cells are available for repair. This issue can influence identification of microenvironments that may harbor true stem cells or merely protect a progenitor cell from injurious agents.

5.4 Tracheobronchial Stem Cells

5.4.1 Evidence in Favor of a Tracheobronchial Stem Cell

The claim that a tissue-specific stem cell maintains and repairs the tracheobronchial region is based on three lines of evidence. First, lineage tracing and ex vivo differentiation were used to identify the basal cell as a multilineage progenitor for the human tracheobronchial and glandular epithelium [55]. These studies detected two multipotential cells with basal cell morphology and suggested direct differentiation of this cell to a secretory lineage progenitor, to a terminally differentiated mucus cell, or to a ciliated cell. Second, identification of label-retaining cells and their spatial restriction to the gland duct junction or intercartilaginous regions [57] substantiated the existence of a region-specific stem cell in mice. Finally, lineage-tracing analysis identified a multipotential keratin 14 expressing subset of mouse basal cells [52, 20]. These studies resulted in the arrangement of tracheobronchial progenitor cells into a classic hierarchy [62] (Fig. 1.2).

5.4.2 Distinctions Between the Human and Mouse Data Sets

Comparison of the human and mouse tracheobronchial stem cell hierarchies identified several distinctions. First, the human hierarchy contained self-renewing

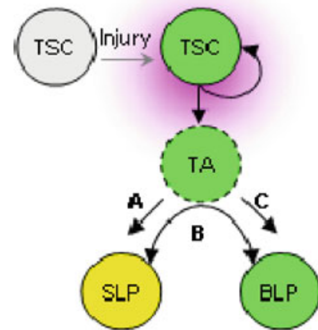

Fig. 1.2 Classic organization of tracheobronchial stem and progenitor cells. The ciliated cell which is a product of secretory lineage progenitor (*SLP*) proliferation is not shown. Differentiation pathways *A–C* are defined in the text. *TSC* tissue-specific stem cell, *TA* transit amplifying cell, *BLP* basal lineage progenitor

bipotential basal cell progenitors, whereas the mouse model included a non-self-renewing secretory/ciliated progenitor. Second, the human hierarchy lacked a transit amplifying cell population. Although analysis of purified nasal polyp cells suggested the existence of a transit amplifying cell [56], lineage tracing methods were not used to distinguish a population effect from proliferation and differentiation of a single cell. In the mouse lineage-tracing experiments, secretory/ciliated only colonies were detected. This result was accommodated by inclusion of a keratin 14 expressing transit amplifying cell in the mouse tracheobronchial stem cell hierarchy (Fig. 1.2). It was postulated that differentiation of this transit amplifying cell was context-dependent. Asymmetric cell division would result in generation of both a secretory and a basal lineage progenitor (Fig. 1.2, pathway B), whereas symmetric cell division would result in generation of clones containing only basal cells (Fig. 1.2, pathway C). On the basis of pulse–chase studies [28, 29], symmetric division of the transit amplifying cell was also thought to generate colonies containing secretory lineage progenitor that self-renewed and generated ciliated cell progeny (Fig. 1.2, pathway A). Finally, the putative human tissue-specific stem cell was defined by morphological criteria (basal-like) or by expression of a type 1 keratin, keratin 14. Neither of these definitions could account for variation in cellular and molecular phenotype following injury in the mouse [20]. Differences in the two data sets suggested that the two systems were fundamentally distinct or that proliferation and differentiation were influenced by other parameters.

Analysis of tracheobronchial stem and facultative progenitor cells has been advanced by clonal analysis methods [63], differentiation in vitro [56, 64, 65, 66], and repopulation of tracheal xenografts ex vivo [55, 15, 56]. These methods have been used to evaluate the self-renewal and differentiation potential of highly enriched subsets of embryonic human bronchial [67], adult bronchial [55], and nasal polyp [54] cells. The results indicate that the surface phenotype of cell populations capable of generating a ciliated epithelium varies among these subcompartments or lesions. However, the analysis of bronchial and nasal polyp cells did not determine

whether single cells could generate all cell types characteristic of the proximal airway and consequently falls short of a final demonstration of the tissue stem cell surface phenotype.

5.4.3 Predictions of the Classic Stem Cell Model

The hierarchical model makes several important predictions. First, if the keratin 14+ cell is a tissue-specific stem cell, it should be a persistent cell type and relatively rare. Second, the keratin 14+ cell and its direct descendant the transit amplifying cell should be spatially restricted. On the basis of analysis of tracheal regeneration in the mouse, these cells should be located in the gland duct junction and intercartilaginous regions of the distal trachea. Consequently, stem-cell-mediated repair of the tracheal epithelium should result in regenerative units that are centered on the position of the tissue stem cell. Third, keratin 14+ cells should be relatively quiescent in the steady state and activated by injury. Finally, lineage-traced steady-state keratin 14 expressing cells should be multipotential and only generate clones containing all epithelial phenotypes.

5.4.4 Revision of the Classic Hierarchical Organization

Alternative explanations for repair of the injured epithelium and the demonstration that basal cells altered their molecular phenotype after injury focused attention on the steady-state basal cell. Consequently proliferation and differentiation were evaluated under normal conditions. Histomorphometric analysis indicated that basal cells constituted approximately half the mitotic pool in the steady state (Cole, in press). Lineage tracing of steady-state keratin 14+ cells demonstrated that the differentiation potential of such cells is limited to self-renewal (Ghosh, M & Reynolds, S.D. unpublished data). In the steady state, keratin 14+ cells generated only basal cells. However, the differentiation potential of these cells was expanded to include secretory and ciliated cells following naphthalene injury. These studies also detected bipotential keratin 14+ cells that generated either Clara-like cells or ciliated cells. These direct basal-to-secretory cell and basal-to-ciliated cell differentiation patterns were in accord with the analysis of human basal cell differentiation potential and with the findings of developmental studies. Further, these studies demonstrated that injury had a significant impact on cellular functions: proliferation rate and differentiation potential.

Reanalysis of multipotential mouse clones generated from steady-state or reparative keratin 14 expressing cells was stimulated by the direct differentiation finding. These studies demonstrated that secretory and ciliated cells were minor constituents of the multipotential clone and that the epithelial structure was rarely stratified. The studies indicated that basal cells differentiated directly into secretory or ciliated cells after naphthalene injury. These data also suggested that differentiation of keratin 14+ cells was regulated by short-range signals that impinged on a single cell and that mechanisms leading to replacement of secretory and ciliated cells required cooperative interactions between basal cells and the reparative macroenvironment.

5.4.5 A Temporally Regulated Tissue-Specific Stem Cell Hierarchy

The demonstration that steady-state keratin 14+ cells are unipotential and that keratin 14 expression in reparative cells is a gain-of-function phenotype may be viewed as being in conflict with reports of spatially restricted label-retaining stem-cell-like cells in the acid and detergent injury models [57] and identification of multipotential cells by clonal analysis [55], by fluorescence-activated cell sorting of homogenous populations of basal cells [54, 65, 68], and by lineage tracing [52, 20]. In contrast, we propose a revised view of tracheal repair and reevaluation of the vertically organized tissue-specific stem cell hierarchy thought to maintain this region of the conducting airway.

A modified view of tracheal progenitor cells is represented in Fig. 1.3b and c. In the steady state, basal and secretory lineage progenitor cells function autonomously to replace cells lost through attrition. Thus, the steady-state tracheobronchial epithelium is maintained by two independent progenitor cell pools, the basal and secretory cell facultative progenitors. The basal cell facultative progenitor pool is divided into two subpopulations (Fig. 1.3b, only the basal cell progenitors are depicted). Lineage tracing of steady-state keratin 14 expressing cells demonstrated that these cells self-renew in the steady state and gave rise to keratin 14–/5+ cells. The presence of an intact secretory/ciliated cell layer and/or the basement membrane may limit basal cell progenitor function to self-renewal.

Following naphthalene-mediated depletion of the secretory cell facultative progenitor, the two basal cell progenitor pools participate in restitution of the

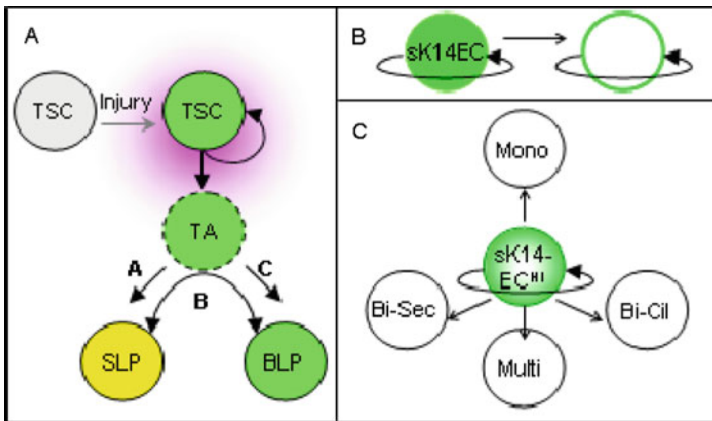

Fig. 1.3 Revised lineage relationships for the tracheobronchial epithelium. **a** Repeated from Fig. 1.1 for comparison purposes. **b** Lineage relationships in the steady state. **c** Expanded differentiation potential of basal cells after injury. Differentiation potential is described by the types of cells found within lineage-traced colonies. The precursor to these colony types may be an activated basal cell, a steady-state keratin 14 expressing cell (*sK14EC*) with high keratin 14 levels. *Mono* only basal cells, *Bi-Sec* basal and secretory cells, *Bi-Cil* basal and ciliated cells, *Multi* all three cell types

epithelium. Both cell types upregulate keratin 14 and are broadly distributed, abundant, and mitotic. Lineage tracing of steady-state keratin 14 expressing cells demonstrates that the differentiation potential of these cells expands in response to injury (Fig. 1.3c). Derivatives of steady-state keratin 14 expressing cells can include basal cells, secretory cells, ciliated cells, or all cell types. However, the type(s) of progeny produced is context-dependent. Comparison of the present study with lineage tracing of reparative keratin 14 expressing cells suggests that upregulation of keratin 14 in steady-state keratin 5+/14– cells generates an activated cell type that can differentiate in response to environmental cues. On the basis of these data we propose that the fate of tracheal basal cells is regulated by two interacting processes: an activation state as indicated by keratin 14 expression level and injury-induced modification of the microenvironment.

To account for differences in the differentiation potential of steady-state and reparative keratin 14 expressing cells, we suggest that an optimal keratin 14 expression level is necessary to attain responsiveness to the microenvironment. Steady-state keratin 14 expressing cells enter the reparative state at a moderate level of activation (Fig. 1.4). A rapid rate of change in keratin 14 expression (thin white arrow) results in generation of an activated cell at a time when the microenvironment is at its most instructive state (Fig. 1.4, represented by the blue portion of the triangle). A slower rate of change in keratin 14 expression (Fig. 1.4, medium white arrow) results in later attainment of the activated state and interaction with a less permissive niche. Keratin 14– cells can achieve the multipotential activation state if they rapidly increase expression of keratin 14 (Fig. 1.4, thin gray arrow). However, the majority of keratin 14– cells upregulate keratin 14 expression at the standard rate (Fig. 1.3, medium gray arrow) and as a consequence are activated during the time when the microenvironment has reduced inductive capacity. Consequently, these cells have a more limited differentiation potential. Finally, the majority of reparative keratin 14 expressing cells are activated late in repair and miss interaction with the reparative microenvironment (Fig. 1.4, thick gray arrow). These cells enter the default differentiation pathway, basal cell generation.

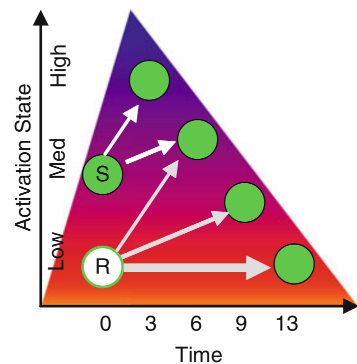

Fig. 1.4 Model for basal cell activation after injury. See the text for details

5.5 Bronchiolar Stem Cells

5.5.1 Evidence in Favor of a Bronchiolar Stem Cell

The concept of a bronchiolar region-specific stem cell is based on identification of a functionally distinct cell: the naphthalene-resistant Clara cell. Injury/repair studies demonstrated that this cell type is rare, sequestered in specific microenvironments (the NEB microenvironment or bronchoalveolar duct junction) [42], slow-cycling [41], relatively undifferentiated (cytochrome P450 2F2 low) [58], and expresses the gene Clara cell secretory protein, CCSP [41]. Thus, the bronchiolar tissue-specific stem cell was termed the "variant CCSP-expressing cell," the vCE [41] (Fig. 1.1).

5.5.2 Caveats to the "Stemness" Claim

Several issues prevent a decisive answer to the question, is there a bronchiolar stem cell? These include compartmental integrity after injury, phenotypic plasticity within the facultative progenitor cell pool, identification of multiple label-retaining bronchiolar cell types (neuroepithelial and differentiated Clara cells), identical differentiation potential of the vCE and the Clara cell, and failure to fulfill Koch's postulates through isolation of putative stem cells and functional analysis in vivo.

5.5.3 Compartmental Boundaries

Lineage tracing studies demonstrate that differentiation of the vCE is limited to bronchiolar cell fates in vivo [16, 69, 17]. However, a broader differentiation potential has been suggested by in vitro assays and analysis of adenocarcinomas induced by transgenic expression of proto-oncogenes [70]. Analysis of bleomycin-injured [71] and ozone-injured [72] mice also suggests that compartmental borders are breached under chronic injury conditions and that the differentiation potential of the stem cell and/or facultative progenitor cell can be expanded in response to depletion of reparative cells specific to adjacent compartments. Consequently, analysis of bronchiolar stem cells is complicated by use of injury to stimulate stem cell activity and by the possibility that injury may alter the differentiation potential of the stem cell itself and interactions between adjacent epithelial compartments.

5.5.4 Phenotypic Plasticity

The concept that the lung epithelium is maintained and repaired through highly specialized regionally restricted, tissue-specific stem and facultative progenitor cells is confounded by the demonstration of phenotypic plasticity of epithelial cells in response to injury [30, 31, 45]. Proliferation of facultative progenitor cells is associated with morphological and functional modifications that render them less differentiated (decreased rough endoplasmic reticulum and secretory granules) and

consequently more stem-cell-like [29] (see Sect. 1.3, Fig. 1.1). Regeneration of the bronchiolar facultative progenitor cell pool is also associated with establishment of a nascent cell type characterized by an intermediate molecular phenotype. Within the terminal bronchiolar epithelium, these cells coexpress airway markers such as CCSP and alveolar cell markers, including surfactant protein B [45] and/or pro-surfactant protein C [70, 17]. Increased numbers of intermediate cells have been equated with stem cell amplification [70]; however, these cells may simply be secretory cells that exhibit a reactive phenotype necessary for patency of small airways or one emblematic of nascent cells that have not yet completed the maturation process [17].

5.5.5 Fulfilling Koch's Postulates

Methods for prospective purification of bronchiolar stem cells have relied on methods/markers validated for isolation of hematopoietic stem cells. Results utilizing the Hoechst efflux strategy have been inconsistent and may reflect the toxic properties of DNA intercalating dyes in cells with limited phase III metabolism [73, 74, 63, 75]. Surface markers identified as diagnostic for hematopoietic stem cells have also been used [70, 76, 77]. As with other methods, the results vary among laboratories and may reflect differences in cell isolation methods, flow cytometry parameters, or cellular adaptation to noxious stimuli or injury [63]. Despite these difficulties, in vitro culture on feeder layers suggested that an alveolar-biased cell preparation selected for $Sca1^{Hi}/CD34^{+}$ cells contained colony-forming cells and that these colonies contained progeny that expressed bronchiolar, and alveolar type 1 and type 2 cell markers. Such cells were termed bronchoalveolar stem cells, but cells with this differentiation potential have not been identified in vivo.

6 Summary

Adaptation of classic stem cell theory to meet the unique characteristics of the conducing airway epithelium was discussed in this chapter. The conducting airway epithelium serves as the interface between the lung and the environment. This role is reflected in the structural and functional diversity found along its proximal to distal axis. Evidence suggests that the subdomains of the epithelium are maintained by multiple progenitor cell pools. These pools include tissue-specific lung stem cells and facultative progenitor cells; however, only subtle differences distinguish these two cell types. The magnitude of these differences may increase or decrease in response to injury and is dependent on type of tissue stem cell. Evaluation of stem cell origin and analysis of the relationship(s) between development and injury/repair is confounded by the long lifespan of lung epithelial cells. Similarly, the absence of robust stem cell purification methods and functional assays has prevented analysis of the molecular mechanisms that differentiate tissue-specific stem cell and facultative progenitor cell function. Clearly new methods that take into account the nuances of the lung epithelium are needed to further the field of lung stem cell biology. Despite

these limitations, the existing data indicate that the lung epithelium is maintained by a hierarchy of progenitor cells that is distinct from that identified in the intestine and hematopoietic systems.

7 Future Directions

Consideration of the following questions is suggested as a basis for the design of future studies. First, what evidence supports or refutes identification of one or several types of lung stem cell? Second, what are the caveats to organization of lung progenitor cells as a classic stem cell hierarchy? Third, in the face of the large facultative progenitor cell pool, is a stem cell needed to maintain the lung epithelium? Fourth, could loss of the putative stem cell or alteration of stem cell function contribute to lung disease? Fifth, how could lung stem cells and facultative progenitor cells be used to treat acute and chronic lung disease?

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Chapter 2
Mesenchymal Stem Cells for Lung Repair and Regeneration

Daniel J. Weiss

1 Introduction

Mesenchymal stem cells (MSCs) are cells of stromal origin that can self-renew and have the ability to differentiate into a variety of cell lineages. Initially described in a population of bone marrow stromal cells, they were first described as fibroblastic colony-forming units [1], subsequently as marrow stromal cells, then as MSCs [2], and most recently as multipotent mesenchymal stromal cells or MSCs [3]. MSCs have now been isolated from a wide variety of tissues, including umbilical cord blood, Wharton's jelly, placenta, and adipose tissue [4–15]. Most recently, MSCs have been isolated from adult mouse lungs [16] and from lungs of both neonates and lung-transplant recipients [17, 18]. MSCs isolated from each of these sources generally express comparable cell surface markers and differentiate along recognized lineage pathways. However, differences in gene expression, lineage tendencies, and other properties have been described among MSCs isolated from the different sources [19–26]. Further, many of the published studies utilized different definitions and characterizations of MSCs. This has complicated comparative assessments of published studies. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has recently updated the minimal criteria for defining (human) MSCs [3]. It is hoped that rigorous adherence to these criteria will help to focus comparative investigations of their potential utility in lung diseases. Nonetheless, the field remains complex as MSC characteristics can change with culture conditions and the microenvironment [27–32]. Further, there is growing evidence that MSCs are heterogeneous and that different MSC subtypes exist, even in cells isolated from the same tissue [33–35].

D.J. Weiss (✉)
Division of Pulmonary and Critical Care, Vermont Lung Center, University of Vermont College of Medicine, Burlington, VT 05405, USA
e-mail: dweiss@uvm.edu

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2 Identification, Characterization, and Culture of MSCs

In the original descriptions of MSCs, they were characterized by tight adherence to tissue culture surfaces, spindlelike shape, multipotential differentiation capacity both in culture and in vivo, and the capacity to generate single-cell-derived colonies [1, 2]. As such, the original description of the cells was as fibroblastic colony-forming units. Confluent cultures of MSCs were subsequently found to be useful as feeder layers for hematopoietic stem cells, hence the description as marrow stromal cells [36]. In parallel, exploration of their potential for multilineage differentiation resulted in the designation as MSCs. The most recent attempt by the International Society for Cytotherapy to resolve the confusion has resulted in the designation as multipotent mesenchymal stromal cells [3]. The designation of "MSC" is thus used with differing degrees of rigor, specificity, and consistency, which complicates both comparative assessments of the literature as well as simply searching the literature itself to find all relevant studies.

The situation is further complicated by differing degrees of rigor for isolation of MSCs. In the simplest approaches, bone marrow aspirates or mononuclear fractions of different tissue homogenates are allowed to adhere to plastic tissue culture dishes. The adherent cells can be mostly, but not completely, composed of cells with characteristics of MSCs. However, caution has to be observed as other cell types may adhere, including fibroblasts and CD34+ progenitor cells, which may overlap with MSCs in expression of certain cell-surface epitopes or in differentiation capacities. For example, human bronchial fibroblasts have also been described to be capable of exhibiting properties consistent with MSCs as have adult mouse lung side population cells [37, 38]. Species differences may occur as well. For example, plating and culture of adherent bone-marrow-derived cells from rat or human marrow aspirates will result in more homogenous populations of cells with characteristics of MSCs [39, 40]. In contrast, CD34+ and CD45+ cells from mouse bone marrow tend to be more adherent, and thus with prolonged culture a significant proportion of the resultant cultures may be composed of these cells or their derivatives. It is therefore imperative to apply rigorous positive and negative selection criteria using tools such as magnetic bead immunodepletion for fluorescence-activated cell sorting to obtain purified populations [3]. Further, appropriate differentiation into recognized lineages such as osteoblasts, chondrocytes, and adipocytes needs to be confirmed for different isolates [3]. MSCs can also create their own microenvironmental niches and change substantially on the basis of autocrine and paracrine signaling. A common rule of thumb is to not let MSC cultures become greater than approximately 50% confluent to minimize this signaling and subsequent differentiation into other cell types. This aspect of MSC use is frequently not described in published investigations and further complicates comparative assessments.

An additional problem with long-term culture of MSCs is propensity for chromosomal instability. This has been best characterized in mouse MSCs, but human MSCs are more stable [41–43]. Nonetheless, all cultured cells have the risk of both genotypic and phenotypic alterations with prolonged culture. Further, MSCs appear to undergo senescence with long-term culture and lose the ability to appropriately

differentiate into recognized lineages. Current recommendations are to utilize both freshly isolated MSCs and also MSCs obtained from central sources such as the Texas (formerly Tulane) MSC Core Facility at relatively early passages.

Overall, these many factors complicate both the design and interpretation of comparative experimental studies. Nonetheless, despite these ongoing issues, exciting and compelling data have emerged with respect to use of MSCs for lung injuries.

3 MSCs of Different Tissue Origins: Similarities and Differences

A growing body of literature describes isolation and culture of cells with the general characteristics of MSCs from a wide range of adult tissues in addition to bone marrow. This has been most prominently described for adipose tissue but lung itself may contain one or more populations of endogenous MSCs [16–18]. Tissues involved in fetal growth and development, including placenta, amniotic fluid, Wharton's jelly, and umbilical cord blood, have been described as particularly rich sources of MSCs. In general, MSCs isolated from these tissues express similar defining cell-surface epitopes and the ability to differentiate along standard lineages. However, significant differences can occur in gene expression, lineage tendencies, and most importantly functional ability to participate in tissue repair and regeneration [14, 19–26]. This has been best described for MSC use in connective tissue disease or regeneration of structures such as bone, cartilage, and trachea [22, 26]. Whether these differences represent inherent differences in properties of MSCs isolated from different tissues or differences between isolation and culture techniques is unclear. A current focus of the National Institutes of Health and the Food and Drug Administration in the USA is rigorous comparative preclinical assessments of MSCs from different tissue sources.

The presence of MSCs in a wide variety of tissues leaves open the question as to their origin and functional roles. Most of the tissue-specific MSCs appear to originate in those tissues rather than being recruited from the bone marrow. However, a growing number of studies have demonstrated that MSCs can be mobilized out of bone marrow and localize to areas of tissue injury [44–51]. For example, MSCs are mobilized in large numbers in burn patients, with the numbers of circulating MSCs correlating with the size and degree of the burn [51]. Although the factors that can induce MSC mobilization are poorly understood, mobilization and homing to tissues can be induced by hypoxia and by growth factors and cytokines/chemokines, including vascular endothelial growth factor (VEGF) and stromal-cell-derived factor 1 (SDF-1) [44–47, 50]. Notably, administration of VEGF and a CXCR4 antagonist selectively mobilized MSCs, but not hematopoietic stem cells or neutrophils, in mice [46]. How this might relate to lung injury and repair is not yet clear, although the SDF-1/CXCR4 axis has been implicated in several types of lung injuries.

The function of local tissue-specific MSCs also remains unclear. In bone marrow, MSCs are felt to provide a local environmental niche for both hematopoietic lineage development as well as local immune modulation. Recent descriptions that

perivascular pericytes share many properties with MSCs suggest a potential role in immune surveillance as well [52–54]. The role of resident MSCs in the lung is as yet poorly characterized although lung MSCs share certain immunomodulatory properties with bone-marrow-derived MSCs [55]. Recent data also suggest a correlation of resident lung MSCs with propensity to develop bronchiolitis obliterans following clinical lung transplantation. Elucidation of the role of tissue-specific MSCs remains a rich area for study.

4 Acquisition of an Airway or Alveolar Epithelial Phenotype by MSCs

MSCs can be induced in vitro to express phenotypic markers of airway and/or alveolar epithelial cells (reviewed in [56]). This can be relatively easily accomplished with use of media or growth factors known to promote or support alveolar and airway epithelial lineages [56]. Moreover, MSCs can be induced by the local microenvironment, for example, mixed or Transwell cultures in combination with lung epithelial cells, to adopt phenotypic markers of airway epithelial cells. In an important proof-of-concept demonstration, MSCs isolated from cystic fibrosis patients and transduced ex vivo to express normal cystic fibrosis transmembrane conductance regulator (CFTR) were able to partially correct defective CFTR-dependent chloride current when the transduced cells were mixed in culture with primary airway epithelial cells obtained from cystic fibrosis patients [57]. Acquisition of an airway epithelial phenotype in vitro has been comparably demonstrated with MSCs of bone marrow, umbilical cord blood, and amniotic fluid origins [4, 56–58]. Whether any one tissue source of MSCs is more suitable for acquisition of an airway or alveolar epithelial phenotype in vitro remains less well understood and is the focus of current inquiry.

However, despite the relative ease with which MSCs of different origins can be induced to acquire an airway or alveolar epithelial phenotype in vitro, engraftment as or fusion with airway and/or alveolar epithelium, interstitium, or vascular endothelium in vivo following systemic administration is generally rare and of uncertain physiologic significance (reviewed in [56]). This holds with both systemic and direct intratracheal MSC administration [56]. As such, emphasis for potential use of MSCs in structural lung repair has shifted toward ex vivo bioengineering approaches for lung regeneration. Use of three-dimensional matrices or other artificial scaffolding for growth of functional lung tissue from stem cells ex vivo and in vivo is being increasingly utilized to generate functional lung tissue ex vivo [59–76]. These approaches have been increasingly successfully utilized in regeneration of other tissues, including skin, vasculature, cartilage, and bone. Given the complex three-dimensional architecture of the lung, this is a daunting task; nonetheless, there has been significant progress in several areas. Notably, MSCs isolated from amniotic fluid, umbilical cord blood, or bone marrow can be seeded on biodegradable polyglycolic acid or other biosynthetic scaffolds and generate tracheal cartilage

for use in repair of congenital tracheal defects and also tendon tissue for use in congenital diaphragmatic defects [68–74]. Notably, the extracellular matrix properties of the resulting cartilage can depend on the source of the MSCs [22, 71].

Three-dimensional culture systems have also been utilized as matrices for ex vivo lung parenchymal development and for study of growth factors and mechanical forces on lung remodeling [59–67]. For example, culture of fetal rat lung suspensions in a three-dimensional glycosaminoglycan scaffold resulted in formation of alveolar-like structures in the scaffold [65]. Fetal mouse cells cultured in three-dimensional hydrogels and in synthetic polymer scaffolds resulted in generation of alveolar-like units [60]. Notably, stimulation of fetal mouse cells in polymer scaffolds with different isoforms of fibroblast growth factor stimulated different patterns of development, demonstrating the power of three-dimensional culture systems to evaluate lung development and repair [59] In vivo, a recent study demonstrated that fetal rat lung cells cultured in a biosynthetic gelatin matrix and subsequently injected into normal rat lungs induced the formation of branching, sacculated epithelial structures reminiscent of lung parenchymal architecture [63].

However, there have been few studies evaluating whether stem or progenitor cells isolated from adult bone marrow, umbilical cord blood, or other sources can also comparably form airway or alveolar-like structures when cultivated in a three-dimensional matrix or other scaffolding material. 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 [64]. Structures resembling lung airways and parenchyma developed when impregnated constructs were implanted subcutaneously in nude mice or inserted into the wound cavity following wedge lung resection in sheep. Adipose-derived MSCs, cultured ex vivo in sheets of polyglycolic acid and then applied to wound edges following lung volume reduction surgery in rats, accelerated alveolar and vascular regeneration [76]. More recently, it has been demonstrated that MSCs cultured in a biosynthetic gelatin matrix will spontaneously express pro-surfactant C and other lung epithelial markers [66]. Further, cyclic stretch of MSCs, either on a two-dimensional surface or in the three-dimensional matrix, significantly enhances expression of messenger RNA and protein for airway and alveolar epithelial proteins while concomitantly decreasing expression of collagen and smooth muscle actin [67]. Lung tissue bioengineering with MSCs and other types of stem cells is projected to be an area of intense investigation.

5 Immunomodulation of Lung Injuries by MSCs

In parallel with continued attempts to utilize MSCs for structural lung repair, an increasing number of studies have demonstrated a functional role of MSCs in mouse models of acute lung inflammation and fibrosis in the absence of significant lung engraftment. The rationale for using MSCs in inflammatory and immune lung diseases is based on their potent immunomodulatory and immunoprivileged

attributes as well as on rapidly increasing clinical experience with use of MSCs in other immune and inflammatory diseases. MSCs constitutively express low levels of human leukocyte antigen (HLA) class I molecules and do not express either HLA class II molecules or the costimulatory molecules CD40, CD80, and CD86, which are essential for activation of T-lymphocyte-mediated immune responses (reviewed in [77–80]). In keeping, allogeneic MSCs do not induce T lymphocyte proliferation in in vitro mixed lymphocyte reaction models. As such, these properties render MSCs nonimmunogenic and have been the basis for use of allogeneic MSCs in recent clinical trials for conditions such as graft versus host and Crohn's diseases [81, 82]. These trials have demonstrated both the efficacy and safety of allogeneic MSC administration and importantly neither infusional toxicity nor subsequent significant adverse effects have been observed [81, 82].

The mechanisms of MSC actions in these clinical trials are not fully understood but are believed to reflect the potent immunomodulatory properties of the MSCs. MSCs inhibit the proliferation and function of a broad range of immune effector cells, including T cells, B cells, and natural killer cells, and further can inhibit maturation and activation of antigen-presenting (dendritic) cells [83–93]. Notably, T lymphocyte proliferation, activation, and cytokine release are inhibited in mixed-lymphocyte reaction models in response to either alloantigens or mitogenic stimuli. This appears to be through a dose-dependent direct suppressive effect on proliferation, rather than through induction of tolerance. Further, addition of MSCs to already proliferating lymphocytes inhibits subsequent proliferation. These effects are best described for T cells, where similar effects on proliferation are observed with both CD4+ and CD8+ T cells. Both direct cell–cell contact as well as release of soluble mediators by MSCs have been proposed to play important roles in inhibition of lymphocyte and dendritic cell proliferation and functions in vitro.

These results suggest that MSCs can have significant immunomodulatory effects in the lung in the absence of significant engraftment, although the mechanisms by which this occurs remain largely unknown. However, growing information suggests several possible relevant actions of the MSCs. MSCs produce a wide variety of soluble mediators and can be influenced by specific microenvironments to release different patterns of mediators [27–32, 94–101]. For example, MSCs in bone marrow secrete cytokines and growth factors supportive of hematopoietic cell proliferation and development, including granulocyte colony-stimulating factor (G-CSF), stem cell factor, leukemia inhibitory factor, monocyte colony-stimulating factor (M-CSF), interleukin (IL)-6, and IL-11 [27]. Stimulation of MSCs with IL-1α, a proinflammatory cytokine that enhances bone marrow hematopoiesis, increases secretion of G-CSF, M-CSF, IL-6, and IL-11. The culture system in which MSCs are maintained ex vivo influences release of cytokines and other inflammatory molecules. For example, culturing MSCs in a three-dimensional hyaluronan scaffolding increased release of SDF-1, matrix metalloproteinase 3, and other mediators [30] compared with standard tissue culturing, whereas culturing under hypoxic conditions in a synthetic fibrous matrix increased fibronectin expression [28, 29]. Matrix stiffness can also affect the differentiation of MSCs [31].

These observations indicate the malleability of MSCs and it is likely that release of inflammatory mediators from MSCs is influenced by the type of inflammatory environment found in different conditions of lung injury. MSCs express a wide variety of chemokine and cytokine receptors, including those for tumor necrosis factor α (TNF-α), IL-4, IL-17, and interferon γ (IFN-γ), as well as several toll-like receptors, including the endotoxin receptor TLR4 [102, 103]. The IL-17_1receptor in particular is expressed in high abundance [102, 104] and IL-17 has recently been described as a proliferative stimulus for MSCs [104]. Stimulation with IFN-γ can alter expression of major histocompatibility complex and costimulatory molecules by MSCs. Nonetheless, the effects on MSC secretion of soluble mediators by other cytokines and chemokines by microenvironment conditions found during lung injury have been less well explored.

