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REVIEW

Lung stem cells

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Abstract The lung is a relatively quiescent tissue comprised of infrequently proliferating epithelial, endothelial, and interstitial cell populations. No classical stem cell hierarchy has yet been described for the maintenance of this essential tissue; however, after injury, a number of lung cell types are able to proliferate and reconstitute the lung epithelium. Differentiated mature epithelial cells and newly recognized local epithelial progenitors residing in specialized niches may participate in this repair process. This review summarizes recent discoveries and controversies, in the field of stem cell biology, that are not only challenging, but also advancing an understanding of lung injury and repair. Evidence supporting a role for the numerous cell types believed to contribute to lung epithelial homeostasis is reviewed, and initial studies employing cell-based therapies for lung disease are presented. As a detailed understanding of stem cell biology, lung development, lineage commitment, and epithelial differentiation emerges, an ability to modulate lung injury and repair is likely to follow.

Keywords Stem cells · Lung · Injury · Development · Bone marrow

Introduction

The lung has evolved to conduct air along specialized branching airways into alveoli in which gas exchange occurs. The epithelial, endothelial, and interstitial cells comprising these structures face repeated insults from inhaled microorganisms and toxicants, and potential injuries from inflammatory mediators carried by the blood supply. Although much is known about lung structure and function, an emerging literature is providing new insights into the repair of the lung after injury.

Recent findings suggest that a variety of cell types participate in the repair of the adult lung. Many mature, differentiated local cell types appear to play roles in reconstituting lung structure after injury. In addition, newly recognized local epithelial progenitors and putative stem cells residing in specialized niches may participate in this process. This review introduces basic concepts of stem cell biology and presents a discussion of the way in which these concepts are helping to challenge and advance our understanding of lung injury and repair.

Classical and non-classical stem cell hierarchies

Highly proliferative tissues such as the intestinal epithelium or the hematopoietic compartment of the bone marrow depend on a classical stem cell hierarchy to maintain homeostasis (Marshman et al. 2002; Pinto et al. 2003; Pinto and Clevers 2005; Morrison et al. 1995). In these tissues a quiescent undifferentiated stem cell undergoes an asymmetric cell division resulting in one cell for self-renewal and one cell that functions as a so-called transit-amplifying progenitor. This latter cell proliferates rapidly giving rise to the differentiated mature cell types that comprise the intestinal villi or the circulating blood. In these systems, progenitor cells display a finite self-renewal capacity, whereas rare undifferentiated stem cells cycle infrequently but possess the capacity for extensive and even life-long self-renewal (Morrison et al. 1995).

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In marked contrast to these tissues, adult lung epithelial cells are significantly more quiescent, with turnover times possibly greater than 100 days (Blenkinsopp 1967). Perhaps for this reason, a classical stem cell hierarchy has not been easily identified in the lung. As a result, researchers have focused instead on characterizing the relatively differentiated epithelial cell types that appear to proliferate in response to airway or alveolar injuries (Rawlins and Hogan 2006).

The bulk of reported studies to date suggest that multiple differentiated airway and alveolar epithelial cell types are capable of proliferating in response to epithelial injury. Whereas data from each particular type of injury may differ, a model is emerging in which several local epithelial cell types can function both as fully differentiated functional cells and as transit-amplifying progenitors (Rawlins and Hogan 2006). In this way, the lung may be more similar to other epithelia derived from the foregut endoderm, such as the liver and pancreas. In the liver, for example, mature differentiated epithelial phenotypes, such as hepatocytes, rather than undifferentiated stem cells, appear to be responsible for the reconstitution of the tissue after injury or tissue resection (Forbes et al. 2002). The liver additionally appears to contain "back-up" facultative stem cells, termed oval cells, which may be called into action following some types of injury, particularly if hepatocyte proliferation is inhibited (Forbes et al. 2002; Wang et al. 2003). The adult lung has also been recently proposed as harboring a rare population of multipotent epithelial stem cells resident in specified niches, such as the bronchoalveolar duct junction (BADJ; Kim et al. 2005; Giangreco et al. 2002).

Lung epithelial structure

The lung is a complex branching organ in which multiple anatomic regions, organized along a proximal-distal axis, are each characterized by a unique cellular organization and potentially unique repair mechanisms (for a recent review, see Rawlins and Hogan 2006). The most proximal conducting airways, viz., the trachea and main bronchi, display a columnar epithelium comprised of ciliated cells, secretory cells, basal cells, and submucosal glandular epithelium. More distal conducting airways do not contain basal cells and are populated by an epithelial surface with an increasing ratio of secretory (Clara) cells to ciliated epithelial cells. At the BADJ, the airway epithelium changes to a distal lung epithelium organized into functional alveoli. Alveolar epithelium consists of flat type I cells, which comprise the majority of the gas-exchange surface area of the lung, and cuboidal surfactant-expressing type II cells.

