

## TISSUE ENGINEERING AND THE USE OF STEM/PROGENITOR CELLS FOR AIRWAY EPITHELIUM REPAIR

Godfried M. Roomans\*

School of Health and Medical Sciences, Örebro University, Örebro University Hospital, SE-70185 Örebro, Sweden

### Abstract

Stem/progenitor cells can be used to repair defects in the airway wall, resulting from e.g., tumors, trauma, tissue reactions following long-time intubations, or diseases that are associated with epithelial damage. Several potential sources of cells for airway epithelium have been identified. These can be divided into two groups. The first group consists of endogenous progenitor cells present in the respiratory tract. This group can be subdivided according to location into (a) a ductal cell type in the submucosal glands of the proximal trachea, (b) basal cells in the intercartilaginous zones of the lower trachea and bronchi, (c) variant Clara cells (Clara<sup>v</sup>-cells) in the bronchioles and (d) at the junctions between the bronchioles and the alveolar ducts, and (e) alveolar type II cells. This classification of progenitor cell niches is, however, controversial. The second group consists of exogenous stem cells derived from other tissues in the body. This second group can be subdivided into: (a) embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, or amniotic fluid stem cells, (b) side-population cells from bone marrow or epithelial stem cells present in bone marrow or circulation and (c) fat-derived mesenchymal cells. Airway epithelial cells can be co-cultured in a system that includes a basal lamina equivalent, extracellular factors from mesenchymal fibroblasts, and in an air-liquid interface system. Recently, spheroid-based culture systems have been developed. Several clinical applications have been suggested: cystic fibrosis, acute respiratory distress syndrome, chronic obstructive lung disease, pulmonary fibrosis, pulmonary edema, and pulmonary hypertension. Clinical applications so far are few, but include subglottic stenosis, tracheomalacia, bronchiomalacia, and emphysema.

**Keywords:** Stem cells, progenitor cells, respiratory tract, epithelium, connective tissue, Clara cells, basal cells, side population cells, cystic fibrosis, defect repair, tissue engineering.

### Introduction

The main functions of stem/progenitor cells for the airway epithelium are epithelial homeostasis and the repair of defects in the airway wall (Giangreco *et al.*, 2009). These defects may be due to obstructing tracheal tumors, trauma, or post-intubation tissue reactions that necessitate replacement of tracheal tissue. In addition, the use of tracheo-esophageal silicon rubber speech valves and tracheostoma valves in prosthetic voice rehabilitation is often accompanied by complications that make repair of tissue defects necessary (Ten Hallers *et al.*, 2004). In many airway diseases such as asthma (Bougault *et al.*, 2009; Burgess, 2009), chronic obstructive pulmonary disease (COPD) (James and Wenzel, 2007; Tzortzaki and Siafakas, 2009), obliterative bronchiolitis (a frequent complication of heart-lung transplantation and allogeneic hematopoietic stem cell transplantation) (Qu *et al.*, 2005; Xu *et al.*, 2008; Williams *et al.*, 2009), and cystic fibrosis (CF) (De Rose, 2002; Hajj *et al.*, 2007), the airway wall, in particular the airway epithelium, is damaged and subsequently repaired (“remodeled”) (Coraux *et al.*, 2008). Repair of airway epithelium damaged by disease would potentially be a major field where stem/progenitor cells could be used. However, in contrast to some other tissues, such as the gut, the hematopoietic system, skin, and hair follicles, adult stem cells in the lung are still ill-defined. Finally, it has to be realized that adult stem cells are not immortal, and show decreasing telomere length with increased age, which may result in an inability to repair damage that therefore becomes irreversible late in life. The possibility to manipulate adult stem cells later in life would potentially improve geriatric care.

An anatomical overview of the airways is shown in Fig. 1. The wall of the trachea and bronchi in the normal human airway consists of [1] a surface epithelium (with ciliated, brush, basal, and secretory cells), approximately 50  $\mu\text{m}$  thick, resting on [2] a basement membrane. Then follow layers of [3] connective tissue and [4] smooth muscle (not clearly separated), and finally, [5] cartilage (Fig. 2). The bronchioli lack cartilage. The epithelium in the major bronchi and proximal bronchioles (Fig. 3a) is a ciliated pseudostratified epithelium, with ciliated columnar cells as the predominant cell type; in the distal bronchioles the epithelium consists of a single cell layer (Fig. 3b). The ciliated columnar cells reach the basal lamina with slender basal processes (Shebani *et al.*, 2005). The function of the brush cells of the airway epithelium is not completely known. These cells lack cilia, and may be involved in detoxification, act as a sensor for airway surface liquid, or may have a chemoreceptor function. The pyramid-shaped basal cells rest on the basal lamina but do not reach the airway lumen. The average half-life

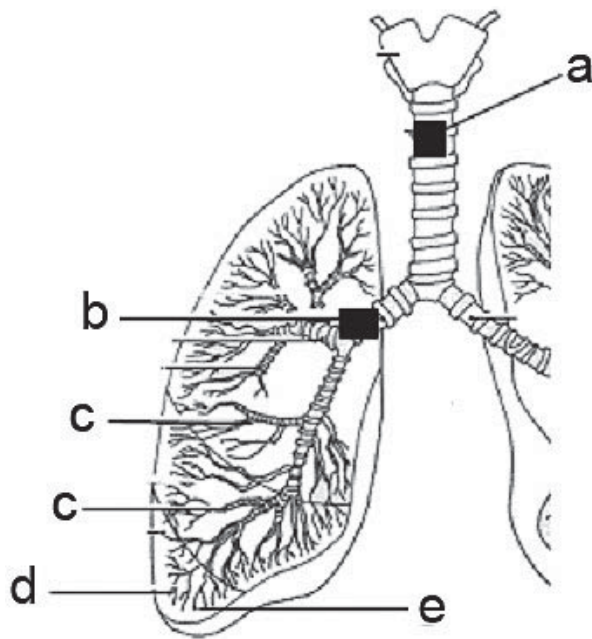
\* Address for correspondence:

Godfried M. Roomans  
School of Health and Medical Sciences,  
Örebro University, Örebro University Hospital,  
SE-70185 Örebro, Sweden

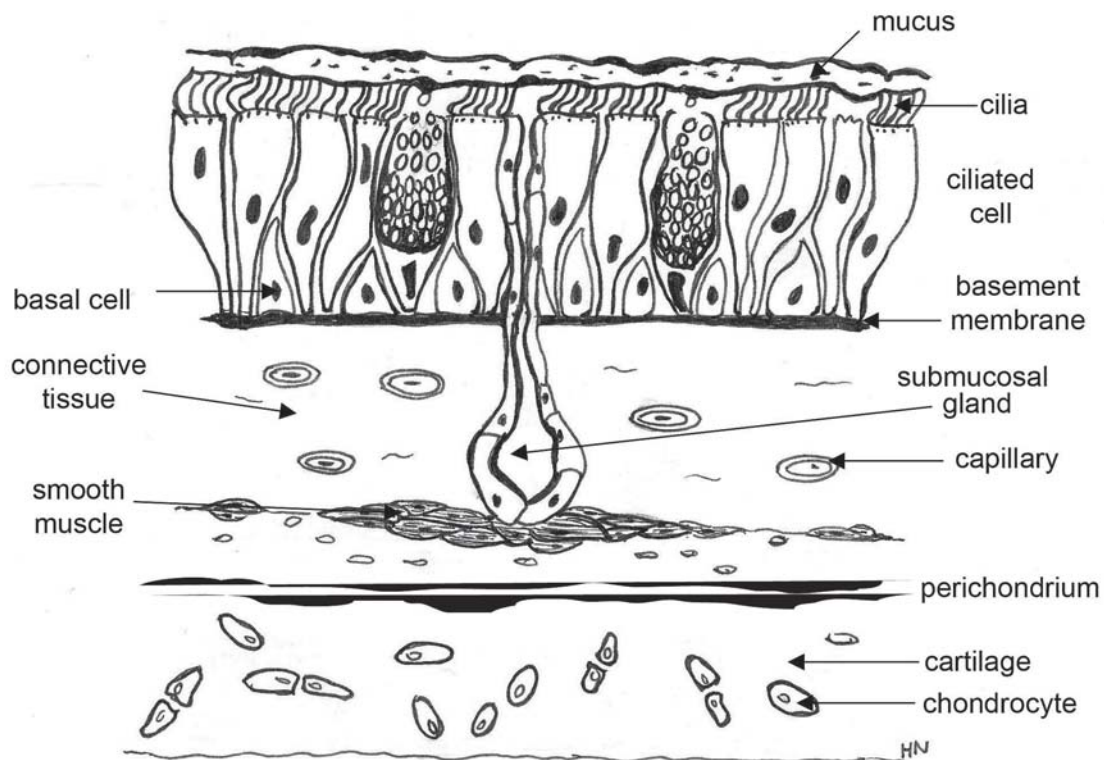
Telephone Number: 0046-19-6026677

FAX Number: 0046-19-6026677

E-mail: godfried.roomans@oru.se



**Fig. 1.** Anatomical overview of the lung and the putative stem/progenitor cell niches in the (mouse) lung: (a) a ductal cell in the submucosal gland, (b) basal cells in the intercartilaginous zones of the lower trachea, (c) Clara<sup>v</sup> cells in the bronchioles, (d) Clara<sup>v</sup> cells at the bronchiolar-alveolar duct junctions, and (e) alveolar type II cells. Note that the mouse only has submucosal glands in the proximal trachea, in contrast to the human. Modified after Liu and Engelhardt (2008).



**Fig. 2.** Overview of the airway wall.

of the ciliated cells has recently been reported to be 6 months in the trachea and 17 months in the lung, which is much longer than previously estimated (Rawlins and Hogan, 2008). This makes the airway epithelium a tissue with a slow turn-over, which is relevant for its repair (Snyder *et al.*, 2009). Different kinds of secretory cells (goblet cells in the trachea, bronchi and proximal bronchioles, Clara cells in the bronchioles, and neuroendocrine cells in the intrapulmonary airways) comprise around 20% of the bronchial and nasal epithelium and produce airway secretion (fluid and mucus). In

addition, the Clara cell secretes Clara cell secretory protein (CCSP), an immunoregulatory protein (Snyder *et al.*, 2009). The wall of the alveoli consists of squamous alveolar type I and surfactant-producing alveolar type II cells (Fig. 3c).