A growing number of studies have demonstrated the effectiveness of both systemic and intratracheal MSC administration for mitigating lung inflammation and injury [105–113]. Notably, systemic administration of MSCs immediately after intratracheal bleomycin administration decreased subsequent lung collagen accumulation, fibrosis, and levels of matrix metalloproteinases [105]. Secretion of IL-1 receptor antagonist by MSCs is hypothesized to account for at least some of these effects [106]. Comparably, intratracheal administration of MSCs 4 h after intratracheal endotoxin administration to mice decreased mortality, tissue inflammation, and concentration of proinflammatory mediators, such as TNF-α and macrophage inflammatory protein 1β, in bronchoalveolar lavage fluid compared with endotoxin-only treated mice [111]. Systemic MSC administration also decreased lung inflammation following endotoxin administration in mice [107, 108, 110]. Notably transduction of the MSCs to express angiopoietin-1 further decreased endotoxin-mediated lung injury, presumably through abrogation of endotoxin-mediated endothelial injury [107]. However, systemic administration of skin fibroblasts transduced to express angiopoietin-1 also decreased acute endotoxin-induced lung injury, suggesting that a variety of cell types might be utilized for cell therapy approaches to acute lung injury [112]. Coculture of MSCs with lung cells obtained from lipopolysaccharide-treated mice resulted in decreased proinflammatory cytokine release from the lung cells [110]. In other lung injury models, intratracheal administration of bone-marrow-derived MSCs decreased pulmonary hypertension and other manifestations of monocrotaline-induced pulmonary vascular injury [109]. Comparable mitigation of acute lung injury and pulmonary edema has recently been observed following administration of MSCs to isolated perfused human lungs injured with endotoxin [113]. Keratinocyte growth factor secreted by the MSCs appears to play an important role in this model. Some data are also available on MSC actions in mouse models of emphysema. In several recent reports, systemic administration of bone-marrow-derived MSCs or of a heterogenous population of autologous adipose-derived stromal cells decreased manifestations of elastase- or papain-induced emphysema in mice and rabbits [114]. Hepatocyte growth factor secreted by the cells was postulated as a potential mechanism of injury repair in one report, but there is little other information available about the mechanisms of action by which MSCs mitigate emphysema. Importantly, no significant

adverse effects of the MSCs were observed in these studies, although longer-term tumorigenesis and toxicology studies are pending.

6 Clinical Use of MSCs in Lung Disease

A recent ground-breaking trial is being conducted in the USA utilizing a commercial preparation of MSCs obtained from bone marrows of healthy volunteers (PROCHYMAL^{TM}, Osiris Therapeutics, Columbia, MD, UDA). In a previous phase I/2, double-blind, placebo-controlled trial of PROCHYMAL^{TM} conducted by Osiris Therapeutics in patients with acute myocardial infarction, an improvement in both forced expiratory volume in 1 s (FEV_1)and forced vital capacity (FVC) was noted in treated patients [115]. Although the mechanisms of improvement in pulmonary function in this patient population are not yet well understood, these observations stimulated a multicenter, double-blind, placebo-controlled phase II trial of PROCHYMAL^{TM} for patients with moderate to severe COPD ($FEV_1/FVC < 0.70$, $30\% \leq FEV_1 \leq 70\%$) 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 six participating US sites. The 6-month interim analysis demonstrated the safety of PROCHYMAL^{TM} administration with no infusional toxicities or significant adverse events related to the infusions reported [116]. Moreover, trends toward improvement in quality of life indices such as 6-min walk and dyspnea scales were observed. These results open a potential door for eventual potential clinical use of MSCs in COPD and other immune and inflammatory lung diseases. However, it will be some time before this therapy becomes potentially available, particularly as longer-term toxicology and tumorigenesis studies are pending. Importantly, one bone marrow aspirate obtained from a normal healthy volunteer can provide thousands of doses of allogeneic MSCs that can be frozen for subsequent use. Use of allogeneic MSCs for inflammatory and immune lung diseases is thus potentially feasible and obviates the need to utilize autologous MSCs for each patient.

7 MSCs and Malignancies: Pros and Cons and Other Cautions for Use

MSCs are also increasingly described as vehicles for delivery of therapeutic genes and proteins [117–121]. Notably, MSCs can home to tumors, through as yet unclear mechanisms, and serve as vehicles for delivery of chemotherapeutic and other antitumor agents [122–125]. This has recently been described in mouse lung tumor models and may provide a viable therapy for lung cancers [126–129]. In contrast, MSCs may also contribute to tumor stroma and influence the behavior

of cancer cells [122, 130–134]. Further, development of lung sarcomas from systemically administered MSCs has been described [41, 42]. Interestingly, this has been described with administration of mouse but not human MSCs and may reflect a greater propensity of mouse MSCs to acquire chromosomal abnormalities with serial passages in culture [42, 43]. However, extensive culture of almost any mammalian cell in culture can lead to crisis, followed by immortalization and then transformation to tumorigenic cells as has been well documented for mouse fibroblasts [135]. Murine MSCs that were extensively expanded in culture through many passages developed chromosomal instability and produced lung sarcomas in mice [41, 42]. Human MSCs that were cultured for 4–7 months underwent similar changes [135, 136]. Whether MSCs or other adult stem or progenitor cell populations contribute to development of epithelial cancers remains an active area of investigation [133, 134].

Some additional cautions with regard to systemic or intratracheal administration of MSCs have been raised. Most culture strategies utilize fetal or bovine calf serum. Despite washing of the cells prior to systemic administration, some bovine antigens may remain adherent to cell surfaces and trigger immune reactions as well as decrease potential engraftment in recipient mice or patients [137]. Culture of MSCs in medium with lower calf serum content, use of heterologous species-specific serum or alternative serum substitutes such as platelet lysate, and removal of calf serum antigens prior to administration are proposed strategies to decrease these potential adverse effects [138–141]. Additionally, following intravenous administration, MSCs initially lodge in the lung vasculature before moving through the pulmonary capillary system and on to other organs. However, depending on the preparative regimens utilized, MSCs can clump and potentially lodge as emboli in lung capillaries [41, 42]. Pretreatment of mice with the vasodilator sodium nitroprusside has been proposed as a mechanism of decreasing MSC trapping in pulmonary capillaries [142]. It is anticipated that additional strategies to maximize therapeutic utility of MSCs while decreasing the chance of any adverse effects will develop over the next several years.

8 Summary

MSCs are as yet still incompletely understood cells that have a broad range of properties. Continuing controversies and unknowns in MSC nomenclature, characterization, comparative properties of MSCs obtained from different tissue sources, and long-term potential tumorigenic effects are areas of current intense research efforts. Nonetheless, progressively accumulating data demonstrate the immunomodulatory effects of MSCs in a variety of inflammatory and immune-mediated lung disease models. In parallel, approaches for lung bioengineering demonstrate the potential utility of MSCs in ex vivo lung regeneration. As such, despite cautions, there is growing promise in the use of MSCs for treatment of lung diseases.

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Chapter 3
The Role of Progenitor Cells in Lung Disease Prognosis

Ellen L. Burnham, Susan Majka, and Marc Moss

1 Introduction and Background

In the past decade, the number of investigations related to the role of stem and progenitor cells in lung repair has grown exponentially. Lung injury has been associated with the release of immature cells into the circulation from bone marrow; complementary to this, research suggests that a functioning bone marrow is necessary to repair lung successfully [1–3] (Fig. 3.1). Bone-marrow-derived cells, including stem cells and progenitor cells, have emerged as candidate markers to prognosticate outcomes during pulmonary disease, as they have for cardiovascular disease, myocardial infarction, atherosclerosis, cerebral vascular diseases, and rheumatoid arthritis [4–7]. In both animal models of disease and human subjects, the relationship of these cells to prognosis has provided clues regarding the underlying pathophysiologic characteristics of different pulmonary disease processes, and direction for future investigations related to lung repair. It has become clearer that not only are these cells capable of contributing to the structure of lung tissue, but also that they may also have paracrine immunomodulatory functions. The ability of a progenitor population to participate in these processes opens the door to further examine the utility of these cells as biomarkers in a variety of lung diseases, including acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), pulmonary arterial hypertension (PAH), emphysema, and asthma [8–10], as well as to validate previous observations related to prognosis in these lung diseases.

A variety of progenitor cell types have been examined for their potential in lung disease prognosis, including hematopoietic progenitor cells, also known as hematopoietic stem cells (HSCs). HSCs are characterized by CD45 and CD34 expression, are typically recruited to the lung when inflammation occurs, and are probably the best described of all progenitor cell types. In response to inflammation and the specific tissue microenvironment, HSCs respond by upregulating receptors,

E.L. Burnham (✉)
Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver School of Medicine, Aurora, CO 80045, USA
e-mail: ellen.burnham@ucdenver.edu

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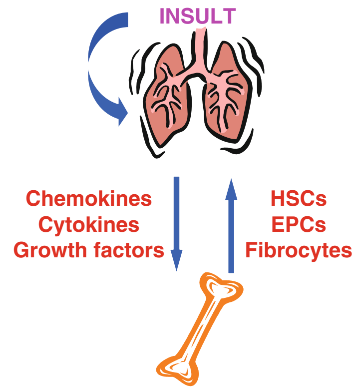

Fig. 3.1 When lung is injured through the effects of infection, chemotherapeutic agents, radiation, or other agents, this may result in the elaboration of chemokines, cytokines, and growth factors by the lung. This in turn leads to the release or "summoning" of stem or progenitor cells from the bone marrow that will then home to the damaged lung

leading to particular lineage differentiation [11]. These receptors include those that specify immune cell, endothelial cell, and mesenchymal lineages.

One HSC type that has been extensively examined for its role in lung disease prognosis is the circulating endothelial progenitor cell (EPC). Circulating EPCs are bone-marrow-derived cells characterized by expression of the cell-surface antigen CD45, along with CD133, CD34, and vascular endothelial growth factor receptor 2 (VEGFR2, also known as Flk-1). Circulating EPCs typically lack differentiated endothelial markers such as CD144 (also known as vascular endothelial cadherin) [12]. These cells are found in very low numbers in the circulation of healthy individuals (less than 1% of all circulating cells). Their functional properties include in vitro differentiation to a phenotypic endothelial cell, as well as 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-labeled acetylated low-density lipoprotein (LDL) uptake and lectin binding. In culture, these cells can form endothelial cell colony-forming units (CFUs). Additionally, they may form angiogenic tubes both in vitro and in vivo. Angiogenic tube formation has been reported to occur both with and without human umbilical vein endothelial cell co-culture (Fig. 3.2). Unfortunately, EPC investigations have been subject to more controversy than mesenchymal stem cell (MSC) investigations, partially owing to the lack of consensus surrounding appropriate EPC identification, and this has impeded advances in this field [13, 14]. For example, flow-cytometric markers chosen to identify EPCs by laboratories often differ greatly, impeding adequate comparison of results between groups. Of note, resident lung EPCs have also been identified using similar cell-surface markers and techniques as for circulating EPCs [15–17]; however, in most cases the true origin of these cells, whether bone marrow or lung, has not been clearly delineated.

Nonhematopoietic stem cells have also been examined in the setting of lung disease. MSCs are nonhematopoietic stem cells with multilineage differentiation potential. These cells are of mesodermal origin and are found within bone marrow, as well as other tissue types, including adipose tissue, tendon, amniotic fluid, and teeth [18]. MSCs have many desirable properties for consideration as cell-based

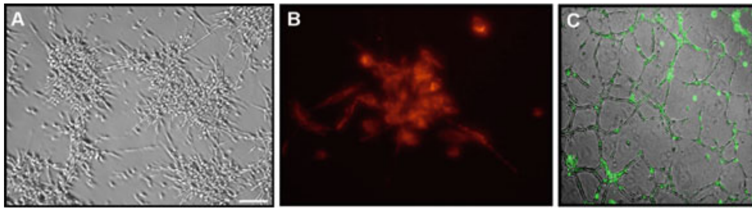

Fig. 3.2 (**a**) Phase-contrast image of endothelial cell colony-forming units (CFU-EC) formed in culture (day 5) by healthy control peripheral blood mononuclear cells (PBMCs) enriched for progenitors. (**b**) CFU-EC display endothelial cell-like characteristics such as 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate labeled acetylated low-density lipoprotein (LDL) uptake and **c** incorporate (CFU-EC labeled *green*) in angiogenesis tubes formed by mature endothelial cells. *Bar* 200 μm

therapies, including a high expansion potential, genotypic and phenotypic stability, and ease in collection. Additionally, MSCs are able to travel to other tissue sites and have immunosuppressive properties that allow them to be utilized in both autologous as well as heterologous transplantation protocols [19]. Most importantly, MSCs have the ability to differentiate into both pulmonary vascular endothelium as well as alveolar epithelium [20, 21]. As such, investigations related to their utility in prognosis bear directly on their development as cell-based therapies for diverse lung diseases.

The fibrocyte has recently emerged as a specific progenitor cell associated with disease prognosis and remodeling [22, 23] with mesenchymal cell properties, but is believed to be a type of HSC. These spindle-shaped cells are defined by the markers collagen, α-smooth muscle actin, vimentin, fibronectin, along with CD13, CD34, CD45, and CD11b positivity. They can differentiate into fibroblast-like cells at sites of injury, and also demonstrate an ability to form fibroblast colonies in culture. Fibrocytes can be potent antigen-presenting cells via their expression of characteristic cell-surface markers, including major histocompatibility markers (HLA-DP, HLA-DQ, HLA-DR), CD80, CD86, CD11a, CD54, and CD58. They also have the ability to prime T cells at significantly higher levels than do monocytes [24]. Further, fibrocytes may promote angiogenesis by enhancing endothelial cell migration via production of matrix metalloprotease 9 along with producing other proangiogenic cytokines, including vascular endothelial growth factor, basic fibroblast growth factor, interleukin (IL)-8, and platelet-derived growth factor [25]. Given this multiplicity of properties, defining the fibrocytes' capabilities in lung diseases could lead to better understanding of disease pathogenesis and development of novel therapies particularly for diseases associated with fibrosis.

Progenitor cells *endogenous to the lung* may similarly have prognostic roles for pulmonary diseases, although sampling and study of such cells in human subjects would undoubtedly pose a challenge. These endogenous cells include the multipotential resident lung progenitor population, also known as lung side population (SP) cells, characterized by their unique "side arm" cytometric profile following Hoechst 33342 staining. Lung SP cells ($CD45^{neg}$) have previously been characterized as

resident lung multipotent MSCs [26, 27] with both epithelial and endothelial differentiation potential. They respond during pediatric and adult murine models of chronic lung disease with changes in cell number and CFU ability [16, 28]. Their role and function in human disease is as of yet unknown.

Although questions remain regarding the hierarchy and abilities of various stem and progenitor cells, correlations between the numbers and functions of these cells and pulmonary disease prognosis have been reported. Here we provide a detailed analysis of the relationship between these progenitors and the course of specific pulmonary diseases.

2 Asthma

Clinical features of asthma include mucous hypersecretion and airway hyperreactivity [29–31]. This disorder is mediated through type 2 T-helper cells (TH-2), and is characterized by airway remodeling with eosinophil infiltration, smooth muscle hyperplasia, and thickening of the basement membrane with collagen deposition. Along with these airway changes, vascular effects of this disease, including an increase in the number and size of vessels in the airway wall, have been reported [8]. Microarray analysis has been performed in patients with asthma to identify diagnostic biomarkers and has confirmed the enhancement of TH-2 cytokine responses dependent on IL-4 and IL-13 [30].

In asthma, circulating CD34^{pos} HSCs have been found to home to sites of pulmonary allergic inflammation and express the IL-5α receptor subunit, specific for eosinophil lineage cells. Further, these CD34^{pos} HSCs cells will form eosinophil and basophil CFUs under appropriate culture conditions. Increased numbers of these cells expressing the IL-5α receptor subunit have been detected in bronchial biopsies of asthmatic patients compared with nonasthmatic control subjects [32]. To better define the function of these cells, Southam et al. [31] postulated that these were effector cells. This group determined that such HSCs would home to the allergen-sensitized airways and undergo in situ differentiation to eosinophils. With use of a murine model of ovalbumin challenge and allergic inflammation, the kinetics of localization for CD34^{pos}/CD45^{pos} lung HSCs was compared with the kinetics of CD34^{pos}/CD45^{pos}/IL-5Rα^{pos} eosinophil lineage-committed cells in terms of eosinophilia and inflammatory cytokine production. They ascertained that CD34^{pos}/CD45^{pos}/IL-5Rα^{pos} cells were elevated following allergen challenge and that these cells could form eosinophil–basophil CFUs in culture, in contrast to what was observed in non-allergen-challenged control mice. In addition, the CD34^{pos}/CD45^{pos}/IL-5Rα^{pos} cells' appearance correlated with production of IL-5 and eotaxin. It was surmised that IL-5 "primed" hematopoietic progenitors to respond to eotaxin by upregulation of CCR3, whereas eotaxin increased IL-5Rα expression by the progenitors [33]. These studies suggest that ascertaining numbers of CD34^{pos} HSCs bearing IL-5α receptors in subjects with asthma could have the potential to help prognosticate disease activity. Interfering with IL-5 signaling might

also be expected to potentially benefit asthmatic subjects as well owing to its ability to limit the effects of the contribution of HSCs to disease pathogenesis.

Subepithelial fibrosis is one pathologic characteristics of asthma that may contribute to refractoriness to standard asthma therapies that is observed in some individuals. A bone-marrow-derived cell that may contribute to this process is the fibrocyte. Schmidt et al. [34] demonstrated that fibrocytes accumulate in the bronchial mucosa of patients with allergic asthma in areas of collagen deposition. These cells expressed CD34, α-smooth muscle actin, and collagen I. Additionally, when cultured in the presence of inflammatory cytokines normally found in asthmatic airways, these cells differentiated into myofibroblasts. In complementary experiments, using a murine model of allergic asthma and labeled fibrocytes, homing of circulating fibrocytes to subepithelial fibrotic bronchial tissue was observed, in contrast to what occurred in non-allergen-challenged controls. Levels of circulating fibrocytes as a marker of disease severity have not been reported, although they could additionally reflect disease activity similar to $CD34^{pos}$ HSCs.

Circulating EPCs may participate in aberrant airway angiogenesis in the setting of asthma. Asosingh et al. [8] demonstrated that $CD34^{pos}/CD133^{pos}$ circulating EPCs are recruited to asthmatic lungs, but not to the lungs of nonasthmatic controls or to the lungs of those with allergic rhinitis, and may participate in the airway angiogenesis observed in asthmatic individuals. These investigators reported that circulating EPC numbers in subjects with asthma not only increase, but that circulating EPCs also exhibit evidence of enhanced proliferation, angiogenic function, and CFU formation. Complementary studies to test the kinetics of circulating EPC recruitment from bone marrow to the lungs were performed using murine models of acute and chronic allergen challenge. Circulating EPC recruitment was observed to depend on type 1 T-helper cell and TH-2 responses and correlated with the establishment of a proangiogenic environment. Specifically, increased microvessel density was observed and was determined to be persistent, ultimately contributing to the pathophysiologic characteristics of asthma through its effects on airway remodeling and edema. As investigators begin to better understand the role of circulating EPCs in angiogenesis within airways, information regarding these cells' numbers or function may be useful prognostically in determining which patients are at highest risk for airway remodeling, and therefore should be targeted for more aggressive available therapies. Alternatively, novel therapies that target these cell types might be useful in modulating disease outcomes.

More recently, a role in asthma for lung SP cells has emerged. Hackett et al. [29] demonstrated a 33-fold increase in the numbers of SP cells isolated in tracheobronchial epithelium from human asthmatic lungs relative to nonasthmatic control lung tissue. $CD45^{neg}$ SP cells from these samples had an enhanced ability to undergo epithelial differentiation relative to non-SP cells that could further contribute to abnormal airway remodeling. Additional studies are necessary to understand the fate of and role of SP cells during asthma prior to making assumptions about their utility in prognosis. These endogenous lung stem cells might have a role in the maintenance of lung homeostasis in the setting of asthma, but overexuberant proliferation or production of mediators by these cells could be deleterious to asthma

control. Importantly, the ability to sample SP cells in humans presents a challenge to the adequate study of these cells. All the lung tissue utilized in the above-mentioned study was obtained from whole lung specimens; this clearly makes the SP cell a less attractive candidate for use as a biomarker, although it is an important one to help better define pathogenesis.

3 Pulmonary Arterial Hypertension

PAH is a disorder characterized by sustained vasoconstriction of both large and small pulmonary arteries along with pulmonary vascular remodeling. Remodeling in this disorder can occur anywhere along the vascular axis, in either large-caliber or small-caliber vessels, including the pulmonary arteries, arterioles, and microvessels. These vascular changes are characterized by increased adventitial inflammation, smooth muscle hypertrophy, and neointimal hyperplasia. Cells implicated in the pathogenesis of this disease process include endothelial cells, smooth muscle cells, and myofibroblasts; however, recent evidence suggests a role for resident lung and circulating bone-marrow-derived progenitors, including the circulating EPCs and fibrocytes [35].

Identifying mutations present in circulating progenitors may ultimately provide prognostic information for PAH. For example, recent investigations have demonstrated that circulating EPCs from patients with idiopathic PAH (iPAH) have deregulation of bone morphogenic protein 2 (BMP-2)-dependent survival responses relative to non-PAH patients [36]. This loss of normal BMP-2 function has previously been reported to affect the endothelium of PAH patients, resulting in increased apoptosis and injury of these cells. When such mutations occur in circulating EPCs, this could lead to abnormal repair processes and ultimately vascular remodeling. These observations have been supported by data from Masri et al. [37], where CD34^{pos}/CD133^{pos} circulating EPCs in iPAH patients have a hyperproliferative, apoptosis-resistant phenotype.

Alterations in numbers and function of circulating progenitors have also been associated with PAH, and may provide clues regarding mechanisms underlying vascular remodeling observed in this disorder. Asosingh et al. [38] demonstrated that iPAH patients have higher numbers of CD34^{pos}/CD133^{pos} circulating EPCs compared with healthy control subjects, and that this cell number correlates with increased pulmonary artery pressures. Majka et al. [39] confirmed this observation, reporting that CD133^{pos} cells were associated with iPAH and familial PAH lesions in postautopsy human tissue specimens (Fig. 3.3). In functional assays, endothelial cell CFUs derived from iPAH patients form disorganized clusters when supplemented with transforming growth factor β or BMP-2. Enhanced tube-forming ability in vitro by circulating EPCs from iPAH subjects has also been reported [38]. Certainly, enumerating circulating EPCs in PAH might provide insight into clinical disease severity, but better understanding of these cells' function might also help to develop directed therapies to modulate disease outcomes.

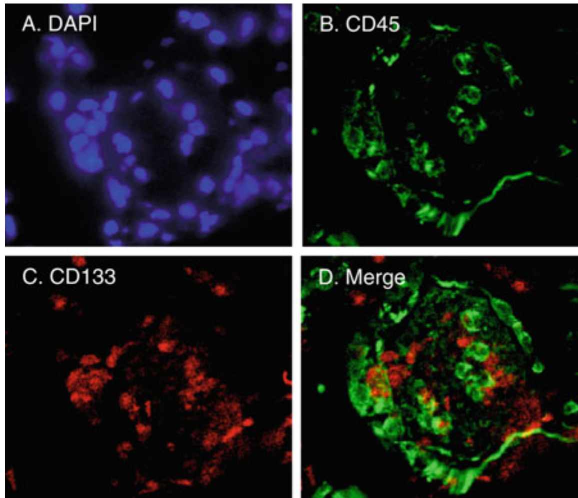

Fig. 3.3 CD133^{pos} and CD45^{pos} cells associate with arteriole structures in human pulmonary arterial hypertension (PAH) tissue. CD133^{pos} progenitor cells were identified in lung tissue by antibody staining to detect CD133 (**c**) and infiltrating immune cells by CD45 (**b**). 4′,6-Diamidino-2-phenylindole (DAPI) was used as the nuclear stain (**a**). CD133 was not detected in control arterioles or parenchymal tissue. In contrast, small vessels from PAH patients displayed high numbers of CD133^{pos} and CD45^{pos} cells (**b–d**). Arterioles from PAH tissue had CD133 and CD45 localized both adjacent to and infiltrating the smooth muscle layers. No significant levels of CD133 coexpression with CD45 was detected and would be indicated by yellow in the merged panel (**d**). *Scale bars* 75 mM

It is important to acknowledge that although circulating EPC numbers have been linked to PAH and that their function appears to differ in this disease state from what is observed in healthy individuals, these cells can also be utilized in a therapeutic role for PAH. Investigations have demonstrated that transplantation of circulating EPCs in canine and rat models of pulmonary hypertension alleviated progression of monocrotaline-induced PAH [40, 41]. Furthermore, when circulating EPCs were transduced ex vivo with human endothelial nitric oxide synthase, the combination of cell and gene therapy proved to be an effective strategy to alleviate the pathophysiologic effects of disease [41]. Interestingly, engraftment of circulating EPCs in these cases was typically low, with the beneficial effects being ascribed to either transient engraftment during repair or unknown paracrine effects of these cells. Again, harnessing the potential of circulating EPCs in PAH could provide novel treatments for this disorder that would benefit prognosis.

An additional population of progenitor cells studied in hypoxia-induced models of PAH includes fibrocytes positive for CD45, CD11b, CD14, CD68, ED1, and ED2 [42]. In this model, these cells have been determined to accumulate within the adventitia and produce collagen. By selective depletion of this population during PAH, vessel remodeling was abrogated. Along with circulating EPCs, this

progenitor cell type may also prove to be an important prognostic marker related to vascular remodeling in PAH, although additional investigations will be necessary to more clearly define its role.

4 Pulmonary Fibrosis

Pulmonary fibrosis is a condition that is insidious in onset, occurring in the setting of repair after ALI, after inhalational injury, in conjunction with connective tissue diseases, or in response to specific environmental exposures. It may also occur without such association, where it is termed “idiopathic pulmonary fibrosis” (IPF). Pulmonary fibrosis is marked by histologic features including collagen deposition in proliferative fibroblast foci, airway and vascular remodeling, and ablation of surfaces for gas exchange in the lung. PAH can also be present secondarily in individuals affected by pulmonary fibrosis. The cells present within the fibroblast foci are hypothesized to be derived either from injured epithelium that undergoes an epithelial–mesenchymal transition or from endogenous lung fibroblasts. More recently, bone-marrow-derived cells that have fibroblastic features have also been implicated in pulmonary fibrosis [43, 44]. Their numbers and activity could have prognostic relevance for patients with this disorder.

Hashimoto et al. [44] reported the bone marrow origin of fibroblasts using a murine model of bleomycin-induced experimental lung fibrosis, and determined that these cells accounted for the majority of the collagen-producing cells in fibroblastic foci. These CD45^{pos}/collagen type I^{pos}/CXCR4^{pos} fibrocytes have been isolated from humans and reintroduced into a model of lung fibrosis in immunocompromised mice. This resulted in trafficking of donor cells to fibroblastic foci [45]. Chemokine receptors including CCR2, CCR7, and CXCR4 have been demonstrated to play a role in recruitment and activation of circulating fibrocytes in murine models of lung fibrosis [46]. These cells may be recruited in response to signals of injury from alveolar epithelium, including ligands for CCR2, CCL2, CCL7, and CCL12. Neutralization of CCL12 is protective during experimental fibrosis [47], suggesting a potential pathway that might be targeted for disease therapy focused on the activity of the fibrocyte. Fibrocytes may also be mobilized into the circulation in response to granulocyte colony-stimulating factor, granulocyte–monocyte colony-stimulating factor, and macrophage colony-stimulating factor [48]. These chemotactic agents are similar between mice and humans and may help to establish a link between experimental and patient disease processes [47]. Methods to intervene in the axis of fibrocyte recruitment in pulmonary fibrosis could represent a method to improve prognosis in these individuals.

More recently, in human subjects, Moeller et al. [43] correlated the level of circulating CD45^{pos}/collagen type I^{pos} fibrocytes with poor prognosis in IPF. These investigators reported a threefold increase in circulating fibrocytes in patients with stable IPF compared with healthy controls; further, in a smaller group of individuals with an acute exacerbation of IPF, the numbers were increased still further.

When all subjects with IPF (both with stable disease and with acute exacerbation) were grouped and stratified by percentage of circulating fibrocytes (more than 5% fibrocytes, or less than 5% fibrocytes), it was observed that subjects in the lower fibrocyte group had significantly longer survival ($p < 0.0001$ by log rank testing). Further, in a model that included pulmonary function testing parameters, 6 min walk test distance, and percentage of circulating fibrocytes, only the percentage of circulating fibrocytes significantly predicted mortality ($p = 0.041$). Unfortunately, these circulating CD45^{pos}/collagen type I^{pos} cells were not functionally analyzed ex vivo for fibroblast CFU activity, proliferation, apoptosis, or collagen production that might have provided some insight into their role in the progression of this disorder.

Other observational studies in humans have determined that circulating EPCs (CD34^{pos}/CD133^{pos}/VEGFR2^{pos}) may be useful in prognosis for patients with restrictive lung diseases, such as pulmonary fibrosis. Fadini et al. [49] demonstrated that subjects with spirometric characteristics of restriction had lower numbers of circulating EPCs than obstructive lung disease subjects. Circulating EPC numbers also correlated with disease severity, including impaired diffusion capacity and reduced lung volumes. Additional investigations of circulating EPCs will be necessary to determine their utility in pulmonary fibrosis prognosis as well as their pathologic role in these individuals.

5 Chronic Obstructive Pulmonary Disease and Emphysema

Chronic obstructive lung disease (COPD) and emphysema are characterized by destruction of lung parenchyma and the loss of surfaces for gas exchange. In severe chronic lung diseases such as these, pulmonary vascular remodeling and secondary PAH may also develop as a consequence of prolonged hypoxia [49]. It is necessary to know the origin of and to characterize the cells that participate in remodeling and repair to determine a role for progenitors in these processes.

Experimental rodent models of elastase-induced emphysema have helped to clarify the function of bone-marrow-derived progenitors in this condition and to determine their prognostic utility. Ishizawa et al. [50] demonstrated that bone-marrow-derived cells are present in alveoli during regeneration of elastase-induced emphysema, and that their presence correlated with lung regeneration. Treatment of these mice with a combination of all-*trans* retinoic acid and granulocyte colony-stimulating factor resulted in a synergistic level of repair and retention of bone marrow cells in lung tissue parenchyma. Other investigations, by Abe et al. [51], using parabiosis (the joining of two murine circulations) tested whether elastase and radiation injury resulted in a hematopoietic contribution to pulmonary regeneration. The studies revealed that hematopoietic cells from the labeled, uninjured donor mice localized as interstitial macrophages, fibroblast-like interstitial cells, and type I epithelium. Building on these data, Ishizawa et al. [52] studied the effects of hepatocyte growth factor on the mobilization of circulating EPCs

following elastase injury in mice. Hepatocyte growth factor mobilized circulating EPCs (Sca-1^{pos}/Flk-1^{pos}/c-kit^{pos}) from the bone marrow that localized to the injured lung and differentiated to vascular endothelial cells during repair. These studies provide evidence that bone-marrow-derived cells might play an important role in both epithelial and endothelial repair in animal models of emphysema/COPD, as they localize to a variety of sites known to be damaged during the pathologic course of this disease. Further, manipulating numbers of these various cell types might improve overall prognosis for this disease.

Human studies of progenitor cells for prognosis in patients with obstructive lung disease are more limited. Fadini et al. [49] performed analyses of circulating EPC numbers (characterized by their expression of CD34^{pos}/CD133^{pos}/VEGFR2^{pos}) in patients with severe lung diseases, including obstructive lung diseases such as COPD. This group determined that patients with the most severe lung disease had significantly lower numbers of circulating EPCs than control subjects; however, in contrast to subjects with restrictive lung diseases, these numbers did not correlate with outcomes. Nevertheless, the observed reduction in EPC number could contribute to the endothelial dysfunction and the lack of endothelial repair observed in obstructive lung disease. Although circulating EPC numbers could potentially serve as markers for disease severity in COPD, additional studies with larger and better-defined patient populations are necessary prior to utilizing cell number or function for this purpose.

6 Acute Lung Injury/Acute Respiratory Distress Syndrome

ALI and ARDS are characterized by both alveolar epithelial (type I cell) and pulmonary vascular endothelial damage. Owing to the fulminant and often unpredictable nature of these illnesses, developing cell-based therapies or utilizing medications to enhance cell numbers to ameliorate disease is challenging. However, prognostic information derived from assaying progenitor cells could be helpful in planning and in family discussions. Yamada et al. [53] were among some of the first investigators to postulate that bone-marrow-derived cells might be important in lung injury repair. Using a lipopolysaccharide (LPS) model of lung injury, they gave wild-type C57Bl/6 mice were given sublethal doses of radiation. This was followed by either LPS or PBS intranasal insufflation. In nonirradiated control animals, the degree of lung injury did not differ whether or not the animals received LPS; however, irradiated mice that were given LPS developed significant emphysematous lesions. In subsequent experiments, when irradiated mice were given LPS followed by an immediate infusion of bone marrow cells, the emphysematous lesions did not develop. GFP^{pos} cells within transplanted animals' lung parenchyma were GFP^{pos}/cytokeratin^{pos}/CD45^{neg} and GFP^{pos}/CD34^{pos}/CD45^{neg}. These observations suggest that these bone-marrow-derived cells can assume certain properties of both epithelial and endothelial cells, and that adequate numbers of such cells in circulation may be important in preventing LPS-induced lung injury. Both the quantity

and the function of these bone-marrow-derived cells have potential importance in lung repair.