Studies of lung epithelial injury and repair

In the proximal tracheobronchial epithelium, bronchiolar epithelium, and distal alveolar epithelium, several differentiated cell types have been shown to participate in repair after injury. In the proximal conducting airways, a role in epithelial repair has been posited for basal cells, secretory (Clara) cells, or cells residing in the submucosal glands (Zepeda et al. 1995; Engelhardt et al. 1991, 1995; Hong et al. 2003; Boers et al. 1998; Breuer et al 1990; Borthwick et al. 2001). Many of these investigations have employed the experimental labeling of proliferating cells by using tritiated thymidine, bromodeoxyuridine (BrdU), or retroviral tagging, thereby allowing the analysis of cells contributing to airway epithelial reconstitution after injury or after xenografting onto tracheas denuded of epithelium (Zepeda et al. 1995; Engelhardt et al. 1991, 1995). Importantly, a large body of data suggests that the type of airway injury is an important determinant of the type of progenitor cell activation. For example, Evans and colleagues have demonstrated that secretory cells of rat airways function as the principal airway epithelial progenitor following injury with nitrogen dioxide or ozone (Evans et al. 1976, 1986). In contrast, following naphthalene injury in the tracheal epithelium, basal cells have been proposed as possessing progenitor capacity (Hong et al. 2003). The results from the rat tracheal xenograft model have similarly been variable, with some studies suggesting that only basal cells (Ford and Terzaghi-Howe 1992), only non-basal columnar cells (Johnson and Hubbs 1990), or both populations (Liu et al. 1994) can restore the proximal airway epithelium.

In cases of extreme and diffuse airway injury such as that suffered after exposure to naphthalene, rare variant-Clara cells residing within neuropepithelial bodies (Hong et al. 2001; Reynolds et al. 2000a, b; Peake et al. 2000) or BADJs (Giangreco et al. 2002) have been shown to proliferate rapidly and to contribute to distal airway epithelial repair. Because naphthalene is toxic only after it is metabolized by the cytochrome P4502F2, Clara cells expressing this cytochrome are selectively killed by naphthalene, whereas variant-Clara cells, which express the Clara cell marker CC10 (also known as CCSP, CCA, or secretoglobin Scgb1a1) but do not express P4502F2, resist injury. Hence, these variant-Clara cells residing within localized anatomical niches appear to function as transit-amplifying progenitors activated after certain types of airway injury (Giangreco et al. 2002; Hong et al. 2001; Reynolds et al. 2000a). However, limited information is available regarding the lineage relationships, self-renewal properties, and clonality of these progenitors, and whether these cells play a role in normal tissue maintenance is also unclear.

Genetic lineage-tagging approaches, such as inducible cre-lox transgenic mouse models, are familiar to developmental biologists (Perl et al. 2002a, b, 2005) but have been lacking until recently in studies of adult lung injury and repair. Two notable studies have adapted this technology to examine the way that the lung repairs itself after naphthalene injury. One group has employed an inducible cre recombinase controlled by a cytokeratin K14 promoter that is selectively active in basal cells of the tracheobronchial epithelium. Lineage tagging following naphthalene injury has suggested that K14-positive cells contribute to multipotent epithelial reconstitution of both secretory and ciliated cells in the proximal airways (Hong et al. 2003). Another group has utilized BrdU labeling and an inducible cre recombinase controlled by the Foxj1 promoter, which is selectively active in ciliated cells. These investigators have found no evidence for ciliated cell proliferation and no evidence of secretory or other airway cells deriving from ciliated cells after recovery from naphthalene injury (Rawlins et al. 2007). These findings have added to the controversy over whether ciliated epithelial cells are able to contribute to airway epithelial reconstitution after injury, as has been suggested by prior studies (Park et al. 2006). There has been general agreement, however, that ciliated airway epithelial cells serve an important function after naphthalene exposure, as they flatten and change their gene expression patterns in order to cover the injured airway following Clara cell ablation (Park et al. 2006; Stripp et al. 1995). Although still controversial, the many animal models employed to examine airway epithelial reconstitution indicate that several airway epithelial cell types are able to give rise to differentiated secretory and ciliated epithelial progeny.