In the human, the embryonic lung starts to develop during the fourth week of embryonic development, when a ventral protuberance in the foregut is formed, the respiratory diverticulum (Fig. 4a) (Larsen, 1993). This entodermal protuberance is covered by mesodermal splanchnopleure, which will develop first into the

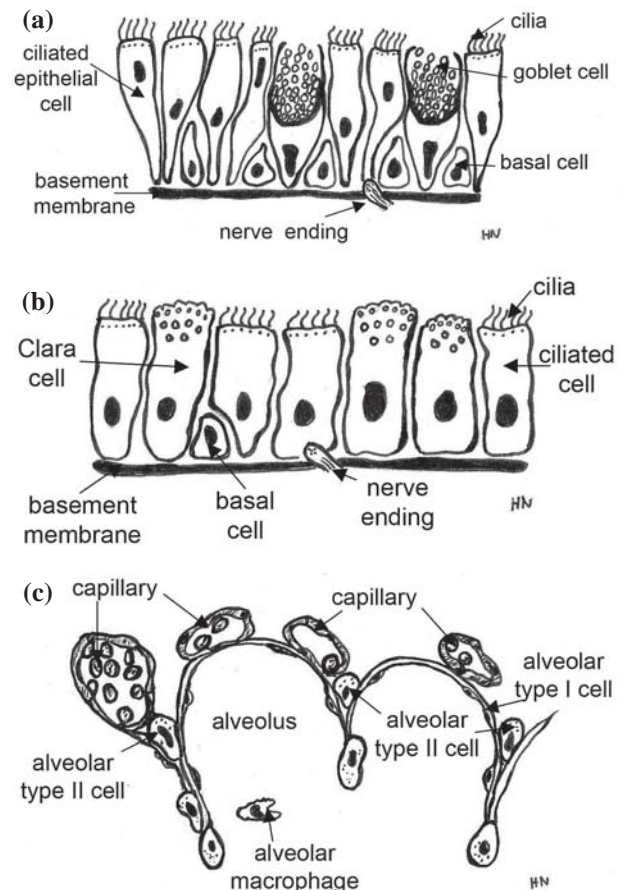
connective tissue lamina propria, and then into the smooth muscle and cartilage of the airway wall, whereas the blood vessels develop from the aortic arch. On day 26-28, the protuberance splits into two primary bronchial protuberances (the origin of the two lungs), and during the fifth week secondary bronchial protuberances (three on the right side and two on the left side: the lung lobes, Fig. 4b) are formed. During the sixth week the tertiary bronchial protuberances are formed that will give rise to the lung segments (Fig. 4c). The protuberances continue to divide during weeks 26-28; the 16th division forms the terminal bronchioles that subsequently give off the respiratory bronchioles and alveolar saccules (week 26) (Fig. 4d). From about week 32, primitive alveoli are formed. Between week 36 and birth, the alveoli (Fig. 4e) mature and from parturition on until the age of 8-10 years, new respiratory bronchioles and alveoli are formed. Only at this age the lungs are fully developed (although the lungs retain regenerative capacity at an advanced age, e.g., after surgical resection).

Organ morphogenesis is, in general, controlled by cell-cell signaling between the epithelium, the mesenchyme, and the vasculature. In practice, therefore, repair of a defect in the airway wall will require (at a minimum) (progenitors of) epithelial cells, basement membrane, and fibroblasts, as well as cells able to form blood vessels.

As stated above, damage to the airway epithelium may be the result of a number of airway diseases. In addition, extraneous chemicals, e.g., air pollution from traffic or industrial activities, may cause damage to the epithelium. However, even under normal conditions, there has to be a turn-over of the cells of the airway wall. Tissues with a relatively slow turn-over, use the self-renewal of differentiated cells as a strategy for tissue regeneration (Rawlins and Hogan, 2006). In addition, damage to the airway epithelium occurs frequently, e.g., due to components of smoke, air pollution, or to bacterial and viral infection, either from direct effects of these microorganisms or due to the inflammatory reaction that follows infection. Normally, in the case of small lesions, the airway epithelium possesses repair mechanisms that can restore the normal structure. If the damage is more extensive, e.g., in asthmatic patients, the repair process is more extensive, and is called airway remodeling (Folli *et al.*, 2008). Airway repair appears to be controlled both by bronchial epithelial cells and by endothelial cells of the perfusing bronchial vasculature (Zani *et al.*, 2008). Of these two cell types, the bronchial epithelial cells have received by far the most attention (Rawlins, 2008; Rawlins *et al.*, 2008; Rawlins *et al.*, 2009), whereas little is yet known about the role of the endothelial cells.

### Airway progenitor cells/stem cells

*In vivo*, the basal cells are widely thought to assume the role of progenitor cells (Rock *et al.*, 2009; Snyder *et al.*, 2009). So far, most experimental studies on lung progenitor cell studies have been performed in murine models. It may be questioned whether this is the best choice for the lung, since there are many structural and physiological



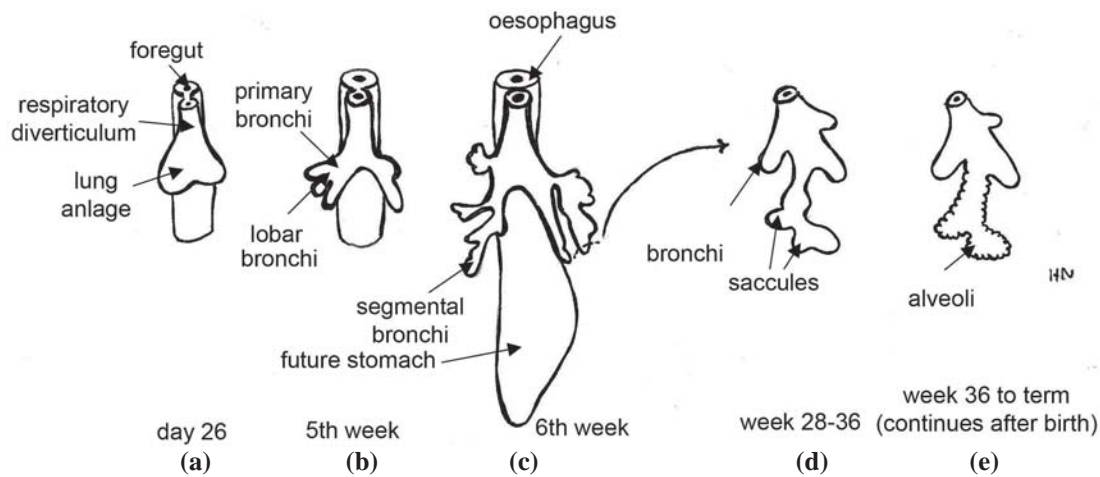
**Fig. 3.** (a) The bronchial epithelium, with ciliated and goblet cells, (b) the bronchiolar epithelium (Clara cells, as depicted here, are often considered to be present only in the most distal bronchioles) and (c) the alveoli.

differences between murine and human airways (Liu and Engelhardt, 2008). The pig is a far better animal model for studies of airway physiology than the mouse (Grubb and Boucher, 1999; Cunningham *et al.*, 2002), but as a large animal it has serious practical drawbacks (e.g., cost of acquisition and maintenance). The airways of the ferret share many similarities with the human airways, with regard to submucosal glands, and fluid transport, in particular CFTR, so the ferret would appear to be a better animal model for airway studies (Sehgal *et al.*, 1996; Wang *et al.*, 2001; Liu and Engelhardt, 2008; Abanses *et al.*, 2009).

Several potential sources of progenitor cells for airway epithelium have been identified. These can be divided into two groups: (1) endogenous stem/progenitor cells present in the respiratory tract and (2) exogenous stem/progenitor cells derived from other tissues in the body; these latter cells can be transported to the lung, where they can divide and grow.

### Endogenous stem/progenitor cells

With regard to the first group, endogenous progenitor cells, one theory has been presented detailing possible stem cell niches in murine airways (Liu and Engelhardt, 2008) (Fig. 1): (1a) a ductal cell type in the submucosal glands of the proximal trachea, (1b) basal cells in the intercartilaginous



**Fig. 4.** Embryonic development of the lungs. (a) Formation of an entodermal diverticulum (respiratory diverticulum) (from the foregut) and the two anlage for the right and left lung, (b) the lung lobes with primary and lobar bronchi, (c) the segmental bronchi; behind the lung are the oesophagus and the stomach, (d) the bronchial tree and the formation of alveolar saccules, (e) the alveoli (development of the alveoli continues after birth until about 8 years of age).

zones of the lower trachea and bronchi, (1c) variant Clara cells (Clara<sup>v</sup>-cells) in the bronchioles, (1d) Clara<sup>v</sup>-cells at the junctions between the bronchioles and the alveolar ducts, and (1e) alveolar type II cells. However, controversy still surrounds the classification of the endogenous stem/progenitor cells, and a definitive classification probably will take several more years.

Stem-cell niches in the submucosal glands and in the basal layer of the intercartilaginous zones of the lower trachea and bronchi (stem cell types 1a and 1b) were first demonstrated by Borthwick *et al.* (2001). These authors found cells expressing high levels of cytokeratin(-5, -14, -18) at these locations in mouse trachea and showed that this cell compartment was rich in bromodeoxyuridine (BrdU) label-retaining cells (LRC) in mice after tracheal damage by intratracheal application of detergent or by having the mice inhale SO<sub>2</sub>. The same result was found after naphthalene-induced tracheal damage (Hong *et al.*, 2004). Similarly, using a variety of cell proliferation markers (Ki-67, cytokeratin-5 and -14, epidermal growth factor receptor), it was concluded that basal(-like) cells constituted the proliferating cell population in cystic fibrosis (CF) airways (Voynow *et al.*, 2005). The fact that the basal cells are the primary stem/progenitor cells in the airway was also indicated by Avril-Delplanque *et al.* (2005), Coraux *et al.* (2005), and Hajj *et al.* (2007), and confirmed by Rock *et al.* (2009), who also developed a method to purify airway basal cells by fluorescence-activated cell sorting (FACS), and demonstrated that these cells could self-renew and generate luminal daughters. In conclusion, the evidence appears overwhelming that basal cells in the airway can act as stem/progenitor cells.