Rojas et al. [3] hypothesized that administering bone-marrow-derived MSCs, purified via enriching fresh bone marrow for CD11b (macrophages/monocytes) and CD45 (hematopoietic cells), would hasten lung repair in a bleomycin lung injury model. In these experiments, some animals were subjected to bone marrow ablation with busulfan prior to bleomycin injury; the animals then received either intravenous MSC infusion or a placebo medium. Lung damage was morphometrically the greatest in animals that had previously received busulfan administration followed by intratracheal administration of bleomycin without concomitant MSC infusion. Conversely, animals with intact bone marrow subjected to bleomycin and immediate MSC infusion had a degree of lung injury comparable to that of controls. Moreover, survival in this latter group was 100% at 16 days after bleomycin injury, whereas in the group receiving busulfan, whose bone marrow was compromised, survival was only 30%. In later experiments by this group [54], utilizing an endotoxin model of ALI, the intravenous administration of similarly purified MSCs to lung-injured animals had beneficial effects in terms of decreased pulmonary edema and inflammatory response, features that have been linked to better outcomes in human ARDS [55, 56]. The *intrapulmonary* administration of MSCs may be similarly beneficial. Gupta et al. [57] purified bone marrow MSCs via in vitro culture, and confirmed that these cells retain the potential to form bone, cartilage, and fat cells. Intrapulmonary administration of these purified cells significantly improved both 48- and 72-h survival of mice after endotoxin inhalation. Further, the pathophysiologic improvements first reported by Rojas et al. were similarly observed in these endotoxin-subjected animals after intrapulmonary administration of MSCs, including decreased pulmonary edema and proinflammatory cytokines. These findings in animal models suggest that if it were possible to either enumerate the MSC number or assess MSC function in human subjects with lung injury, this might provide prognostic information for these individuals. Toward this end, to extrapolate the relevance of MSCs to prognosis in human subjects, cells were obtained from 13 individuals with ALI in an effort to characterize alveolar mesenchymal cells obtained by bronchoalveolar lavage [58]. These investigators observed that the majority (99%) of cells isolated by bronchoalveolar lavage were $CD45^{pos}$ at day 1 after ALI diagnosis. However, by day 4, the phenotype of cells in bronchoalveolar lavage was altered by the presence of a significant number of unique $CD45^{neg}$ cells that possessed the mesenchymal markers prolyl 4-hydroxylase (an enzyme specific for mesenchymal cells), vimentin, and fibronectin. These $CD45^{neg}$ cells would proliferate in cell culture for multiple passages. More recently, Moeller et al. [43] measured circulating fibrocytes in ten subjects with ALI and reported that the numbers did not differ significantly for healthy controls ($n = 7$) or subjects with stable IPF ($n = 51$). Although these studies in ALI are purely observational, and the relevance of MSCs or fibrocytes to prognosis cannot be determined, given the biologic plausibility of these cells having a role in the fibrotic phase of ALI/ARDS, additional investigations to determine the relationship between these cells and outcomes appears warranted.

Other investigators have focused on endothelial repair in ALI and the role of EPCs in its prognosis. Yamada et al. [59] attempted to identify endothelial progenitor cells in the circulation of patients with bacterial pneumonia, a common risk factor for the development of ARDS [60]. EPCs were classified by this group as peripheral blood mononuclear cells that had been cultured for 1 week that stained positively for DiI-labeled acetylated LDL uptake and lectin binding, as well as the markers VEGFR2 and CD31. With use of this method, the number of cells consistent with EPCs in culture was determined to be higher soon after diagnosis and decreased significantly after therapy had been completed. More relevant to prognosis, individuals with low pretreatment EPC counts were determined to have fibrotic changes present on lung CT scanning that persisted into recovery, suggesting that long-term pulmonary dysfunction could be expected in these individuals. In contemporaneous investigations by Burnham et al. [61], EPCs defined similarly (with endothelial-specific culture, and staining for LDL uptake/lectin binding) reported that EPC CFUs (not single EPCs) were significantly higher among patients on mechanical ventilation, including patients with ALI, than in healthy controls (Fig. 3.4). However, when patients with ALI were stratified into a low EPC CFU group or a high EPC CFU group, depending on the numbers of CFUs obtained from blood within 72 h of diagnosis, it was found that survival was significantly better in the group with higher EPC CFUs (70% surviving compared with 35%, $p < 0.03$). Confirmatory studies regarding the utility of the assays in prognosis need to be performed, although it appears feasible that such methods could be useful in subjects with ALI.

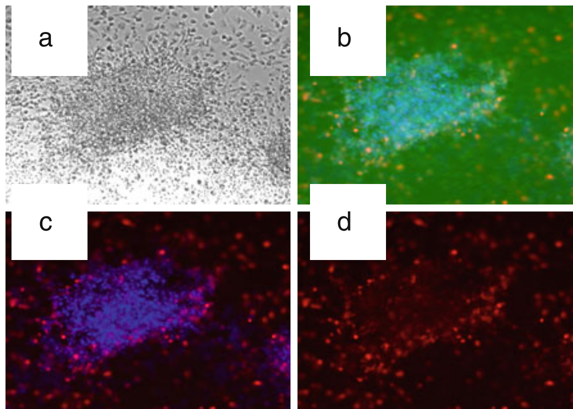

Fig. 3.4 PBMCs from subjects with acute lung injury (ALI) form colony-forming units (CFUs) that correlate with outcome. Photomicrographs of a CFU, derived from 7 days' culture of PBMCs from a subject with ALI. *Green* indicates lectin; *red* indicates LDL, and *blue* indicates DAPI. (**a**) Unstained, bright-field microscopy of CFU. (**b**) CFU stained for lectin binding, LDL uptake, and DAPI nuclear counterstain. (**c**) CFU stained for LDL uptake with DAPI nuclear counterstain. (**d**) CFU stained for LDL uptake

6.1 Sepsis

Endothelial dysfunction is a hallmark of both ARDS and severe sepsis, resulting from a variety of host immune mediators that operate in concert to activate the endothelium [62–64]. The endothelium responds with structural and functional changes, including cytoplasmic swelling and detachment with expression of surface adhesion molecules. These pan-endothelial changes lead to extravasation of intravascular volume to the extravascular space, resulting in hypotension and shock, the clinical features of severe sepsis [62, 65, 66]. Endothelial damage may precede clinical symptoms and end-organ failure. Sepsis is a common risk factor for ALI in medical intensive care unit patients. Mutunga et al. [67] demonstrated several years ago that the severity of sepsis was linked to the number of circulating *mature* endothelial cells. These cells were VEGFR2^{pos} and vWF^{pos}, and could be cultured to confluence in a significant number of subjects with sepsis, but not from the circulation of healthy controls. The correlation between vWF^{pos} circulating cells and the severity of sepsis was significant, suggesting a prognostic value of identifying and enumerating these types of cells. Additionally, in subjects who ultimately succumbed to death with septic shock (the most severe type of sepsis), the numbers of VEGFR2^{pos} cells in circulation were significantly higher. These results suggest that the quantity of circulating *mature* endothelial cells in patients with sepsis relates to the severity of vascular injury. It follows that higher numbers of *immature* endothelial cells, such as EPCs, could portend better outcomes. In healthy human subjects, low-dose endotoxemia has been reported to affect mobilization of endothelial progenitors. In 36 healthy subjects, administration of 2 ng/kg LPS intravenously resulted in a small but significant decrease in VEGFR2^{pos}/CD34^{pos}/CD133^{pos} cells (consistent with circulating EPCs) in the circulation within 4 h after administration [68]. The numbers of circulating EPC CFUs formed by culture of these subjects' peripheral blood mononuclear cells were decreased significantly at 4 h as well. Concentrations of tumor necrosis factor α were significantly elevated prior to the observation of lower EPC numbers, and may have contributed to this observation. In patients with sepsis, Rafat et al. [69] obtained blood within 48 h of diagnosis, as well as blood from nonseptic controls (both in and out of the intensive care unit). They enumerated the number of VEGFR2^{pos}/CD34^{pos}/CD133^{pos} cells present in 1×10^6 peripheral blood mononuclear cells. In parallel to what Mutunga et al. observed in the endothelial cell study, septic patients had a significantly higher percentage of circulating EPCs in their circulation than did nonseptic intensive care unit controls or healthy controls. The prognostic importance of this observation was reflected in the fact that nonsurvivors of sepsis had significantly fewer circulating EPCs present than did survivors. Importantly, this information was readily obtainable via flow cytometry. As this technology is commonly available in hospitals, the possibility of analyzing circulating EPCs in patients with sepsis to aid in prognosis appears to be a realistic goal in the near future. Of note, work by Becchi et al. [70] corroborated these observations. In their investigations, the numbers of circulating EPCs were highest in patients with the most severe sepsis, compared with patients with sepsis of a lesser severity and healthy controls. However, it must be noted that

their definition of circulating EPCs required that the isolated cells only exhibited CD34 to be considered EPCs, and therefore their findings are potentially less specific than those of other investigators. Both of these cell types are obtainable from either bronchoalveolar lavage or blood sampling, and as such would be useful as a potential biomarker.

7 Lung Cancer

After eradication of primary tumors in individuals with malignancies such as lung cancer, patients will frequently develop secondary tumors and subsequent relapse of disease. This may be due in part to small subpopulations of cells within tumors that have somatic stem cell characteristics; namely, the capability for self-renewal, asymmetric division, and multilineage differentiation. Such cells are sometimes referred to as tumor-initiating cells (TICs) [71]. The identity of TICs and their relationship to stem cells is not well established. TICs have a varying degree of phenotypes specific for different organ tissues. One consistent feature of TICs, however, is a high level of CD133 expression. As detailed above, this same cell-surface marker (also known as prominin-1) is found on the cell surface of purported EPCs. TICs positive for CD133 are notably resistant to chemotherapeutic agents and radiation therapy. It is not clear whether this property is a function of CD133 itself or if CD133 is a marker for a particularly resistant cell type. Recently, Eramo et al. [72] evaluated whether $CD133^{pos}$ cancer cells could be found within lung tumors of both small-cell and non-small-cell varieties. Their work revealed that a small but consistent percentage of $CD133^{pos}$ cells were in fact present within *all* lung cancer cell types and this percentage was significantly higher than that observed in normal lung tissue. A low number of freshly isolated lung cancer $CD133^{pos}$ cells were able to reproduce the human tumor of origin in immunocompromised mice, in contrast with the abilities of lung cancer $CD133^{neg}$ cells. The $CD133^{pos}$ cells also displayed other features consistent with stem cells, including an undifferentiated cell phenotype, extensive proliferation, and self-renewal potential. Oct-4 expression has subsequently been determined to be transcriptionally and translationally upregulated in $CD133^{pos}$ lung cancer cells, which may in part explain their observed self-renewal and pluripotent capacity [73]. This regulator can function as a “master switch” during differentiation, and has been found to be endogenously expressed in mouse pulmonary stem cells. It follows that assessing the number of $CD133^{pos}$ cells and their functional abilities might bear on prognosis in patients with lung cancer. This is reflected in work by Dome et al. [74]. These investigators enumerated EPCs in *peripheral blood* of 53 patients with non-small-cell lung cancer by flow cytometry, defining EPCs as those cells that were $CD34^{pos}$/$VEGFR2^{pos}$ or $CD133^{pos}$/$VEGFR2^{pos}$. It was observed that cells with these phenotypes were numerically similar; therefore, flow-cytometric evaluation of cells that were $CD34^{pos}$/$VEGFR2^{pos}$ was used exclusively in the final analyses. With use of these definitions, it was observed that non-small-cell lung cancer patients had significantly higher numbers of EPCs (which were

CD34^{pos}/VEGFR2^{pos}) per milliliter of peripheral blood compared with controls. Moreover, a higher number of EPCs predicted who would not respond to therapy. In post hoc analysis, stratifying the 53 subjects into two groups, those with high numbers of EPCs (more than 1,000 EPCs per milliliter) and low numbers of EPCs (1,000 EPCs or fewer per milliliter) and including variables such as tumor stage, smoking, and histologic type of tumor, in multivariable analysis, higher EPC number independently predicted shorter survival ($p < 0.001$). In 22 of these 53 subjects with non-small-cell lung cancer, tissue was available for immunohistochemical analysis. Nine of these 22 subjects had identifiable CD133^{pos}/VEGFR2^{pos} cells present, mainly in small intratumoral capillaries. Although this study was descriptive, it is possible that these investigators identified TICs within their population of lung cancer patients that correlated with response to therapy, and ultimately to outcome. Importantly, Pircher et al. [75] in a separate cohort of ten subjects with non-small-cell lung cancer confirmed that patients had higher numbers of circulating CD133^{pos} cells than did healthy controls. They also reported that the majority of these lung cancer patients, both preoperatively and postoperatively, had increased numbers of CD133^{pos} cells as determined via immunofluorescent staining. As this particular study was small, no comment was made on correlation of these observations with patient outcomes.

In addition to purported EPCs, other progenitor cells have been identified in the circulation of patients with lung cancer. In work by Ishii et al. [76], pulmonary vein blood obtained after pneumonectomy from 16 of 47 subjects with non-small-cell lung cancer could form ex vivo outgrowth fibroblastic colonies with characteristics of MSCs that were CD44^{pos}/collagen type I^{pos}/sm-actin^{pos}/vimentin^{pos}. These progenitors were recruited into cancer stroma created in immunosuppressed mice using a transplantable human lung cancer cell line, ultimately differentiating into cancer-stromal fibroblasts. Importantly, there were no obvious clinical correlates to the presence of fibroblast progenitors in the circulation. To confirm their observations, separate experiments by this same group [77] further established that pulmonary arterial blood from human subjects after pneumonectomy contained mesenchymal progenitors with the ability to differentiate into osteocytes and adipocytes. When mononuclear cells from these subjects' pulmonary arteries were purified on the basis of cell-surface markers, CD105^{pos} mononuclear cells consistently demonstrated an ability to differentiate into mesenchymal progenitors. The relevance of this observation to outcomes in subjects with lung cancer is uncertain at this time, but it is possible that such cells could support the growth of tumor stromal formation, and the numbers might portend outcomes in lung cancer as well.

8 Additional Considerations

The majority of studies to date regarding stem and progenitor cells that relate to prognosis have utilized animal models whose representation of human disease may not be entirely representative. Further, establishing an analogous progenitor cell

type between animals and humans is not always possible given differences between species. Nevertheless, continued development of novel animal models for various lung diseases is imperative to fully understand the role of progenitors in prognosis and repair. This is particularly important for rare stem cell types such as SP cells, where it is not feasible to obtain adequate amounts of human samples to study given the large number of cells required.

The heterogeneous characteristics of human subjects and variability in disease phenotype can also impede our understanding of the utility for progenitors in lung disease prognosis. Most human studies examining progenitor cells in prognosis have included small numbers of subjects, and are therefore underpowered to draw definitive conclusions, particularly given that the severity of lung injury can vary widely between patients with the same diagnosis. For example, the majority of lung diseases described herein have hypoxemia as a major clinical feature, a factor known to affect stem cell mobilization [35, 42]. Depending on the acuity of illness, or chronicity of a given disease process, the degree of hypoxemia can vary widely between subjects with the same disorder. Depending on when "sampling" of progenitor cells is conducted in the course of a given pulmonary disorder, these fluctuations in oxygenation might affect the numbers or functions of these cells, confounding the ability to use these cells prognostically. Another important consideration in clinical studies of these cells in prognosis is that patients with the same diagnosis can be heterogeneous in terms of other comorbidities. Common comorbidities including age, gender, tobacco use, and diabetes have all been demonstrated to affect the number and function of progenitor cells [78–81]. Adequately powered and well-designed clinical studies are necessary to more clearly define the utility of progenitor cells in prognostication. Further, validating positive correlations found between stem and progenitor cells and lung disease is imperative prior to the widespread use of these techniques for actual prognosis in the clinical setting. The continued investigation of these types of cells should help clinicians and investigators identify potential mechanisms underlying lung diseases, and may ultimately direct future therapeutic efforts that can benefit the population as a whole.

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Chapter 4
The Role of Fibrocytes in Lung Repair and Fibrosis

Ellen C. Keeley, Borna Mehrad, and Robert M. Strieter

1 Introduction

Chronic inflammation is defined as an immune response lasting several months in which inflammation, tissue remodeling, and attempts at repair are occurring simultaneously [1]. In contrast to an acute inflammatory episode characterized by edema, rapidly resolving vascular changes and neutrophil influx, chronic inflammation is characterized by fibrosis and may follow single or repeated bouts of acute inflammation [1]. Chronic inflammation may also begin as an insidious, low-grade, smoldering response without an identifiable preceding episode of acute inflammation. Pathologically, chronic inflammation can result from various stimuli, including infections, autoimmune reactions, toxins, radiation, and mechanical injury. The repair process involves two distinct phases: a regenerative phase (injured cells are replaced by cells of the same type) and a fibrotic phase (connective tissue replaces normal tissue). The fibrotic phase, when maladaptive, results in extensive matrix deposition and replacement of normal tissue with permanent scar tissue [1]. Chronic inflammation is integral to the pathogenesis of diseases such as atherosclerosis, rheumatoid arthritis, tuberculosis, and fibrotic lung diseases (the topic of this chapter). Although the mechanisms underlying the fibrotic response seen in chronic lung disease are similar to those that occur in the orderly healing of cutaneous wounds, the persistence of the initial stimuli and/or immune and autoimmune reactions promote growth factor and cytokine production, inhibit metalloproteinases, and ultimately result in collagen synthesis and deposition [1].

Tissue fibroblasts and myofibroblasts are classically thought to be derived from *resident* tissue fibroblasts that migrate to the injured area, proliferate, and deposit constituents of the extracellular matrix in response to tissue damage [2, 3]. More recently, however, two additional hypotheses have been proposed. The first hypothesis contends that tissue injury induces epithelial cells to transition to a mesenchymal

R.M. Strieter (✉)
Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Virginia, Charlottesville, VA, USA
e-mail: strieter@virginia.edu

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phenotype that subsequently contributes to fibroproliferation (called "epithelial–mesenchymal transition") [4–6]. The second hypothesis contends that fibrocytes (circulating bone-marrow-derived progenitor cells) home to and extravasate into sites of tissue damage, differentiate into fibroblasts/myofibroblasts, and contribute to the generation of extracellular matrix during fibroproliferation [7–9].

In humans, the term "fibrotic lung disease" represents a large group of disorders characterized by varying degrees of inflammation and fibrosis of the lung parenchyma [10]. Most are insidious in onset and the clinical course is usually one of irreversible, progressive replacement of lung tissue with scar tissue, and concomitant clinical deterioration. In this chapter we will discuss idiopathic pulmonary fibrosis (the most common of the fibrotic lung disorders) and aberrant airway remodeling in asthma, and will summarize the evidence supporting the hypothesis that circulating bone-marrow-derived progenitor cells, fibrocytes, play a pivotal role in lung repair and fibrosis in these disorders.

2 The Fibrocyte

Fibrocytes were first identified in 1994 in the context of an experimental model of wound repair [8]: within 1 day following injury, 10% of the cells in the wound chamber were spindle-shaped and expressed collagen, procollagen, and CD34. Given that the recruitment of fibroblasts from the surrounding skin is known to require multiple, time-consuming steps (traversing the permeable plastic layer, entering the wound chamber itself, and begin producing collagen), it was hypothesized that these cells were derived from the bloodstream because their initial appearance in the wound chamber occurred much faster than would be expected [11]. Since these spindle-shaped cells bore markers of connective tissue cells but not of monocytes, macrophages, endothelial cells, or epithelial cells, the word "*fibrocyte*" (a term combining "fibroblast" with "leukocyte") was coined [11]. Fibrocytes constitute 0.1–1% of the nucleated cells in the peripheral blood in healthy hosts [9, 12–14] and have been found in a variety of tissues under both physiologic and pathologic states [9, 15].

2.1 Characteristics Suggesting a Bone Marrow Origin

Fibrocytes exhibit prominent cell-surface projections on scanning electron microscopy, making them morphologically distinct from leukocytes [8, 11], and are defined by their unique growth characteristics and surface phenotype. There is substantial information available supporting the hypothesis that fibrocytes are derived from the bone marrow. Fibrocytes express the hematopoietic stem cell antigen CD34, the common leukocyte marker CD45, major histocompatibility complex II, the myeloid markers CD11b and CD13, and several fibroblast markers, including vimentin, stromal cells (collagen I, collagen III), and fibronectin [7, 8, 12,

Table 4.1 Gene expression of human fibrocytes assessed by microarray analysis

Name of gene	Relative gene expression
Glyceraldehyde 3-phosphate dehydrogenase	204,078
Chemokine receptors	
CCR2	91
CCR5	353
CCR7	385
CXCR4	7,086
Collagen	
Type I, alpha 1	223,848
Type I, alpha 2	30,141
Type III, alpha 1	122,958
Type IV, alpha 2	120,161

13, 16–21]. Microarray analysis has shown that fibrocytes express high levels of collagens I, III, and IV (Table 4.1): the constitutive expression of collagen genes is associated with fibrocyte production of soluble collagen in vitro as compared with lung fibroblasts and peripheral blood monocytes, and is augmented in the presence of transforming growth factor (TGF)-β (Table 4.2). The coexpression of collagen production and the other hematologic markers (such as CD45) is frequently used to identify fibrocytes: early in culture, fibrocytes are associated with expression of CD34, CD45, collagen I, and vimentin. Fibrocytes also express a number of other markers, including chemokine receptors and adhesion molecules [9, 13]. They do not, however, express T-cell markers (CD3, CD4, and CD8), B-cell markers (CD19), the interleukin (IL)-2 receptor chain CD25, the low-affinity Fc gamma receptor III (CD16), and myeloid markers (CD14 and nonspecific esterase) [7–9, 12, 13, 22].

Table 4.2 In vitro collagen production by fibrocytes

Time point	Fibrocytes	Fibroblasts	Monocytes
Unstimulated			
Week 1	80.9	72.9	ND
Week 2	86.7	86.7	ND
Week 3	95.8	102.6	ND
Stimulated[a]			
Week 1	144.9	138.7	ND
Week 2	182.8	196.8	ND
Week 3	206.5	225.1	ND

Soluble collagen (Sircol; ug/ml in conditioned medium)
ND none detected
[a]Stimulated with 10 ng/ml of Transforming Growth Factor-β

Some studies suggest that fibrocytes can differentiate from CD14+ peripheral blood monocytes that express the receptors for the Fc portion of IgG, CD64, and CD32 [17, 18, 20, 23]. Circulating fibrocytes may be present in a subset of CD14+

CD16– monocytes that carry the chemokine receptor CCR2 on their surface [24, 25]. At the time of tissue injury, this monocyte subset is released from the bone marrow into the peripheral blood and migrates to inflamed sites via a CCR2-mediated pathway [24, 25]. Human fibrocytes may, however, represent an intermediate stage of differentiation of this monocyte subset into mature fibroblasts and myofibroblasts in tissue [26]. This hypothesis is supported by the fact that fibrocytes express major histocompatibility complex class I and class II, CD80, and CD86 [8, 17, 18, 27, 28], exhibit antigen-presenting activity [28], and activate CD4+ and CD8+ lymphocytes [27, 28], but do not express markers of monocyte-derived dendritic cells such as CD1a, CD10, and CD83. Although fibrocytes are most likely of myeloid lineage, more information is needed to determine whether they are derived from a CD14+ progenitor cell. Regarding the expression of CD14, our laboratory has found that the vast majority (two thirds) of circulating fibrocytes are CD14– (data not shown).

2.2 Diverse Functions That Promote Tissue Repair and Fibrosis

Circulating fibrocytes perform many diverse functions that are crucial for both tissue repair and fibrosis. Fibrocytes participate in tissue remodeling by producing extracellular matrix proteins (collagen I, collagen III, and vimentin), and by secreting matrix metalloproteinases [29]. Fibrocytes also contribute to autocrine and paracrine signals by serving as an important source of inflammatory cytokines, chemokines, and growth factors that provide intercellular signals locally (some chemokine signals have been identified), and those that recruit them into distant sites of tissue injury and propagate the fibrotic response [9, 13, 29]: in a wound chamber model fibrocytes were found to express messenger RNA for IL-1β, IL-10, tumor necrosis factor α, CCL2, CCL3, CCL4, CXCL2, platelet-derived growth factor A, TGF-β_1, and macrophage colony-stimulating factor.

Fibrocytes can differentiate into lung fibroblasts and myofibroblasts [7–9, 12, 13, 18, 22]: Fibrocytes can also differentiate into adipocytes both in vitro and in vivo, via a process that can be inhibited by TGF-β via downregulation of peroxisome-proliferator-activated receptor γ [30, 31]. The differentiation of fibrocytes into myofibroblasts is augmented in the presence of TGF-β or endothelin-1, and results in cells that produce fibronectin and collagen, and express the myofibroblast marker α-smooth muscle actin [7, 9, 12, 13, 22]. Moreover, in a wound repair model, bone marrow transplantation from green fluorescent protein (GFP)-transgenic animals to wild-type animals showed that the cells in the wound coexpressed GFP and α-smooth muscle actin, indicating that the myofibroblasts present in the wound were derived from the bone marrow [32]. We recently obtained similar results in a murine model of pulmonary fibrosis [33].

Fibrocytes spontaneously gain expression of α-smooth muscle actin in culture, and gradually lose the expression of CD34 and CD45 over time depending on the inflammatory milieu [11]: this response can be augmented by

exposure of the fibrocytes to TGF-β or endothelin, resulting in differentiation into myofibroblast-type cells [12, 13, 22, 34]. Although the definition of a fibrocyte is based on the presence of the classic markers CD45, CD34, collagen I, and vimentin, it likely underestimates the number of fibrocytes in tissue.

Recently, it has been shown that the profibrotic cytokines IL-4 and IL-13 promote fibrocyte differentiation from CD14+ peripheral blood monocytes without inducing proliferation, whereas the antifibrotic cytokines IL-12 and interferon (IFN)-γ inhibit fibrocyte differentiation [35]: IL-4, IL-13, and IFN-γ were found to regulate fibrocyte differentiation through a direct effect on monocytes, whereas IL-12 was found to have an indirect effect, possibly through CD16+ natural killer cells. Although a number of growth factors have been shown to play an important role in the pathogenesis of fibrotic lung disease [36–39], little is known about their effect on fibrocytes. Fibrocyte differentiation appears to be influenced by a complex profile of cytokines, chemokines, and plasma proteins within the area of tissue injury. Although the concept that fibrocytes can differentiate from monocytes is interesting, we have recently measured fibrocytes in the bone marrow that are collagen I+ [33].

Lastly, fibrocytes are capable of promoting antigen-specific immunity by functioning as antigen-presenting cells, and inducing the clonal proliferation of T cells [28]. Lastly, fibrocytes promote angiogenesis in vivo through the generation of proangiogenic signals [9, 13, 40].

2.3 Homing of Fibrocytes and the CXCR4–CXCL12 Axis

According to the disease process and organ involved, fibrocytes can use different chemokine ligand–receptor pairs for tissue homing. Human fibrocytes express several chemokine receptors, including CCR3, CCR5, CCR7, and CXCR4; in contrast, mouse fibrocytes express CXCR4 and CCR7, but also CCR2 [7, 12, 13, 33, 41]. Fibrocyte migration into wound sites can be quantified by labeling the cells ex vivo with fluorescent probes: when chemokines that bind to CCR7 or CXCR4 (such as CCL21, chemokine ligand to CCR7, and CXCL12, chemokine ligand to CXCR4) were injected intradermally, fluorescently labeled fibrocytes migrated to the site of injection [7].

The CXCR4–CXCL12 axis plays an important role in the homing of bone-marrow-derived progenitor cells [42]: CXCR4 is an important chemokine receptor in stem cell trafficking, and the differential expression of CXCL12 in tissues creates the gradient required for trafficking of CXCR4+ cells. Although an early study reported little chemotaxis of fibrocytes to CXCL12 [7], our group detected substantial in vitro and in vivo chemotaxis of these cells to CXCL12 [12], and consider the earlier results to be due to methodological differences between the experiments, especially since hypoxia plays an important role for CXCR4 expression and chemotaxis [33]. In a murine system, fibrocytes were found to express CXCR4 and migrate in response to CXCL12 in vitro, and in the setting of bleomycin-induced pulmonary fibrosis in vivo [12, 19].

Several murine models of pulmonary fibrosis have been studied, including radiation-induced pulmonary fibrosis [43], intrapulmonary fluorescein isothiocyanate (FITC) administration [41], and intrapulmonary bleomycin administration [12, 33, 44, 45]. Although no animal model completely replicates human idiopathic pulmonary fibrosis, the intrapulmonary administration of bleomycin to mice closely simulates this disease process, resulting in epithelial cell necrosis, acute alveolitis, and intense interstitial inflammation associated with fibroblast proliferation and extracellular matrix synthesis [46, 47]. Despite its shortcomings (namely, the fact that it is the result of a single insult, is acute, and self-limiting compared with human disease), it represents the best and most widely available model. In the context of this model, fibrocytes have been shown to home to the lungs and contribute to fibrosis [12]. Human fibrocytes that were administered intravenously to severe combined immunodeficient mice, previously treated with either bleomycin or saline, preferentially homed to the lungs in animals treated with bleomycin. Similarly, in immunocompetent bleomycin-treated mice, the magnitude of lung procollagen I and procollagen III upregulation correlated with the number of CD45+ collagen I+ CXCR4+ fibrocytes in the bone marrow, blood, and lung [12]. Moreover, CXCL12 was significantly increased in the lungs of mice that were treated with bleomycin, supporting the notion that a CXCL12 gradient between the lungs and the plasma promoted the recruitment of the CD45+ collagen I+ CXCR4+ fibrocytes to the injured lung. Importantly, the administration of neutralizing anti-CXCL12 antibodies to bleomycin-treated mice resulted in significantly reduced fibrocyte extravasation into the lung, reduced collagen deposition in the lungs, and reduced immunohistochemical expression of α-smooth muscle actin, but did not affect the numbers of other leukocyte populations in the lungs [12].

To further investigate the trafficking of bone-marrow-derived fibrocytes to the lung during the pathogenesis of pulmonary fibrosis, we transplanted bone marrow from GFP+ C57BL/6 transgenic mice that express GFP under the direction of the ubiquitin C promotor into lethally irradiated wild-type mice, and subsequently exposed them to intratracheal bleomycin. The mice were euthanized on day 8 and single-cell suspensions of their lungs were performed to assess for the presence of fibrocytes (Table 4.3) and α-smooth muscle actin+ fibrocytes (Table 4.4) in their lungs by quantitative fluorescence activated cell sorting analysis. We found that although bone marrow transplantation was effective in repopulating the bone marrow with CD45+ GFP+ cells (Table 4.3) and cells that can differentiate into GFP+ α-smooth muscle actin+ cells (Table 4.4), residual recipient CD45+ GFP– cells can be found in the bone marrow (Table 4.3) and can differentiate into GFP– α-smooth muscle actin+ cells (Table 4.4) in these mice even after lethal total body irradiation. Several groups have since corroborated these findings in the context of mouse models of lung fibrosis: bone-marrow-derived mesenchymal stem cells home to the lung in response to injury, develop an epithelium-like phenotype, and reduce inflammation and collagen deposition [44, 45].

In the FITC-induced model of pulmonary fibrosis, fibrocytes isolated from the bronchoalveolar lavage fluid and whole lung samples were found to express CCR2, CCR5, CCR7, as well as CXCR4 [41]: the fibrocytes isolated from the lung

Table 4.3 Bone-marrow-derived green fluorescent protein (*GFP*)+ fibrocytes contribute to bleomycin-induced pulmonary fibrosis

Fibrocytes	Condition	
Bone marrow		
	Saline [cells/BM ($\times 10^5$)]	Bleomycin [cells/BM ($\times 10^5$)]
CD45+ Col1+	0.95 ± 0.08	3.7 ± 0.12
CD45+ Col1+ GFP+	0.92 ± 0.09	3.2 ± 0.2[a]
CD45+ Col1+ GFP–	0.03 ± 0.001	0.5 ± 0.015
Lung		
	Saline [cells/lung ($\times 10^6$)]	Bleomycin [cells/lung ($\times 10^6$)]
CD45+ Col1+	1.5 ± 0.09	3.3 ± 0.12
CD45+ Col1+ GFP+	0.97 ± 0.2	2.26 ± 0.15[a]
CD45+ Col1+ GFP–	0.48 ± 0.1	1.1 ± 0.48

BM bone marrow, *Col* collagen
[a]A larger population of CD45+ Col1+ GFP+ cells and a smaller population of CD45+ Col1+ GFP– cells is found both in the bone marrow and lungs of chimeric mice on day 8 following exposure to bleomycin

Table 4.4 Bone-marrow-derived GFP+ fibrocytes differentiate into α-smooth muscle actin+ (α*SMA*+) cells

Fibrocytes (αSMA+)	Condition	
Bone marrow		
	Saline [cells/BM ($\times 10^5$)]	Bleomycin [cells/BM ($\times 10^5$)]
CD45+ Col1+ αSMA+	0.46 ± 0.05	1.8 ± 0.082
CD45+ Col1+ αSMA+ GFP+	0.42 ± 0.05	1.4 ± 0.08[a]
CD45+ Col1+ αSMA+ GFP–	0.04 ± 0.05	0.4 ± 0.04
Lung		
	Saline [cells/lung ($\times 10^5$)]	bleomycin [cells/lung ($\times 10^5$)]
CD45+ Col1+ αSMA+	1.0 ± 0.2	2.4 ± 0.1
CD45+ Col1+ αSMA+ GFP+	0.68 ± 0.1	1.6 ± 0.1[a]
CD45+ Col1+ αSMA+ GFP–	0.34 ± 0.12	0.77 ± 0.005

[a]The majority of cells in both the bone marrow and the lungs of chimeric mice on day 8 following exposure to bleomycin are CD45+ Col1+ αSMA+ GFP+, when compared with the number of CD45+ Col1+ αSMA+ GFP– cells

expressed CCR2, migrated toward CCL2 and CCL12 ligands, and lost expression of CCR2 when cultured in vitro to a differentiated fibroblast. In CCR2-deficient mice challenged with intrapulmonarly administered FITC, fibrocyte recruitment to the lungs was reduced. Moreover, wild-type mice that received CCR2–/– bone marrow had reduced recruitment of fibrocytes to the lung and a reduction in pulmonary fibrosis. Transplantation of bone marrow cells from the wild-type mice into irradiated CCR2–/– mice once again restored the ability to grow fibrocytes from whole lung homogenates and the susceptibility to FITC-induced lung fibrosis [41]. Although these results were not replicated in CCL2-deficient mice,

immunoneutralization of CCL12 resulted in reduced recruitment of fibrocytes to the lung and attenuated pulmonary fibrosis.