In the gas-exchange distal air sacs (alveoli), the cuboidal type II cell is thought to function as the progenitor of the alveolar epithelium based on a capacity to replenish itself and to give rise to terminally differentiated flat type I cells (Brody and Williams 1992; Mason and Williams 1977). This model has evolved from classic in vivo thymidine incorporation studies demonstrating serial progression of labeling from type II into type I cell nuclei after lung injury (Evans et al. 1973, 1975; Adamson and Bowden 1974, 1979). In addition, in vitro studies have shown that cells with a type I cell phenotype arise during the culture of primary type II cells (Brody and Williams 1992; Dobbs et al. 1985; Mason et al. 1975; Danto et al. 1992, 1995). Recently, several investigators have suggested that heterogeneity may exist amongst type II cells. For example, one group has found E-cadherin-positive and -negative fractions of type II cells (Reddy et al. 2004), whereas others have described heterogeneity of transgene expression in type II cells of genetically engineered mice (Perl et al. 2005; Roper et al. 2003). Despite these intriguing observations, uncertainty remains as to whether subtypes of type II cells occur with differing progenitor or other functional capacities.

A unique model for studying lung regeneration in adult animals in the absence of direct injury is the post-pneumonectomy compensatory lung growth model first described in rats by Brody (1975). In this model, resection of one lung results in the rapid growth of the contralateral lung until there is regeneration of the initial starting mass, volume, and DNA and RNA content of both lungs (Brody 1975; Brody et al. 1976; Rannels et al. 1979; Kuboi et al. 1992; Brown et al. 2001; Sakamaki et al. 2002; Hsia 2004a, 2004b). Analagous to other compensatory growth models, such as partial hepatectomy (Forbes et al. 2002) or partial pancreatectomy (Dor et al. 2004), compensatory lung growth in rodents following pneumonectomy appears to involve the proliferation of mature endogenous differentiated cell types (Voswinckel et al. 2004). This model is considered a pure model of post-natal lung growth as inflammation is notably absent. The model has been used to demonstrate that bone-marrow-derived cells do not appear to contribute to the pulmonary endothelium during compensatory lung growth (Voswinckel et al. 2003). To date, however, this model has not been applied for the study or detection of endogenous lung progenitor populations. It is also not yet clear whether humans display compensatory lung growth, although pulmonary function tests demonstrate that, in humans, recovery in lung function after pneumonectomy or partial lung resection is greater than would be expected based solely on mechanical changes (Laros and Westermann 1987, 1989; Werner et al. 1993; Eren et al. 2003).

Novel endogenous stem cell candidates for lung epithelium

The question of whether a multipotent lung stem cell exists able to self-renew and to reconstitute all epithelial phenotypes, including airway and alveolar structures, has been addressed in a recent investigation (Kim et al. 2005). Kim et al. (2005) have focused on the BADJ region first proposed by Stripp and colleagues as playing a key role in repair after naphthalene-induced lung injury (Giangreco et al. 2002). Employing immunofluorescence microscopy, this group has identified rare cells that lie at the BADJ and that appear to co-express both the alveolar type II cell marker, surfactant protein-C (SPC), and the Clara cell marker, CC10 (Kim et al. 2005). Following naphthalene exposure, these cells resist injury and begin to proliferate suggesting a role in repair. Although rare, the authors suggest that SPC+/CC10+ cells can be purified by flow cytometry from proteolytically digested murine lung tissue based on the surface marker profile: Sca1+CD45-CD31-

CD34+. Sorted single cells can be expanded in culture, and *ex vivo* clonogenic assays have been used to demonstrate the stem cell properties of self-renewal and multipotent differentiation into cells expressing markers of airway and alveolar epithelium. The cells have been termed "broncho-alveolar stem cells" (BASCs) by this group of researchers who have extrapolated their in vitro findings to suggest that BASCs possess similar in vivo self-renewal and multipotent capacity. In addition, based on studies employing a mouse model of lung adenocarcinoma induced by activated K-ras, BASCs have been hypothesized to be the cell of origin for some types of lung cancer (Kim et al. 2005). Whether BASCs bear any relationship to the BADJ variant-Clara cells identified by Stripp and colleagues (Giangreco et al. 2002) remains unclear. Whereas initial work characterizing these putative stem cell populations is promising, future studies employing in vivo lineage-tagging methods will be required to define the true in vivo differentiative repertoire of BASCs or BADJ variant-Clara cells.