The LRCs in the submucosal gland have not yet been characterized, although preliminary data indicate that they may be naphthalene-resistant, Clara cell secretory protein (CCSP)-positive, and associated with calcitonin gene related peptide (CGRP)-expressing cells (Liu and Engelhardt, 2008). Questions also remain about the epithelial-mesenchymal interactions that are responsible

for epithelial invagination, ductal elongation and branching in the submucosal glands. Data from other bud-forming organs indicate that a number of signaling pathways, such as wingless + int-1 (Wnt, e.g., the lymphoid enhancer binding factor (Lef-1)), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Notch, and Hedgehog, may be involved in the morphogenesis of submucosal glands (Watkins *et al.*, 2003).

A number of findings have pointed to a possible role of Clara cells as progenitor cells for the bronchial epithelium (Hong *et al.*, 2001; Stripp, 2008; Reynolds and Malkinson, 2010). A subset of Clara cells in the bronchioles (Clara<sup>v</sup>-cells) appears to function as local stem/progenitor cells in that region of the airways (Snyder *et al.*, 2009). This subset (stem cell type 1c) consisted of LRCs that were CCSP-positive, resistant to naphthalene, could exclude the DNA-binding dye Hoechst 33342, and expressed stem-cell antigen (Sca)-1 (Giangreco *et al.*, 2004). In addition, there is a subset of naphthalene-resistant, CCSP and surfactant protein C double positive, CGRP-negative Clara<sup>v</sup> cells at the bronchioalveolar duct junction of the adult mouse lung (stem cell type 1d) (Kim *et al.*, 2005). Finally, a subset of alveolar type II cells (stem cell type 1e) has the ability to replicate and to differentiate into squamous alveolar type I cells after injury (Reddy *et al.*, 2004). (It should be noted that during embryonic development the alveolar type II cells are formed first in the alveolar lining, and that some of these type II cells undergo terminal differentiation to alveolar type I cells, whereas other type I cells may develop from a pool of epithelial precursor cells in the alveolar lining (Carlsson, 1999)).

A different theory on stem/progenitor cell populations has been presented by Warburton *et al.* (2008), who distinguish (at least) two populations of stem/progenitor cells: (a) the laryno-tracheal portion of the airways, and (b) the distal lung below the first bronchial bifurcation; this population appears to correspond largely to stem cell types 1d (Clara<sup>v</sup> cells at the junctions of the bronchioles

and the alveolar ducts) and 1e (alveolar type II cells) described above. When the alveolar epithelium is damaged, it is likely that at least one progenitor cell per alveolus must be present to achieve rapid repair of the alveolus (Warburton *et al.*, 2008). After acute oxygen injury, the expression of the enzyme telomerase, which is a marker for progenitor cells, is upregulated in type II alveolar epithelial cells (AEC) during the recovery phase (Driscoll *et al.*, 2000). AEC positive for telomerase, with reduced levels of E-cadherin, can be isolated by flow cytometry from the lungs of rats during the recovery phase from sublethal hyperoxia, and may be responsible for the repair of the damaged alveolar epithelium (Reddy *et al.*, 2004; Lee *et al.*, 2006).

Progenitor cells can be isolated from normal human adult airways after mechanical and enzymatic digestion. The isolated cells form multicellular spheroids where the cells have alveolar type II or Clara cell features and display both epithelial and mesenchymal cell characteristics (Tesei *et al.*, 2009). Another CCSP-positive bronchiolar stem/progenitor cell was described by Teisanu *et al.* (2009), with a defining phenotype of CD45(-) CD31(-) CD34(-) Sca-1 (stem cell antigen-1)(low) AF (autofluorescence) (low); these cells could be distinguished from the more abundant pool of Clara cells that were characterized by AF(high), but not by Sca-1 levels, whereas CD34(+) cells did not belong to the airway epithelial cell lineage at all (CD34 cells are mainly found as hematopoietic cells in bone marrow and umbilical cord).

### Exogenous stem cells

Examples of the second group, the exogenous stem cells, are: (2a) embryonic stem (ES) cells derived from the inner cell mass of a blastocyst-stage embryo (Coraux *et al.*, 2005), or (human) amniotic fluid stem cells (Carraro *et al.*, 2008), (2b) side-population (SP) cells from bone marrow, isolated by virtue of their ability to exclude the DNA-binding dye Hoechst 33342 (MacPherson *et al.*, 2005; MacPherson *et al.*, 2006), and epithelial stem cells present in bone marrow or in the circulation (Gomperts *et al.*, 2006; Gomperts *et al.*, 2007), (2c) fat-derived mesenchymal cells (Suzuki *et al.*, 2008), and (2d) induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Geoghegan and Byrnes, 2008).

ES cells propagate readily and generate a great variety of specialized cell types, including (murine and human) airway progenitor cells (Nishimura *et al.*, 2006; Rippon *et al.*, 2006; Wang *et al.*, 2007; Winkler *et al.*, 2008). However, gaining human ES cells implies destruction of the human embryo, and hence carries obvious ethical problems. In addition, use of ES cells carries the risk of teratoma formation (Cao *et al.*, 2007; Lee *et al.*, 2009). These problems are absent from amniotic fluid stem cells that are isolated from discarded amniocentesis specimens (Furth and Atala, 2009). These cells can differentiate into specific cell types, and preliminary results have shown that the cells can be injected in the blood stream and then end up in the lung. Since the output of the entire right side of the heart goes to the narrow alveolar capillary bed in the lung, more rigid cells than erythrocytes and leukocytes

are easily trapped in the capillary bed. However, transport of the stem cells from the capillaries to the lung parenchyma/airway wall ("homing") is inefficient in the absence of lung damage, and even in the presence of lung damage the efficiency of integration is less than 2% (Warburton *et al.*, 2008). Hence, improving the efficiency of uptake and integration will be an important prerequisite before clinical studies with exogenous stem/progenitor cells can be successfully carried out. So far, studies on homing of exogenous stem cells in the lung are rare. It has been shown that signals from the damaged lung, such as granulocyte macrophage-colony stimulating factor (GM-CSF), stimulate the bone marrow to release large numbers of mesenchymal cells, that could serve as exogenous stem cells. These cells may make, however, a major contribution to bleomycin-induced pulmonary fibrosis in mice, which is a complication that may have to be taken into account (Liu *et al.*, 2007).

Adult stem cells from bone marrow have previously been reported to have the potential to give rise to cells of multiple lineage (Krause *et al.*, 2001), and hence it appeared reasonable to investigate whether they also could give rise to cell lineages in the respiratory tract (MacPherson *et al.*, 2005). SP-cells are cells with a blood stem cell phenotype, found in small numbers in the bone marrow (Goodell *et al.*, 1996). As stated above, SP cells are defined by their ability to exclude the DNA-binding dye Hoechst 33342, and therefore also called Hoechst low cells. It was, however, found that putative stem cells from solid tissues also possessed the SP phenotype, and SP cells were isolated from a wide variety of mammalian tissues based on the same dye efflux phenomenon, and in many cases this cell population was shown to contain multipotent stem cells (Challen and Little, 2006). Indeed, the presence of SP cells in the airways was later demonstrated (Reynolds *et al.*, 2007; Hackett *et al.*, 2008).

A possible role of exogenous SP cells in lung repair was demonstrated by studies of MacPherson *et al.* (2005, 2006). SP cells were isolated from the bone marrow of male mice, and seeded on tracheae from female mice, after the epithelium had been removed by treatment with 2% povidone-iodine. One week after removal of the original epithelium, the epithelium had regrown, and it could be shown that a small proportion of cells had a Y chromosome and hence was of donor origin ("sex mismatch chimera") (MacPherson *et al.*, 2005). The donor cells had epithelial characteristics as shown by cytokeratin expression, but also hematopoietic characteristics as shown by CD45 expression (MacPherson *et al.*, 2006).

Gomperts *et al.* (2006) found another population of progenitor epithelial cells in bone marrow and in the circulation in mice. This population was positive for the early epithelial marker cytokeratin 5 (CK5) and the chemokine receptor CXCR4. CK5+ circulating progenitor epithelial cells were found to contribute to re-epithelialization of the airway and re-establishment of the pseudostratified epithelium, dependent on the presence of CXCL12 in the tracheal transplant. The keratinocyte growth factor receptor (KGF) co-localized with a population of CK5+ basal cells in the repairing airway.

Systemic administration of keratinocyte growth factor (KGF) resulted in a significant increase in mobilization of CK5+ progenitor cells and more rapid repair of the tracheal epithelium. Hence, KGF is an important growth factor for local resident progenitor epithelial cell repair (Gomperts *et al.*, 2007).

Yet another bone marrow-derived cell population capable of repairing injured airways was described by Wong *et al.* (2009). This sub-population expressed Clara-cell secretory protein (CCSP) (like the endogenous stem cells described by Liu and Engelhardt (2008) and mentioned above), type I and type II alveolar markers as well as basal cell markers and epithelial sodium channels (ENaC). The cells could be delivered to the airway intratracheally and homed preferentially to areas with naphthalene-induced lung damage.

Fat-derived stem cells have been used in the repair of bone (Bessa *et al.*, 2008; Betz *et al.*, 2010), and are regarded as potentially suitable for the repair of soft-tissue defects (Vallée *et al.*, 2009). Applications to the repair of airway defects are, however, so far few. Adipose-derived stem cells (ASCs) with multilineage differentiation capability were implanted by Suzuki *et al.* (2008) as part of a bioengineered scaffold into tracheal defects in rats. On day 14 after implantation, a pseudostratified columnar epithelium with well-differentiated ciliated and goblet cells and neovascularization was observed in the rats.