Additional data from the same investigators suggest that CCR2 ligands play a key role in the accumulation of fibrocytes triggered by intrapulmonary administration of FITC [48], and may be involved in the accumulation of fibrocytes in human diseases since the recruitment of human fibrocyte precursors (CD14+ CD16– monocytes) into areas of inflammation is dependent on CCR2 [25]. Using the FITC murine model of pulmonary fibrosis, these investigators studied the effect of gammaherpesvirus infection: mice exposed to murine gammaherpesvirus 68 infection had increased total lung collagen, histologic changes of acute lung injury, and diminished lung function compared with control mice [49]. The gammaherpesvirus increased fibrocyte recruitment to the lung in wild-type but not CCR2–/– mice, suggesting that upregulation of chemokines during viral infections and subsequent recruitment of fibrocytes are associated with worsening pulmonary fibrosis. Lastly, in a murine model of renal fibrosis, the chemokine receptors CCR2 and CCR7 played an important role in the recruitment of fibrocytes [50]. These results highlight the importance of chemokine-mediated fibrocyte influx in the pathogenesis of pulmonary fibrosis.

2.4 Role of Fibrocytes in Human Idiopathic Pulmonary Fibrosis

Several lines of evidence support the role of circulating fibrocytes in the development of human lung fibrosis [14, 15, 33, 51]. In one study, the numbers of CD45+, collagen I+, CXCR4+ circulating fibrocytes were markedly higher in patients with fibrotic interstitial lung disease than in healthy controls [14]: CXCL12 ligand expression was also found to be markedly elevated in the lung and plasma of patients with lung fibrosis. In another study, fibrocytes were identified in tissue from eight out of nine fibrotic lungs in patients with idiopathic pulmonary fibrosis. Although there was a positive correlation between the abundance of fibrotic foci and the number of lung fibrocytes ($r = 0.79$; $p < 0.02$), no fibrocytes were identified in normal lungs [15]. Moreover, the level of CXCL12 was increased in the plasma of the patients with idiopathic pulmonary fibrosis (and present in about half of their bronchoalveolar samples); and the chemokine level directly correlated with disease severity, with higher CXCL12 levels associated with worse gas exchange [15].

The CXCR4–CXCL12 axis also appears important in the homing of human fibrocytes in the setting of lung fibrosis: in a study of patients with fibrotic interstitial lung disease, the numbers of CD45+, collagen I+, CXCR4+ fibrocytes were an order of magnitude higher than in healthy controls, with fibrocytes comprising between 6 and 10% of the leukocyte population in patients with fibrotic interstitial lung disease compared with 0.5% in healthy controls [14]. In addition, CXCL12 ligand expression was markedly elevated in the lung and plasma of patients with lung fibrosis, and the predominant cell type in the lungs that expressed CXCL12 was the hyperplastic type II pneumocyte [14]. These findings have been confirmed in a more recent study of patients with idiopathic pulmonary fibrosis [15]: immunofluorescence and

confocal microscopy of fibrotic lung tissue from patients with idiopathic pulmonary fibrosis using immunoflorescent CXCR4 stained more fibrocytes than combinations using immunoflorescent CD34 or CD45RO. In addition, the CXCL12 level was significantly increased in the plasma of patients with idiopathic pulmonary fibrosis compared with healthy controls; and CXCL12 was detectable in the bronchoalveolar lavage fluid in 40% of patients with pulmonary fibrosis (but not in controls). Moreover, CXCL12 was strongly expressed by alveolar epithelial cells within the lung; and the predominant cell that expressed CXCL12 was the hyperplastic type II pneumocyte, similar to the results previously reported by our group [14]. Lastly, the fibrocyte has been found to be a clinically important predictor of disease severity in patients with idiopathic pulmonary fibrosis. In a recently published study, patients with idiopathic pulmonary fibrosis, both patients with a stable clinical course and those with an acute exacerbation, had significantly increased levels of circulating fibrocytes compared with normal controls [51]. Moreover, the increase in fibrocyte levels directly correlated with episodes of disease exacerbation: specifically, patients in whom fibrocytes constituted more than 5% of the circulating nucleated cell population had increased mortality compared with those in whom fibrocytes constituted less than 5% of the circulating nucleated cell population, suggesting that fibrocytes could serve as a biomarker both in the diagnosis of exacerbation and in the prognosis of idiopathic pulmonary fibrosis [33, 51].

The results of these human studies underscore the importance of chemokine-mediated fibrocyte influx in lung fibrosis, and indicate that circulating fibrocytes, likely recruited through the CXCR4–CXCL12 axis, may contribute to the expansion of the fibroblast/myofibroblast population in idiopathic pulmonary fibrosis.

2.5 Effect of Hypoxia on the Circulating Fibrocyte

CXCR4 expression is known to be regulated by hypoxia inducible factor (HIF)-1α, and to be enhanced in a hypoxic milieu in cancer cells [52, 53]. Since the bone marrow is known to be hypoxic compared with arterial blood [54, 55], we tested the expression of CXCR4 in human fibrocytes cultured in hypoxic compared with normoxic conditions [33]. When compared with human fibrocytes cultured in normoxic conditions, human fibrocytes cultured in hypoxic conditions showed a marked induction of CXCR4 messenger RNA, increased surface expression of CXCR4, and enhanced chemotaxis to CXCL12 that were associated with an increase in intranuclear and cytosolic levels of HIF-1α [33].

Since the CXCR4–CXCL12 axis appears to play a pivotal role in the recruitment of fibrocytes to the lung, the factors that regulate CXCR4 may be important in fibrocyte trafficking. It is already known that HIF-1α is a major transcriptional factor for induction of CXCR4 [52, 53, 56], and activation of the phosphatidylinositol 3-kinase (PI3-kinase)/phosphatase and tensin homolog (PTEN)/Akt/mammalian target of rapamycin (mTOR) pathway in the setting of hypoxia results in upregulation of the expression and function of CXCR4 on lung cancer cells in an HIF-1α-dependent manner [52]. We sought to determine whether in vivo pharmacological

inhibition of the PI3-kinase/mTOR pathway with rapamycin could be used to impair homing of fibrocytes in mice challenged with intratracheal bleomycin or saline. Rapamycin treatment resulted in a reduction in the number of circulating fibrocytes that expressed CXCR4, and an attenuated bleomycin-induced CXCR4+ fibrocyte infiltration into the lungs that was associated with a reduction in lung collagen deposition compared with vehicle controls (Fig. 4.1) [33]: treatment with rapamycin resulted in a 50% decrease in lung tissue levels of CXCL12. Interestingly, the inhibition of fibrocyte homing to the lungs was even more significant than our previous observation with depletion of CXCL12 [12]. These data support the hypothesis that the relative hypoxic environment of the bone marrow is an important factor for promoting the expression of CXCR4 on fibrocytes.

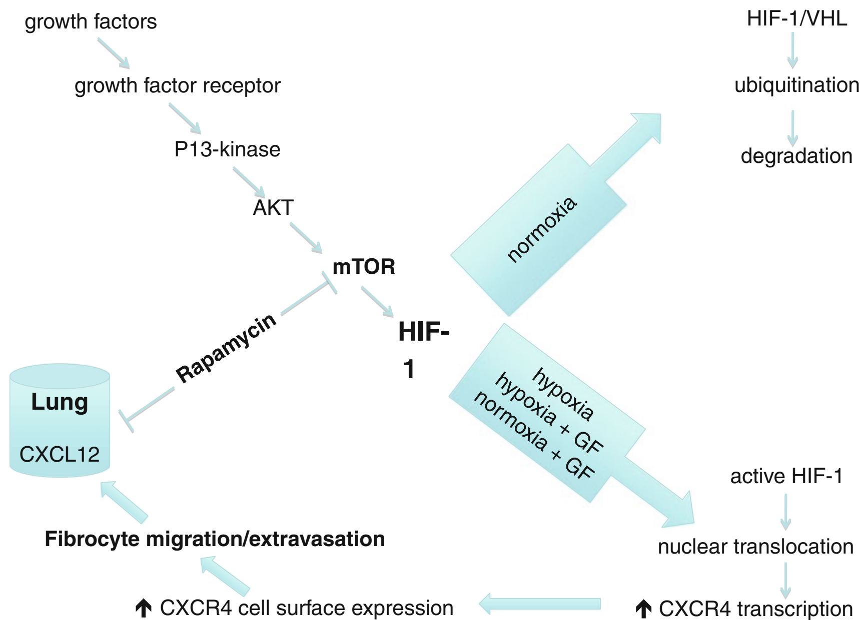

Fig. 4.1 The chemokine receptor–chemokine (CXCR4–CXCL12) biological axis plays a critical role in the homing of circulating fibrocytes to the lungs during the pathogenesis of pulmonary fibrosis. In the presence of hypoxia, activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway promotes the homing of CXCR4+ fibrocytes to the lung in response to CXCL12. *GF* growth factor, *VHL* von Hippel–Lindau tumor suppressor

2.6 Role of Fibrocytes in Human Asthma

Repetitive episodes of airway inflammation and aberrant airway remodeling are the basis for the pathologic findings in asthma. Airway remodeling refers to a diverse set of structural changes including epithelial cell metaplasia, subepithelial fibrosis, angiogenesis, and smooth muscle hyperplasia. In one study, airway biopsies from patients with asthma had a marked accumulation of CD34+ collagen I+ cells, and a

smaller number of CD34+ α-smooth muscle actin+ cells below the basement membranes within 24 h after allergen exposure [22]. Others have shown an increased number of fibrocytes expressing CD34, CD45, α-smooth muscle actin, and procollagen I in bronchial biopsies from steroid-naïve patients with mild asthma, and localization of these cells to areas close to the basement membrane when compared with controls: the fibrocyte levels correlated to the thickness of the basement membrane, suggesting that these cells may participate in airway wall remodeling [57]. The expression of CCR7 in the myofibroblasts in the bronchial mucosa in asthmatic patients in another study provides further evidence that these cells may be of fibrocyte origin [58]. Moreover, these investigators also observed increased production of CCL19 and its receptor CCR7, suggesting that the CCL19–CCR7 axis may be important in fibrocyte recruitment in asthma [58]. It has also been shown that in asthmatic patients with chronic airflow obstruction the numbers of circulating fibrocytes are increased, and the circulating fibrocytes can be differentiated into myofibroblasts by exposure to TGF-β [59]. More recently, markedly elevated levels of fibrocytes have been found in bronchial biopsy specimens (in the airway smooth muscle compartment) and in the peripheral blood samples from patients with all severities of asthma when compared with healthy controls [60]. Moreover, these investigators found that in vitro airway smooth muscle promoted fibrocyte chemotaxis and chemokinesis. However, the fibrocyte numbers in the peripheral blood and in the bronchial biopsy specimens were not correlated with lung function or airway smooth muscle mass. Collectively, these studies underscore the potential role of fibrocytes in the development of asthma.

3 Conclusion

Regeneration and fibrosis are integral parts of the recovery process from tissue injury, and dysfunction of these mechanisms is a hallmark of many chronic fibrotic diseases, including idiopathic pulmonary fibrosis and asthma. Data suggest that circulating bone-marrow-derived progenitor cells, *fibrocytes*, play a pivotal role in the repair of injured tissue, including fibrotic lung disease. Fibrocytes exhibit characteristics of monocytes, fibroblasts, and hematopoietic stem cells, and serve as an important source of fibroblasts and myofibroblasts during both physiologic and pathologic remodeling and repair processes. A better understanding of fibrocytes has the potential to allow for therapeutic manipulation of tissue repair and fibrosis, the hallmark of fibrotic lung disease.

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Chapter 5
Stem Cells and Cell–Matrix Interactions in Lung

Viranuj Sueblinvong and Jesse Roman

1 Introduction

The acellular material localized between cells in tissues is termed the "extracellular matrix" (ECM). This material contains glycoproteins, collagenous proteins, proteoglycans, and other components secreted and deposited into insoluble matrices by surrounding cells (e.g., epithelial and endothelial cells, fibroblasts). The ECM serves to mark distinct cellular components (as is the case for basement membranes) and provide a scaffold for cell organization and migration. Although the ECM was initially thought to only provide structural support to organs, data generated during the past two decades clearly demonstrate that it also contains important information that affects cellular behavior. Cells recognize the ECM through cell-surface receptors (e.g., integrins) that are linked to their intracellular signaling and transcriptional machinery. Thus, alterations in the amount and relative composition of the ECM influence many cell functions, including cell adhesion and migration, proliferation, and differentiation.

Because of the critical role the ECM plays in modulating cell functions, it is no surprise that attention is given to its role in stem cell biology, and that deciphering the effects of ECM components on diverse stem cells during health and disease has turned into an important goal. This becomes even more relevant when considering that, under certain conditions, stem cells leave their natural habitat and home to other tissues, requiring their adjustment to the new microenvironment. Injured organs, for example, are characterized by the release of proinflammatory factors and other mediators that typically affect ECM composition through changes in ECM gene expression and matrix turnover, and influence cellular recognition of the ECM through alterations in matrix receptor expression.

Here, we will summarize the limited data available regarding the roles of the ECM in stem cell biology. This area of investigation is relatively new and, therefore, much of the current knowledge available about stem cells and the ECM was derived

J. Roman (✉)
Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine,
Emory University School of Medicine, Atlanta, GA 30322, USA
e-mail: jroman@emory.edu

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from experiments performed in vitro or in artificial systems, whereas data in animals are limited, and data in humans are essentially unavailable. Consequently, the effects that individual ECM components have on distinct stem cells are unknown, and how stem cell–stroma interactions influence tissue repair after injury remains to be elucidated. Nevertheless, information obtained from relevant systems allows for the development of testable paradigms. We summarize this information as it relates to the lung on the basis of data generated in rodent models of lung injury in the hope that the discussion will stimulate research in stem cell biology as it relates to this organ.

2 Extracellular Matrix in Lung

2.1 Lung Extracellular Matrix in Health and Disease

The lung is composed of organized fiber ECM networks that support the airways and alveolar epithelium, endothelium, bronchial and vascular smooth muscle, nerves, and visceral pleura [1, 2]. This fiber network represents the lung "skeleton" that supports the organ. However, with its many ECM components distributed in distinct relative concentrations throughout the lung, it also provides spatial information to cells, while influencing their behavior. During lung development, alterations in ECM composition coincide temporally and spatially with important embryological processes needed for the formation of the mature lung. For example, studies performed in embryonic lungs examined ex vivo and in genetically engineered animals revealed important roles for ECMs in lung branching morphogenesis during which the pattern of the primitive airway structure is formed. ECMs are also considered important for vasculogenesis and angiogenesis, processes needed for lung vascularization. Finally, ECMs are considered important for the differentiation of terminal air sacs termed "alveoli" which are necessary for efficient gas exchange [3].

Although there is significant turnover, the relative composition of the lung ECM is very much established by the time of birth. However, lung injury triggers a reactivation of developmental and repair pathways that lead to dramatic alterations in ECM composition. For the most part, lung injury triggers the increased expression of certain ECM components (e.g., fibronectin), which are thought to provide temporary scaffolds for the re-epithelialization of denuded basement membranes, among other functions. Tissue injury is also characterized by increased expression and/or activation of proteases (e.g., matrix metalloproteinases) that serve to degrade connective tissue matrices. Together with other processes, increased matrix expression and turnover in injured organs is thought to accelerate wound healing. Unfortunately, more often than not, these mechanisms may become disengaged from normal counterregulatory mechanisms, leading to permanent alterations in the relative composition of the ECM, and often promoting excessive ECM deposition and fibrosis or excessive matrix degradation, resulting in the destruction of the original architecture and loss of lung function.

Considering the above observations, it is evident that a better understanding of the factors responsible for and the mechanisms that lead to alterations in ECM expression, organization, and degradation in lung is necessary to develop strategies that promote controlled wound healing ensuring maintenance of normal lung structure and function. Furthermore, a clearer definition of the factors influencing ECM recognition by resident (e.g., lung fibroblasts) and incoming (e.g., immune cells) cells in injured lungs is needed for the same purpose. Since stem cells present in lung or attracted to it during injury are also likely to be influenced by alterations in ECM composition, a better understanding of how these events affect stem cells is needed. Below, we succinctly describe information about certain ECM components that are considered important in regulation of cell functions during development, injury, and repair.

2.1.1 Collagens

Collagens account for over 20% of the dry weight of the lung. These are helical molecules that consist of three α-chains, which form a ropelike triple helix [4]. There are 11 different types of collagens in the lung [5]. In general, collagens are classified into three categories: fibrillar, nonfibrillar, and low molecular weight collagens [4]. Fibrillar collagens (collagen types I, II, and III) constitute over 65% of all lung collagens, and associate laterally to form thick cables that provide strength to the lung connective tissue. Nonfibrillar collagens, such as collagen type IV, are essential components of basement membranes [4]. Collagens and other matrices are degraded by matrix metalloproteinases and other enzymes in several pulmonary disorders (e.g., cavity formation in tuberculosis), whereas excessive collagen deposition is noted in fibrotic lung disorders.

2.1.2 Elastin

Elastin gives elasticity to tissues, allowing them to stretch when needed and then return to their original state, whereas collagen provides strength and rigidity. Elastin accounts for over 40% of the lung dry weight [2]. This polymeric molecule is predominant in the pleura, alveolar septa, and walls of blood vessels and airways [2]. Maintenance of the natural structure/function of elastin is essential for lung function [6]. In contrast, in the setting of emphysema, loss of elastin is associated with alveolar wall destruction, which appears to be due to uncontrolled degradation of the elastic fibers accompanied by nonfunctional replacement as a response to injury [7].

2.1.3 Proteoglycans

Proteoglycans are high molecular weight molecules composed of a protein core linked to polysaccharide chains of chondroitin, dermatan, keratin, and heparan sulfates. Proteoglycans form flexible bridges between the fibrillar macromolecules and fill spaces between collagen bundles to help stabilize the three-dimensional

matrix and provide resistance to tissue compression. Aside from supporting the lung structure, proteoglycans, in particular heparan sulfate proteoglycans, have been shown to regulate epithelial cell movements during tube formations and branching, likely through multiple signaling pathways, including bone morphogenetic proteins, fibroblast growth factors, transforming growth factor β, and hedgehog pathways that are active during lung morphogenesis [6, 8]. Further, proteoglycans also mediate lung mesenchymal cell differentiation, migration, and matrix production [6]. In addition, proteoglycans are highly expressed on cell surfaces, where they can mediate cell–matrix adhesion, epithelial cell migration, proliferation, and gene expression through binding with other matrix molecules (such as hyaluronic acid, fibronectin, and collagens) [9, 8].

2.1.4 Laminins

Laminins are a family of heterotrimeric glycoproteins that represent the majority of glycoproteins in basement membranes. Laminins are composed of three polypeptide chains: α, β_1, and γ, accounting for at least 15 laminin isoforms [1]. Laminins are large (400–900 kDa) cruciform-shaped molecules with one long arm composed of segments of each of the three chains in a coil, with two or three short arms each composed of a segment of the constituent chains [1]. Like other ECM components, laminins play important roles in morphogenesis, including lung development [10, 11]. This is highlighted by the observation that lack of laminin α_2 results in demyelination of motor nerves of both humans and mice despite retention of laminin α_4, which is normally expressed during nerve development, but is not present at maturity [12]. Laminin α_5 null embryos have defects in multiple organs, including kidneys, limbs, placenta, intestines, hair follicles, and lung [13, 14]. In lung, all five laminin α chains are present during early embryonic development, but normal adult lung tissue contains primarily laminin α_3, α_4, and α_5 chains [15]. Laminin is usually found associated with heparan sulfate proteoglycan, and this association appears to be needed for epithelial cell polarization and lumen formation of lung cells in culture [16].

2.1.5 Fibronectins

Fibronectins are high molecular weight, multidomain glycoproteins that are assembled into insoluble multimeric matrices by fibroblasts and other cell types [17]. Fibronectins are composed of homologous type I, II, and III motifs. The so-called *plasma fibronectins* are produced by hepatocytes, whereas *cellular fibronectins* contain an extra type III repeat and are produced by large vascular structures and cells within the lung, including fibroblasts and alveolar macrophages. There are over 21 splicing variants of fibronectin, which result from alternative splicing of a single fibronectin gene [18]. Alternative splicing is developmentally regulated, leading to increased expression of ED-A, ED-B, and IIICS isoforms in fetal tissues and tumor cells [19]. In adult cells, especially in cells induced to terminally differentiate, there is a decrease in fibronectin ED-A [20]. The polymerization of soluble

fibronectin into insoluble fibrils within the ECM is a dynamic, cell-dependent process that is mediated by a series of events involving the actin cytoskeleton and integrin receptors [21]. Fibronectins and other ECM components interact with each other covalently and noncovalently to form insoluble heterogenous matrices important for the maintenance of tissue integrity. Once deposited, fibronectin modulates cell contractility, collagen gel contraction, and cell migration. In injured lungs, its deposition may accelerate the re-epithelialization of denuded basement membranes [22, 23]. Alterations in cell–fibronectin interactions may promote abnormal tissue remodeling by stimulating the proliferation of fibroblasts [24], by promoting myofibroblast differentiation [25], and by facilitating the excessive deposition of other matrix components such as collagens [26]. How these events are modulated by fibronectin and its variants remains incompletely understood, but new information has uncovered a role for fibronectin ED-A in lung injury and repair. Transforming growth factor β_1 has been implicated in the regulation of ED-A and ED-B splicing since treating fibroblasts with this growth factor results in an increase in both ED-A and ED-B messenger RNAs [20]. This is important since transforming growth factor β_1 expression is invariably increased in lung injury where myofibroblast transdifferentiation is common [27]. Further, in clinical specimens of lung from patients with pulmonary fibrosis, there is a higher proportion of ED-A protein expression than in normal controls [28]. Furthermore, mice that lack ED-A expression fail to develop fibrosis following bleomycin-induced lung injury [28].

2.2 Extracellular Matrix Recognition Through Integrins

The previously described alterations in ECM composition in developing, healthy, and diseased lungs undoubtedly affect the function of resident (e.g., fibroblasts) and incoming (e.g., neutrophils) lung cells. Virtually every cell of muticellular organisms expresses functional matrix-binding receptors capable of signal transduction. An important group of matrix binding receptors is known as *integrins*. Integrins are a large family of transmembrane receptors which mediate cell–matrix and cell–cell adhesion. Integrins were initially identified as receptors for ECMs [29], but were later demonstrated to also bind to cell-surface counterreceptors, soluble plasma proteins, and microorganisms extracellularly and signaling proteins (e.g., protein kinase C and cytoskeleton) intracellularly [30, 31]. Further, some cellular processes (e.g., adhesion and migration of cells) are regulated through the combination of signals from both growth-factor-mediated receptors and integrin receptors. Through these interactions, it has been shown that integrins can modulate every aspect of cell behavior, including migration, growth, survival, and differentiation [30]. Because of their key role in mediating cell–matrix interactions, many investigations have focused on elucidating their role in specific disorders with the intention of unveiling potential new targets for therapeutic intervention. These studies have revealed a role for integrins in embryogenesis, thrombosis, tissue fibrogenesis, and cancer, among many other disorders. For example, studies in the 1960s showed that normal

cells require attachment to a substrate to proliferate as compared with tumor cells, which proliferate when cultured in suspension [31]. During embryogenesis, cells require activation of integrins at the leading edge and inactivation at the rear edge to migrate to appropriate sites for normal development [32]. During vascular injury, platelet integrins, $\alpha_{IIb}\beta_3$, are activated, resulting in platelet adhesion and aggregation at the site of injury to stop bleeding.

Integrins are heterodimeric receptors consisting of one α and one β subunit, both with single transmembrane and intracytoplasmic domains (Fig. 5.1). Currently, 18 α (α_1–α_{11}, α_{IIb}, α_E, α_L, α_M, α_X, and α_v) and eight β (β_1–β_8) subunits have been identified and form at least 24 different heterodimers in mammals [31]. The main function of integrins is to mediate communication with the environment through so-called *outside-in* signal transduction. In addition to sensing the environment, integrins often change their conformation in response to intracellular signals, thereby affecting ligand binding through so-called *inside-out* signal transduction.

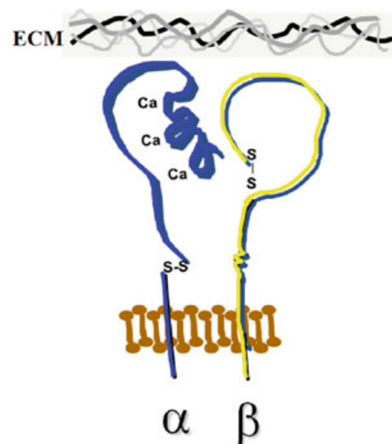

Fig. 5.1 Integrin receptors. Integrins are heterodimeric receptors composed of α and β subunits assembled noncovalently at the cell surface. Each subunit has a long extracellular domain that interacts with ligands (e.g., extracellular matrix; ECM) and intracellular domains that interact with intracellular molecules such as cytoskeletal proteins and signaling molecules

Outside-in signal transduction occurs when integrins bind to their ligands (i.e., fibronectin), leading to integrin clustering at the cell surface followed by the formation of focal contacts or focal adhesion contacts. At these sites, the cytoplasmic domain of integrins interacts with actin filaments and other cytoskeletal components (such as tensin, vinculin, paxillin,and α-actinin), resulting in rearrangements of the intracellular cytoskeleton and initiation of many intracellular signaling cascades which help establish cell polarity, and influence cell migration and cell survival functions [33]. One very important intracellular signaling protein that links the extracellular ligand to intracellular signaling is a member of the protein tyrosine kinase family, called focal adhesion kinase (FAK). FAK binds to β-subunit cytoplasmic domains and to other components of focal adhesions [34]. FAK also has binding sites for the SH2 domain of the *ras*-associated protein Grb2, the SH2 domains of src, phosphatidylinositol 3-kinase (PI3-K), phospholipase C-γ, and other proteins which are downstream targets of integrin signaling [35, 36]. Other proteins which

have been shown to be involved in integrin signaling include v-Src, v-Abl (associated with oncogenes), sodium–proton antiporters (regulate intracellular pH), and protein kinase C [31]. Thus, ligand binding triggers integrin clustering and activation, which, in turn, results in the recruitment to the cell membrane of many cytoskeletal and signaling molecules that become organized into a reversible "signaling organelle." Dissociation of the ligand from the integrin leads to dissolution of this signaling apparatus.

Inside-out signal transduction refers to the idea that integrins can also sense intracellular signals which affect their ability to bind extracellular ligands. Integrin receptors on leukocytes and platelets are regulated by this mechanism. In the resting state of these cells, integrins are present on the cell surface, but their extracellular domains are in such a conformation that inhibits ligand binding and activation. This prevents spontaneous adhesion of platelets and leukocytes within the circulation or to blood vessel walls. In the presence of agonists (i.e., fibrinogen, chemokines), integrins are activated, resulting in cell adhesion and migration. This process is regulated by their cytoplasmic tail binding activity [31]. One of the factors capable of interacting with integrins intracellularly, GTPas Rap1, has been shown to increase the activity of leukocyte function-associated antigen 1 (LFA-1 or $\alpha_L\beta_2$ integrin) on the membrane of lymphocytes in response to infection and inflammation [37]. Another example of inside-out signaling was described in experiments showing that activation of protein kinase C affects integrin receptor expression and/or activation in monocytic cells [38].

3 Stem Cell–Extracellular Matrix Interactions

Stem cells are defined as undifferentiated cells capable of unlimited self-renewal and multilineage differentiation [39, 40]. Stem cells are traditionally classified into two major categories: *embryonic stem cells* (ESCs) and *extraembryonic or adult stem cells*. This is best described in previous chapters. Briefly, ESCs are pluripotent cells derived from the inner cell mass of the blastocyst during early embryogenesis [41]. Extraembryonic or adult stem cells are derived from differentiated postnatal tissues; examples of these are adult bone-marrow-derived mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and umbilical-cord-blood-derived MSCs [42]. Two broad categories of adult stem cells exist: *tissue endogenous stem cells* and *exogenous stem cells* [43]. Tissue endogenous stem cells have been identified in multiple tissues, including crypt cells in intestine [44], astrocytes in brain [45], satellite cells in muscle [46], and cells at bronchoalveolar duct junctions in lung [47]. These are cells thought to be tissue-specific and give rise to mature cells of tissues in which they reside and help maintain normal homeostasis and repair following injury [47]. Exogenous stem cells are stem cells which can be recruited to specific tissues; these cells are mostly referred to as bone-marrow-derived stem or progenitor cells. Bone marrow contains multiple populations of progenitor cells [48]. In culture, these cells can be divided into two general populations; those that remain

floating in culture, referred to as classic HSCs, and the other cells that adhere to plastic [49]. Plastic-adherent cells represent a heterogeneous population that include cells of CD-31 positive endothelial stem cell lineage, mac-1/CD11b/CD45 positive myeloid stem cell lineage, and fibroblast-like cells [50]. The latter cell type has been classified as MSCs and can serve as precursors for bone, muscle, fat, and cartilage tissue [51, 50]. Both HSCs and MSCs have been shown to differentiate into mature cells of nonhematopoietic and nonmesodermal cell lineages under certain experimental conditions [52, 53].

Like non-stem cells, stem cells express receptors for soluble and insoluble mediators that potentially affect their function. In particular, stem cells express a repertoire of integrins and other matrix-binding receptors which are influenced by the local microenvironment. This is likely important since stem cells initiate their existence in an organ with specific characteristics, but are programmed to exit their immediate microenvironment in response to certain signals, and home at another site (sometimes another organ) where they might assist in the repair of tissues through phenotypic differentiation and the release of soluble mediators. Consequently, one must assume that stem cell functions (e.g., mobilization, homing, survival, and differentiation) are tightly regulated by their surrounding microenvironment (i.e., ECM) [54, 55], which, in turn, is recognized through cell-surface receptors such as integrins (Fig. 5.2). For example, the adherence of HSCs to ECM components through integrin receptors may not only serve to anchor progenitor cells to the microenvironment, but also to directly alter their proliferative potential. HSCs express a variety of integrin receptors, including members of the β_1 integrin family [very late antigen (VLA)-1–VLA-6] and of the β_2 family or leukocyte integrins [i.e., such as LFA-1, $\beta_2\alpha_L$, or CD18/CD11a, and Mac-1 ($\beta_2\alpha$M or CD18/CD11b)] [56]. Integrin $\alpha_4\beta_1$ (also known as VLA-4 or CD49d) was found to modulate HSC migration into the circulation and to have a direct role in controlling the late stage of erythroid differentiation [56]. Further, $\alpha_4\beta_1$ played an important role in homing of

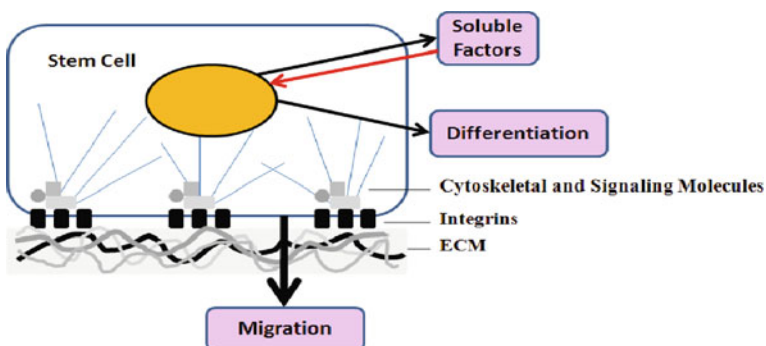

Fig. 5.2 The ECM signals through cell-surface integrin receptors and regulates cell functions. Stem cell functions, including mobilization and homing, adhesion to scaffolds, the release of survival signals, and differentiation, are tightly regulated by the surrounding microenvironment, which includes growth factors, chemokines/cytokines, and the ECM

transplanted HSCs to the bone marrow [56]. These studies suggest that abnormalities in their function might lead to continuous proliferation of the progenitor cell population and the premature release of progenitor cells into the circulation, which is one of the characteristics of malignant processes, including those involved in the development of chronic myeloid leukemia [57].

Several in vitro studies have shown that both murine and human ESCs can be driven to express airway epithelial cell markers [58, 59, 60, 61]. These studies have provided further insight into the importance of the microenvironment in stem cell manipulation. For example, ESCs cultured as a hanging droplet show higher expression of Clara cell secretory protein as compared with cells in the traditional two-dimensional culture system [62]. When placed at an air–liquid interface, they form cell layers similar to tracheal epithelium with ciliated and nonciliated cells and a basal cell layer [62]. Similarly, when cultured in differentiated basal medium (without leukemia inhibitory factor) within Matrigel®, a protein substrate produced by cultured tumor cells rich in basement membrane proteins such as laminin and nidogen, these cells show a tendency to differentiate into alveolar epithelial cells with the expression of surfactant proteins and the formation of lamellar bodies [61].

3.1 Stem Cells Recognize and Influence the Extracellular Matrix

Not only can stem cells recognize the ECM, but they can also influence ECM composition. This was shown in studies demonstrating that the maintenance of ESCs in an undifferentiated state requires culturing these cells in tight adherent colonies in contact with feeder layers; i.e., mouse embryonic fibroblasts or in pooled ECM products [63]. Furthermore, ESC lines H1 and H9 express specific subtypes of laminin (laminin 511), collagen type IV, and nidogen-1 [64]; nidogen-1 is a linker protein which is important in basement membrane organization through its ability to bind with laminin 511 ($\alpha_5\beta_1\gamma_1$) and laminin 111 ($\alpha_1\beta_1\gamma_1$) [65]. Of interest, both undifferentiated H1 and H9 human ESCs also express laminin receptors, $\alpha_6\beta_1$ integrin, suggesting their capability to recognize basement membrane ECM proteins [64]. Together, these observations suggest that the expression of nidogen-1, laminin 511, and $\alpha_6\beta_1$ integrin may be essential for ESC survival, colony formation, and maintenance of undifferentiated states, but this needs further exploration.