Controversies regarding the role of non-local cells in lung repair

Classical models of lineage commitment and differentiation posit that each germ layer (ectoderm, endoderm, and mesoderm) is specified during early embryonic development (Tam and Behringer 1997). Accordingly, mature adult cells are thought to be replenished only by precursor cells that originate from the same germ layer.

A series of studies in the late 1990s has argued against this dogma by suggesting that circulating cells derived from the bone marrow, a mesodermal tissue, directly reconstitute non-hematopoietic tissues, such as neurons (ectoderm) or hepatocytes (endoderm; Eglitis and Mezey 1997; Mezey et al. 2000; Petersen et al. 1999; Lagasse et al. 2000; Gussoni et al. 1999; Herzog et al. 2003). These initial studies were typically based on models of bone marrow transplantation in which genetically labeled bone marrow cells obtained from donor mice were transplanted into recipients, and the subsequent engraftment in recipient tissues was analyzed (for extensive reviews, see Herzog et al. 2003; Wagers and Weissman 2004). In most cases, the recipient mice were irradiated prior to bone marrow transplantation in order to ablate the marrow of the recipients, thereby allowing the establishment of mice with chimeric bone marrow and blood systems. In 2001, Krause and colleagues analyzed the lungs of chimeric mice, 11 months after transplantation of a single bone-marrow-derived hematopoietic stem cell (HSC; Krause et al. 2001). Contrary to the traditional tri-lineage germ layer boundaries established by developmental biologists, this study found, by histologic analyses, that 20% of the type II alveolar epithelial cells and 4% of the airway epithelial cells were derived from the transplanted donor marrow cell. Soon after this report, our group showed that intravenous injections of plastic-adherent cultured marrow cells after bleomycin-induced lung injury resulted in the rare engraftment of flat cells in the alveolar wall, the flat cells appearing to co-express a lacZ tracking marker and the type I alveolar cell marker, T1α (Kotton et al. 2001). Bleomycin injury seemed to facilitate the engraftment of these cells in lung tissue. Although this study suggests that, under rare experimental conditions, manipulated marrow cells may assume some aspects of a distal lung epithelial phenotype, it in no way supports the concept that lung epithelial cells normally arise from recruited bone marrow cells.

Since that time, numerous studies, employing transplantation of either unfractionated marrow cells or various purified marrow-derived populations, have reported engraftment of a variety of pulmonary epithelial (Herzog et al. 2003, 2006; Grove et al. 2002; Theise et al. 2002; Harris et al. 2004; Wang et al. 2005; Loi et al. 2006), endothelial (Hilbe et al. 2004; Bull et al. 2003; Zhao et al. 2005; Davie et al. 2004; Bailey et al. 2004), or interstitial (Phillips et al. 2004; Hashimoto et al. 2004; Schmidt et al. 2003) phenotypes derived from bone marrow (for an extensive review, see Weiss et al. 2006). Many of these studies have utilized some form of lung injury (e.g., bleomycin: Kotton et al. 2001; Ortiz et al. 2003; Rojas et al. 2005; elastase: Ishizawa et al. 2004; Abe et al. 2004; endotoxin: Beckett et al. 2005; Xu et al. 2007; naphthalene: Loi et al. 2006; radiation injury: Theise et al. 2002; Herzog et al. 2006) in order to study the reconstituting potential of the transplanted marrow- or blood-derived cells. Notably, some of these reports have involved the transplantation of marrow cell populations purified by prolonged culture on plastic (such as mesenchymal stem cells [MSCs]: Kotton et al. 2001; Ortiz et al. 2003; Rojas et al. 2005; Xu et al. 2007; multipotent adult progenitor cells: Jiang et al. 2002; fibrocytes: Phillips et al. 2004; Schmidt et al. 2003). Use of plastic-adherent culture raises the possibility that the culturing process triggers an alternative potential for these cells, a potential that may not normally occur without culturing.

Because transplantation experiments necessarily require the artificial introduction, into the circulation, of cells that may not normally enter the blood stream, some investigators have employed a parabiotic mouse model to test the role of circulating cells in lung repair without the use of cell transplantation (Abe et al. 2004; Wagers et al. 2002). This model involves joining two mice (analogous to a donor and recipient) by suturing together their flanks so that their circulatory systems gradually merge. After elastase-induced lung injury to this model, one group found lung engraft-

ment resembling fibroblasts and type I alveolar epithelial cells derived from the circulation of the parabiotic partner (Abe et al. 2004). In marked contrast, another group employed the parabiotic mouse model and concluded that there was no contribution of cells from the circulation to any non-hematopoietic lung lineage (Wagers et al. 2002).