It has been shown to be possible to directly reprogram somatic cells to an embryonic stem cell-like pluripotent state (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). These induced pluripotent stem (iPS) cells present an important advantage over “real” embryonic stem cells, in that they do not require an embryo to be sacrificed, and that they ultimately will allow autologous transplantation of stem cells to repair damaged tissues (Park *et al.*, 2008). The reprogramming process requires retroviral transduction with at least three genes: Oct3/4, Sox2 and Klf4 (Geoghegan and Byrnes, 2008; Hong *et al.*, 2009), and may require suppression of the tumor suppressor genes p53 and p21 (Hong *et al.*, 2009). Although initially experiments were carried out on mice, it has been shown to be possible to prepare human iPS cells (Hong *et al.*, 2009). While no direct applications to airway cells have yet been reported, it appears well possible that such applications will become possible in the near future.

### Tissue engineering of airway epithelium

Reconstruction of tracheal defects requires in the first place the availability of airway epithelial cells. In addition, it may require the presence of fibroblasts or fibroblast-derived substances. Whether fibroblasts themselves or products produced by fibroblasts are required is not yet resolved. Airway epithelial cells should be co-cultured in a system that includes a basal lamina equivalent, extracellular factors of mesenchymal fibroblasts, and the presence of an air-liquid interface system for proliferation and differentiation of the epithelial cells (MacPherson *et al.*, 2005; Pfenninger *et al.*, 2007). The presence of fibroblasts markedly speeds up the growth of epithelial

cells (Kobayashi *et al.*, 2006; Kobayashi *et al.*, 2007). However, the effect depends on the source of the fibroblasts. Gingival fibroblasts could induce morphologically and functionally normalized tracheal epithelium (comparable to the epithelium induced by tracheal fibroblasts), whereas nasal and dermal fibroblasts could not (Kobayashi *et al.*, 2007).

Pioneering work using human tracheal xenografts was carried out in immunodeficient mice (severe combined immunodeficiency (SCID) mice and nude mice), where it was shown that human airway epithelial cells implanted in such conditioned host grafts can regenerate a well-differentiated and functional human epithelium (Dupuit *et al.*, 2000; Puchelle and Peault, 2000; Escotte *et al.*, 2004). Native human respiratory epithelial cells grow slowly, so the same group improved on this model by using human airway epithelial cells transduced by a lentivirus vector encoding the enhanced green fluorescent protein (eGFP). After lentiviral transduction the fetal airway epithelial cells rapidly reassembled as three-dimensional spheroid structures, that developed mature cilia, and possessed normal chloride channels and tight junctions; the cells could be kept in culture for (at least) 80 days (Castillon *et al.*, 2004).

Epithelial cell sheets can be directly transplanted to host tissues without the use of scaffolding or carrier materials. Yang *et al.* (2006) developed cell sheet engineering using temperature-responsive culture dishes, which allows for the harvest of cultured cells as intact sheets along with their formed extracellular matrix; these sheets can be used for several types of transplantation and surgery, e.g., tracheal replacement and corneal surface transplantation. Oxidant-induced apoptosis of airway epithelial cells, due to activation of caspase-3 and caspase-9, was inhibited by the mucolytic agent carbocysteine (Yoshida *et al.*, 2009), which may be used to protect transplanted epithelium from cell death. However, the slowness of epithelial cell growth within an animal or on a collagenous artificial trachea (see below) remains a problem. In a series of studies, Nomoto and coworkers therefore optimized methods of culturing airway epithelium. In a first study (Nomoto *et al.*, 2006), isolated rat tracheal epithelial cells were seeded on a collagenous gel that was stratified on a collagenous sponge. The next step was to co-culture tracheal epithelial cells with fibroblasts, which led to the observation that fibroblasts activated epithelial cell proliferation and migration. In co-culture with fibroblasts, epithelial cells reconstructed a pseudostratified epithelium, which was composed of ciliated, goblet, and basal cells. Also, a basement membrane was reconstructed below the epithelium (Kobayashi *et al.*, 2006). It then was found, that the source of fibroblasts was critical, and that gingival fibroblasts were better suited than nasal and dermal fibroblasts (Kobayashi *et al.*, 2007). Also Heikal *et al.* (2008) prepared a tissue-engineered respiratory epithelium construct including autologous (ovine) respiratory epithelial cells, fibroblasts and autologous fibrin, which resulted in the development of cells with immature cilia. Also from studies of physiological airway repair, it is well-known, that the

interaction between epithelium and mesenchymal elements such as fibroblasts is essential, and an abnormal response of this epithelial-mesenchymal trophic unit has been proposed to be central to the airway pathology and physiology characteristics of asthma (Folli *et al.*, 2008).

Further progress in tissue engineering of airways was obtained by the use of a bipotential collagen scaffold, developed by conjugating a collagen vitrigel membrane to a collagen sponge (Tada *et al.*, 2008). Nomoto *et al.* (2008) suspended isolated rat tracheal epithelial cells in a collagenous gel. This collagenous gel with fibroblasts was layered on a collagenous sponge, and the grafts of this "bioengineered trachea" were implanted into tracheal defects of rats. Already seven to fourteen days after implantation, epithelium had covered most of the defect, and the presence of fibroblasts had a positive effect on epithelial growth, even though the fibroblasts disappeared in the end.

The fact that fibroblasts had a positive effect on airway epithelial cell growth emphasizes the fact that the airway wall is not a simple structure, and that epithelial-mesenchymal interactions are important. A complicated system was proposed by Wallis *et al.* (2004), who used 10- to 15-cm-long acellularized pig jejunal segments with their own vascular pedicle. Autologous costal chondrocytes, smooth muscle cells, and respiratory epithelium and endothelial progenitor cells were cultured *in vitro* and disseminated on this matrix. The endothelial progenitor cells re-endothelialized the matrix and the vascularized scaffold was seeded with smooth muscle cells and viable ciliated respiratory epithelium. Chondrocyte growth and production of extracellular cartilaginous matrix was observed as soon as 2 weeks after their culture.

### Tissue engineering of trachea

To repair larger defects, it may be necessary to grow the airway epithelium (plus fibroblasts) on a sturdy, solid, biocompatible substrate, e.g., polymer-coated wires (Servetnyk *et al.*, 2006). Repair of larger defects may also make it necessary to use cartilage implants, and this has been investigated primarily for use in laryngotracheal reconstruction (LTR) after stenosis. In a series of studies by Kojima and coworkers, chondrocytes and fibroblasts were isolated and seeded onto separate non-woven meshes of polyglycolic acid. The chondrocyte-seeded mesh was wound around a helical template and then covered with the fibroblast-seeded mesh. Gross morphology and tissue morphology of tissue-engineered tracheas (TET) was similar to that of native tracheas. Collagen and cell contents in TET were elevated compared with that of normal tracheas, whereas proteoglycan content was less than in normal tracheas (Kojima *et al.*, 2002; Kojima *et al.*, 2003a). Kojima *et al.* (2003b) showed that the properties of tissue-engineered trachea using nasal chondrocytes were similar to that obtained using tracheal chondrocytes, which has the advantage of facilitating use of an easily obtainable autologous source of chondrocytes for repair of segmental tracheal defects. A composite engineered cylindrical tracheal equivalent constructed from autologous tissue

could be lined with nasal epithelial cells in a nude-mouse model. However, some problems with the mechanical stability of the tracheal implant were reported (Kojima and Vacanti, 2004). Weidenbecher *et al.* (2007) used Hyalograft C combined with autologous chondrocytes to engineer cartilage grafts for LTR in rabbits. However, all tissue-engineered grafts and empty scaffolds revealed marked signs of an unspecific foreign body reaction, leading to a complete degradation of the neocartilage, whether implanted para- or intralaryngeally. This group also implanted autologous neotracheal constructs in the abdomen of rabbits, using scaffold-free cartilage sheets made of auricular chondrocytes. A muscle flap raised from the external abdominal oblique muscle and the engineered cartilage were wrapped around a silicone stent to fabricate a vascularized neotrachea *in vivo*. It was found that scaffold-free engineered cartilage can successfully fabricate a well-vascularized, autologous neotrachea (Weidenbecher *et al.*, 2008; Weidenbecher *et al.*, 2009; Gilpin *et al.*, 2010).

Recent studies in animals have pointed out the usefulness of combining epithelial stem/progenitor cells and mesenchymal stem cell-derived chondrocytes to repair airway defects (Baiguero *et al.*, 2010; Go *et al.*, 2010). In order to repair tracheal stenosis, Komura *et al.* (2008) designed an engineered tracheal graft from a biodegradable scaffold using chondrocytes for rabbit auricular cartilage. The cells were seeded on a scaffold consisting of collagen, a polyglycolic acid mesh, and an L-lactide/ $\epsilon$ -caprolactone mesh; basic fibroblast growth factor was released into the graft from gelatin sponges.

A different approach was taken by Yamashita *et al.* (2007) who regenerated tracheal epithelium in an experimentally-induced defect in canine trachea. A polypropylene and collagen scaffold preclotted with peripheral blood was inserted into the defect, and this resulted after 8-12 months in a regenerated mucosa with ciliated epithelium and capillaries, as well as newly formed cartilage.

### Developmental signaling molecules of relevance for tissue engineering

It is evident that knowledge about the development of the respiratory system in the embryo is relevant for tissue engineering to repair the airways in the adult, particularly details on the regulation of cell proliferation and differentiation. As stated above, the lung starts to develop as a ventral protuberance in the foregut, and separation of this protuberance (the future larynx and trachea) from the foregut (the future esophagus, stomach and duodenum) is a necessary step for the subsequent separation of the foregut tube into trachea and esophagus. Defects in foregut morphogenesis underlie the human birth defects known as esophageal atresia and tracheo-esophageal fistula. Que *et al.* (2006) showed that most mouse embryos homozygous null for *Nog*, the gene encoding noggin, a bone morphogenetic protein (Bmp) antagonist, have esophageal atresia or tracheo-esophageal fistulas, as well as defects in lung branching, but that these abnormalities

can be rescued by reducing the gene dose of *Bmp4* by 50%. Hence, normal foregut morphogenesis requires that the level of *Bmp4* activity is carefully controlled by means of antagonists such as noggin.