The adult bone marrow consists of a number of cell types, including HSCs and MSCs. Several factors have been shown to control stem cell behavior or stem cell fate, including cell–cell contact (MSC–HSC), cytokine expression, and cell–ECM interactions [54, 55]. Regarding the latter, the adherence of HSCs to matrix components may not only serve to anchor progenitor cells to the microenvironment, but also to directly alter their proliferative potential. HSCs express a variety of integrin receptors as noted earlier. As mentioned above, integrin $\alpha_4\beta_1$ was found to modulate HSC migration into the circulation, to have a direct role in controlling the late stage of erythroid differentiation, and to play important roles in homing of transplanted HSCs to the bone marrow [56].

Several integrin receptors have also been identified in MSCs, including, but not limited to, α_4, α_5, and β_1, and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [66]. However, contradictory results exist and it is still unclear if all β1 integrin subunits are expressed by human MSCs [67]. What is clear is that, aside from adhesion of cells to ECMs, integrins also serve as signal transducers capable of modulating MSC differentiation. For example, $\alpha_5\beta_1$ regulates human MSC osteogenic and chondrogenic differentiation [66, 67]. Moreover, the repertoire of MSC integrins changes as cells differentiate or are exposed to different matrices. For example, the expression of the collagen/laminin integrin receptor, $\alpha_2\beta_1$, increased when cultured human MSCs were subjected to osteogenic differentiation factors and microgravity. Interestingly, the downstream activation of mitogen-activated protein and FAKs was reduced under such conditions [68]. When human MSCs were cultured on plates coated with laminin 5, there was an increase in integrin $\alpha_3\beta_1$ expression followed by phosphorylation of FAK and runt-related transcription factor 2 (Runx2), one of several key osteogenic and chondrogenic transcription factors [69]. These and other studies highlight the relationship between the ECM microenvironment and stem cell differentiation. However, how ECM components, individually or together, and their matrix receptors influence these events remains unclear.

3.2 Extracellular Matrix and Stem Cells in Lung Development

Lung development is regulated by networks of transcriptional factors, growth factors, matrix components, physical forces, and interactions between the mesoderm-derived mesenchyme and the endoderm-derived epithelium. The lung derives from the foregut at the level of the fourth and sixth pharyngeal arches, which contains at least two populations of progenitor cells, one giving rise to the larynx and trachea and the other to the peripheral bronchi and alveoli [70]. It undergoes development through processes classified within the following stages: (1) the *pseudoglandular stage* during which the conducting airway system develops through branching morphogenesis, (2) the *canalicular stage* when the lung becomes vascularized through vasculogenesis and angiogenesis, and the alveolar air sacs develop along with the differentiation of pulmonary epithelium, formation of the air–blood barrier, and synthesis of surfactant, and (3) the *terminal saccular stage*, which is characterized by widening of the air spaces and rearrangement of the capillaries. During this last stage, thinning of intrasaccular and intraductal septae is accompanied by deposition of elastin. Finally, alveolarization and alveolar septation in human continues at least up to 7 years of age [71].

The ECM contained within the mesenchyme is important for progenitor cell migration and differentiation in normal lung development. In the absence of mesenchyme, lung epithelia collapse to form unbranched structures in which individual cells lose orientation with each other and die [72]. Further, there is a correlation between the processes of bud outgrowth and epithelial proliferation, and the localization of specific basement membrane components nidogen, laminin 1, fibronectin,

and collagen IV [72]. The embryonic lung has abundant laminin isoforms and several studies suggest that different laminin isoforms have unique functions in lung development. For example, a study using murine lung bud explants showed that laminin α_1 and laminin 111 ($\alpha_1\beta_1\gamma_1$), which are expressed exclusively during early lung development, play roles in branching morphogenesis and in epithelial cell polarity, and that laminin α_2 and laminin 211 ($\alpha_2\beta_1\gamma_1$) influence smooth muscle cell differentiation through downregulation of RhoA activity [1]. Other laminins are also found to be important during later stages of lung development. For instant, laminin α_5 expression is found in early lung development and persists into adulthood, where it is found in airway, alveolar, endothelial, and visceral pleura basement membranes. Studies of laminin α_5-deficient mice indicate that this laminin chain, found in laminins 511 ($\alpha_5\beta_1\gamma_1$) and 521 ($\alpha_5\beta_2\gamma_1$), is essential for normal lobar septation in early lung development, and in normal alveolarization and distal epithelial cell differentiation and maturation in late lung development [1].

Like laminin, collagen type IV and nidogen are also components of basement membranes. Nidogen and collagen IV are localized throughout the entire epithelial–mesenchymal basement membrane [73, 74]. Also similar to laminin, nidogen and collagen type IV expression localizes within the epithelial–mesenchymal basement membrane and is reduced in areas of bud outgrowth [72]. These data suggest that collagen type IV and nidogen have a role during branching morphogenesis. Fibronectins are also important for lung branching morphogenesis [33] and the branching of other organs [75]. Studies in mouse, chick, and rabbit have demonstrated that expression of fibronectin is developmentally regulated during lung morphogenesis, with higher expression during the pseudoglandular stage followed by a decline after the onset of the alveolization stage as compared with laminin. The fibronectin integrin receptor, $\alpha_5\beta_1$, was found to be expressed in the mesenchyme and parabronchial cells early during the pseudoglandular stage, but its expression decreased during the canalicular stage, coinciding with the expression of fibronectin [33]. Similar studies also suggest a role for fibronectin lung in vasculogeneis, angiogenesis and alveolarization [33].

Critical interactions between the aforementioned matrix components and integrins are important for normal lung development as demonstrated in work showing that the treatment of murine lung explants with synthetic peptides with the amino acid sequence arginine–glycine–aspartic acid, known for their ability to inhibit ligand binding to certain integrins, prevents branching morphogenesis [76].

3.3 Extracellular Matrix and Lung Tissue Bioengineering

The successful establishment of tissue involves selecting the correct cell sources and the presence of soluble mediators such as growth factors in addition to establishing scaffolds or matrices to support cell growth and differentiation. Regarding the latter, the ECM is critical for the generation of tissue. For example, laminin was found to enhance neural progenitor generation, expansion, and differentiation in neurons

from human ESCs [77]. Others found that when stem cells are delivered into injured mouse brain within scaffolds containing laminin or fibronectin, they become more widely distributed as compared with cells delivered in media alone, and this was associated with increased survival and performance on spatial learning tasks [78]. In relation to lung, ESCs cultured in Matrigel® show spontaneous differentiation into type II alveolar epithelial cells with expression of surfactant proteins A, B, and C and the formation of lamellar bodies [61]. Similarly, culturing MSCs on different substrates can affect the phenotype of differentiated cells. For example, culturing MSCs on poly(lactide-*co*-glycolide)(PLGA) results in upregulation of osteocalcin gene expression as compared with culturing on polycaprolactone) (PCL), and this is likely mediated through MSC recognition of collagen type I in PLGA versus vitronectin in PCL [79].

In view of the above observations, ECMs are considered important when establishing three-dimensional matrices or other artificial scaffolding for the growth of functional lung tissue from stem cells through tissue bioengineering ex vivo and in vivo. These approaches have been increasingly successful when utilized for the regeneration of skin, vasculature, cartilage, and bone. Studies in both animals and humans demonstrated that MSCs from various sources can be seeded onto biodegradable scaffolds, where they generate tracheal cartilage that can be used for the repair of congenital tracheal and diaphragmatic defects [80, 81]. More recently, these approaches were successful in the preparation of a bioengineered trachea and bronchi that could be used in the clinical arena [82, 83].

Given the complex three-dimensional architecture of the lung, engineering functional lung parenchyma ex vivo is a more complicated task. However, both in vitro and in vivo studies utilizing mixed fetal lung cells cultured in three-dimensional scaffolds resulted in the formation of alveolar-like structures [84, 85]. Notably, 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 three-dimensional culture systems to evaluate lung development and repair [86]. A recent study demonstrated that fetal rat lung cells cultured in a biodegradable gelatin sponge, and subsequently injected into normal rat lungs, induced formation of branching with sacculated epithelial structures reminiscent of lung parenchymal architectures [84]. Mixed murine fetal lung cells admixed with Matrigel® and injected subcutaneously into the abdominal wall of adult mice induced the expression of prosurfactant protein C after 1 week [86]. There have been few studies evaluating whether stem or progenitor cells isolated from adult bone marrow, umbilical cord blood, or other sources can also form airway or alveolar-like structures when cultivated in a three-dimensional matrix or other scaffolding material, and whether stem or progenitor cells cultured in such fashion can be utilized for functional lung regeneration in vivo. Of note, these studies reiterated the importance of matrices and their influence on stem cell behavior, including differentiation [79, 84–85]. These studies suggest that tissue bioengineering from adult-derived MSCs is feasible and that selected matrices may support cell growth, which represents one of many important factors for successful tissue construction [84–86]. Studies in lung tissue bioengineering from adult-derived stem cells are still lacking.

3.4 Extracellular Matrix in Cancer Stem Cells

The growth and metastasis of malignant tumors require a sequence of events, including the ability of neoplastic cells to adhere to themselves, to normal surrounding cells, or to the ECM. Integrins and the ECM have been implicated in cancer cell biology, including their role in cancer cell proliferation and metastasis [87, 88]. It has been shown that inhibition of β_1 and β_4 integrins results in attenuation of growth and metastasis of breast cancer in a mouse model [89].

Cancer stem cells are cancer cells with stem-cell-like properties, including the ability to fully recapitulate the tumor of origin when transplanted into immunodeficient mice, to be serially transplanted or undergo self-renewal, and to express high levels of ATP-binding-cassette-type transporters which allow the cells to be resistant to drug treatment and radiation. These cells are believed to drive cancer and provide a reservoir for recurrent disease after therapy, and are associated with poor prognosis. Interestingly, cancer stem cells from prostate and breast tumors express several integrins, including β_1 and β_3 integrins [90]. Data suggest that hyaluronan is an essential component of the cancer stem cell niche and modulates the function of cancer stem cells [91]. This is supported by a study in acute myeloid leukemia which showed that administration of antibodies against CD44, a hyaluronan receptor, resulted in significantly lower rates of disease onset [91]. This suggests that CD44 and its ligand are essential in leukemic stem cell homing, adhesion to the niche, and stem cell survival [91]. Although there are essentially no data on the role of lung cancer stem cell–stroma interactions in lung cancer growth and metastasis, there are many data to indicate a role for the ECM in lung cancer progression. For example, in non-small-cell lung cancer, the interaction between fibronectin and integrin $\alpha_5\beta_1$ has been speculated to promote lung cancer cell proliferation, tissue invasion, and metastasis through activation of both extracellular-signal-regulated kinases and the PI3-K/Akt pathways with subsequent increase in AP-1 binding activity, and expression of c-Fos and matrix metalloproteinase 9 [92]. Furthermore, integrin-mediated signals include the induction of the mammalian target of rapamycin pathway through PI3-K/Akt activation which has been implicated in cell cycle progression and cell proliferation, and is found to be upregulated in human cancers [92]. Little is known, however, regarding the true role of lung cancer stem cell niches, ECMs, and their role in lung cancer progression.

3.5 Extracellular Matrix and Stem Cells in Lung Injury

Stem cells are considered important in the process of wound healing. This is highlighted in studies showing that the intravenous injection of MSCs into animals exposed to bleomycin, an injurious agent that causes severe lung inflammation and fibrosis, is protective [93]. In contrast, progenitor cells of another type termed “fibrocytes” are considered effector cells in fibrotic lung disorders [94]. Thus, different stem cells play distinct roles in tissue injury and repair. It is intriguing to

postulate that the way in which these cells influence wound healing and repair might be driven, at least in part, by signals provided by their surrounding stroma; specifically, the composition of the ECM. Although few data exist to support this idea, several in vitro studies are consistent with it. For example, the type 2 alveolar epithelial cell is thought to be one of the lung endogenous stem cells in view of its ability to give rise to type 1 alveolar epithelial cells in the setting of lung injury [70]. In vitro studies using type 2 alveolar epithelial cells showed that lung epithelial permeability after injury is regulated by integrin $\alpha_v\beta_6$ [95] and that matrix composition may affect cell–cell interactions through effects of claudins [96]. Further, there is indirect evidence for the role of integrins in maintaining lung epithelial integrity following lung injury, as well as in pulmonary fibrosis. However, there are limited data on the expression dynamics of integrins on type 2 alveolar epithelial cells. Another population of lung endogenous stem cells has recently been identified and are called "bronchoalveolar stem cells." Bronchoalveolar stem cells are thought to have the capacity to give rise to both columnar epithelial cells and alveolar epithelial cells [97]. Ongoing research is focusing on identifying and characterizing the functions of these cells. To date, no data regarding the repertoire of integrins expressed by these cells and their potential functional regulation have been reported. Further understanding of how these cells interact with the ECM and surrounding cells and how this impacts cell functions in health and disease is crucial.

4 Research Needs

Over the past decade, interest has risen regarding the use of stem cells for cell-based therapy in a number of diseases, including chronic lung diseases. The administration of stem cells in vivo would result in stem cells interacting with other cells as well as with ECMs. Unfortunately, the number of studies investigating the role of stem cell–stroma interactions in physiological and pathological processes is limited. The data available to date indicate that stem cells express specific receptors (i.e., integrins) that allow them to recognize individual ECMs, and that these interactions influence stem cell function in many settings. However, a better understanding of the true role of these interactions can only come after carefully designed studies that identify functional cell-surface integrins expressed in different stem cells (e.g., ESCs, HSCs, MSCs) under different experimental conditions both in vitro and in vivo. Once the stem cell integrin repertoire has been defined, efforts should be directed at examining the signals activated by stem cell–stroma interactions and, most importantly, the effects these interactions have on differential gene expression and cellular functions. This information is needed in view of the increasing interest in the use of stem cells as therapeutic tools. Equally important is to better understand how the ECM composition changes with developmental state and in the setting of injury and cancer. Aging, for example, appears to affect lung ECM composition (Sueblinvong et al., unpublished observations), yet how these events impact the reparative effects of stem cells remains unknown. The most

informative work will come from studies performed in vivo that examine the true effects of matrix-binding integrins and related receptors on stem cells using animal models of embryogenesis, injury and repair, and oncogenesis, among others. The latter studies will be difficult in view of animals deficient in specific matrix glycoproteins (e.g., fibronectin, collagens) or their integrin receptors (e.g., $\alpha_5\beta_1$) being embryonically lethal [98]. Nevertheless, they might be useful to examine initial specification of precursor cells [99]. Furthermore, the last decade has seen the emergence of new technologies that could facilitate further work in this area. For example, silencing of specific integrin receptors via small interfering RNA and short hairpin RNA technology is now feasible. Animals with conditional expression of specific integrin or matrix genes are also available. Finally, some animals genetically engineered to only express specific splicing variants of matrix glycoproteins (e.g., fibronectin ED-A) live for prolong periods of time, allowing for more detailed investigations [28]. Once the effects of stem cell–stroma interactions have been elucidated, studies designed to modulate these interactions in vivo with the purpose of generating new and effective therapeutic modalities should be considered.

5 Conclusion

Intimate relationships between cells and their surrounding stroma are needed throughout human life starting at conception. The same is true for stem cells and their interactions with the ECM. In utero, the ECM directs stem cells to achieve normal organogenesis. In adult human life, the ECM continues to influence the behavior of stem and progenitor cells, among other cells, both during normal homeostasis and in diseased states. These interactions are mediated by specific matrix-binding integrin receptors in addition to other cell-surface receptors capable of matrix recognition. Although many of the signaling events triggered by integrin binding have been elucidated in other cells, the surface repertoire of integrin receptors and the signaling events triggered by ligand binding in stem cells remain unclear. Furthermore, since the field of stem cell biology is relatively young, much still needs to be learned about the role of stem cells in physiological and pathological processes. Undoubtedly, new information regarding the above points will prompt investigations into the role stem cell–stroma interactions play in these processes. It is our hope that this new information will reveal ways to control stem and progenitor cell fate. More importantly, these studies are expected to unveil how stem cells can be used as vehicles for therapies or as targets for therapeutic approaches to common disorders.

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Chapter 6
Mobilization of Stem Cells/Progenitor Cells by Physical Activity

Patrick Wahl and Wilhelm Bloch

1 Introduction

Tissue damage due to degenerative disease and injury is a major problem in terms of health care, lost economic productivity, diminished quality of life, and premature death. Advances in cell, developmental, and molecular biology and the discovery of regeneration-competent cells in many nonregenerating mammalian tissues have given impetus to systematic investigations that will enable us to regenerate tissues by the induction of regeneration from the body's own tissues [1]. Growth, regeneration, and repair of tissues are dependent on the addition of new differentiated cells. These cells are mainly derived from undifferentiated cells which can proliferate and differentiate. These cells are called "stem cells" or "progenitor cells." These cells are sources for tissue regeneration and repair as well as tissue growth [2]. Initially uncommitted, following specific signals, stem and progenitor cells have the capacity to differentiate into lineage-committed cells. The stem and progenitor cells reside in different tissues and organs and participate to various extents to the replenishment of mature cells responsible for the specialized functional properties of the tissue in which they reside. Residing stem and progenitor cells are found in the skin, skeletal muscle, fat, liver, kidney, heart, brain, and other tissues [3, 4]. The degree of restriction of the developmental potential of stem/progenitor cells to the specific tissue/niche in which they reside is open to debate and is difficult to resolve since pluripotent and multipotent stem cells and progenitor cells circulate continuously in the blood and lymphatic vasculature. A major source of these circulating stem and progenitor cells is the bone marrow, with its different stem and progenitor cell populations, such as hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) as well as endothelial progenitor cells (EPCs) [5, 6]. Independent of the source, stem and progenitor cells must be activated and mobilized before they can help to regenerate, repair, or expand tissue. Stem and progenitor cells can be activated and mobilized by different stimuli and mechanisms [7, 8, 9].

W. Bloch (✉)
Institute of Cardiovascular Research and Sport Medicine, The German Research Center of Elite Sport, German Sport University, Cologne, Germany
e-mail: w.bloch@dshs-koeln.de

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Physical activity is one of the inducers of different stem and progenitor cells. However, the types of stem cells (or progenitor cells) and the mechanisms by which these cells are activated and mobilized by physical activity to induce regeneration or growth differ depending on the respective organ or tissue and have only been partially investigated [10]. Exercise reveals a number of stimuli: mechanical, metabolic, and hypoxic (Fig. 6.1). It also induces the release of various growth factors, cytokines, and hormones. With the help of these processes, different mechanisms lead to the activation of stem and progenitor cells. Exercise induces molecular adaptations that improve physical performance, fitness, and/or health whether under performance sport conditions or in leisure sport, prevention, or rehabilitation. The exercise-induced activation and mobilization of stem cells and progenitor cells is not restricted to the skeletal muscle; it can also lead to the activation and mobilization of stem cells from bone marrow, brain, and other sources [11, 10]. These cells are involved in the repair of different tissues (e.g. neuronal, cardiovascular, and muscular) [2]. The current knowledge in the field of activation and mobilization of stem and progenitor cells by physical activity is reviewed here.

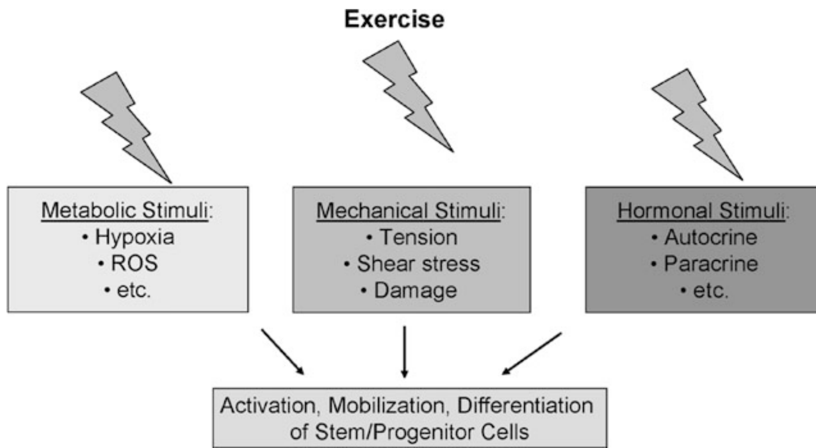

Fig. 6.1 Physical activity induces a variety of stimuli (metabolic, mechanical, and hormonal) which might by responsible for the activation, mobilization, differentiation, and homing of stem and progenitor cells. Another important factor of exercise which might influence the release of stem cells could be inflammatory processes due to tissue damage. The activation, mobilization, and differentiation of stem and progenitor cells might improve adaptational and regenerative processes. *ROS* reactive oxygen species

2 Characterization of Stem and Progenitor Cells

Stem and progenitor cells are cell sources for tissue regeneration and repair as well as tissue growth. They have two common properties: the capacity for self-renewal and the potential to differentiate into one or more specialized cell types. In

general, they can be divided into two broad categories: adult (somatic) stem cells and embryonic stem cells. The stem cells can also be classified into three principal categories depending on their differentiation potential: (1) the totipotent cells, which can form a whole organism such as a human; (2) the pluripotent cells, which have lost the capacity to from a whole organism, but have the ability to differentiate to most cell types existing in the organism; and (3) the multipotent cells, which form a limited number of specialized cell types, and generally function locally to replace fully differentiated cells lost through depletion or damage [12]. Progenitor cells have lost the capacity for self-renewal, but have preserved the pluripotent or multipotent capacity to differentiate in a more or less wide range of specialized cells in different tissues and organs which are important for tissue regeneration and growth throughout the whole life. In the adult organism, pluripotent and multipotent stem and progenitor cells from different sources are found. These stem cells can produce more stem cells and cells that differentiate by undergoing asymmetrical cell division; both tasks can be accomplished in a single step. Equally well, stem cell numbers would remain constant if only symmetrical divisions occurred, provided that each time a stem cell gave rise to two daughter tissue-specific cell, another stem cell gave rise to two daughter stem cells. Stem cells residing in tissues and organs participate to various extents in the replenishment of mature cells responsible for the specialized functional properties of the tissue. The stem and progenitor cells seem strongly influenced by the local environment, cellular and extracellular, which can maintain stem and progenitor cells in a quiescent stage or can induce their activation. Alteration of the local environment, as can be induced by physical activity, can change the behavior of stem and progenitor cells.

3 Stem Cell Sources

For a long time it was believed that stem cells are restricted to tissues with high turnover rates such as the blood (bone marrow), gut, and skin. Today, stem and progenitor cells are thought to be also involved in controlling homeostasis in tissues with low turnover rates and were once thought to be essentially postmitotic such as in the brain or the myocardium. Since many differentiated cell types are inherently short-lived, or are lost through general wear and tear, and injury, stem cells must function throughout the lifetime of the organism to maintain tissue homeostasis and avoid the onset of atrophy and aplasia. There is evidence that aged tissues have a strongly reduced capacity to maintain homeostasis, or return to a homeostatic state after exposure to stress or injury and possibly physical activity. This has implicated stem cell decline in the aging process [13].

In the adult organism stem cells and progenitor cells from different sources are thought to be activated and mobilized by physical activity. The sources where stem and progenitor cells are possibly activated by physical activity are bone marrow, skeletal muscle, heart, and brain [14, 11, 15]. Less is known about the activation

from other sources. Therefore, this review concentrates on stem/progenitor cells originally derived from bone marrow, muscle, and brain.

Adult skeletal muscle contains an abundant and highly accessible population of muscle stem and progenitor cells called "satellite cells." They have long been regarded as a population of muscle-specific committed progenitors that are responsible for the postnatal maintenance, growth, repair, and regeneration of skeletal muscles. Satellite cells are characterized anatomically by their direct attachment to muscle fibers under the basal lamina and functionally by their myogenic differentiation. They are quiescent under normal physiological conditions. However, in response to activation signals resulting from exercise or injuries, satellite cells are activated, then proliferate, undergo self-renewal, and differentiate into mature muscle cells [16].

For many years bone marrow was primarily regarded as the source of HSCs, which are the stem cells for all differentiated blood cells. More recently, it was recognized that bone marrow contains not only HSCs but also heterogeneous non-HSCs, which were assigned different names (e.g., MSCs, multipotent adult progenitor cells, or marrow-isolated adult multilineage inducible cells). Furthermore, the bone marrow contains endothelial precursors (EPCs). The EPCs residing in bone marrow could be released/mobilized into peripheral blood as a source of cells able to play a role in the vascularization of regenerating and growing organs [5, 17]. But the phenotypic characterization and derivation of EPCs remains controversial. It seems to be extremely difficult to accurately characterize these cells because of the many different possible origins of EPCs [17]. Additionally, specific surface markers found on EPCs are fractionally expressed on other cell types.

The following general definition of EPCs has been given:

1. EPCs are circulating bone-marrow-derived stem cells that are functional and phenotypically distinct from mature endothelial cells.
2. EPCs can differentiate into endothelial cells.
3. EPCs can contribute to postnatal vasculogenesis and to vascular homeostasis [18].

This rather general definition does not take into account that EPCs can derive from different cell populations:

1. HSCs [19, 20, 21, 22–24, 18, 25, 26, 27].
2. Monocytes/macrophages [28, 24, 29, 30, 31, 32].
3. MSCs (multipotent adult progenitor cells) [33, 34]. The accurate characterization of EPCs has been confounded by the presence of non-bone-marrow-derived circulating endothelial cells, which can also contribute to neoangiogenesis/vasculogenesis [35].
4. Myoendothelial cell progenitors. Other putative myogenic and endothelial cell progenitors were identified in the interstitial spaces of murine skeletal muscle [36, 37].

In general, bone marrow may contain heterogeneous non-HSCs, which could support the regeneration of various tissues/organs, including bone, cartilage, tendon, adipose tissue, skeletal muscle, cardiac muscle, and brain. For all of these bone-marrow-derived stem and progenitor cells it was demonstrated that physical activity is involved in activation and mobilization of these cells from their host tissue [14, 38, 10].

In the past few years it has been established that the heart contains a reservoir of stem and progenitor cells, which makes the heart a self-renewing organ. These cells are positive for various stem/progenitor cell markers (Kit, Sca-1, Isl-1, and side population properties). The relationship between the various cardiac stem cells and progenitor cells described awaits clarification. Cardiac stem cells are composed of clonally derived cells, consisting of proliferating c-Kit positive cells primarily in their core and differentiating state cells express cardiac and endothelial cell markers on their periphery. Although the intracardiac origin of adult myocytes has been unequivocally documented, the potential of an extracardiac source of cells able to repopulate the lost cardiac stem cells in pathological conditions (infarct) cannot be excluded [39, 40]. This resident population of multipotent undifferentiated cells gives rise to myocytes, endothelial cells, smooth muscle cells, and fibroblasts, which would allow a complex cardiac repair replacing damaged coronary arteries, arterioles, and capillaries, and substitution of hypertrophied poorly contracting myocytes with smaller better functioning parenchymal cells [40]. Up to now, knowledge of the biology of cardiac progenitor cells and their fate following pathological and physiological stimuli, e.g., infarction and physical activity is very limited.

Recent studies have expanded our knowledge of progenitor cells that continue to reside in the adult nervous system, and their respective roles in the maintenance of the brain and spinal cord. In the adult, neural stem cells persist within the forebrain ventricular zone, and give rise to a variety of more restricted progenitor phenotypes. The major progenitor pools of the adult human brain include ventricular zone neuronal progenitor cells, hippocampal neuronal progenitors, and parenchymal glial progenitor cells. These cells are located in specific local environmental niches [41], where they reside as transit amplifying cell pools up to the point where they will be mobilized to restore neurons lost and induce neurogenesis [42].

The resident neuronal stem and progenitor cells might be mobilized and induced by specific stimuli (caloric restriction and physical activity) to differentiate in vivo mediated by the actions of factors such as neurotrophic factors, neurotransmitter receptors, protein chaperones, and mitochondrial biosynthesis regulators [43].

Physical activity is one stimulus which can increase the release of growth factors and can change the metabolic environment leading to neurogenesis in the brain. The discovery that exercise regulates adult hippocampal neurogenesis, which is the production of new neurons in the adult brain, was surprising news and changed quite fundamentally our view of how physical activity affects the brain. But current knowledge of how this exercise-induced regulation of neurogenesis might work needs substantial addition [11].

4 Principal Mechanism of Stem and Progenitor Cell Activation/Mobilization

The types of stem cells (or progenitor cells) and the mechanisms by which these cells are activated to induce regeneration or growth differ depending on the respective organ or tissue. Although a multitude of stem and progenitor cell types exist in the adult organism and a multitude of factors and stimuli have been described which can influence the activation and mobilization of these cells, some principal mechanisms have been identified as necessary for stem cell activation and mobilization. Interaction of stem and progenitor cells with their specific microenvironments, known as stem cell niches, is critical for maintaining stem/progenitor cell properties, including the self-renewal capacity of stem cells and the ability of stem and progenitor cells to differentiate into multiple lineages. Therefore, activation and mobilization needs a dynamic change of the specific microenvironment, e.g., by a change of cytokines, growth factors, oxygen tension, metabolic situation, cell–cell interactions, and mechanical forces [44, 10]. The first step in stem cell mobilization is the release of stem cells from the stem cell niche and the subsequent activation from the quiescent to an active stage, allowing the cell to proliferate and differentiate as well as to move to the target tissue, e.g., as is shown for bone marrow stem cells [45]. The mobilization of stem cells from marrow is such a dynamic process, regulated by shear stress imparted by blood flow, and the activation of metalloproteinases that induce the release of Kit ligand, facilitating egress from the marrow to the circulation [8], and seems to be a model for understanding stem cell activation and mobilization. After mobilization from their niche, resident stem cells can migrate to their final location, whereas nonresident stem and progenitor cells must be guided to move from their host tissue to the target tissue; during this process transmigration over the endothelial barrier and invasion of the extracellular matrix of the target tissue are important steps [46, 47]. Physical activity can support the mobilization and the guidance of stem and progenitor cells by a change of the local environment and the release of factors which can activate and guide stem and progenitor cells (Fig. 6.2) [10].

5 Activation of Stem and Progenitor Cells by Physical Activity

A number of studies have shown that exercise improves the function and regeneration of the cardiovascular system and skeletal muscle by activating and mobilizing organ-resident stem cells [48, 49, 50] or by recruiting blood-circulating stem or progenitor cells [51, 52, 53, 54]. However, the types of stem cells (or progenitor cells) and the mechanisms by which these cells are activated to induce regeneration or growth differ depending on the respective organ or tissue. Exercise provokes a number of stimuli: mechanical, metabolic, and hypoxic. It also induces the release of various growth factors, cytokines, and hormones. Physical activity may also induce

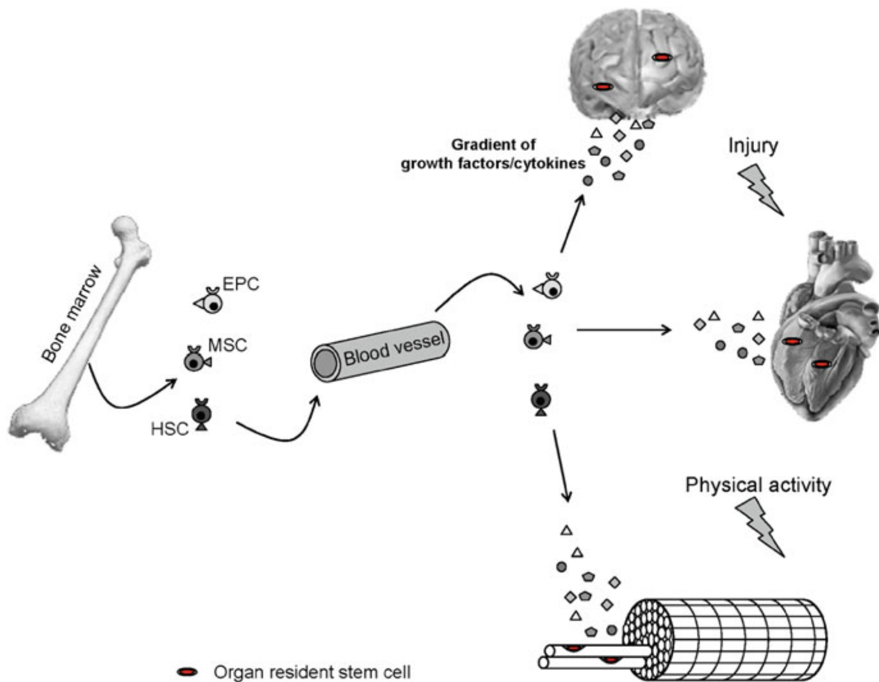

Fig. 6.2 Proposed relationship between bone-marrow-derived stem cells and organ-resident stem cells. Endothelial progenitor cells (*EPC*), mesenchymal stem cells (*MSC*), and hematopoietic stem cells (*HSC*) are released from bone marrow to peripheral blood in response to certain stimuli (exercise, injuries). These stem cells are guided by gradients of growth factors and cytokines (released by the damaged or loaded tissue) to the sites where they are needed and migrate into various tissues and organs. These bone-marrow-derived stem cells might support organ-resident stem cells or perform different functions for the regeneration and adaptation of the tissue

injuries/traumata and inflammatory processes in various tissues. All these stimuli and processes may cause an activation, mobilization, and differentiation of stem and progenitor cells in response to exercise (Fig. 6.1).