The tracheal xenograft model has also been used to test the potential of bone marrow cells or circulating cells to contribute to repopulation of the tracheobronchial epithelium. For example, two reports examined repopulation of tracheas denuded of epithelium. One study delivered HSCs purified from mouse bone marrow by the Hoechst-efflux method and found no evidence of epithelial reconstitution of the tracheal xenograft (MacPherson et al. 2005), although low level engraftment in host tracheas was detectable in marrow chimeras in vivo. A more recent study suggested that circulating bone-marrow-derived cells expressing CXCR4 and cytokeratin 5 could contribute to epithelial reconstitution of a denuded grafted trachea (Gomperts et al. 2006).

Krause and colleagues have addressed whether cell fusion might account for the observed contribution of marrow-derived cells to lung epithelium. This group employed a cre-lox transgenic mouse model and found no evidence for cell fusion in the lung tissue of uninjured recipients after bone marrow transplantation (Harris et al. 2004). As has been reported by others, fusion events were observed in injured muscle tissue, which served as a positive-control tissue.

In contrast, a follow-up study by the same group employed transplantation of wild-type marrow into sex-mismatched SPC-null recipients and found that rare fusion events between donor marrow-derived cells and the diseased recipient alveolar epithelium were detectable. The authors proposed that these fusion events resulted in heterokaryon formation, occasional Y-chromosome loss, and reconstitution of SPC gene expression in approximately 1 per 1,000 type II alveolar epithelial cells (Herzog et al. 2007). Other groups have used co-culture experiments to demonstrate cell fusion in vitro between bone marrow MSCs and lung epithelial cells (Spees et al. 2003), but whether this process explains reports of in vivo lung epithelial engraftment deriving from infusions of MSCs remains in question (Loi et al. 2006).

Evidence against lung epithelial reconstitution by bone-marrow-derived cells

At least three separate reports have called into question the reproducibility of lung epithelial reconstitution by marrow-derived cells. Wagers et al. (2002) have employed single HSC transplantion and parabiotic mouse models to assess whether marrow-derived cells contribute to non-hematopoietic tissues. This group has found that all bone marrow- or blood-derived cells in the lung tissue of recipients are actually hematopoietic in phenotype rather than epithelial. By co-staining for the green fluorescent protein (GFP) tracking marker and the pan-hematopoietic marker, CD45, this study has shown that all donor marrow-derived cells in the lung express CD45, a marker not expressed in the airway or alveolar epithelium (Wagers et al. 2002).

Two reports from our group have attempted to determine whether marrow-derived cells are capable of contributing to the alveolar epithelium in neonatal, adult, or lung-injured mice after bone marrow transplantation (Kotton et al. 2005b; Chang et al. 2005). In contrast to our earlier work (Kotton et al. 2001), freshly purified cells, rather than plastic-adherent cultured cells, were employed in these experiments. We utilized bone marrow from transgenic mice lines in which the GFP reporter gene was expressed either ubiquitously or under the regulatory control of a human SPC promoter. One study employed deconvolution fluorescence microscopy and found that the appearance of marrow-derived type II alveolar epithelial cells on tissue sections resulted from overlapping fluorescent signals of endogenous SPC+ type II cells and donor-derived GFP+ cells (Chang et al. 2005). In a separate study employing transplantation of single stem cells, purified HSCs, or bulk bone marrow followed by bleomycin-induced lung injury, we observed that autofluorescence or rare non-specific binding of fluorescently tagged antibodies could introduce rare false-positive artifacts. When analysis of engraftment was performed by methods that did not depend on antibody or probe binding and that allowed exclusion of dead or autofluorescent cells, no reconstitution of type II alveolar cells by bone-marrow-derived cells was detected (Kotton et al. 2005b).

The many conflicting reports regarding whether cells derived from the bone marrow contribute structurally to the lung epithelium will take time and careful repeated study to reach consensus. While still controversial, the picture emerging from the use of more sophisticated techniques (Loi et al. 2006; Beckett et al. 2005; Kotton et al. 2005b; Chang et al. 2005) is that local cells within the lung are primarily responsible for maintaining or reconstituting the lung epithelium, and that bone-marrow-derived cells contribute few, if any, cells directly to the structure of the airway or alveolar epithelium.