Several gene factors may influence the process of airway epithelial growth. The tumor suppressor gene *Pten*, which is mutated in many human cancers, appears to be essential for the homeostasis of bronchioalveolar stem cells and hence for normal lung morphogenesis and the prevention of lung carcinogenesis (Yanagi *et al.*, 2007). Deletion of this gene caused inhibition of the differentiation of various lung epithelial cell lines, with decreased numbers of terminally differentiated cells (Tiozzo *et al.*, 2009). Another tumor suppressor gene, *p63*, related to the *p53* gene, is present mainly in the basal layer of the developing trachea (and esophagus), but decreases towards the more superficial cells (Daniely *et al.*, 2004); this gene appears in general to be involved in the development of stratified epithelia (Koster *et al.*, 2004; Senoo *et al.*, 2007). In contrast to basal cells, ciliated cells express the HNF-3/ forkhead homologue-4 (HFH-4), a transcription factor of the winged-helix/forkhead family (Blatt *et al.*, 1999; Tichelaar *et al.*, 1999).

The *Sox2* gene is important for the branching of the airways; it appears that downregulation of this gene is required for branching to take place (Gontan *et al.*, 2008). Investigations of the significance of the *Sox2* gene (Que *et al.*, 2009) were carried out on mutants with a conditionally deleted *Sox2* gene in the ventral epithelial domain of the early anterior foregut, which gives rise to the future trachea and lung buds. All mutants died of respiratory distress at birth, and most had a short trachea, which means that the primary budding site of the lung shifted anteriorly. Conditional deletion of *Sox2* was also important in the maintenance of adult epithelium, since epithelial cells lacking *Sox2* showed a reduced capacity to proliferate in culture and to repair after injury *in vivo*. *Sox17*, a gene required for early endoderm formation, reduced the expression of transforming growth factor (TGF)- $\beta$  cell cycle inhibitors, and hence activated the cell cycle, and reinitiated multipotent progenitor cell behavior in mature lung cells (Lange *et al.*, 2009). The Notch gene is instrumental in the differentiation of cells in the proximal airways, silencing the ciliated program in those cells that will differentiate into secretory cells (Tsao *et al.*, 2009).

### The connective tissue compartment

The important role of tracheal mesenchymal cells for the growth of the respiratory epithelium has been discussed above. During embryonic development, mesenchyme and vascular smooth muscle develop from mesothelium (Que *et al.*, 2008). As stated previously, in the bioengineered lung, mesenchyme cells can be obtained from a variety of fibroblast sources, but tracheal mesenchyme cells can also be isolated from the lungs of premature infants undergoing mechanical ventilation (Henrick *et al.*, 2007). In addition to endogenous mesenchymal cells, also exogenous mesenchymal cells, such as bone marrow-derived mesenchymal stem cells (BMSCs) can be used (Van

Haafte *et al.*, 2009). In animal experiments (rats), it could be shown, that BMSCs developed characteristics of type II alveolar epithelial cells (AEC2) when co-cultured with lung tissue, and were preferentially attracted towards oxygen-damaged lung vs normal lung. *In vivo*, intratracheal delivery of BMSCs in newborn rats improved survival of the animals while attenuating alveolar and lung vascular injury and pulmonary hypertension. However, it was doubtful if this was due to BMSCs acting as stem/progenitor cells, because of their small numbers; a more likely explanation of the effect was felt to be a paracrine activity of the BMSCs (Van Haafte *et al.*, 2009).

Since airway mesodermal cells, such as myofibroblasts and airway smooth muscle cells, contribute to the pathology of asthma by excessive proliferation, it was investigated whether mesenchymal cells from asthmatics would be more suitable for use in tracheal reconstruction than mesenchymal cells from healthy donors; this was not found to be the case (Ward *et al.*, 2008).

Apart from their importance for epithelial growth, airway mesenchymal cells also have a wider significance. The administration of mesenchymal stem cells (MSCs) has been proposed for the treatment of pulmonary hypertension. Baber *et al.* (2007) studied the effect of intratracheal administration of rat MSCs (rMSCs) on monocrotaline-induced pulmonary hypertension and impaired endothelium-dependent responses. Intratracheal injection of rMSCs after administration of monocrotaline attenuated the rise in pulmonary arterial pressure and pulmonary vascular resistance, restored pulmonary responses to acetylcholine toward normal values, and decreased the right ventricular hypertrophy induced by monocrotaline. Transplanted rMSCs were found widespread in the lung parenchyma surrounding the airways in monocrotaline-treated rats and retained markers specific for endothelial and smooth muscle phenotypes, but were not detected in the wall of pulmonary vessels.

Treatment of experimental animals with bleomycin or alcohol is used as an animal model for fibrosis. In rats treated with bleomycin, it was investigated whether MSC engraftment could protect the lungs against bleomycin-induced injury (Zhao *et al.*, 2008). Some MSCs positive for pan-cytokeratin staining, an indicator of alveolar epithelial cells, were present in the injured lung tissue. Bleomycin injection increased the content of hydroxyproline in lung tissue, as well as laminin and hyaluronan in bronchoalveolar lavage fluid, markers for lung injury and fibrosis. These effects were attenuated by MSC treatment. Lung fibrosis also is associated with increased synthesis of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), platelet-derived growth factor (PDGF)- $\alpha$ , PDGF- $\beta$ , and insulin-like growth factor (IGF)-I. Also this effect of bleomycin was significantly decreased by MSC treatment (Zhao *et al.*, 2008).

### Clinical applications

Concurrent with the ongoing search for stem cells that can be applied to human treatment, precise delivery and homing (to the site of the disease) must be ensured for



successful therapy. Stem cells can safely be instilled via the trachea. This involves a skin incision below the larynx, opening of the tracheal lumen, and caudal insertion of a cannula into and along the tracheal lumen, and injection of a stem cell vehicle mixture into the airways. The airway epithelium will confine the stem cells initially to the airway lumen, and subsequently, lung expansion and negative pressure during inhalation may assist in stem cell integration in the epithelium (Peter, 2007). In a murine model of epithelial airway injury, up to about 5% of the injected stem cells survived, but survival was drastically reduced when the airway epithelium was intact (Leblond *et al.*, 2009). There will certainly also be obstacles involving physiologic barriers such as mucus and airway-surface or alveolar liquid (Roy and Vij, 2010).

A common feature of lung disorders with poor treatment options, including emphysema, is a failure to initiate a repair process of the alveolar epithelium. As stated previously, several putative stem cell niches in the lung thought to be involved in lung homeostasis have been described. (Liu and Engelhardt, 2008). However, under pathophysiological conditions resident stem cell niches in the lung thought to be involved in lung homeostasis are unable to recover damaged alveolar epithelium, in particular in emphysema. (Emphysema is a subtype of COPD, defined as dilatation and destruction of lung tissue distal to the terminal bronchiole). Recently, it has been shown that all-*trans*-retinoic acid (ATRA), an analogue of vitamin A, has a potential therapeutic effect on various resident lung progenitor cells (Lenssen and Stolk, 2007). Experiments on rat and mice models of emphysema have shown that ATRA inhibited progression of airway obstruction (Tepper *et al.*, 2000) and promoted lung regeneration after injury (Ishizawa *et al.*, 2004). Retinoic acid reduced the damage induced by elastase to cultured airway epithelial cells (cell lines and primary cultures) (Nakajoh *et al.*, 2003). Elastase treatment of laboratory animals is an established model in experimental studies of emphysema (Hayes *et al.*, 1975; Kaplan *et al.*, 1973). The first study with retinoic acid on humans, however, failed to show any positive effects on the emphysema, but did show mild negative side-effects (including skin changes, transient headache, hyperlipidemia, transaminites, and musculoskeletal pains) (Mao *et al.*, 2002). The absence of positive effects was confirmed in a later study (Roth *et al.*, 2006). Nonetheless, a further clinical study is in progress (Lenssen and Stolk, 2007). However, additional studies on mice have shown strain-specific differences in the response to retinoic acid (Stinchcombe and Maden, 2008). The potential effect of retinoic acid on emphysema is hence at the moment, unclear.

For a number of serious airway diseases, stem/progenitor cell therapy has been suggested as a possible cure. For none of these diseases there is yet experimental support that stem/progenitor cells would be helpful. The prime disease mentioned in this respect is cystic fibrosis (Olsson *et al.*, 2007; Weiss, 2008), but also acute respiratory distress syndrome (ARDS) (Jiang and Li, 2009), COPD (Puchelle *et al.*, 2006; Ohnishi and Nagaya, 2008), pulmonary fibrosis, pulmonary edema (Olsson *et*

*al.*, 2007), and pulmonary hypertension (Sueblinvong and Weiss, 2009). It has also been suggested to use stem/progenitor cells to promote perinatal lung growth (Roszell *et al.*, 2009).

Chen *et al.* (2008) reported a successful attempt at transplanting stem cells in two patients with mucopolysaccharidosis (MPS), a lysosomal storage disease caused by the absence or malfunctioning of the enzymes needed to break down glycosaminoglycans, and commonly associated with obstructive airway disease and obstructive sleep apnea (Yeung *et al.*, 2009). The two patients were (at the time of transplantation) a 23-month MPS type IH patient and an 18-month old MPS type VI patient. Surprisingly, the authors also reported that the cornea, which normally is clouded in MPS patients, became clear and that the patient's heart condition improved.