5.1 Satellite Cells/Myogenic Stem Cells

Both skeletal muscle and bone marrow tissue contain myogenic stem cells. Thereby, the population residing in muscles is more heterogenic than was previously thought. The typical satellite cells are the predominant population, but another population is a group of multipotent muscle stem cells which, at least in part, are derived from bone marrow. These cells are tracked by gradients of growth factors, etc. released from the muscle owing to injury or exercise (Fig. 6.2). In this section only satellite cells will be described. Bone-marrow-derived stem cells will be described in the next section.

Regeneration and growth of skeletal muscle are mainly managed by resident stem cells, the so-called satellite cells. Satellite cells occupy a sublaminar position between the basal lamina and sarcolemma [55]. In contrast to adult stem cells, which by definition are multipotent cells, with considerable proliferative potential, satellite cells are only unipotent stem cells and have a limited capacity for self-renewal.

Upon activation, satellite cells increase their cytoplasm content and the numbers of organelles and reduce the amount of heterochromatin. Skeletal muscle satellite cells supporting growth or regeneration are thought to be activated and incorporated into growing myofibers by endocrine and locally expressed autocrine and paracrine growth factors, the latter being load-sensitive, e.g., vascular endothelial growth factor (VEGF) [56], insulin-like growth factor (IGF)-I [57], nitric oxide, hepatocyte growth factor [58], and fibroblast growth factor [59]. Very interestingly, the levels of many of these autocrine/paracrine factors are also systemically increased in situations of enhanced exercise and thus they may contribute to activation of the satellite cells. They may also initiate or activate other stem-cell-dependent regeneration processes, e.g., vascular development (see below). Special attention should be drawn to the release of IGF-I regarding muscle regeneration [60]. It seemed relevant to measure expression levels of two insulin-like splice variants following imposed local damage. These were the systemic IGF-IEa and an autocrine splice variant produced by muscle. The latter was recently cloned from stretched, stimulated muscle. Because of this, and since it has a sequence different from that of systemic IGF-I, it has been called mechano-growth factor (MGF). IGF-I is reportedly involved in satellite cell activation [61], although these in vitro studies may not accurately reflect what is happening in vivo, particularly in mature muscle when subjected to damage. Recent in vivo studies have indicated that MGF has different expression kinetics than IGF-IEa [62]. This and other studies [63] suggest they have different modes of action.

There are several reports that indicate exercise activates satellite cells in mature skeletal muscle cells and induces their differentiation, leading to muscle hypertrophy [64, 65, 66]. Exercise also activates myogenin protein expression [66].

To distinguish the respective potential of endurance and resistance training to increase the satellite cell pool, Verney et al. [67] investigated the effects of 14 weeks of concurrent lower body endurance and upper body resistance training (three sessions per week) on vastus lateralis and deltoid muscles. After 14 weeks of training, the satellite cell pool increased similarly (+38%) in both muscles, mainly in type II muscle fibers. No significant change in myonuclear number or myonuclear domain in either muscle was found. Sixteen weeks of knee extensor resistance training in three groups (extreme responders, modest responders, and nonresponders) resulted in myofiber hypertrophy, which averaged 58, 28, and 0%, respectively. The satellite cell number increased robustly during training in extreme responders only and myonuclear addition was most effectively accomplished in extreme responders as well (26%). After training, extreme responders had more myonuclei per fiber than nonresponders (23%) and tended to have more than modest responders (19%). These findings strongly suggest that myonuclear addition via satellite

cell recruitment may be required to achieve substantial myofiber hypertrophy in humans [50].

This exercise-induced activation of satellite cells seems to be specifically attributed to eccentric exercise, i.e., to a situation when the muscle is activated while it is stretched. It is interesting to note that the forces generated by activation combined with stretching exceed even those of maximal isometric contraction. In the muscle fibers involved, the sarcomeres might be pulled out to such a degree that there is no longer any overlap of the actin and myosin filaments, thus causing damage [68]. Immediately following injury, myofibers hyalinize, vacuolate, and lyse. Interstitial edema with neurophils releases trophic factors to activate the satellite cells within 2 h of injury. Factors such as MyoD and Myf6 are induced within 2–6 h of injury, corresponding to their role in activation of satellite cells. Following activation of the satellite cell population, they re-enter the cell cycle and demonstrate a significant proliferative capacity between 2 and 3 days following injury. Transcriptional regulators such as Pax 3, Pax 7, and forkhead are upregulated during the repair and regenerative period. This period is followed by a differentiation phase where myoblasts withdraw from the cell cycle and form small centronucleated myotubes. Fusion of myoblasts and further growth of the centronucleated myofibers result in restoration of the cellular architecture within an approximately 2-week period [69]. However, exercise-induced activation of satellite cells seems to be age- and gender-dependent. It was recently reported that myofiber hypertrophy with resistance training is greater in young men than in young women and older adults [70]. In another study it was shown that a single bout of maximal eccentric exercise increases satellite cell numbers in young and old men, with a significantly greater response among the young men [71]. Taken together, these data suggest that age-related changes in satellite cell recruitment may contribute to muscle regeneration deficits among the elderly. This issue should be taken into account when thinking of age-related rehabilitation and prevention programs.

5.2 Bone-Marrow-Derived Stem and Progenitor Cells (Mesenchymal)

Mesenchymal stem cells (MSCs) are involved in the repair of damaged tissues and thus they play a crucial role in regenerative medicine and exercise physiology. MSCs reside in several adult tissues, such as bone marrow, adipose tissue, cartilage, and skin [72] and can give rise to osteoblasts, chondroblasts, adipocytes [73], skeletal muscle cells [74, 75], cardiomyocytes [76, 77], and smooth muscle cells [78]. Under several physiological and pathological conditions, they migrate toward organs in which they do not normally reside [79]. This requires MSCs circulate in the blood to their final destination. Afterward, MSCs must transmigrate across the endothelium and invade their target tissue. This transmigration requires among other adhesion molecules the interaction of vascular cell adhesion molecule 1 and very late antigen 4 and triggers the clustering of β_1 integrins [47]. MSCs also secrete

cytokines and matrix metalloproteinase 2, which allows them to cross the barrier [47]. Our group was able to show that MSCs emit soluble factors that alter the NO and calcium levels of endothelial cells and may be important to facilitate crossing the endothelial barrier. When they have arrived in the target tissue, the cells are exposed to a new local environment including cell–cell contacts and soluble factors secreted by the tissue. This local environment influences the differentiation process of the stem cell.

It is known that exercise increases the number of stem cells within the circulation; however, the exact mechanisms of mobilization and homing of circulating stem cells still need to be elucidated. An acute bout of exercise (21 km of running) leading to muscle injury as well as a chronic muscle injury (McArdle disease) caused a mobilization of MSCs and increases in the number of circulating cells [80]. These circulating cells may migrate, at least to a certain extent, to the damaged muscle to participate in the process of muscle repair [81, 82, 83, 80, 84]. Hence muscle injury might be one molecular mediator of MSC mobilization [80]. To answer the question whether the circulating cells increase their migratory activity after exercise, we analyzed the influence of athletes' blood sera, taken before and after exercise, on human MSCs. The exercise-conditioned sera significantly increased the migratory activity of MSCs after exercise without affecting proliferation and apoptosis, showing that exercise does not only increase the number of cells within the circulation but also increases the migratory activity of these cells, which is a prerequisite for the invasion of stem cells and therefore the repair and buildup of new tissue [38]. Generally, it is recognized, that damaged or exercising (muscle) tissue releases signals (growth factors, cytokines) which, on the basis of chemical gradients, attract circulating cells [85, 86]. These signals include hepatocyte growth factor/scatter factor (HGF), stromal-cell-derived factor 1 (SDF-1), leukemia inhibitory factor (LIF), IGF-I and the appropriate receptors, such as c-met (HGF), CXCR4 (SDF-1), and LIF-R (LIF). Migration of cells outside the blood vessel is mediated by adhesion molecules such as integrins or selectins on the cell surface. Exposed to the local environment, stem cells start to differentiate [87].

Palermo et al. [88] were able to show that the contribution of bone-marrow-derived stem cells to muscle is enhanced in response to increased muscle activity resulting from muscle overload or forced exercise. The delivery of IGF-I to adult skeletal muscle increases the integration of bone-marrow-derived stem cells. The number of fusion events was substantially augmented by IGF-I, which seems to be sufficient to enhance the fusion of bone marrow derivatives with adult skeletal muscle [89]. It is suggested that stem cells finding a niche in skeletal muscle are derived from bone marrow side population (the term "side population" does not specify the type of cell in detail) rather than bone marrow main population [90]. It was also shown that the incorporation of bone marrow cells depends on the muscle type [91].

But MSCs might also play a crucial role in orthogenesis and chondrogenesis. In these cases, mechanical strain, also induced by exercise of various tissues, might be an inherent stimulus for chondrogenic and/or osteogenic differentiation in undifferentiated MSCs. It was shown that the application of cyclic tensile strain significantly stimulates the expression levels of the early chondrogenic and osteogenic marker

genes in MSCs and promotes MSC proliferation [92, 93]. The results of Ocarino et al. [94] suggest that nitric oxide, produced by endothelial cells and erythrocytes under exercise conditions, stimulates osteogenic differentiation of MSCs and that nitric oxide mediates the beneficial effects of physical activity upon osteogenic differentiation of MSCs. The inhibition of nitric oxide synthase in vivo had a negative effect upon the osteogenic potential of MSCs. Further studies suggest that the positive effects of exercise on bone and fat may occur during mesenchymal lineage selection. Mechanical strain enhanced the potential for MSCs to enter the osteoblast lineage despite exposure to adipogenic conditions. MSC commitment to adipogenesis can be suppressed by mechanical signals, allowing other signals to promote osteoblastogenesis [95].

5.3 Hematopoietic Stem/Progenitor Cells

As previously described, several studies showed that cells from bone marrow (MSCs) can give rise to differentiated skeletal muscle fibers (see Sect. 5.2). Other results indicated that, besides their normal function of regulating blood cells, HSCs (such as CD45+:Sca1+ cells) are also involved in skeletal muscle regeneration [96]. Dormant HSCs are also located in niches at the endosteum, whereas activated HSCs are in close contact with sinusoids of the bone marrow microvasculature [97]. Generally, it was shown that exercise (20 min of moderate-to-vigorous cycle ergometer exercise) can affect HSC and HSC mediators (SDF-1, granulocyte colony-stimulating factor) [98]. Supramaximal exercise doubled the number of circulating CD34+ cells [99]. Thijssen et al. [100] investigated the effects of training and aging on HSCs. Acute exercise significantly increased the number of HSCs. Older men showed significantly lower baseline and exercise-induced levels of HSCs than young men. Therefore it seems that advancing age results in lower circulating numbers of HSCs and attenuates the acute-exercise-induced increase in the number of HSCs.

It is likely that HSCs can migrate into skeletal muscle and constitute, at least in part, a multipotent muscle-derived stem cell population [87]. Abedi et al. [101] used an immunocompetent and an immunocompromised model of bone marrow transplantation to characterize the type of marrow cells participating in regenerating skeletal muscle fibers. They found that MSCs rather than mesenchymal cells or more differentiated hematopoietic cells are responsible for the formation of muscle fibers after injury. In contrast, Fukuda and Fujita [102] showed that MSCs but not HSCs were mobilized and differentiated into cardiomyocytes after myocardial infarction in mice.

In conclusion, the results document that normal muscle regeneration is dependent on bone-marrow-derived cells. Furthermore, macrophages are a likely bone-marrow-derived cell type responsible for modulating muscle regeneration, although other bone-marrow-derived progenitor cells, such as HSCs or inflammatory/immune cells, may also contribute. Future studies in regenerative medicine must include consideration of the role of bone-marrow-derived cells (HSCs/possibly macrophages) that regulate effective skeletal muscle regeneration [103].

5.4 Endothelial Progenitor Cells

Especially in endurance exercise, muscles need to be well provided with O_2 and nutrients. Adaptation processes in response to endurance training are well known. Capillarization is one important adaptation. For many years it was believed that the sole mechanism for growth of new blood vessels in response to training was angiogenesis. But in recent years several groups were able to show the positive effects of training on EPCs [51, 104, 52, 53, 105], which means that vasculogenesis might also contribute to the growth of new blood vessels. EPCs are bone-marrow-derived progenitor cells that, if required, are released into peripheral circulation. The release of EPCs from bone marrow is regulated by a variety of growth factors, enzymes, ligands, and surface receptors. Postnatal vasculogenesis is mainly induced by ischemia/hypoxia. Ischemia and hypoxia are the most potent physiological stimuli known to trigger growth factor secretion and accordingly increase the number of circulating EPCs [51, 106]. Hypoxia alters vascular endothelium, causing EPCs to arrest in these regions. EPC adhesion was significantly increased in hypoxic endothelium. The exposure of EPCs to hypoxia stimulates proliferation and the organization of cell clusters. These cell clusters align in the direction of the ischemic gradient and form vascular-like cords [106]. Davie et al. [107] showed c-kit+ cells are mobilized from bone marrow to the circulation in response to hypoxia. Circulating mononuclear cells exposed to hypoxia differentiated into endothelial cells. In the studies of Tepper et al. [106] the extent of recruitment of EPCs was directly proportional to the degree of tissue ischemia. But the question which is still controversially discussed in the literature is: Does exercise cause local hypoxia, e.g., in trained muscles? Ischemia as well as training increased the serum concentrations of VEGF [51, 52, 106]. Several studies were able to show an increase of EPCs in peripheral blood in response to exercise [51, 104, 52, 53, 105]. All the studies used the same methods (flow cytometry and cultivation of mononuclear cells with acetylated LDL and *Ulex* lectin) to quantify and determine EPCs before and after exercise, whereas different surface markers were used in flow cytometry by the investigators [51, 104, 52, 53, 105]. Laufs et al. [104] even distinguished between three types of EPCs: $CD34^+/VEGFR2^+$, $CD34^+/CD133^+$, and $CD34^+/CD117^+$. In addition they analyzed the migratory activity of EPCs with a modified Boyden chamber and quantified colony-forming units. Rehman et al. [53] differentiated between EPCs [$CD133^+$/vascular endothelial (VE)-cadherin$^+$] and HSCs ($CD133^+$/VE-cadherin–) both analyzed by flow cytometry and circulating angiogenic cells (mononuclear cells) cultivated with acetylated LDL and *Ulex* lectin. Laufs et al. [52] as well as Adams et al. [51], Steiner et al. [105], and Rehman et al. [53] studied the effects of exercise on patients with coronary artery disease or patients with cardiovascular risk factors and not on healthy subjects; Adams et al. and Rehman et al. investigated the short-term effects of exercise within hours, whereas Laufs et al. and Steiner et al. determined the long-term effects of a 4- and 12-week training period respectively. In the study of Adams et al. [51], the number of EPCs increased only in patients with exercise-induced myocardial ischemia 24 and 48 h after exercise and not in patients without induced ischemia or in healthy.

Rehman et al. [53]. showed an increase in the number of EPCs and HSCs (analyzed by flow cytometry) and of circulating angiogenic cells quantified by cell culture assay 10 min after exercise

Laufs et al. [52] demonstrated an increase in the number of EPCs in peripheral blood and bone marrow (flow cytometry) and of mononuclear cells (cultivation) after 7, 14, and 28 days of training in mice. In coronary artery disease patients the number of EPCs was augmented after 28 days of exercise. A 12-week training period produced a 2.9 ± 0.4-fold increase in the number of EPCs in peripheral blood [105].

In a recent study Laufs et al. [104] investigated the effects of running for different duration and intensity in healthy individuals. Intensive running for 30 min at the individual anaerobic threshold (IAT) as well as moderate running at 80% of the IAT caused an increase of circulating EPC numbers to 235 ± 93% and 263 ± 106%. Exercise also increased EPC migratory and colony-forming capacity 10 and 30 min after exercise. Exercise for 10 min at 80% of the IAT caused no changes.

All five studies determined potential EPC mobilizing proinflammatory cytokines such as granulocyte/monocyte colony-stimulating factor and granulocyte colony-stimulating factor, growth factors such as VEGF and erythropoietin, chemokines such as stromal-cell-derived factor 1, and hormones such as cortisol [51, 104, 52, 53, 105]. But only two investigations were able to show an increase in VEGF plasma levels, but in the study of Laufs et al. the increase was only apparent in patients with exercise-induced myocardial ischemia and not in nonischemic coronary artery disease patients or in healthy subjects [51, 52].

Besides these five studies which measured the direct effect of training on EPCs, there are some other studies with relevance to sport. Shear stress plays an important role in blood-flow-dependent phenomena such as angiogenesis. Shear stress is known to modulate the function and gene expression of endothelial cells [108]. Blood flow increases during physical exercise and accordingly increases shear stress on endothelial cells and EPCs. Yamamoto et al. [109] showed that shear stress generated by blood flow or tissue-fluid flow can accelerate the proliferation, differentiation, and capillary-like tube formation of EPCs. Shear stress augmented the expression of VEGFR2 and VE-cadherin on EPCs.

All the results show that exercise seems to affect EPCs. Adaptation processes in sports might be explainable on cellular and molecular levels. Thus, the following remains an open question: What kind of exercise (duration, intensity) under what kind of environmental conditions (high-altitude training) may have the ideal effect on EPCs and vasculogenesis for adaptation and regeneration? Further results might help to improve the control of training.

5.5 Neuronal Stem Cells

Research in humans and animals has shown that exercise improves mood and cognition [110]. Hippocampal neurogenesis is positively regulated by voluntary exercise [111] and physical exercise is known to promote adult neurogenesis [112]. Exercise

can increase neurogenesis and affect gene expression in the brains of adult rats. Lou et al. [113] found that exercise influenced neurogenesis in an intensity-dependent manner. One week of low- or moderate-intensity exercise in a treadmill running task enhanced neurogenesis in the dentate gyrus of hippocampus. Gene expression levels (brain-derived neurotrophic factor, NMDAR1, Flk-1, messenger RNA) in the low-intensity exercise group were greater than those in the high-intensity group for these four molecules.

Recent investigations showed that neuronal stem cells may be involved in the processes of neurogenesis and might be influenced by physical activity. Five weeks of training not only promoted the maturation and survival of immature neurons in middle-aged mice but also increased neural stem/precursor cell proliferation and the number of immature neurons. Physical activity restored the age-dependent decline of brain-derived neurotrophic factor and its receptor, TrkB, which are known to promote neuronal differentiation and survival. Wu et al. [114] assumed that running exercise alters the brain chemistries of middle-aged animals toward an environment that is favorable to neural stem/precursor cell proliferation, survival, and maturation. The acute upregulating effect of voluntary wheel running on precursor cell proliferation decreases with continued exercise, but continued exercise reduces and prevents the age-dependent decline in adult neurogenesis and precursor cell activity [115].

Glucocorticoid (corticosterone in rodents) is a factor that is known to affect neurogenesis. As physical exercise modulates corticosterone secretion, Chang et al. [112] hypothesized that corticosterone signaling is involved in exercise-induced adult neurogenesis. Five weeks of training increased the doublecortin-positive neuronal progenitor cells in adult hippocampus and transiently increased the serum corticosterone level at the end of the training protocol. They concluded that the induction of neuronal progenitor cells in the dentate area of adult hippocampus by training is partly due to the downregulation of glucocorticoid/mineralocorticoid receptor signaling, which subsequently enhances differentiation along a neuronal lineage and/or neuronal progenitor cell survival. Physical activity might not only be important for neurogenesis in healthy persons but might also be important as a therapeutic tool for regeneration after injuries or application of radiation.

Cell therapy and exercise training may be options for spinal cord regeneration. Carvalho et al. [116] investigated the effects of autologous bone marrow stem cell [CD45(+)/CD34(–)] transplantation in acute spinal cord injury in exercise training and in sedentary rats. The animals underwent a 60-min swimming session six times per week for six consecutive weeks. The combination of bone marrow stem cell therapy [CD45(+)/CD34(–)] and exercise training resulted in significant functional improvement in acute spinal cord injury. The findings of another study support the positive effects of exercise on neuron regeneration. Naylor et al. [111] investigated acute effects of irradiation and the effects of voluntary running on hippocampal neurogenesis and behavior 3 months after irradiation. Voluntary running significantly restored precursor cell and neurogenesis levels after a clinically relevant, moderate dose of radiation. The radiation perturbed the structural integration of immature

neurons in the hippocampus. This perturbation was reversed by voluntary exercise. These results support the usefulness of physical exercise for functional and structural recovery from radiation-induced injury to the juvenile brain, and they suggest exercise should be evaluated in rehabilitation therapy of childhood cancer survivors.

5.6 Stem Cells from Other Sources

Physical exercise improves cardiac function. This improvement of cardiac function has been shown to be attributed at least partially to an increase in cardiac hypertrophy (for reviews see [117, 118, 119]) and an improvement in cardiac capillarization [120, 17]. Regarding the development of cardiac hypertrophy, recent research has shown that exercise-induced cardiac hypertrophy involves several signaling pathways, including those mediated by Akt [121, 122].

In the last few years, evidence has emerged that the heart is not a terminally differentiated organ but has an intrinsic regenerative potential. The replacement of cardiac cells (cardiomyocytes, fibroblasts, endothelial and vascular cells) seems to take place by an activation of cardiac-resident stem cells, which are located in cardiac stem cell niches [123, 124, 125, 126, 127], or by the recruitment of blood circulating progenitor cells [51, 52, 53, 54]. Resident cardiac stem cells have been identified as cells that are positive for various stem or progenitor cell markers (e.g., Kit, Sca-1, Isl-1, and side population properties) [128, 125, 126, 127]. Cardiac stem cells have been described to divide symmetrically and asymmetrically, with the symmetrical division predominating. Thus, the replicating cardiac stem cell gives rise to one daughter and one daughter committed cardiac stem cell. By this mechanism of growth kinetics, the pool of primitive cardiac stem cells is preserved, and a myocyte progeny is generated together with endothelial and smooth muscle cells [127]. Up to now nothing has been written on whether physical activity may improve or influence the cardiac stem cell pool.

Although research on the self-renewing capacity of the heart is still lacking, the self-repair capacity of the cardiac muscle seems to be limited. In most cases the damage to cardiomyocytes resulting from ischemic injury is irreversible and leads to the development of progressive heart failure, which is characterized by the loss of functional cardiomyocytes. In these cases, cell-based transplantation therapy provides a potential alternative approach for replacing damaged myocardial tissue and restoring cardiac function [125]. There is evidence that physical activity increases the number of circulating bone-marrow-derived progenitor cells [51, 52, 53] and also improves their migratory capacity in patients after myocardial infarction [129], as well as that short intensive exercise can increase the migratory activity of MSCs (Schmidt et al. in press). It has not been shown whether this improvement of the stem/progenitor cell activation may be attributed to an increased homing, transmigration, and differentiation of the circulating progenitor cells into cardiomyocytes.

In conclusion, although evidence exists for a self-renewing capacity of the cardiac muscle by resident as well as circulating stem cells, the mechanisms underlying these processes have to be further investigated. Moreover, although preliminary evidence suggests physical activity may be involved in stem-cell-mediated myocardial adaptation and repair, further research is necessary to evaluate the role of physical activity in detail.

6 Future Perspectives

All the results show that exercise seems to affect stem cells. Adaptation processes in sports might be explainable on cellular and molecular levels. Thus, the following remains an open question: What kind of exercise (duration, intensity) under what kind of environmental conditions (high-altitude training) may have the ideal effect on stem cells for adaptation and regeneration? Further results might help to improve the control of training.

However, various questions need to be addressed in the future. Mobilization of stem cells by exercise may be a possible novel therapeutic option to enhance regeneration. Probably, exercise could be used instead of, or in combination with, a medicamentous therapy to induce regeneration by stem cells after, e.g., degeneration of tissues and therefore could reduce drug application. The exogenous application of stem cells represents a new therapeutic option for the treatment of cardiac and skeletal muscle diseases as well as for the treatment of vascular impairment (Fig. 6.3). Although less is known about the influence of physical activity on the self-renewing capability of cardiac muscle, it seems possible that, similar to what has been described in skeletal muscle, physical activity may contribute to an increased predifferentiation of resident cardiac stem cells. In a very recent study it was demonstrated that exercise training for 3 weeks after acute myocardial infarction leads to a significant mobilization and increase in functional activation of bone-marrow-derived circulating progenitor cells in humans [129]. The findings of other studies support the positive effects of stem cell therapies and exercise. Physical exercise might be used as an adjuvant therapy for patients undergoing stem cell transplantation [130]. The combination of bone marrow stem cell therapy [CD45(+)/CD34(–)] and exercise training resulted in significant functional improvement in acute spinal cord injury [116]. Physical activity rescues adult hippocampal neurogenesis after irradiation of the young mouse brain and should be evaluated in rehabilitation therapy of childhood cancer survivors.

Hypoxia, shear stress, and strain may represent first-line mediators of complex pathways in exercise-induced stem cell tissue replacement. In addition, exercise may support stem-cell-induced regeneration by preconditioning and optimizing the microenvironment (e.g., pH alterations or a prearrangement of the extracellular matrix). A better understanding of these mechanisms may make physical activity a useful tool for the regulation of stem cell proliferation and differentiation also in minimally invasive stem cell transplantation therapy.

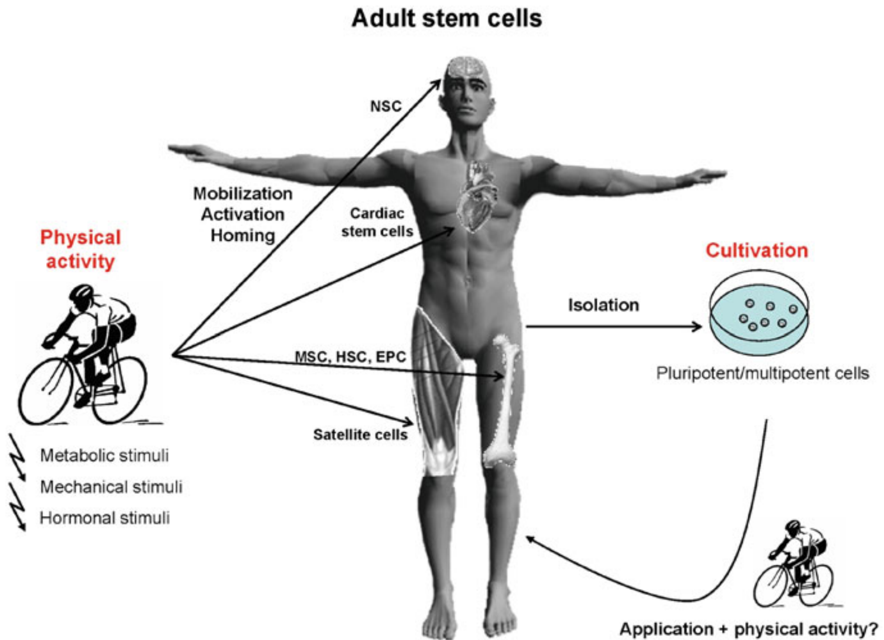

Fig. 6.3 Physical activity influences/activates a variety of adult stem cells which might be released into the circulation or might be activated in their organ-resident state. Thereby, a variety of stimuli such as metabolic, mechanical, and hormonal stimuli might by responsible for the mobilization. These processes might improve adaptational and regenerative processes. Physical activity could also enhance the success of stem cell applications in stem cell therapies by improving the microenvironment for stem cells to home to the site of regeneration. *NSC* neuronal stem cells, *MSC* mesenchymal stem cells, *HSC* hematopoietic stem cells, *EPC* endothelial progenitor cell

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Chapter 7
Mesenchymal Stem Cells for Acute Lung Injury

Jae W. Lee, Naveen Gupta, and Michael A. Matthay

1 Introduction

Morbidity and mortality have declined only modestly in patients with clinical acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) despite extensive research into the pathophysiology [1–3]. Treatment remains primarily supportive with lung protective ventilation and a fluid conservative strategy [4, 5]. Within the past decade, Erickson et al. [6] found in a retrospective cohort of patients enrolled in the Acute Respiratory Distress Syndrome Network a decline in crude mortality from 35 to 26%. However, there were no statistically significant temporal trends in 60-day mortality for the most common causes of lung injury (pneumonia, sepsis, aspiration, and trauma), suggesting that other advancements in critical care, aside from low tidal ventilation, accounted for the improvement in survival. Currently, pharmacologic therapies that reduce the severity of lung injury in vivo and in vitro have not yet been translated into effective clinical treatment options. Consequently, innovative therapies are needed.

Recent studies have suggested that bone marrow-derived multipotent mesenchymal stem cells (MSCs) may have therapeutic applications in several clinical disorders, including myocardial infarction [7–9], diabetes [10], sepsis [11], hepatic failure [12], and acute renal failure [13]. MSCs have also been studied in several in vivo animal models of lung disease. Cell-based therapy with MSCs for the treatment of ALI is very attractive. MSCs, owing to their multipotent nature and their ability to secrete multiple paracrine factors such as growth factors, factors regulating endothelial and epithelial permeability, and anti-inflammatory cytokines, can potentially treat the major abnormalities that underlie ALI, such as impaired alveolar fluid clearance (i.e., resolution of pulmonary edema) and altered lung endothelial permeability. This chapter focuses first on describing the existing experimental literature that has tested the use of MSC in models of ALI/ARDS, and then on describing the

J.W. Lee (✉)
Departments of Medicine and Anesthesiology, University of California, San Francisco, CA 94143, USA
e-mail: leejw@anesthesia.ucsf.edu

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potential mechanisms underlying their therapeutic use with an emphasis on secreted paracrine soluble factors. There will also be a discussion of current challenges [14] that should be resolved prior to clinical trials.

2 Background

Adult stem cells are tissue-specific cells that have retained the ability for self-renewal and to differentiate into a variety of cell lineages. Although adult stem cells do not possess the full range of plasticity of embryonic stem cells, they offer practical advantages including ease of isolation and propagation, and they are not associated with the ethical controversy that surrounds embryonic stem cell research. One class of adult stem cells that has been of particular interest is MSCs. MSCs, also called "marrow stromal stem cells," were first discovered in 1968 by Friedenstein [15], who found bone marrow stromal cells that were adherent, clonogenic, and fibroblastic in appearance. Adult MSCs can now be isolated from almost every type of connective tissue, such as the bone marrow, placenta, and adipose tissue [16].

Bone marrow-derived MSCs reside near the sinusoids and function as support cells for hematopoietic stem cells. Although MSC comprise less than 0.1% of all bone marrow cells, they can be isolated from whole bone marrow aspirates because of their ability to adhere to plastic and form colonies. Currently, there are no MSC-specific cell-surface markers. Consequently, in 2006, the International Society of Cellular Therapy defined MSCs by three criteria: [1] MSCs must be adherent to plastic under standard tissue culture conditions; [2] MSCs must express certain cell-surface markers such as CD105, CD90, and CD73, but must not express other markers, including CD45, CD34, CD14, and CD11b; and [3] MSCs must have the capacity to differentiate into mesenchymal lineages including osteoblasts, adipocytes, and chrondoblasts under in vitro conditions [17]. Use of these cells for therapeutic purposes in a variety of diseases has attracted considerable attention owing to their low immunogenicity, their immunomodulatory effects, and their ability to secrete anti-inflammatory cytokines and endothelial and epithelial growth factors (Table 7.1).

Allogeneic MSCs are able to evade clearance by the host immune system through a variety of mechanisms, including low expression of the major histocompatibility complex (MHC) I and II proteins as well as lack of the T cell costimulatory molecules CD80 and CD86 [18]. This property makes MSCs attractive for cell-based therapy because they can be administered to patients without human leukocyte antigen matching. However, recent literature has shown that MSCs can express higher levels of the MHC class proteins than originally thought. Specifically, at low levels of exposure to interferon-γ, MSCs upregulate expression of MHC II and possess some immunostimulatory properties [19–21]. In addition, recent studies have demonstrated that infusion of allogeneic MSCs can elicit a host response and lead to graft rejection [22]. Therefore, the original belief that MSCs have low immunogenicity is not entirely correct. These cells have complex interactions with the innate and adaptive immune systems.

Table 7.1 Paracrine factors secreted by mesenchymal stem cells (MSCs) with a potential role in acute lung injury/acute respiratory distress syndrome (ALI/ARDS)

Soluble factors[a]	Important in
Keratinocyte growth factor	Apoptosis
Hepatocyte growth factor	Surfactant synthesis
Epidermal growth factor	Alveolar fluid transport
	Endothelial permeability?
Angiopoietin-1	Epithelial and endothelial permeability?
Interleukin-1 receptor antagonist	Anti-inflammatory Activity
Interleukin-10	
Prostaglandin E_2	
To be determined	Antibacterial Activity

A correlation between the level of cytokines, growth factors, and antipermeability factors secreted will need to be made with the therapeutic efficacy in animal and human models of ALI/ARDS. For example, will MSCs improve alveolar fluid clearance, lung water or lung endothelial permeability to protein or reduce the inflammatory milieu within the injured alveolus.
[a]Secretion of some soluble factors may be dependent on cell–cell contact or the alveolar milieu itself, such as interleukin-10 or prostaglandin E_2

3 MSCs in Animal and Human ALI Models

Allogeneic MSCs have been studied in several in vivo models of lung disease [23–32]. In bleomycin-induced lung injury and fibrosis, MSCs improved survival and lung inflammation when given intravenously. These beneficial effects were not accounted for by lung engraftment rates (less than 5%) but rather through a paracrine mechanism [24, 27]. In a follow-up study, Ortiz et al. [25] found that a subpopulation of mouse MSCs produced interleukin (IL)-1ra that was capable of attenuating the severity of bleomycin-induced lung injury. In the same study, these authors also isolated a subpopulation of human MSCs, approximately 5%, that produced high levels of IL-1ra. To determine the effect of MSCs on ALI, several groups studied the therapeutic effect of MSCs following intraperitoneal [28] or intratracheal administration of *Escherichia coli* endotoxin [30, 32]. Xu et al. [28] found that intravenous administration of MSC following intraperitoneal administration of lipopolysaccharide (LPS) prevented endotoxin-induced pulmonary inflammation, injury, and edema as well as the influx of neutrophils into the injured alveoli. In addition, Xu et al. [32] and Mei et al. [30] also discovered that transfection of MSCs with human angiopoietin-1 (Ang1) further reduced the parameters of *E. coli* endotoxin-induced lung injury.