Circulating cells and paracrine effects

Despite the controversy fueling the debate concerning bone marrow plasticity, most investigators agree that cells derived from the bone marrow appear to play some role

during lung repair after injury. Still in question is what this role may be. Although direct structural contributions to lung epithelial cells may not readily occur, some form of significant supportive or paracrine function probably arises from circulating cells originating from the bone marrow (De Palma et al. 2005; Grunewald et al. 2006). For example, bone-marrow-derived MSCs expanded in culture possess a number of immunomodulatory properties, and two investigative teams have employed intravenous infusions of MSCs following bleomycin-induced lung injury to demonstrate amelioration of pulmonary fibrosis (Ortiz et al. 2003; Rojas et al. 2005). In clinical trials, infusions of MSCs result in anti-inflammatory effects, thereby abrogating graft versus host disease in some patients (Le Blanc et al. 2004). Xu et al. (2007) have found that infusions of MSCs prevent endotoxin-induced lung inflammation, injury, and edema in mice. Potential mechanisms accounting for the immunomodulatory effects of MSCs have been studied *ex vivo* (Aggarwal and Pittenger 2005). During co-culturing with a variety of leukocyte subpopulations, for example, MSCs have been shown to secrete factors that modulate TH1 and TH2 phenotypes toward tolerance. In addition, MSCs decrease the secretion of inflammatory cytokines by co-cultured leukocytes and promote the formation of T regulatory cells (Aggarwal and Pittenger 2005).

Functional rescue of lung injury has also been demonstrated after infusion of heterogeneous populations of bulk bone marrow or cells derived from cultured fat cells (Yuhgetsu et al. 2006; Shigemura et al. 2006a, b). These cell-based therapies have been tested in a mouse model of emphysema induced by intratracheal elastase. Unfractionated bone marrow cells intratracheally instilled at the time of elastase injury (Yuhgetsu et al. 2006) or cultured fat-derived stromal cells infused after elastase injury (Shigemura et al. 2006b) each appear to diminish the severity of subsequent emphysema as quantified by mean linear intercepts. The mechanism for this protective effect is not known but has been suggested by the authors to involve paracrine effects (such as the secretion of hepatocyte growth factor) rather than direct structural repair in view of the rarity of engrafted cells detected (Shigemura et al. 2006b).

New strategies to identify resident lung stem cells

Recent work has shown that small populations of resident cells within solid organs display phenotypic features that typify known stem cell populations, such as the expression of certain surface antigens or the ability to efflux vital dyes. Taking this into account, investigators have attempted to isolate and characterize tissue stem cells by identifying cells with these features.

One such strategy is to identify organ cells that express "stem cell antigens", such as Sca-1, c-kit, or CD34 (Oh et al. 2003; Asakura et al. 2002; Tamaki et al. 2002; Welm et al. 2002; Quaini et al. 2002; Petersen et al. 2003). This approach has proven limited because these antigens in isolation do not specifically identify stem cells. For example, in lung tissue, Sca-1 may be expressed on rare BASCs but is also expressed throughout the endothelium of pulmonary arteries, veins, and capillaries (Kotton et al. 2003). Moreover, c-kit and CD34 are similarly expressed on many cells in lung tissue, including subtypes of leukocytes and endothelial cells (Dong et al. 1997).

Another strategy is to subject purified populations of lung cells to *ex vivo* assays designed to test multipotency. For example, well-defined culture conditions have been developed to display the potential of bone marrow MSCs to self-renew or differentiate into bone, cartilage, and fat (Pittenger et al. 1999). These same culture conditions have been employed to demonstrate that the adult lung contains an endogenous population of rare cells with similar *ex vivo* capacity (Summer et al. 2007; Lama et al. 2007). Whereas the role of these rare lung-derived MSCs in tissue homeostasis or disease pathogenesis remains unclear, they can be purified from murine lung tissue based on their capacity to efflux Hoechst dye in combination with a CD45-/CD31- surface phenotype (Summer et al. 2007). In adult humans, cells with MSC features have been retrieved by broncho-alveolar lavage (Lama et al. 2007). In both species, the investigators have found the cells to be resident within the host lung tissue rather than being derived from the bone marrow or circulation.

The capacity of stem cell populations to efflux Hoechst dye has also been employed to isolate rare CD45+ HSCs from a variety of tissues, including muscle, liver, and lung (Gussoni et al. 1999; Goodell et al. 2001; Kotton et al. 2005a; Summer et al. 2005). The role of these resident HSCs in organ homeostasis is unknown. Notably, some organ-derived HSCs possess long-term blood-reconstituting capacity approaching that of bone-marrow-derived HSCs (Kotton et al. 2005a).