Subglottic stenosis is characterized by the obliteration of the tracheal lumen due to excessive formation of connective tissue. Tracheal injury triggers the early production of transforming growth factor-beta1 (TGF- $\beta$ 1), a factor implicated in fibroproliferative disorders. TGF- $\beta$ 1 stimulates the transformation of tracheal fibroblasts into myofibroblasts with increased matrix production and scar contraction (Jarmuz *et al.*, 2004). Such a condition is, as stated above, a prime example where repair of the airway by stem cells could be used. Omori *et al.* (2008) developed a tissue scaffold made from a mesh tube covered by collagen sponge, which was implanted to repair the larynx and trachea in 4 patients (1 with subglottic stenosis and 3 with thyroid cancer), where the cartilage of the cervical trachea and laryngeal cartilages were resected and reconstructed by use of the scaffold. This was reported to result in a well-epithelialized airway lumen without any obstruction.

The most interesting series of studies on bioengineered trachea was initialized by Macchiarini *et al.* (2004), who prepared a bioartificial patch starting from muscle cells and fibroblasts isolated from a biopsy obtained from the patient (i.e., the future recipient of the transplant). These cells were seeded onto a collagen network obtained from a decellularized porcine jejunal segment (the porcine tissue was gradually replaced by autologous connective tissue) (Wallis *et al.*, 2004). The thus obtained patch was after some weeks of culture transplanted to the patient. After the transplantation, the patch was reseeded with the patient's own ciliated respiratory epithelium. The first human tissue-engineered trachea replacement was performed by Macchiarini *et al.* (2008), who removed cells and thereby major histocompatibility complex (MHC) antigens from a tracheal segment of a deceased human donor (a 51-year old female), which was then readily colonised by epithelial cells and mesenchymal stem-cell-derived chondrocytes that had been cultured from cells taken from the recipient (a 30-year old woman with end-stage bronchomalacia). This graft was then used to replace the recipient's left main bronchus. The graft immediately provided the recipient with a functional airway, improved her quality of life, and had a normal appearance and mechanical properties at 4 months. Since this was transplantation with autologous epithelial cells and

chondrocytes, the patient had no anti-donor antibodies and did not need immunosuppressive drugs. At 18 months after the surgery, the patient was reported to lead a near-normal life without immunosuppression (Bader and Macchiarini, 2010).

Finally, it has been proposed that since chronic lung diseases, such as obliterative bronchiolitis, COPD, and asthma are characterized by epithelial and mesenchymal remodeling (with as main features chronic injury to the airway epithelium, decreased number of Clara cells, goblet cell hyperplasia, mucous cell metaplasia, basement membrane thickening, and smooth muscle hypertrophy), a more thorough understanding of the role of stem/progenitor cells in airway biology could yield important insights into these chronic disease processes (Snyder *et al.*, 2009).

### Conclusion

In theory, stem cells can be used to repair defects in the airways, and this could be used to treat airway diseases varying from common (COPD, pulmonary edema, repair of tumor-connected defects) to less common (ARDS, bronchomalacia, CF, pulmonary hypertension). Given the fact that (chronic) airway diseases occupy four of the top-ten places in the list of global causes of death ([www.who.int/mediacentre/factsheets/fs310](http://www.who.int/mediacentre/factsheets/fs310)), the importance of finding a cure for these diseases cannot be overestimated. Stem cell therapy provides a potentially novel approach to this problem, and deserves to be seriously explored. There are many potential sources for suitable stem cells, each with their own advantages and disadvantages: some can be isolated from the lung, and thus resemble closely the cell type needed for repair, but here the problem may be to obtain a sufficient number of stem cells. Other stem cells can more easily, and in greater quantity, be obtained from other tissues, such as bone marrow or adipose tissue, but steps are needed to “convert” these cells to airway cells. Cells suitable for transplantation can be isolated from the patient that is to be treated (e.g., Macchiarini *et al.*, 2008), which is a definite advantage, because it avoids the need for immunosuppressive drugs. It is also clear that much work is needed before stem cell therapy in respiratory medicine can be done in practice. “Homing” of the stem cells that act as a replacement still is a problem, because it appears to require extensive epithelial damage of the cells that have to be replaced. At the moment, practical results are scarce, but the interest in this field is growing.

### Acknowledgements

I am grateful to Dr Harriet Nilsson for the schematic drawings. Supported by grants from the Swedish Research Council and the Swedish Heart Lung Foundation.

### References

- Abanses JC, Arima S, Rubin BK (2009) Vicks vapoRub induces mucin secretion, decreases ciliary beat frequency, and increases tracheal mucus transport in the ferret trachea. *Chest* **135**: 143-148.
- Avril-Delplanque A, Casal I, Castillon N, Hinnrasky J, Puchelle E, Péault B (2005) Aquaporin-3 expression in human fetal airway epithelial progenitor cells. *Stem Cells* **23**: 992-1001.
- Baber SR, Deng W, Master RG, Bunnell BA, Taylor BK, Murthy SN, Hyman AL, Kadowitz PJ (2007) Intratracheal mesenchymal stem cell administration attenuates monocrotaline-induced pulmonary hypertension and endothelial dysfunction. *Am J Physiol Heart Circ Physiol* **292**: H1120-H1128.
- Bader A, Macchiarini P (2010) Moving towards in situ tracheal regeneration: the bionic tissue engineered transplantation approach. *J Cell Mol Med*, in press.
- Baiguero S, Birchall MA, Macchiarini P (2010) Tissue-engineered tracheal transplantation. *Transplantation* **89**: 485-491.
- Bessa PC, Pedro AJ, Klösch B, Nobre A, van Griensven M, Reis RL, Casal M (2008) Osteoinduction in human fat-derived stem cells by recombinant human bone morphogenetic protein-2 produced in *Escherichia coli*. *Biotechnol Lett* **30**: 15-21.
- Betz OB, Betz VM, Abdulazim A, Penzkofer R, Schmitt B, Schröder C, Mayer-Wagner S, Augat P, Jansson V, Müller PE (2010) The repair of critical size bone defects using expedited, autologous BMP-2 gene activated fat implants. *Tissue Eng Part A* **16**: 1093-1101.
- Blatt EN, Yan XH, Wuerffel MK, Hamilos DL, Brody SL (1999) Forkhead transcription factor HFH-4 expression is temporally related to ciliogenesis. *Am J Respir Cell Mol Biol* **21**: 168-176.
- Borthwick DW, Shahbazian M, Krantz QT, Dorin JR, Randell SH (2001) Evidence for stem-cell niches in the tracheal epithelium. *Am J Respir Cell Mol Biol* **24**: 662-670.
- Bougault V, Turmel J, St-Laurent J, Bertrand M, Boulet LP (2009) Asthma, airway inflammation and epithelial damage in swimmers and cold-air athletes. *Eur Respir J* **33**: 740-746.
- Burgess JK (2009) The role of the extracellular matrix and specific growth factors in the regulation of inflammation and remodelling in asthma. *Pharmacol Ther* **122**: 19-29.
- Cao F, van der Bogt KE, Sadrzadeh A, Xie X, Sheikh AY, Wang H, Connolly AJ, Robbins RC, Wu JC (2007) Spatial and temporal kinetics of teratoma formation from murine embryonic stem cell transplantation. *Stem Cells Dev* **16**: 883-891.
- Carlsson BM (1999) *Human Embryology & Developmental Biology*. 2<sup>nd</sup> ed. Mosby, St. Louis, pp. 344-347.
- Carraro G, Perin L, Sedrakyan S, Giuliani S, Tiozzo C, Lee J, Turcatel G, De Langhe SP, Driscoll B, Bellusci S, Minoo P, Atala A, De Filippo RE, Warburton D (2008) Human amniotic fluid stem cells can integrate and

differentiate into epithelial lung lineages. *Stem Cells* **26**: 2902-2911.

Castillon N, Avril-Delplanque A, Coraux C, Delenda C, Péault B, Danos O, Puchelle E (2004) Regeneration of a well-differentiated human airway surface epithelium by spheroid and lentivirus vector-transduced airway cells. *J Gene Med* **6**: 846-856.

Challen GA, Little MH (2006) A side order of stem cells: the SP phenotype. *Stem Cells* **24**: 3-12.

Chen J, Jiang H, Dong L, Wang Y, Luo C, Zhou M, Zhang W, Huang S, Gu X, Qiu W, Zhang H, Gu L (2008) [Treatment of 2 children with mucopolysaccharidosis by allogeneic hematopoietic stem cell transplantation] *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* **25**: 675-677.

Coraux C, Nawrocki-Raby B, Hinnrasky J, Kileztky C, Gaillard D, Dani C, Puchelle E (2005) Embryonic stem cells generate airway epithelial tissue. *Am J Respir Cell Mol Biol* **32**: 87-92.

Coraux C, Roux J, Jolly T, Birembaut P (2008) Epithelial cell-extracellular matrix interactions and stem cells in airway epithelial regeneration. *Proc Am Thorac Soc* **5**: 689-694.

Cunningham S, Meng QH, Klein N, McAnulty RJ, Hart SL (2002) Evaluation of a porcine model for pulmonary gene transfer using a novel synthetic vector. *J Gene Med* **4**: 438-446.

Daniely Y, Liao G, Dixon D, Linnoila RI, Lori A, Randell SH, Oren M, Jetten AM (2004) Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. *Am J Physiol Cell Physiol* **287**: C171-C181.

De Rose V (2002) Mechanisms and markers of airway inflammation in cystic fibrosis. *Eur Respir J* **19**: 333-340.

Driscoll B, Buckley S, Bui KC, Anderson KD, Warburton D (2000) Telomerase in alveolar epithelial development and repair. *Am J Physiol Lung Cell Mol Physiol* **279**: L1191-L1198.

Dupuit F, Gaillard D, Hinnrasky J, Mongodin E, de Bentzmann S, Copreni E, Puchelle E (2000) Differentiated and functional human airway epithelium regeneration in tracheal xenografts. *Am J Physiol Lung Cell Mol Physiol* **278**: L165-L176.

Escotte S, Catusse C, Coraux C, Puchelle E (2004) Reconstitution of human airway tissue in the humanized xenograft model. *J Cyst Fibros* **3 Suppl 2**: 63-65.

Folli C, Descalzi D, Scordamaglia F, Riccio AM, Gamalero C, Canonica GW (2008) New insights into airway remodelling in asthma and its possible modulation. *Curr Opin Allergy Clin Immunol* **8**: 367-375.