Despite the promising results, little was known regarding the effect of bone marrow-derived MSCs as therapy in experimental models of ALI and pulmonary edema. Most prior studies did not adequately evaluate MSCs as a treatment modality; the cells were given concurrently with the injury or before the injury, primarily by the intravenous route of delivery. However, we reported that intrapulmonary (via

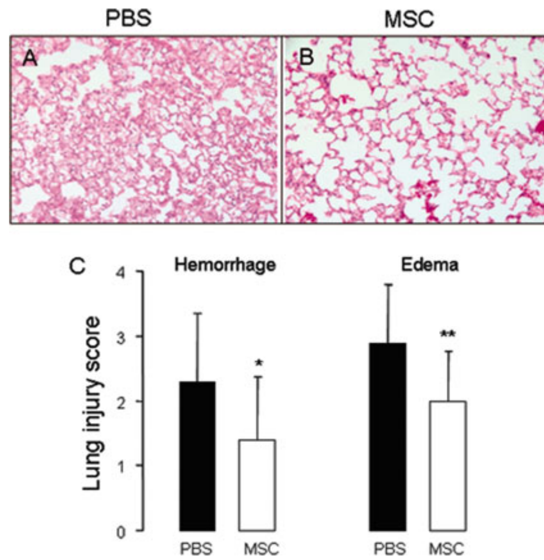

Fig. 7.1 Mesenchymal stem cells (MSCs) improved lung injury as assessed by histologic methods despite the low levels of engraftment detected. Hematoxylin and eosin staining of lung sections demonstrated attenuated lung injury in the MSC group (**b**) compared with the phosphate buffered saline (PBS) group (**a**) at 48 h after instillation of endotoxin. (**c**) Quantification of lung injury showed a significant reduction in the degree of hemorrhage and edema in the mice receiving MSCs (*$p < 0.05$; **$p < 0.01$). The data are expressed as the mean ± the standard deviation. (From Gupta et al. [29], reprinted with permission, *Journal of Immunology*, copyright 2007 The American Association of Immunologists, Inc)

the trachea) treatment with MSCs 4 h *after* endotoxin delivery to the lung improved survival and reduced the extent of pulmonary edema formation in *E. coli* endotoxin-induced ALI in mice [29] (Figs. 7.1 and 7.2). MSC therapy reduced the plasma and bronchoalveolar lavage levels of proinflammatory cytokines and increased the levels of several anti-inflammatory cytokines, including IL-10. In addition, treatment with MSCs in endotoxin-induced lung injury in mice reduced the levels of a type I alveolar epithelial antigen, the receptor for advanced glycation end products [33].

To further define the therapeutic potential of MSCs, we have developed two human models of ALI: [1] an ex vivo human lung preparation perfused partially with human blood injured by *E. coli* endotoxin and [2] primary cultures of human alveolar epithelial type II cells grown in a Transwell plate with an air–liquid interface injured by an inflammatory insult (Fig. 7.3). In the ex vivo perfused human lung, the intrabronchial instillation of human MSCs 1 h following endotoxin-induced lung injury restored alveolar fluid clearance (i.e., the ability to resolve pulmonary edema), in part by the secretion of keratinocyte growth factor (KGF) [34, 35]. In primary cultures of human alveolar type II cells, human MSCs grown in the bottom chamber of a Transwell plate and separated from the type II cells restored the increase in epithelial permeability to protein caused by exposure to inflammatory cytokines in

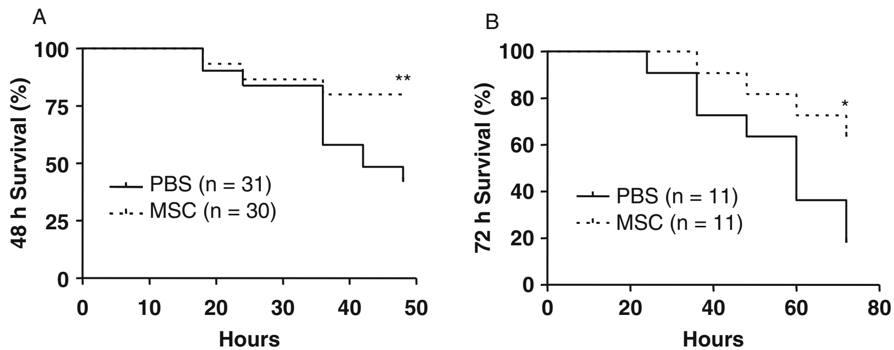

Fig. 7.2 Intratracheal treatment with MSCs improved 48- and 72-h survival in an endotoxin model of acute ling injury in mice. MSC or PBS was administered intratracheally 4 h after intratracheal instillation of endotoxin (5 mg/kg). The 48-h survival was 80% in the MSC group and 42% in the PBS group [$n = 30$ for the MSC group, $n = 31$ for the PBS group, $^{**}p < 0.01$ using a log-rank test (**a**)]. At 72 h, survival was 64% in the MSC group and 18% in the PBS group [$n = 11$ per group, $^{*}p < 0.05$ using a log-rank test (**b**)]. (From Gupta et al. [29], reprinted with permission, *Journal of Immunology*, copyright 2007 The American Association of Immunologists, Inc)

part by the secretion of Ang1 [36]. Further studies are in progress to further define the mechanisms of benefit in both models.

4 Mechanism (Engraftment)

Much of the initial interest in MSC therapy stemmed from the multipotent properties of the cells. Krause et al. [37] found that a single bone-marrow-derived cell could give rise to cells of multiple different organs, including the lung. They reported up to 20% engraftment of bone marrow-derived cells in the lung, including epithelial cells, from a single hematopoietic precursor. Kotton et al. [38] found that plate-adherent cultured bone marrow cells when given intravenously in wild-type mice following bleomycin-induced lung injury engrafted into the recipient lung parenchyma with a morphologic and molecular phenotype of alveolar type I pneumocytes. This gave rise to intensive investigation into the possibility that bone marrow-derived stem cells, MSCs specifically, may be able to regenerate the lung epithelium and/or endothelium [39–41]. However, these results were questioned by multiple groups who observed only engraftment of leukocyte lineages [42], or low engraftment rates in lung injury models with observed rates of less than 1% [24, 27, 43, 44]. Despite initial interest in the multipotent properties of MSCs, engraftment in the lung now does not appear to play a major beneficial role. The beneficial effect of MSCs appears to derive more from their capacity to secrete paracrine soluble factors that modulate immune responses as well as alter the responses of endothelium or epithelium to injury through the release of growth factors [11, 35, 36, 45–51].

However, the role of stem cell engraftment in repair following lung injury requires further research, as suggested by several recent publications. Sueblinvong

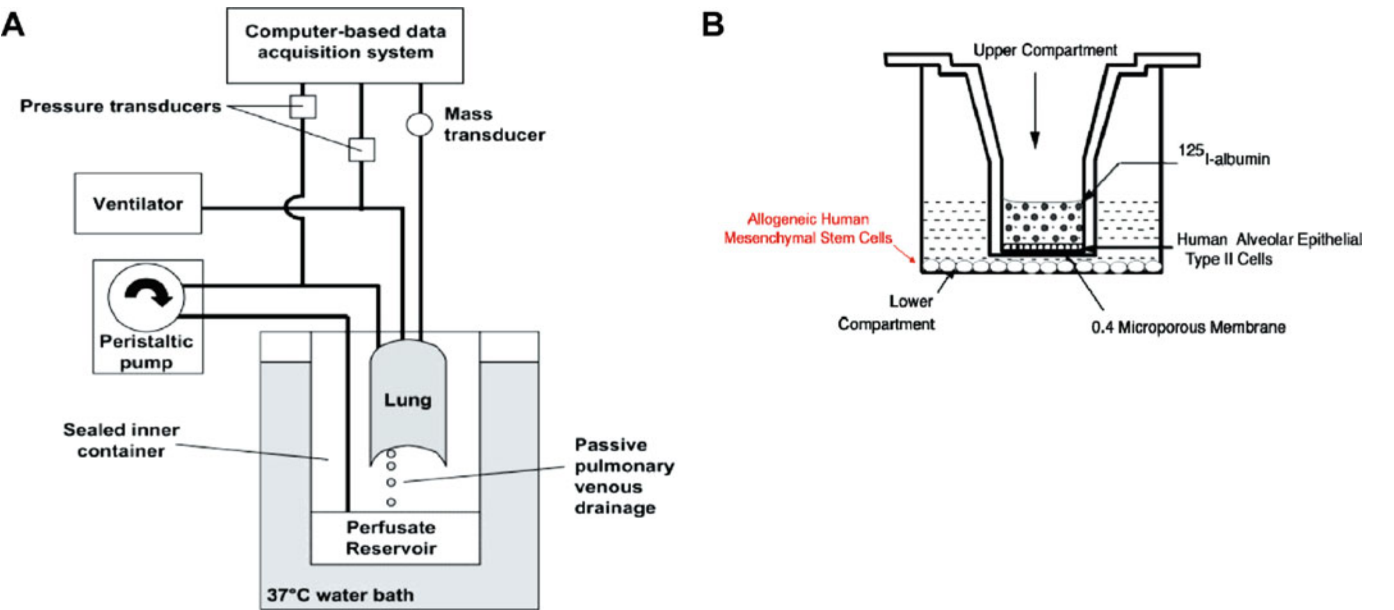

Fig. 7.3 The ex vivo perfused human lung (**a**) and primary cultures of human alveolar epithelial type II cells grown on a Transwell plate with an air–liquid interface (**b**). (**a**) The right or left human lung was selected for perfusion if the total ischemic time was less than 48 h and if the selection criteria were met. The lung was initially rewarmed and perfused with medium (DME H-21) containing 5% albumin over 1 h and inflated and oxygenated with 10 cm H_2O continuous positive airway pressure (Fraction of Inspired Oxygen, FiO_2 0.95). The perfusion rate was set at 0.30–0.4 l/min and the left atrial pressure at 0 mmHg. Following rewarming, alveolar fluid clearance (AFC) was measured in the right or left upper lobe as described [34]. If AFC >10%/h, 100 ml of fresh human blood was added to the perfusate for a final hematocrit level of 4%, and *Escherichia coli* endotoxin was instilled in the right middle or left lower lobe. Allogeneic human MSCs or control lung fibroblasts were added to the endotoxin-injured lung lobe 1 h after the endotoxin instillation. (**b**) Human alveolar epithelial type II cells were isolated from human donor lungs that were declined for transplantation by the Northern California Transplant Donor Network [82] and plated on collagen I-coated 24-well plates (Biocoat, BD Biosciences) at 5% CO_2 at 37°C at a concentration of 1.0×10^6 cells per well. Net fluid transport was measured across human type II cells on Transwell plates (0.4-μm pore size and collagen I-coated, CoStar, Corning) following an inflammatory injury (cytomix) with and without MSCs in the bottom chamber as previously described [82]. (**a**) From Frank et al. [34], reprinted with permission, *American Journal of Physiology. Lung Cellullar and Molecular Physiology*, copyright 2007 from the American Physiological Society)

et al. [52] found that human umbilical cord MSCs when cultured in vitro with specialized growth medium/growth factors expressed Clara cell secretory protein (CCSP), surfactant protein C (SP-C), and cystic fibrosis transmembrane conductance regulator (CFTR). More significantly, after systemic administration to immunotolerant, NOD-SCID mice, rare cells were localized in the lung airway epithelium that expressed cytokeratin and human CFTR. Wong et al. [53, 54] found a subpopulation of adherent human and murine bone marrow cells that expressed CCSP as well, and when cultured ex vivo with an air–liquid interface, these CCSP+ cells expressed alveolar type I and II markers such as pro-SP-C, CFTR, and epithelial sodium channel (ENaC). CCSP+ cells preferentially homed to naphthalene-damaged airways when delivered transtracheally or intravenously. Interestingly, these bone marrow cells expressed CD45 and the MSC markers CD73, CD90, and CD105 [17].

Recently, we obtained and characterized MSCs from bone marrow of green fluorescent protein (GFP) transgenic mice and developed an in vitro model to study the endodermal differentiation of MSCs using co-cultures of MSCs and transformed lung epithelial (A549) cells. MSCs in co-culture experiments with A549 were separated by a cell-impermeable membrane to eliminate the possibility of cell fusion. Under these conditions, MSCs expressed several lung epithelial markers (cytokeratins 5, 8, 14, 18, and 19, pro-SP-C, ZO-1), detected using quantitative reverse transcriptase polymerase chain reaction and Western blot. β-Catenin signaling was activated in MSCs. Treatment of MSCs with 10–20 mM lithium chloride activated the β-catenin pathway and enhanced expression of epithelial markers, although this activation was transient. We concluded that A549 cells could trigger endodermal epithelial differentiation of MSCs by a paracrine mechanism that may include activation of β-catenin signaling [55]. We further investigated the participation of bone marrow cells in the process of airway epithelial restoration after naphthalene-induced injury. We transplanted sex-mismatched GFP-tagged bone marrow-derived cultured plastic-adherent MSCs into 5-Gy-irradiated C57BL/6 mice recipients. After 1 month of recovery, experimental animals were subjected to 250 mg/kg naphthalene intraperitoneal (IP). Animals were killed at 2–30 days after naphthalene administration. By immunofluorescence, immunohistochemistry, and in situ hybridization for the Y chromosome, there were patches of donor-derived cells in the large and small conducting airways, mostly at 2–6 days after injury. GFP^+ cells in the epithelium of airways were positive for pancytokeratin and some other epithelial markers. Although rare, GFP^+ cells formed clear isolated patches of the bronchial epithelium, consistent with clone formation. Some cells were also positive for proliferating cell nuclear antigen, a marker of proliferating cells [56].

5 Mechanism (Immunomodulation)

A major characteristic of MSCs is the immunomodulatory properties of the cells. Multiple studies have demonstrated that MSCs possess potent immunosuppressive effects by inhibiting the activity of both innate and adaptive immune cells [46, 47, 57, 58]. This immunosuppression is mediated by cell-contact-dependent

and cell-contact-independent mechanisms through the release of soluble factors. The list of candidate mediators released or induced by MSCs includes transforming growth factor (TGF)-β, prostaglandin E_2 (PGE_2), indoleamine 2,3-dioxygenase, hepatocyte growth factor (HGF), IL-10, and IL-1ra among others. In a model of sepsis following cecal ligation and puncture in mice, Nemeth et al. [11] found that bone marrow-derived MSCs, activated by LPS or tumor necrosis factor α (TNF-α), secreted PGE_2, which reprogrammed alveolar macrophages to secrete IL-10. The beneficial effect of MSCs on mortality and improved organ function following sepsis (cecal ligation and puncture) was eliminated by macrophage depletion or pretreatment with antibodies to IL-10 or the IL-10 receptor, suggesting an essential role for IL-10 in these experiments; IL-10 is a cytokine secreted predominantly by monocytes that downregulates the expression of Th1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages (Fig. 7.4). Interestingly, in co-culture experiments, cell contact between MSCs and macrophages was required to stimulate IL-10 production following LPS stimulation; MSCs separated by a Transwell plate or MSC-conditioned medium could not induce IL-10 production [11]. In a model of ALI following intratracheal administration of *E. coli* endotoxin in mice [29], intrapulmonary administration of MSCs improved survival and lung injury in association with a decrease in macrophase inflammatory protein 2 and TNF-α levels in the bronchoalveolar lavage fluid and elevated levels of IL-10 in both the plasma and the bronchoalveolar lavage fluids. In bleomycin-induced lung injury and fibrosis in mice, Ortiz et al. [25] found that MSCs decreased subsequent lung collagen accumulation, fibrosis ,and levels of matrix metalloproteinases in part by IL-1ra secretion; IL-1ra is a cytokine that competitively competes with IL-1β for IL-1 receptor binding. IL-1β is one of the major inflammatory cytokines in pulmonary edema fluid in patients with ALI/ARDS [59]. These results confirmed the anti-inflammatory effect of MSCs in multiple lung injury experiments in mice [24, 27, 28, 30, 32].

Despite the well-documented immunosuppressive effects of MSCs, recent literature described a dual role for MSCs as immunostimulatory cells as well [21]. As explained above, some studies have reported that MSCs can upregulate expression of MHC II when exposed to low levels of inflammation and function as antigen-presenting cells stimulating the adaptive immune system [19, 20]. Recent evidence has also shown that MSCs can secrete IL-6 and induce production of IgG by B lymphocytes in an in vitro setting [60]. In addition, MSCs can prevent neutrophil apoptosis and degranulation in culture without inhibiting their phagocytic or chemotactic capabilities [61]. Thus, recent studies have demonstrated that MSCs have more complex effects on the immune system than their classic role as immune suppressor cells. Understanding the mechanisms responsible for these apparently paradoxical roles that MSCs play in the immune response will be important in developing cell-based therapy for clinical use.

A safety concern with MSC-based therapy, particularly in treating ARDS, is the effect of MSCs on host defense against bacterial infection. Bacterial pneumonia and sepsis from a nonpulmonary cause are two of the most common origins of ARDS [2]. Given the preponderance of literature that describes the immunosuppressive effect of MSCs, there is concern that this effect may impede the host's ability to

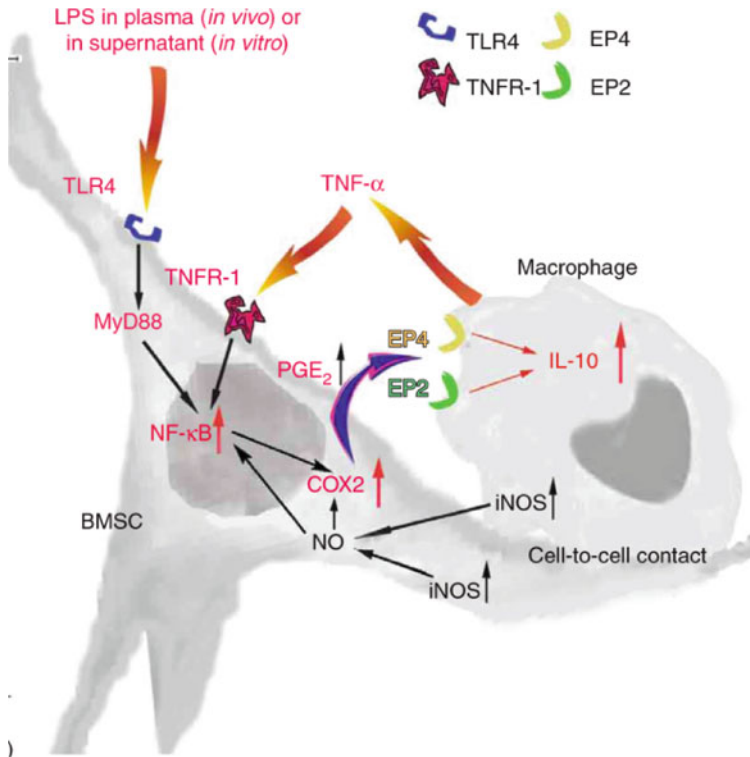

Fig. 7.4 The potential mechanism underlying the interaction between MSCs and alveolar macrophages in the cecal ligation and puncture sepsis model leading to its therapeutic efficacy. Bacterial toxins (lipopolysaccharide) and circulating tumor necrosis factor (TNF)-α act on TLR4 and TNFR-1 of the MSCs, respectively. This results in the translocation of nuclear factor κB (NF-κB) into the nucleus; an activation process, which may be nitric oxide dependent. Activated NF-κB induces the production of COX2, resulting in increased production and release of prostaglandin E_2. Prostaglandin E_2 binds to EP2 and EP4 receptors on the macrophage, increasing its interleukin-10 secretion and reducing inflammation. (From Nemeth et al. [11], adapted by permission from Macmillan Publishers Ltd: *Nature Medicine*, PMID 19098906, copyright 2009)

clear an infection. However, as mentioned previously, there is new work describing a dual role for MSCs in regulating the immune system and their immunostimulatory effects. Furthermore, there is a recent report demonstrating a protective effect of systemically administered MSCs in a mouse model of bacterial sepsis [11] as well as preliminary data from our own group that MSCs are associated with a reduction in the number of live bacteria in *E. coli* pneumonia in mice [62]. Additional work is needed to better define the effects of MSCs in the setting of a bacterial infection before MSC-based therapy can be used in patients with ALI/ARDS.

Another safety concern with administering MSCs to patients is the potential for the MSCs to undergo malignant transformation or to propagate existing tumors. Although human MSCs have not been shown to cause malignancy, mouse MSCs

have been shown to induce malignant tumors in mice [63, 64]. There remains concern that MSCs may transform after repeated passage in vitro since studies have demonstrated that some of the cells develop abnormal karyotypes, which predispose the cells to malignant transformation [65]. In addition, in mice models, MSCs have been found to enhance the metastatic potential of solid tumors, such as breast cancer [66, 67]. However, a recent report has provided evidence that MSCs may have anti-angiogenic properties as well that could inhibit tumor growth [68]. Ironically, because MSCs may have greater immunogenicity they may prove to be beneficial with respect to the concerns of malignancy, since their eventual recognition and clearance by the host immune system would make the development of tumors less likely.

6 Mechanism (Alveolar Fluid Clearance)

Impaired alveolar fluid clearance (i.e., the resolution of pulmonary edema) is common in patients with ALI/ARDS. The level of alveolar fluid clearance impairment has a significant prognostic value in determining morbidity and mortality [69, 70]. Several experimental studies have investigated the mechanisms that reduce alveolar fluid clearance in ALI, and several pathways have been implicated [71, 72]. In the alveolar environment, basal alveolar fluid clearance is determined predominately by amiloride-sensitive and amiloride-insensitive sodium channels and the activity of the Na-K ATPase [71, 73–76]. Several catecholamine-dependent and catecholamine-independent factors can upregulate alveolar fluid clearance, including β-adrenergic agonists via cyclic AMP (cAMP)-dependent mechanisms [71, 72]. In the mouse and the human lung, cAMP-dependent alveolar epithelial fluid transport is dependent on CFTR activity, especially in mediating β-adrenergic receptor driven alveolar epithelial fluid transport [77–79].

In ALI, we and other investigators have reported that pulmonary edema fluid contained high levels of several proinflammatory cytokines, including IL-1β, IL-8, TNF-α, and TGF-β_1 [80–82]. Several of these proinflammatory cytokines have been studied in experimental fluid transport experiments. For example, TNF-α decreased the expression of ENaC (α, β, and γ subunits) messenger RNAs (mRNAs) and protein levels as well as the amiloride-sensitive current and ouabain-sensitive Rb^+ uptake in rat alveolar epithelial cells [83]. Similarly, IL-1β decreased dexamethasone-induced αENaC mRNA and protein levels and the amiloride-sensitive fraction of the transepithelial current and sodium transport across rat type II cell monolayers [84]. More recently, we reported that TGF-β_1 decreased the amiloride-sensitive fraction of Na^+ uptake and fluid transport across monolayers of rat and human type II cells as well as αENaC mRNA and protein expression [85]. In chronic inflammation associated with nasal polyposis, TGF-β_1 downregulated CFTR mRNA and protein expression as well as the cAMP-dependent current in human nasal epithelial cells [86].

Bone marrow-derived MSCs are known to produce several epithelial specific growth factors, specifically KGF, the seventh member of the fibroblast growth factor (FGF) family. We have been particularly interested in KGF because of work from

our group as well as other investigators who have reported that KGF can reduce lung injury in small animal models of pulmonary edema. Recombinant KGF pre-treatment reduced mortality following intratracheal instillation of hydrochloric acid [87, 88], bleomycin [89, 90], hyperoxia [91, 92], and *Pseudomonas aeruginosa* [93]. In rat lung, KGF improved alveolar fluid transport in part by upregulating αENaC gene expression [94] and Na-K ATPase activity [95].

In the ex vivo perfused human lung, intrabronchial instillation of human MSCs 1 h following endotoxin-induced lung injury restored alveolar fluid clearance in part by the secretion of KGF [35]. Several properties of KGF could explain the therapeutic effect of human MSCs on restoring alveolar fluid clearance, including alveolar epithelial type II cell hyperplasia and differentiation, surfactant production [96], anti-apoptotic effects [97], and increased transcription and/or translation of the major sodium and chloride transport proteins [94]. Because the effect of MSC therapy in the *E. coli* endotoxin-induced lung injury in the ex vivo perfused human lung occurred over a 3-h time period, the therapeutic benefit of KGF in these experiments is less likely explained by type II cell hyperplasia or transcriptional effects. Alternatively, an increase in vectorial fluid transport across the alveolar epithelium can be mediated by an increase in trafficking of sodium transport proteins to the cell surface [98, 99].

7 Mechanism (Lung Endothelial Permeability)

Another possible mechanism through which MSCs may be potentially beneficial is through therapeutic effects on the injured lung endothelium. The integrity of the lung microvascular endothelium is essential to prevent the influx of protein-rich fluid from the plasma as well as inflammatory cells which may further aggravate the ability of the lung epithelium to reduce alveolar edema. Several paracrine soluble factors, such as Ang1 and KGF, are potentially interesting. Ang1, a ligand for the endothelial Tie2 receptor, is a known endothelial survival [100] and vascular stabilization factor that reduces endothelial permeability and inhibits leukocyte–endothelium interactions by modifying endothelial cell adhesion molecules and cell junctions [101–104]. MSCs or MSCs (used as a vehicle for gene delivery) transfected with the human Ang1 gene reduced both pulmonary vascular endothelial injury and the recruitment of inflammatory cells into the lung in mice injured by LPS-induced lung injury [30, 32, 105]. In the study by Mei et al. [30], the transfection of Ang1 further reduced lung inflammation and nearly completely reversed the LPS-induced increase in lung permeability. We recently found that allogeneic human MSCs secrete a significant amount of Ang1. In addition, using small interfering RNA technology, the secretion of Ang1 was responsible for the therapeutic effect on epithelial protein permeability among primary cultures of human alveolar epithelial type II cells injured by an inflammatory insult [36].

MSCs produce several epithelial specific growth factors, such as KGF. In models of acute permeability edema such as α-naphthylthiourea [95, 106], *P. aeruginosa* [93], and ventilator-induced lung injury [107], KGF reduced lung edema and bronchoalveolar lavage protein levels. Cultured allogeneic human MSCs produced

substantial quantities of KGF. The role of KGF is intriguing given the findings of previous studies of ALI in animal models and a recent study by Murakami et al. [108], who reported that FGF2, FGF4, and FGF8, which are specific for both FGF receptors 1IIIc and 3IIIc, are responsible for the maintenance of endothelial barrier homeostasis. Another epithelial specific growth factor secreted by MSCs is HGF. Previously, HGF was found to induce pulmonary endothelial cell integrity by the inhibition of Rho GTPase and the prevention of actin stress fiber formation and paracellular gaps among pulmonary endothelial cells injured by thrombin [109, 110]. In the future, it will be important to understand the contribution of each soluble factor secreted by MSCs to lung endothelial permeability in ALI.

The potential role of other bone marrow-derived cells such as endothelial progenitor cells in ALI as therapy has been studied in animal experiments [111] as well as in some clinical trials [112, 113]. In an oleic acid lung injury in rabbits, Lam et al. [111] found that autologous transplantation of endothelial progenitor cells preserved not only the pulmonary alveolar-capillary barrier but also pulmonary vascular endothelial-dependent relaxation. In the future, the role of engraftment, transdifferentiation or fusion with both alveolar epithelial and lung endothelial cells will need to be studied further.

8 Challenges

The isolation and classification of human MSCs must be further defined, particularly concerning the issue of potency. Are all MSCs the same despite different isolation techniques, organ niches and growth conditions, passage number, and therapeutic use? This is particularly important since MSCs lack a specific cell-surface marker which distinguishes them from other cells (based on the classification of the International Society of Cellular Therapy) [17]. For example, although confluent cultures of MSCs have a uniform fibroblast-like appearance, two types of cells exist within the culture, type 1 or rapidly self-renewing MSCs and type 2 or slowly replicating MSCs, which have different patterns of gene expression, surface epitopes, clonogenicity, potential to differentiate, and tendency to generate lethal pulmonary emboli following intravenous infusion [114]. A potency assay should be developed to compare and contrast MSCs currently in use. Some secreted paracrine soluble factors that may constitute such a potency assay are included in Table 7.1. Perhaps more significantly, a functional assay specific to the clinical disease entity being studied must be correlated with the levels of the secreted factors. The behavior of MSCs from different tissues may depend on the niche to which they are being subjected. For example, Koga et al. [115] found that MSCs from synovial tissue were more effective in the repair of cartilage defects of the knee in rabbits than MSCs from bone marrow, muscle, or fat.

Second, a more precise understanding of the mechanisms underlying the therapeutic effect of MSCs in models of lung injury is needed. Although most investigators have invoked both the immunomodulatory and the growth factor production properties of MSCs to explain the protective effects, the exact mechanisms

responsible for these effects remain unclear. For example, MSCs secrete or induce production of a variety of soluble factors, such as IL-10, PGE_2, TGF-β, and KGF, but it is not known which of these factors is essential in the protection provided by MSCs. In addition, another major question is whether the effect is produced predominantly through cell-contact-dependent or cell-contact-independent mechanisms or both (Fig. 7.4), and whether or not the functional behavior of the cells changes depending on the alveolar milieu. Answering these questions will determine whether the effect of MSCs can be replicated with a mixture of recombinant soluble factors secreted by MSCs or with MSC-conditioned medium alone.

Lastly, although the focus of this review has been predominantly on the potential use of bone marrow-derived MSCs, other adult stem cells, such as MSCs derived from placental or amniotic and endothelial progenitor cells, may have more therapeutic potential [116]. As we began to understand further the mechanisms underlying the therapy efficacy, we will need to expand our experiments to determine the optimal source for the most effective stem cell.

9 Conclusions

ALI/ARDS is the most common cause of acute hypoxemic respiratory failure in critically ill patients. Current treatment for ALI/ARDS is supportive and therefore new treatments are needed. MSCs are adult stem cells most commonly isolated from the bone marrow that possess unique immunomodulatory and paracrine properties which make them attractive for cell-based therapy. There has been rapidly emerging literature demonstrating the therapeutic potential of MSCs in various organ injury models, such as myocardial infarction [7–9], diabetes [10], sepsis [11], hepatic failure [12], and acute renal failure [13]. Recently, some investigators have also reported that MSCs have beneficial effects in experimental models of ALI in both animals [29, 30, 32] and human tissue [35, 36]. Given the promising initial results obtained with the use of MSCs in experimental models of ALI/ARDS, there has been enthusiasm to advance cell-based therapy to patients with ALI/ARDS. Although clinical trials of MSC-based therapy have been initiated in patients with cardiac, renal, and autoimmune diseases, there are several questions that need to be addressed before cell-based therapy can be tested in patients with ALI/ARDS. Future research in this field should continue to focus on elucidating the basic mechanisms responsible for the beneficial effects of MSCs, as well as determining the practical issues involved in translating a cell-based therapy for patients prior into clinical trials.

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Chapter 8
Animal Models of Lung Injury: Role for Mesenchymal Stem Cells

Mauricio Rojas, Smita Iyer, Carter Co, and Kenneth L. Brigham

1 Introduction

Adult stem cells are specific cells that have retained the ability to differentiate into a variety of cell lineages, thereby making them multipotent. Although adult stem cells do not possess the full range of plasticity of embryonic stem cells, they offer practical advantages including ease of isolation and propagation. Mesenchymal stem cells (MSC) are emerging as a therapeutic modality in various lung diseases, including chronic obstructive pulmonary disease (COPD), acute lung injury (ALI), and cystic fibrosis. MSCs have been isolated from multiple tissues, including adipose tissue [1], skeletal muscle [2], synovium [3], spleen, thymus [4], blood, lung, fetal blood [5], and amniotic fluid [6]. The most accessible and by far the best characterized source of MSCs is the bone marrow, and much of what we know about MSCs in lung repair is based on studies in bone marrow MSCs [7, 8].

In this chapter, we describe that, according to studies in animal models, MSCs and their immunomodulatory properties can confer substantial protection in the setting of lung diseases.

2 MSCs and Animal Models of ALI

The ability of MSCs to create a tolerogenic niche by direct interaction with immune cells and by secretion of regulatory molecules makes them attractive therapeutic candidates for regulating the inflammatory response to infection or injury. Several studies, including studies by our group, have demonstrated compelling benefits from the administration of MSCs in animal models of lung injury (Fig. 8.1).