An important remaining question is whether the property of Hoechst dye efflux may be used to isolate lung cells with epithelial stem cell capacity. Intriguing work by one group has examined rare CD45- dye-effluxing cells within single cell suspensions prepared from lung airway digests; a portion of these cells appear to express the phenotype of variant-Clara cells of the neuroepithelial body (Giangreco et al. 2003).

Embryonic stem cells and lung epithelial differentiation

The discovery of pluripotent embryonic stem (ES) cells offers unprecedented opportunities for the study of the

molecular pathways involved in the development and differentiation of lung epithelium. The potential to derive lung epithelium from ES cells in culture has also raised the exciting possibility of future cell-based therapies designed to reconstitute injured lung epithelium.

ES cells are derived from the inner cell mass of the mammalian blastocyst and can be maintained and expanded in culture in a pluripotent state (Evans and Kaufman 1981). When transplanted into blastocyst-stage embryos, ES cells are able to contribute to all somatic tissue lineages, including all pulmonary epithelial cell types. Upon addition of specified inducing substances, ES cells are also able to generate a broad spectrum of differentiated cell lineages in culture (Keller 1995).

Because the initial developmental stages in the ES cell system appear to recapitulate those in the mouse embryo, investigators have focused on using ES cells to study the molecular control of early developmental events, such as establishment of the three primitive germ layers. In this regard, after being cultured under appropriate conditions, ES cells differentiate into embryoid bodies, which contain derivatives of all three germ layers (Gadue et al. 2005). To date the majority of studies have centered on mesoderm and ectoderm development and, as a consequence, conditions have been established for the efficient differentiation of hematopoietic, vascular, muscle, and neural lineages (Wiles and Keller 1991; Keller et al. 1993; Rohwedel et al. 1994; Hescheler et al. 1997; Kramer et al. 2000; Nakano et al. 1994; Okabe et al. 1996; Vittet et al. 1996; Nishikawa et al. 1998; Czyz and Wobus 2001).

Far fewer studies have successfully derived endodermal lineages, such as lung epithelium, from ES cells in vitro. Initial proof-of-principle reports have demonstrated the derivation of insulin-expressing cells and cells with hepatocyte characteristics from undifferentiated ES cells (Lumelsky et al. 2001; Abe et al. 1996; Hamazaki et al. 2001; Hori et al. 2002; Jones et al. 2002). With regard to the differentiation of lung lineages, Ali and colleagues (2002) first demonstrated that the expression of SPC mRNA and protein could be detected after mouse ES cells had differentiated into embryoid bodies followed by prolonged culture periods in proprietary "small airway growth medium". These investigators also applied their protocol to generate SPC expression from human ES cells (Samadikuchaksaraei et al. 2006). Subsequent differentiation of mouse ES cells into cells expressing the type I alveolar epithelial marker, aquaporin 5, was also reported (Qin et al. 2005). In addition, Coraux and colleagues (2005) demonstrated the derivation, from mouse ES cells, of epithelial cells with characteristics of tracheobronchial epithelium, including a Clara-cell-like phenotype, after culture under conditions that included an air-liquid interface. Taking into account the important patterning signals secreted by developing lung mesenchyme, investigators