Furth ME, Atala A (2009) Stem cell sources to treat diabetes. *J Cell Biochem* **106**: 507-511.

Geoghegan E, Byrnes L (2008) Mouse induced pluripotent stem cells. *Int J Dev Biol* **52**: 1015-1022.

Giangreco A, Shen H, Reynolds SD, Stripp BR (2004) Molecular phenotype of airway side population cells. *Am J Physiol Lung Cell Mol Physiol* **286**: L624-L630.

Giangreco A, Arwert EN, Rosewell IR, Snyder J, Watt FM, Stripp BR (2009) Stem cells are dispensable for lung homeostasis but restore airways after injury. *Proc Natl Acad Sci USA*. **106**: 9286-9291.

Gilpin DA, Weidenbecher MS, Dennis JE (2010) Scaffold-free tissue-engineered cartilage implants for laryngotracheal reconstruction. *Laryngoscope* **120**: 612-617.

Go T, Jungebluth P, Baiguero S, Asnaghi A, Martorell J, Ostertag H, Mantero S, Birchall M, Bader A, Macchiarini P (2010) Both epithelial cells and mesenchymal stem cell-derived chondrocytes contribute to the survival of tissue-engineered airway transplants in pigs. *J Thorac Cardiovasc Surg* **139**: 437-443.

Gomperts BN, Belperio JA, Rao PN, Randell SH, Fishbein MC, Burdick MD, Strieter RM (2006) Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury. *J Immunol* **176**: 1916-1927.

Gomperts BN, Belperio JA, Fishbein MC, Keane MP, Burdick MD, Strieter RM (2007) Keratinocyte growth factor improves repair in the injured tracheal epithelium. *Am J Respir Cell Mol Biol* **37**: 48-56.

Gontan C, de Munck A, Vermeij M, Grosveld F, Tibboel D, Rottier R (2008) Sox2 is important for two crucial processes in lung development: branching morphogenesis and epithelial cell differentiation. *Dev Biol* **317**: 296-309.

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* **183**: 1797-1806.

Grubb BR, Boucher RC (1999) Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiol Rev* **79**: S193-S214.

Hackett TL, Shaheen F, Johnson A, Wadsworth S, Pechkovsky DV, Jacoby DB, Kicic A, Stick SM, Knight DA (2008) Characterization of side population cells from human airway epithelium. *Stem Cells* **26**: 2576-2585.

Hajj R, Lesimple P, Nawrocki-Raby B, Birembaut P, Puchelle E, Coraux C (2007) Human airway surface epithelial regeneration is delayed and abnormal in cystic fibrosis. *J Pathol* **211**: 340-350.

Hayes JA, Korthy A, Snider GL (1975) Pathology of elastase-induced panacinar emphysema in hamsters. *J Pathol* **117**: 1-14.

Heikal MY, Aminuddin BS, Jeevanan J, Chen HC, Sharifah S, Ruszymah BH (2008) A scanning electron microscopic study of *in vivo* tissue engineered respiratory epithelium in sheep. *Med J Malaysia* **63 Suppl A**: 34.

Henrick KT, Keeton AG, Nanua S, Kijek TG, Goldsmith AM, Sajjan US, Bentley JK, Lama VN, Moore BB, Schumacher RE, Thannickal VJ, Hershenson MB (2007) Lung cells from neonates show a mesenchymal stem cell phenotype. *Am J Respir Crit Care Med* **175**: 1158-1164.

Hong H, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nahagawa M, Okita K, Yamanaka S (2009) Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* **460**: 1132-1135.

Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR (2001) Clara cell protein-expressing cells of the airway neuroepithelial body environment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *Am J Respir Cell Mol Biol* **24**: 671-681.

- Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR (2004) *In vivo* differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations. *Am J Physiol Lung Cell Mol Physiol* **286**: L643-L649.
- Ishizawa K, Kubo H, Yamada M, Kobayashi S, Numasaki M, Ueda S, Suzuki T, Sasaki H (2004) Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema. *FEBS Lett* **556**: 249-252.
- James AL, Wenzel S (2007) Clinical relevance of airway remodelling in airway diseases. *Eur Respir J* **30**: 134-155.
- Jarmuz T, Roser S, Rivera H, Gal A, Roman J (2004) Transforming growth factor-beta1, myofibroblasts, and tissue remodeling in the pathogenesis of tracheal injury: potential role of gastroesophageal reflux. *Ann Otol Rhinol Laryngol* **113**: 488-497.
- Jiang JX, Li L (2009) Potential therapeutic application of adult stem cells in acute respiratory distress syndrome. *Chin J Traumatol* **12**: 228-233.
- Kaplan PD, Kuhn C, Pierce JA (1973) The induction of emphysema with elastase: I. The evolution of the lesion and the influence of serum. *J Lab Clin Med* **82**: 349-356.
- Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* **121**: 823-835.
- Kobayashi K, Nomoto Y, Suzuki T, Tada Y, Miyake M, Hazama A, Kanemaru S, Nakamura T, Omori K (2006) Effect of fibroblasts on tracheal epithelial regeneration *in vitro*. *Tissue Eng* **12**: 2619-2628.
- Kobayashi K, Suzuki T, Nomoto Y, Tada Y, Miyake M, Hazama A, Nakamura T, Omori K (2007) Potential of heterotopic fibroblasts as autologous transplanted cells for tracheal epithelial regeneration. *Tissue Eng* **13**: 2175-2184.
- Kojima K, Vacanti CA (2004) Generation of a tissue-engineered tracheal equivalent. *Biotechnol Appl Biochem* **39**: 257-262.
- Kojima K, Bonassar LJ, Roy AK, Vacanti CA, Cortiella J (2002) Autologous tissue-engineered trachea with sheep nasal chondrocytes. *J Thorac Cardiovasc Surg* **123**: 1177-1184.
- Kojima K, Bonassar LJ, Roy AK, Mizuno H, Cortiella J, Vacanti CA (2003a) A composite tissue-engineered trachea using sheep nasal chondrocyte and epithelial cells. *FASEB J* **17**: 823-828.
- Kojima K, Bonassar LJ, Ignatz RA, Syed K, Cortiella J, Vacanti CA (2003b) Comparison of tracheal and nasal chondrocytes for tissue engineering of the trachea. *Ann Thorac Surg* **76**: 1884-1888.
- Komura M, Komura H, Kanamori Y, Tanaka Y, Suzuki K, Sugiyama M, Nakahara S, Kawashima H, Hatanaka A, Hoshi K, Ikada Y, Tabata Y, Iwanaka T (2008) An animal model study for tissue-engineered trachea fabricated from a biodegradable scaffold using chondrocytes to augment repair of tracheal stenosis. *J Pediatr Surg* **43**: 2141-2146.
- Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR (2004) p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev* **18**: 126-131.
- Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**: 369-377.
- Lange AW, Keiser AR, Wells JM, Zorn AM, Whitsett JA (2009) Sox17 promotes cell cycle progression and inhibits TGF- $\beta$ /Smad3 signaling to initiate progenitor cell behavior in the respiratory epithelium. *PLoS One* **4**: e5711
- Larsen WJ (1993) *Human Embryology*. Churchill Livingstone, New York.
- Leblond AL, Naud P, Forest V, Gourden C, Sagan C, Romefort B, Mathieu E, Delorme B, Collin C, Pagès JC, Sensebé L, Pitard B, Lemarchand P (2009) Developing cell therapy techniques for respiratory disease: Intratracheal delivery of genetically engineered stem cells in a murine model of airway injury. *Hum Gene Ther*, in press.
- Lee AS, Tang C, Cao F, Xie X, van der Bogt K, Hwang A, Connolly AJ, Robbins RC, Wu JC (2009) Effects of cell number on teratoma formation by human embryonic stem cells. *Cell Cycle* **8**: 2608-2612.
- Lee J, Reddy R, Barsky L, Winberg K, Driscoll B (2006) Contribution of proliferation and DNA damage repair to alveolar type 2 cell recovery from hyperoxia. *Am J Physiol Lung Cell Mol Physiol* **290**: L714-L720.
- Lenzen J, Stolk J (2007) Pulmonary stem cells and the induction of tissue regeneration in the treatment of emphysema. *Int J COPD* **2**: 131-139.
- Liu T, Chung MJ, Ullenbruch M, Yu H, Jin H, Hu B, Choi YY, Ishikawa F, Phan SH (2007) Telomerase activity is required for bleomycin-induced pulmonary fibrosis in mice. *J Clin Invest* **117**: 3800-3809.
- Liu X, Engelhardt JF (2008) The glandular stem/progenitor cell niche in airway development and repair. *Proc Am Thorac Soc* **5**: 682-688.
- Macchiarini P, Wallis T, Biancosino C, Mertsing H (2004) First human transplantation of bioengineered airway tissue. *J Thorac Cardiovasc Surg* **128**: 638-641.
- Macchiarini P, Jungebluth P, Go T, Asnaghi MA, Rees LE, Cogan TA, Dodson A, Martorell J, Bellini S, Parnigotto PP, Dickinson SC, Hollander AP, Mantero S, Conconi MT, Birchall MA (2008) Clinical transplantation of a tissue-engineered airway. *Lancet* **372**: 2023-2030.
- MacPherson H, Keir P, Webb S, Samuel K, Boyle S, Bickmore W, Forrester L, Dorin J (2005) Bone marrow-derived SP cells can contribute to the respiratory tract of mice *in vivo*. *J Cell Sci* **118**: 2441-2450.
- MacPherson H, Keir PA, Edwards CJ, Webb S, Dorin J (2006) Following damage, the majority of bone marrow-derived airway cells express an epithelial marker. *Respir Res* **7**: 145.
- Mao JT, Goldin JG, Dermand J, Ibrahim G, Brown MS, Emerick A, McNitt-Gray MF, Gjertson DW, Estrada F, Tashkin DP, Roth MD (2002) A pilot study of all-trans-retinoic acid for the treatment of human emphysema. *Am J Respir Crit Care Med* **165**: 718-723.
- Nakajoh M, Fukushima T, Suzuki T, Yamaya M, Nakayama K, Sekizawa K, Sasaki H (2003) Retinoic acid inhibits elastase-induced injury in human lung epithelial cell lines. *Am J Respir Cell Mol Biol* **28**: 296-304.