M. Rojas (✉)
Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Center for Translational Research in the Lung, McKelvey Center for Lung Transplantation, Emory University, Atlanta, GA 30322, USA
e-mail: mrojas@emory.edu

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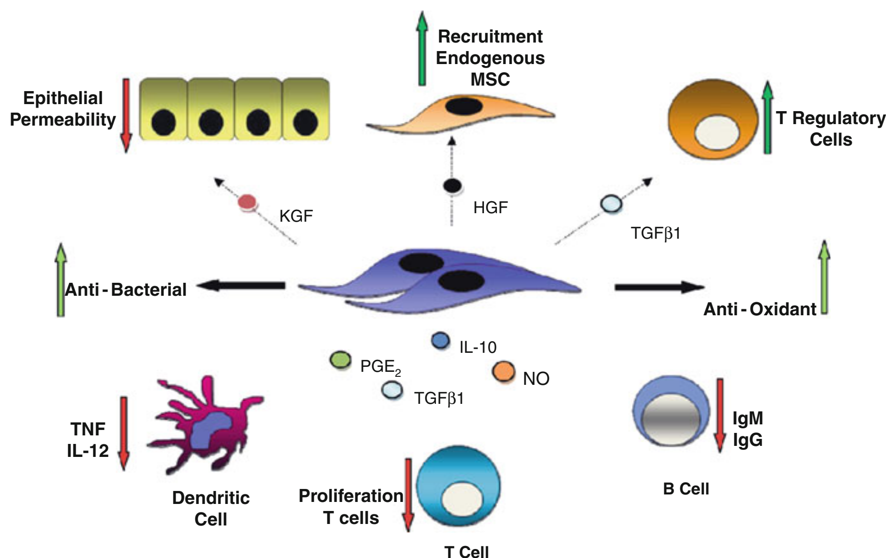

Fig. 8.1 Effector molecules produced by mesenchymal stem cell (MSCs) that inhibit lymphocyte proliferation. MSCs inhibit T-lymphocyte proliferation via soluble effector molecules. MSCs constitutively express tolerogenic mediators such as hepatocyte growth factor, transforming growth factor β_1 (TGF-β_1), and interleukin (IL)-10. MSCs also express COX2, the enzyme involved in prostanoid synthesis, and Prostaglandin E_2 (PGE_2) can be detected in unstimulated MSCs. PGE_2 inhibits T-lymphocyte proliferation via accumulation of cyclic AMP. Stimulated MSCs produce nitric oxide, which suppresses lymphocyte proliferation by inhibiting stat5 phosphorylation. MSCs also interact with other immune cells. At high concentrations MSCs inhibit B-cell proliferation, immunoglobulin secretion, and chemokine receptor expression. MSCs inhibit production of tumor necrosis factor α and IL-12 in stimulated dendritic cells. MSCs increase proliferation of T-regulatory cells by elaborating TGF-β_1 production. In addition, an antibacterial and antioxidant effect has been recently described for MSCs

In humans, ALI is initiated by an acute inflammatory response to physical trauma or infection [9], most commonly sepsis [10], and often leads to severe respiratory failure termed "acute respiratory distress syndrome" (ARDS) [11, 12]. ALI is characterized by sequestration of inflammatory cells in the lung, pulmonary edema, and upregulation of proinflammatory mediators both systemically and locally. Administration of lipopolysaccharide (LPS) to mice initiates the cascade of events leading to the pathophysiological changes similar to those seen in human ALI.

Using this murine model of ALI, we have demonstrated that exogenous MSCs from syngeneic donors infused intravenously immediately after intraperitoneal administration of LPS (1 mg LPS/kg body weight) decreased the systemic inflammatory response and attenuated lung injury. After the administration of LPS, the physiological and structural alterations in the lung can be best observed between 6 and 48 h; after the latter time point, it has been shown that the injury begins to resolve by itself [13]. The MSC infusion protected the injured mice from the development of pulmonary edema and histological examination of lung sections also

demonstrated that there was decreased neutrophil infiltration into the lung. Plasma levels of proinflammatory cytokines, interleukin (IL)-1β, interferon (IFN)-γ, IL-6, and macrophage inflammatory protein (MIP)-1α were all significantly decreased with MSC infusion compared with the levels in LPS-treated mice. Levels of IL-10 were maintained and the levels of granulocyte colony-stimulating factor increased acutely. We observed that the protection conferred by MSC stems from the modulation of the inflammatory response, was not related to clearance of endotoxin, and appeared to be at least partly independent of the amount of MSC engraftment into the lung.

Gupta et al. [14] recently reported similar findings of decreased lung injury and improved survival by intrapulmonary delivery of MSCs in a mouse endotoxin model. They instilled MSCs into the trachea 4 h after giving LPS via the same route and demonstrated decreased lung edema at 24 and 48 h. Protein infiltration into the lung, a measure of leakiness of the alveolar capillary barrier, was decreased at 48 h, but not at 24 h. They also found a decrease in MIP-2 levels in the lung lining fluid by 24 h followed by a decrease in tumor necrosis factor (TNF)-α levels at 48 h. An acute increase (8 h) in the levels of IL-10 was noted in the plasma and lining fluid with MSC infusion. Significant histological improvement in lung injury was observed despite low levels of donor MSC engraftment in the lung. It is evident that both intravenous and intratracheal infusion of MSCs curbs the severe acute inflammatory response systemically and in the lung, and significantly attenuates lung injury.

Several paracrine soluble factors, such as keratinocyte growth factor, are potentially important in these effects, reducing lung edema and bronchoalveolar lavage protein levels [15]. Using an innovative cell-based and gene-based approach, Mei et al. [16] reported that the protective effect of MSCs in the LPS-injured mouse lung is greatly potentiated by infusion of MSC overexpressing angiopoietin 1 (ANGPT1), a vasculoprotective gene. MSC infusion given 30 min after intravenous administration of LPS, significantly decreased airspace neutrophil count 3 days after onset of endotoxemia. In mice given MSCs expressing ANGPT1 (MSC-pANGPT1), the level of inflammation was further reduced and was not significantly different from that in control animals. Infusion of MSC-pANGPT1 resulted in a significantly greater suppression of IFN-γ and IL-1β compared with giving MSCs alone. Furthermore, the level of TNF-α in the lining fluid was significantly reduced only with MSC-pANGPT1 infusion. Recently, Lee et al. [17] described an experimental human model of ALI consisting of an ex vivo human lung preparation perfused partially with human blood and injured by *Escherichia coli* endotoxin. In this model, the intrabronchial instillation of human MSC 1 h following endotoxin-induced lung injury restored alveolar fluid clearance, in part by the secretion of keratinocyte growth factor.

Given the preponderance of literature that describes the immunosuppressive effect of MSCs, there is concern that this effect may impede the host's ability to clear an infection. Furthermore, there has been a recent report demonstrating a protective effect of systemically administered MSCs in a mouse model of bacterial sepsis [18] as well as data from Lee et al. [17] indicating that MSCs are associated with a reduction in the number of live bacteria in *E. coli* pneumonia in mice.

In line with our observations and those of other groups, the protective effect seen with MSC therapy again did not require a high level of engraftment in the lung. These promising results demonstrate that protection conferred by MSCs can be further augmented by gene therapy approaches, where a synergy of the anti-inflammatory effect of MSCs with improved preservation of endothelial function can improve outcomes. Taken together, these findings demonstrate a role of MSCs in mitigating the inflammatory response to LPS and, as a consequence, in attenuating lung injury. These results indicate the potential for MSCs as a therapy in ALI, a disease with high mortality and limited treatment options.

3 MSCs and Cystic Fibrosis

Defects in the cystic fibrosis transmembrane conductance regulator (CFTR) gene lead to the manifestations of cystic fibrosis in many patients. With defective/deficient CFTR, there is decreased chloride secretion/increased sodium absorption, leading to bronchial obstruction, infection, inflammation, and eventual airway destruction. A logical candidate for the use of MSCs as cell therapy is cystic fibrosis. Replacement of alveolar epithelial cells defective in CFTR by normal derived MSCs might result in the improvement of the disease [19]. In this context, Wang et al. [20] isolated bone marrow MSCs from cystic fibrosis patients and transduced them with a viral vector for expression of CFTR. In coculture in vitro experiments, the CFTR-corrected MSCs from patients with cystic fibrosis could correct the defect in Cl^- of airway epithelial cells obtained from cystic fibrosis patients in response to cyclic AMP agonist stimulation. These studies support the capacity of MSCs to acquire an airway epithelial cell phenotype and provide a proof of concept for potential cell-based therapy of cystic fibrosis. However, when CFTR-corrected MSCs were in vivo infused into CFTR-deficient mice, only small numbers of MSCs engrafted into the lungs [20]. These observations confirm that engraftment of MSCs does occur but at low frequency and that appropriate signals are required for the homing and differentiation of MSCs. These signals occur during injury but they are absent under normal conditions.

4 MSCs and COPD

COPD, specifically emphysema, is frequently progressive, resulting in destruction of alveolar septa, leading to airspace enlargement and a consequent decrease in functional alveolar surface area [21]. COPD was ranked sixth among the causes of death globally in 1990 but is projected to be the third most common cause of death by 2020 [22]. In the USA, COPD is currently the fourth leading cause of death.

Cigarette smoking and air pollution are the major risk factors associated with development of emphysematous changes in the adult lung. Although numerous

aspects of the pathogenesis of emphysema remain to be understood, a salient aspect of the disease is the upregulation of inflammatory processes from smoking leading to apoptosis of epithelial cells and proteolysis of the terminal airspaces and lung extracellular matrix components. MSCs are being considered as a therapy in COPD both because of their ability to regenerate type I and type II cells in the airspaces and owing to their immunomodulatory effects.

Recently, one study investigated the effects of MSC administration in a rat model of emphysema [23]. Rats were exposed to ^{60}Co radiation and intratracheal papain treatment to induce emphysema; afterward bone-marrow-derived MSCs were infused intravenously. Lungs were harvested after 28 days and histological changes in the lung were compared between the different treatment groups. As expected, emphysematous changes in the lung quantified by mean linear intercept were increased in irradiated, papain-treated rats. On the other hand, the rats who received an infusion of MSCs were significantly protected against airspace destruction. Furthermore, the percentage of apoptotic cells measured was also significantly decreased following MSC treatment. Immunohistochemical analysis of lung sections revealed costaining of engrafted MSC with the type II epithelial cell marker, surfactant protein C, suggesting that MSCs may have differentiated into pneumocytes to participate in lung regeneration. These data suggest that MSCs can protect against progression of emphysema by mechanisms related to epithelial cell regeneration and also owing to paracrine effects resulting in decreased alveolar apoptosis.

5 MSCs and Pulmonary Hypertension

Pulmonary hypertension is a rapidly progressive and often fatal disease characterized by increased pulmonary arterial pressure, dysfunction of the right side of the heart, and lung vascular remodeling leading to loss of the distal pulmonary vasculature [24]. Strategies aimed at promoting neovascularization and regeneration of the lost vasculature are of immense therapeutic interest in patients with pulmonary hypertension. Because MSCs produce growth factors such as vascular endothelial growth factor that promote neovascularization, there is growing interest in utilizing MSC-based therapies in pulmonary hypertension. Furthermore, it has been shown that MSCs are mobilized from the bone marrow to the peripheral blood during chronic hypoxia [25]. This raises the possibility that exogenous infusion of MSCs may bolster endogenous reparative mechanisms.

Studies by Haynesworth et al. [26] demonstrated that administration of MSCs attenuates monocrotaline-induced pulmonary hypertension in rats. MSCs were infused intratracheally, 14 days after intravenous challenge with monocrotaline. The results showed that MSC infusion attenuated the monocrotaline-induced increase in pulmonary arterial pressure and improved pulmonary vascular resistance. Immunohistochemical analysis of lung sections revealed that immunolabeled MSCs were detected in the lung parenchyma surrounding the airways, but not in

the pulmonary vessel walls. Therefore, the benefits were attributed to paracrine effects of MSCs in the lung parenchyma resulting in improved endothelial function, rather than local engraftment into the vessel walls and regeneration of endothelial cells. This finding is corroborated by studies of Kanki-Horimoto et al. [27], who found that intravenous infusion of MSCs 7 days after subcutaneous administration of monocrotaline improved right ventricular hypertrophy. Interestingly, infusion of MSCs overexpressing endothelial nitric oxide synthase further improved right ventricular hypertrophy. These results suggest that protection conferred by MSCs can be augmented by different gene therapy methods, where a synergy of the paracrine effects of MSCs with improved preservation of vascular function by overexpressing endothelial nitric oxide synthase can improve outcomes in the rat model.

Together with the observation that the peripheral MSC population is significantly augmented during hypoxia [25], these in vivo data suggest that MSCs home into the lung and produce growth factors during hypoxic conditions. Ex vivo studies have demonstrated that treating pulmonary artery rings with conditioned medium from hypoxia-stressed MSCs prior to subjecting arteries to low oxygen conditions attenuates the hypoxia-induced vasoconstriction [28]. This raises the possibility that hypoxia-stressed MSCs secrete soluble factors that improve hypoxia-induced alterations in pulmonary vasoreactivity. Further research into identifying MSC-derived factors will aid not only in identifying therapeutic targets but will also lead to better understanding of the disease process. However, it should be noted that mesenchymal precursors from the monocyte/macrophage lineage termed "fibrocytes" are also recruited to the vasculature during hypoxic vascular remodeling [29]. It has also been shown that MSCs cultured in the presence of a demethylating agent increase the expression of fibrocyte cell surface markers [30]. Fibrocytes play a role in fibrotic tissue remodeling by increasing expression of matrix components such as collagen and fibronectin. Because MSCs and fibrocytes are both adherent populations, care must be taken to distinguish these cell types by the presence of markers such as CD45 prior to in vivo infusion.

6 MSCs and Asthma and Allergy

Another area in pulmonary medicine where the immune regulatory potential of MSCs is being actively investigated is asthma. Asthma is one of the most common chronic inflammatory diseases, affecting an estimated 300 million people worldwide [31]. Its pathogenesis stems from the complex interplay between the allergic response, inflammatory/immune cells, and airway hyperresponsiveness, which leads to bronchoconstriction and eventually airway remodeling. To date, multiple studies have proven that MSCs have immune regulatory properties and can reduce acute inflammation. Now, investigators are looking at the ability of bone-marrow-derived MSCs to decrease the inflammatory response in an ovalbumin-induced asthma mouse model. Weiss et al. [32] found that administration of MSCs

significantly attenuated ovalbumin-induced increases in airway hyperresponsiveness as well as the number of eosinophils in bronchoalveolar lavage fluid after ovalbumin challenge. In addition, they saw a significant decrease in the levels of T_H2 cytokines in the lungs of MSC-treated mice. The anti-inflammatory effects were seen with both syngeneic and allogeneic MSC administration, consistent with the concept of MSC immunoprivilege. These findings, though preliminary, open yet another door for potential therapy of asthma.

Ongoing studies are also investigating the mechanisms of MSCs affecting dendritic cell activation and antigen presentation, and their effects on T-cell lineage commitment and T-cell effector function [32]. The above mechanisms involve the acute phase of asthma, and if left unchecked, the resulting uncontrolled inflammation eventually leads to airway remodeling and fibrosis characteristic of severe uncontrolled asthma. The pathogenesis of chronic airway remodeling involves effector molecules such as transforming growth factor (TGF)-β and vascular endothelial growth factor, along with other cytokines and inflammatory cells [33]. We speculate that MSCs through the regulation of these molecules not only can control the inflammatory acute phase, but potentially can also affect the process of remodeling. However, studies providing proof of this concept are needed.

In a recent study, Cho et al. [34] demonstrated that in a mouse model, adipose-tissue-derived stem cells (ASCs) inhibit the allergic response. They were able to simulate experimental allergic rhinitis by sensitizing BALB/c mice to ovalbumin. The treatment involved intravenous infusion of cultured ASCs pooled from allogenic mice prior to ovalbumin sensitization. Following infusion and sensitization, blood was collected for analysis of IgE, IgG_1, and IgG_{2a} in the serum. They also harvested splenocytes for cytokine determination and nasal mucosa for immunohistochemistry. The results showed significantly lower levels of IgE, IgG_1, IgG_{2a}, and IgG_1/IgG_{2a}, along with decreased IL-4 and IL-5 and elevated IFN-γ levels in the ovalbumin-sensitized mice treated with ASCs compared with untreated mice. Histological analysis of nasal mucosal sections of ASC-treated, ovalbumin-sensitized mice showed migration of ASCs into the tissues along with markedly less inflammatory cell and eosinophil infiltration of the mucosal layers. Interestingly, the investigators also observed significantly fewer symptoms of sneezing and nose rubbing in the ovalbumin–ASC-treated mice. These animal experiments are examples that serve as the groundwork for expanding the growing number of applications for stem cells to include not only inflammatory but also allergic pulmonary disease.

7 MSCs and Fibrotic Lung Disorders

Although the previous studies demonstrate an acute protective effect of MSCs in ameliorating the systemic cytokine storm induced by LPS, we and others have shown that MSC infusion also protects the lung from localized inflammation and aberrant repair induced by bleomycin [13, 35, 36]. Endotracheal administration of

bleomycin leads to lung fibrosis and occurs in three stages. Bleomycin-induced cytotoxicity leads to apoptosis and necrosis of the alveolar epithelial cells, followed by an inflammatory phase characterized by infiltration of neutrophils and macrophages in the lung microenvironment which peaks at day 7. An aberrant repair and remodeling process ensues, resulting in enhanced deposition of matrix molecules such as collagen at day 14. The fibrosis together with impaired re-epithelialization of the alveolar wall is a hallmark of the fibrotic process [37]. The protective effect of MSCs in reducing inflammation and moderating the fibrotic lung remodeling in response to bleomycin was first reported by Ortiz et al. [35]. They obtained MSCs from male mice and infused the MSCs into female mice immediately following bleomycin challenge. Donor MSC engraftment into the injured lung was determined by copurification of MSCs with type II epithelial cells. The results show that MSC infusion decreased the lung matrix metalloproteinase messenger RNA and lung collagen content. The protective effects of MSCs were minimal when infusion was done at 7 days after bleomycin challenge. In this study, the systemic changes in mediator production after MSC infusion were not determined.

To obtain additional insights into the mechanisms by which MSCs confer protection, we felt that a thorough characterization of the local and systemic response to MSC infusion would be necessary. We studied this by administering bleomycin to myelosuppressed mice, and to mice with a normal, intact bone marrow. A subgroup of mice within each group received an additional infusion of green fluorescent protein (GFP)-positive MSCs 6 h after bleomycin-treatment. Our results showed that the MSC infusion conferred a substantial survival benefit in myelosuppressed bleomycin-treated mice. Morphometric analysis of the lung at day 14 revealed that the MSC infusion protected against bleomycin-induced lung injury. Engraftment of MSCs in the lung was quantified at day 14, and the intensity of GFP staining in the lung was greater in myelosuppressed animals that received MSC than in mice that had intact bone marrow. We found messenger RNA levels of T_H1 cytokines (IL-2, IL-1β, IFN-γ) were significantly decreased in the lung 14 days after bleomycin administration, and IL-4 expression was also upregulated. MSC infusion also increased circulating levels of granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor at day 14. These results indicate MSCs alter the cytokine milieu in favor of repair and together with evidence form Ortiz et al. support a role of MSCs in ameliorating the local inflammatory response to bleomycin-induced lung injury.

Although MSCs have been shown consistently to attenuate inflammation in numerous experimental models of injury, it is important to recognize that the time window is a critical factor in optimizing the protective effect of MSC transplantation. Recent data obtained by Yan et al. [38] indicate that infusion of MSCs at a later stage of lung injury can in fact be deleterious. They tested early and late time points wherein GFP^+ MSCs were infused at 4 h, 60 days, or 120 days after lung irradiation. Cells that were infused early (4 h) engrafted into the lung at low levels and were distributed around alveolar and bronchial epithelium. In contrast, cells injected at a later stage (60 and 120 days) were detected in the interstitium as myofibroblasts, suggesting that differentiation of MSCs occurred in response to mediators

produced in the injured tissue. These data point to the conclusion that infusion of MSCs during an ongoing fibrotic response may worsen the disease process and augment scarring in injured tissue rather than reversing it. Thus, the time window for MSC infusion is a critical factor and must be given due consideration to optimize the protective effects of MSCs in lung injury.

8 MSCs and Lung Transplant

In end-stage pulmonary diseases, lung transplantation is the last viable treatment option. Preventing the development of obliterative bronchiolitis becomes very important in posttransplant patients as it reduces survival, and accounts for 30% of deaths after the third year. In an animal model of heterotopic tracheal transplantation (unpublished observations), we demonstrated that systemic administration of MSCs prevents the development of obliterative bronchiolitis. We observed a complete inhibition of inflammation and fibrosis when mice received a single dose of MSCs immediately after tracheal transplant. This effect was independent of the strain of mice from which the MSCs were obtained. Because proliferation of T-regulatory cells represents a mechanism by which MSCs can attenuate graft rejection, we studied the interaction of MSCs with T-regulatory cells in vitro and found that MSCs induced the proliferation of T-regulatory cells via the secretion of soluble factors such as TGF-β_1 and IL1RN. In summary, in vivo studies in animal models of injury have shown that exogenously administered MSCs can protect from injury mostly by modulating the inflammatory response, yet at the same time may also participate in the repair process by differentiation and engraftment into the injured organ.

9 MSCs and Oxidative Stress

Oxidative stress is a hallmark of inflammation [39, 40], and studies in patients with various inflammatory lung diseases have shown that increased oxidant burden is associated with the progression and severity of the disease process [41–43]. Because oxidative stress is intimately related to inflammation and tissue injury, the role of MSCs in modulating the redox environment is a rapidly emerging area of interest. The potential redox modulatory effects of MSCs are especially relevant to ALI, a disease characterized by dramatic perturbations in the systemic redox environment.

9.1 Oxidative Stress in ALI

The idea that highly reactive oxygen metabolites, produced by activated leukocytes, cause tissue injury [44] was advanced before the clinical description of ALI in 1967 [45]. The acute pulmonary injury caused by these reactive oxygen species (ROS) was believed to occur in a pathway that was parallel to the ongoing inflammatory

response. We now know that ROS and redox signaling pathways, that are not strictly ROS-mediated, converge with cellular and humoral components of the immune system and this interaction appears to be a key pathway in the pathogenesis of ALI and ARDS.

Over the years, studies in humans have consistently shown two things. Firstly, patients with ALI and ARDS show increased levels of oxidative stress compared with healthy controls and, secondly, higher levels of oxidative stress in ARDS patients correlate with poorer outcomes. For instance, elevation of the level of plasma hypoxanthine, a substrate for superoxide and hydrogen peroxide, is associated with increased mortality in ARDS patients [46]. In this study, hypoxanthine was highly negatively correlated with loss of protein thiol groups in the plasma, indicating oxidative modification of extracellular protein thiols. The redox state of thiol residues on extracellular proteins is regulated by two, low molecular weight thiol/disulfide control systems, cysteine (Cys) and its disulfide cystine (CySS) and glutathione (GSH) and glutathione disulfide (GSSG) [47].

Cys and GSH are critical determinants of cytokine expression during activation of the immune system, and alteration in Cys and GSH homeostasis is a central feature of inflammation and tissue injury [48]. Therefore, understanding the regulation of Cys and GSH redox systems during inflammation by MSCs is important not only to fully delineate the systemic effects of MSCs but also to identify potential therapeutic targets. In this section, we identify known redox regulatory and antioxidant defense systems in MSCs. Several studies have investigated the redox-dependent effects on MSCs in response to extracellular signals and these are addressed. Finally, we discuss emerging evidence from our laboratory that suggests MSCs can attenuate oxidation of Cys and GSH redox systems in vivo. Together these discoveries are contributing to the novel concept of MSCs as a therapeutic modality in attenuating oxidative stress in inflammatory lung diseases.

9.2 Antioxidant Defense Systems in MSCs

The inflammatory state is characterized by elevated levels of ROS and reactive nitrogen species, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and nitric oxide (NO) [49]. These reactive species serve multiple functions, including killing phagocytosed microorganisms, removal of cell and tissue debris, and induction of signaling events related to inflammation and repair. However, ROS can also cause tissue necrosis and inflammation, which contribute to increased tissue injury and destruction. Indeed, elevated oxidant burden is purported to be a pathogenic component in numerous inflammatory lung diseases, including ALI, idiopathic pulmonary fibrosis, and COPD.

Existing evidence suggests that MSCs may also be enriched for the presence of antioxidant protective genes. Comparison of the human bone-marrow-derived fibroblast cell line V54/2 with the peripheral blood-derived fibroblast cell line L87/4 revealed that V54/2 cells expressed higher levels of glutathione *S*-transferase, an

enzyme system involved in the detoxification by electrophiles by conjugation to GSH [50]. Detailed comparisons of antioxidant defense systems between MSCs and other stromal cells such as lung fibroblasts, as well as cell types of epidermal and endodermal origin will lead to a better understanding of the antioxidant capacity of MSCs.

A number of studies have also investigated the effects of various stressors on ROS production and antioxidant levels in MSCs (Fig. 8.2). Takahata et al. [51] reported that stimulation of the MSC line C3H10T1/2 with adrenalin led to an increase in cellular GSH levels via a process involving nuclear factor E2 p45-related factor 2 (Nrf-2)-mediated activation of the CySS/glutamate antiporter, xCT. This suggests β_2 adrenergic stimulation of MSC can lead to increased influx of CySS from the extracellular compartment into the cells for the purpose of GSH synthesis. In addition to hormones, temperature has also been studied as a stressor. Stolzing and Scutt investigated whether culturing MSCs under reduced temperature conditions impacts ROS production and antioxidant defense systems [52]. MSCs were derived from the bone marrow and were cultured at either 32 or 37°C. MSCs cultured at 32°C expressed higher levels of glutathione peroxidase, lower levels of ROS, and demonstrated decreased nitric oxide levels and a decrease in markers of oxidative stress such as malondialdehyde and protein carbonyl content. Interestingly, the decrease in oxidant stress was associated with a decrease in apoptosis. Similarly studies by Ebert et al. have shown that supplementing telomerase-immortalized human MSCs with selenium decreases ROS production and increases glutathione peroxidase activity, whereas cells cultured under selenium-deficient conditions demonstrated increased DNA damage as evidenced by formation of micronuclei [53].

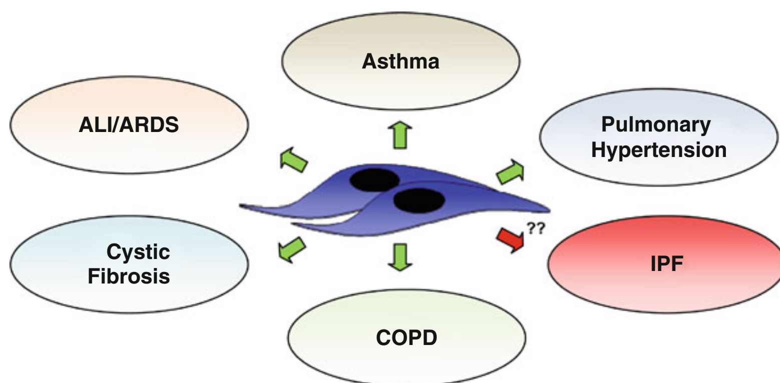

Fig. 8.2 MSCs in inflammatory lung diseases. MSCs are emerging as a therapeutic modality in various inflammatory lung diseases such as acute lung injury, chronic obstructive pulmonary disease, cystic fibrosis, pulmonary hypertension, and allergic diseases such as asthma. Owing to their potential fibrotic effects in vivo, MSCs may not represent a therapeutic option in patients with fibrotic lung disorders such idiopathic pulmonary fibrosis

In addition to the role of exogenous insults and nutrient deficiency on antioxidant capacity of MSCs, in vitro studies have demonstrated that MSCs may have the capacity to modulate the redox environment. For instance, adipose tissue derived MSC conditioned medium (ADCM) demonstrated antioxidant capacity comparable to 100 μM ascorbic acid. Furthermore, culturing *tert*-butyl hydroperoxide treated dermal fibroblasts with ADCM improved cell viability. This study suggests that MSCs actively secrete antioxidant factors which may confer protection in the setting of inflammatory lung diseases [54]. However, studies in Matrigel angiogenesis assay have demonstrated that direct contact of MSCs with endothelial cells (endothelial cell to MSC ratio 1:1–1:3) led to increased ROS production, resulting in endothelial cell apoptosis and ultimately to capillary degeneration. A drop in cytotoxicity was observed when the MSC numbers were decreased by an order of magnitude. These studies indicate that in vivo effects of MSCs may vary depending on the number of MSCs and on the interacting cell population [55].

9.3 MSCs and Thiol/Disulfide Redox State

Studies from our laboratory suggest a reciprocal interaction between MSCs and the extracellular thiol/disulfide redox state. In unpublished observations, we have found that MSCs exposed to an oxidized extracellular Cys/CySS redox state in vitro demonstrate a greater than twofold upregulation in cellular ROS production. Because the Cys/CySS redox state is oxidized in the setting of endotoxin-induced lung injury, understanding the effects of Cys/CySS redox on MSC function and anti-inflammatory effects is paramount. Indeed, preliminary observations from our laboratory suggest that production of IL-1 antagonist is decreased in endotoxin-stimulated cells that are exposed to an oxidized Cys/CySS redox state compared with the physiological redox state. These findings suggest that therapies to preserve oxidation of Cys/CySS during ALI may improve the anti-inflammatory effects of MSCs in vivo.

Although redox state changes can impact critical signaling events in MSCs, studies in a mouse model of endotoxin-induced lung injury are revealing that MSC infusion improves Cys and GSH homeostasis. What these data show is that in addition to modulating the systemic inflammatory environment, infusion of MSCs alters the systemic redox environment to a less oxidizing value. Although the mechanistic basis for these changes is unclear, it is likely that processes related to increased recycling and transport of thiols and disulfides may be involved.

MSCs represent not only an emerging therapeutic modality, but also a paradigm for the resolution of inflammation. Therefore, identifying the mechanisms by which MSCs modulate thiol/disulfide redox status will aid in understanding the regulation of these systems during the resolution of inflammation, and may unveil potential therapeutic targets. Furthermore, determining whether the anti-inflammatory effects of MSCs can be augmented by dietary or pharmacological interventions to preserve the Cys/CySS and GSH/GSSG redox state represents a therapeutic strategy that can be readily translated to the clinic.

10 MSCs and the Alcoholic Lung

10.1 Alcohol Abuse and the Lung

Alcohol is one of the most commonly used and abused beverages throughout history. Numerous epidemiology and experimental studies have described alcohol's health-related benefits, such as cardio- and neuroprotection when consumed in moderation [56]. However, long-term alcohol abuse leads to dependency, followed by multiple complications affecting different organ systems. One devastating consequence of alcohol abuse is immune and bone marrow suppression [57]. To date, a multitude of studies have shown that alcohol can alter the immune system by quantitatively and qualitatively disrupting both cytokine signaling and the immune regulatory cells [58–60].

The link between alcohol and its detrimental effects on the lung date back a century to observations made by William Osler, who noted that alcoholics had increased risk of having lung infections such as pneumonia and tuberculosis. This predisposition was initially attributed to alterations of immune function along with disruption of upper-airway defenses resulting in unopposed entry of pathogens into the lungs [61]. It was not until the study by Moss et al. [62], showing alcoholism as an independent risk factor for developing ARDS, that the concept of alcohol serving as a priming agent for inflammatory lung injury was born. This intriguing relationship later spawned investigations ultimately leading to the concept of the "alcoholic lung" [63].

Multiple mechanisms are involved in the pathogenesis of the alcoholic lung. These pathological processes involve interactions between the different cellular components and the inflammatory mediators, including [63, 64] perturbations in granulocyte–macrophage colony-stimulating factor signaling by alveolar macrophages leading to epithelial barrier dysfunction and impaired innate immunity, depletion of glutathione [65–67], increased angiotensin II [68], followed by increased nitric oxide [69], superoxide, and NADPH oxidase [70] in the epithelial lining, resulting in increased oxidative stress and cellular apoptosis, increased TGF-β_1 [71] and fibronectin [72], worsening proinflammatory cytokine production, upregulated soluble endothelial selectin [73], and changes in epithelial tight junctions (or claudins) [74], disrupting the endothelial–alveolar capillary barrier integrity, leading to "leaky" alveoli. Other pathways include impairment of the immune cell proliferation and functions leading to alterations in TNF-α, ILs, IFN-γ, nuclear factor κB, immunoglobulins, etc., further worsening the inflammatory response and weakening the host defense to infections. These are discussed in further detail in [58].

10.2 MSCs and the Alcoholic Lung

The effects of alcohol on stem cells have not been fully characterized. The orthopedic literature reports effects of alcohol on MSC differentiation and that exposure to alcohol enhances adipogenic differentiation [75] and inhibits osteogenesis [76].

So far, there have been no studies on the effects of alcohol on MSC immune modulation, nor are there investigations on MSCs regulating alcohol-mediated inflammatory injury.

Preliminary in vitro experiments in our institution have shown that chronic alcohol exposure of stem cells increases their expression of TGF-β_1 and fibronectin, suggesting a shift toward a proinflammatory and, possibly, a profibrotic state. Acute exposure on the other hand, decreases TNF-α secretion, consistent with an anti-inflammatory effect. We speculate that part of alcohol's detrimental effects on the immune system could be related to the bone marrow suppression of MSC proliferation. Given the significant influence of MSCs on immune regulation, their depletion could exacerbate the dysfunctional inflammatory response observed in alcoholics. Could replacement of these cells modulate the alcohol-induced proinflammatory state? Can cellular therapy reverse the effects of chronic alcoholism on the immune system? Can MSCs attenuate ARDS in the alcoholic lung? At this time, these questions remain unanswered. However, as discussed and illustrated above, the mechanisms whereby alcohol exerts its immune dysregulation are also pathways influenced by MSCs. Further studies are needed to expand and correlate these findings with the goal of translating them into clinical application.

11 Conclusions

From the description of their immunomodulatory effects in vitro to their current applications in animal models of inflammatory lung diseases, MSCs illustrate a bench to bedside paradigm. Opportunity exists to extend the in vivo findings to the clinic to test whether MSC-based interventions are beneficial in patients with cystic fibrosis, ALI, pulmonary hypertension, and asthma (Fig. 8.2).

At the same time, however, studies are needed to fill gaps in our understanding of the mechanistic role and the potential efficacy of MSCs in inflammatory lung diseases. It must be noted that in most of the in vivo studies of ALI, MSCs were administered either before or immediately after the inflammatory challenge. Care should be taken to design experiments to test the efficacy of MSCs when administered during the progression of lung injury/inflammation so that improved clinical outcomes can be ascertained.

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