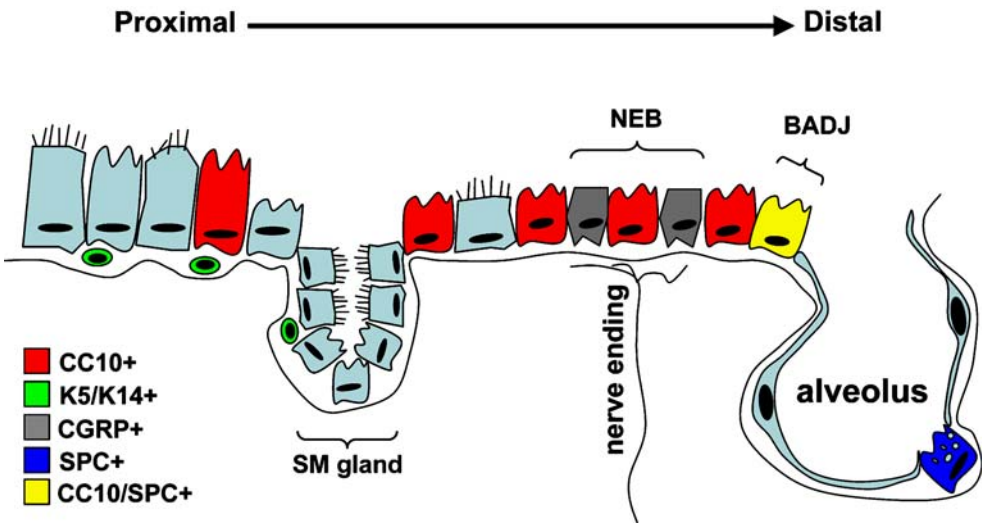

Fig. 1 Summary of cells with putative lung epithelial progenitor potential. The composition of the lung epithelium changes significantly along a proximal to distal axis. Cell markers and localized anatomic niches used to identify cells with lung epithelial regenerative potential are illustrated and color-coded. In the proximal airways (trachea and large bronchi), basal (*green*; expressing keratins K5 and K14) and non-basal cells and cells within the submucosal gland (*SM gland*) duct have each been proposed as having airway epithelial regenerative capacity. In the distal bronchiolar airways, Clara cells (*red*; CC10+) are able to proliferate after injury. Subsets of CC10+ cells (variant-Clara cells) within the localized anatomic niches of neuroendocrine bodies (*NEB*) or broncho-alveolar duct junctions (*BADJ*) resist naphthalene-induced lung injury and possess putative airway epithelial regenerative capacity. The NEB niche is innervated and typically identified by staining for the marker calcitonin gene-related peptide (*CGRP*; *gray*), although the variant-Clara cells themselves do not express this neuroendocrine marker. In the alveolus, the type II alveolar epithelial cell (*blue*; expressing surfactant protein-C, *SPC*) serves as an alveolar progenitor able to self-renew and to differentiate into flat type I alveolar epithelial cells. Some investigators have proposed that a variant-Clara cell in the BADJ niche co-expresses both CC10 and SPC and functions as a multipotent broncho-alveolar stem cell (*yellow*)

have also successfully derived SPC+ cells by co-culturing ES cells with suspensions of digested fetal lung mesenchyme (Denham et al. 2006; Van Vranken et al. 2005).

Kubo et al. (2004) have significantly advanced the study of ES-derived endoderm by demonstrating that activin A can stimulate nodal signaling in ES cells, thereby enabling, for the first time, the efficient and robust derivation of definitive endoderm. Subsequent reports by this and other groups have confirmed that several endodermal lineages, including liver, lung, intestine, and pancreatic epithelia, can be derived both in vitro and in vivo from ES cells cultured by using this approach (Gouon-Evans et al. 2006; Gadue et al. 2006; Yasunaga et al. 2005; D'Amour et al. 2005; Rippon et al. 2006). The signaling pathways responsible for this differentiation appear to involve activin/nodal/transforming growth factor-β signaling, canonical wnt signaling activated by wnt3a, and the presence of bone morphogenetic protein 4 (Gouon-Evans et al. 2006; Gadue et al. 2006).

Recently, a pure population of cells resembling type II alveolar epithelium has been isolated from human ES cells engineered to express an antibiotic-resistance cassette under regulatory control of the SPC promoter (Wang et al. 2007). Under relatively simple culture conditions with serum and puromycin antibiotic, ES cells differentiate, and antibiotic-resistant cells can be selected until a highly pure (>99%) population of cells expressing a variety of type II alveolar epithelial markers is obtained.

Lessons from developmental biology

Advances in ES cell research highlight the importance of applying the lessons of developmental biology to the study of lung epithelial differentiation. A detailed understanding of lung development also has relevance to the comprehension of similar signaling pathways activated during adult lung injury and repair (Park et al. 2006). Much can also be learned from the developing lung in which a small number of true epithelial progenitors in the prospective lung field of the embryonic foregut endoderm appear to give rise rapidly to the entire adult lung epithelium (Cardoso and Lu 2006). Sophisticated lineage-tagging strategies have recently been employed in order to reveal where and when these progenitors are active during development (Perl et al. 2002a, 2005). Whether any epithelial progenitors of similar phenotype remain and can be re-derived in the adult lung are important questions that may take many years to answer.

Concluding remarks

In summary, mature differentiated lung epithelial cells, endogenous progenitors, and putative stem cell niches all appear likely to participate in the response of the lung to injury (Fig. 1). Manipulation of endogenous lung progenitors and delivery of exogenous cells are two potential therapeutic approaches currently being explored in animal models of lung injury. In order for these therapies to succeed, significant hurdles will need to be surmounted. As a detailed understanding of stem cell biology, lung development, lineage commitment, and epithelial differentiation emerges, an ability to modulate lung injury and repair is likely to follow.

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