- Nishimura Y, Hamazaki TS, Komazaki S, Kamimura S, Okochi H, Asashima M (2006) Ciliated cells differentiated from mouse embryonic stem cells. *Stem Cells* **24**: 1381-1388.
- Nomoto Y, Suzuki T, Tada Y, Kobayashi K, Miyake M, Hazama A, Wada I, Kanemaru S, Nakamura T, Omori K (2006) Tissue engineering for regeneration of the tracheal epithelium. *Ann Otol Rhinol Laryngol* **115**: 501-506.
- Nomoto Y, Kobayashi K, Tada Y, Wada I, Nakamura T, Omori K (2008) Effect of fibroblasts on epithelial regeneration on the surface of a bioengineered trachea. *Ann Otol Rhinol Laryngol* **117**: 59-64.
- Ohnishi S, Nagaya N (2008) Tissue regeneration as next-generation therapy for COPD – potential applications. *Int J COPD* **3**: 509-514.
- Olsson F, Denham M, Cole TJ, Hooper SB, Mollard R (2007) Deriving respiratory cell types from stem cells. *Curr Stem Cell Res Ther* **2**: 197-208.
- Omori K, Tada Y, Suzuki T, Nomoto Y, Matsuzuka T, Kobayashi K, Nakamura T, Kanemaru S, Yamashita M, Asato R (2008) Clinical application of in situ tissue engineering using a scaffolding technique for reconstruction of the larynx and trachea. *Ann Otol Rhinol Laryngol* **117**: 673-678.
- Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ (2008) Disease-specific induced pluripotent stem cells. *Cell* **134**: 877-886.
- Peter Y (2007) Tracheotomy: a method for transplantation of stem cells to the lung. *J Vis Exp* **25**: 163 (video).
- Pfenninger C, Leinhase I, Endres M, Rotter N, Loch A, Ringe J, Sittlinger M (2007) Tracheal remodeling: comparison of different composite cultures consisting of human respiratory epithelial cells and human chondrocytes. *In Vitro Cell Dev Biol Anim* **43**: 28-36.
- Puchelle E, Peault B (2000) Human airway xenograft models of epithelial cell regeneration. *Respir Res* **1**: 125-128.
- Puchelle E, Zahm JM, Tournier JM, Coraux C (2006) Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* **3**: 726-733.
- Qu N, de Vos P, Schelfhorst M, de Haan A, Timens W, Prop J (2005) Integrity of airway epithelium is essential against obliterative airway disease in transplanted rat tracheas. *J Heart Lung Transplant* **24**: 882-890.
- Que J, Choi M, Ziel JW, Klingensmith J, Hogan BL (2006) Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps. *Differentiation* **74**: 422-437.
- Que J, Wilm B, Hasegawa H, Wang F, Bader D, Hogan BLM (2008) Mesothelium contributes to vascular smooth muscle and mesenchyme during lung development. *Proc Natl Acad Sci USA* **105**: 16626-16630.
- Que J, Luo X, Schwartz RJ, Hogan BL (2009) Multiple roles for Sox2 in the developing and adult mouse trachea. *Development* **136**: 1899-1907.
- Rawlins EL (2008) Lung epithelial progenitor cells. Lessons from development. *Proc Am Thorac Soc* **5**: 675-681.
- Rawlins EL, Hogan BL (2006) Epithelial stem cells of the lung: privileged few or opportunities for many? *Development* **133**: 2455-2465.
- Rawlins EL, Hogan BL (2008) Ciliated epithelial cell lifespan in the mouse trachea and lung. *Am J Physiol Lung Cell Mol Physiol* **295**: L231-234.
- Rawlins EL, Okubo T, Que J, Xue Y, Clark C, Luo X, Hogan BL (2008) Epithelial stem/progenitor cells in lung postnatal growth, maintenance, and repair. *Cold Spring Harb Symp Quant Biol* **73**: 291-295.
- Rawlins EL, Okubo T, Xue Y, Brass DM, Auten RL, Hasegawa H, Wang F, Hogan BL (2009) The role of Scgbl1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. *Cell Stem Cell* **4**: 525-534.
- Reddy R, Buckley S, Doerken M, Barsky L, Weinberg K, Anderson KD, Warburton D, Driscoll B (2004) *Am J Physiol Lung Cell Mol Physiol* **286**: L658-L667.
- Reynolds SD, Malkinson AM (2010) Clara cell: Progenitor for the bronchiolar epithelium. *Int J Biochem Cell Biol* **42**: 1-4.
- Reynolds SD, Shen H, Reynolds PR, Betsuyaku T, Pilewski JM, Gambelli F, Di Giuseppe M, Ortiz LA, Stripp BR (2007) Molecular and functional properties of lung SP cells. *Am J Physiol Lung Cell Mol Physiol* **292**: L972-L983.
- Rippon HJ, Polak JM, Qin M, Bishop AE (2006) Derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step differentiation protocol. *Stem Cells* **24**: 1389-1398.
- Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL (2009) Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci USA* **106**: 12771-12775.
- Roszell B, Seaton A, Fong GH, Finck CM (2009) Cell-based therapy improves cell viability and increases airway size in an explant model. *Exp Lung Res* **35**: 501-513.
- Roth MD, Connett JE, D'Armiento JM, Foronjy RF, Friedman PJ, Goldin JG, Louis TA, Mao JT, Muindi JR, O'Connor GT, Ramsdell JW, Ries AL, Scharf SM, Schluger NW, Sciruba FC, Skeans MA, Walter RE, Wendt CH, Wise RA, FORTE Study Investigators (2006) Feasibility of retinoids for the treatment of emphysema study. *Chest* **130**: 1334-1345.
- Roy I, Vij N (2010) Nanodelivery in airway diseases: Challenges and therapeutic applications. *Nanomedicine* **6**: 237-244.
- Sehgal A, Presente A, Dudus L, Engelhardt JF (1996) Isolation of differentially expressed cDNAs during ferret tracheal development: application of differential display PCR. *Exp Lung Res* **22**: 419-434.
- Senoo M, Pinto F, Crum CP, McKeon F (2007) p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* **129**: 523-536.
- Servetnyk Z, Kozlova I, Knetsch ML, Koole LH, Roomans GM (2006) Culture of respiratory epithelial cells on copolymer-coated wires. *J Cystic Fibrosis* **5**: S10 (abstr).
- Shebani E, Shahana S, Jansson C, Roomans GM, BHR

- group (2005) Attachment of columnar epithelial cells in asthma. *Tissue & Cell* **37**: 145-152.
- Snyder JC, Teisanu RM, Stripp BR (2009) Endogenous lung stem cells and contribution to disease. *J Pathol* **217**: 254-264.
- Stinchcombe SV, Maden M (2008) Retinoic acid induced alveolar regeneration: critical differences in strain sensitivity. *Am J Respir Cell Mol Biol* **38**: 185-191.
- Stripp BR (2008) Hierarchical organization of lung progenitor cells. Is there an adult lung tissue stem cell? *Proc Am Thorac Soc* **5**: 695-698.
- Sueblinvong V, Weiss DJ (2009) Cell therapy approaches for lung diseases: current status. *Curr Opin Pharmacol* **9**: 268-273.
- Suzuki T, Kobayashi K, Tada Y, Suzuki Y, Wada I, Nakamura T, Omori K (2008) Regeneration of the trachea using a bioengineered scaffold with adipose-derived stem cells. *Ann Otol Rhinol Laryngol* **117**: 453-463.
- Tada Y, Suzuki T, Takezawa T, Nomoto Y, Kobayashi K, Nakamura T, Omori K (2008) Regeneration of tracheal epithelium utilizing a novel bipotential collagen scaffold. *Ann Otol Rhinol Laryngol* **117**: 359-365.
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**: 663-676.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**: 861-872.
- Teisanu RM, Lagasse E, Whitesides JF, Stripp BR (2009) Prospective isolation of bronchiolar stem cells based upon immunophenotypic and autofluorescence characteristics. *Stem Cells* **27**: 612-622.
- Ten Hallers EJ, Rakhorst G, Marres HA, Jansen JA, van Kooten TG, Schutte HK, van Loon JP, van der Houwen EB, Verkerke GJ (2004) Animal models for tracheal research. *Biomaterials* **25**: 1533-1543.
- Tepper J, Pfeiffer J, Aldrich M, Tumas D, Kern J, Hoffman E, McLennan G, Hyde D (2000) Can retinoic acid ameliorate the physiologic and morphologic effects of elastase instillation in the rat? *Chest* **117**: 242S-244S.
- Tesei A, Zoli W, Arienti C, Storci G, Granato AM, Pasquinelli G, Valente S, Orrico C, Rosetti M, Vannini I, Dubini A, Dell'Amore D, Amadori D, Bonafè M (2009) Isolation of stem/progenitor cells from normal lung tissue of adult humans. *Cell Prolif* **42**: 298-308.
- Tichelaar JW, Wert SE, Costa RH, Kimura S, Whitsett JA (1999) HNF-3/forkhead homologue-4 (HFH-4) is expressed in ciliated epithelial cells in the developing mouse lung. *J Histochem Cytochem* **47**: 823-832.
- Tiozzo C, De Langhe S, Yu M, Londhe VA, Carraro G, Li M, Li C, Xing Y, Anderson S, Borok Z, Bellusci S, Minoo P (2009) Deletion of Pten expands lung epithelial progenitor pools and confers resistance to airway injury. *Am J Respir Crit Care Med*, in press.
- Tsao PN, Vasconcelos M, Izvolsky KI, Qian J, Lu J, Cardoso WV (2009) Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. *Development* **136**: 2297-2307.
- Tzortzaki EG, Siafakas NM (2009) A hypothesis for the initiation of COPD. *Eur Respir J* **34**: 310-315.
- Vallée M, Côté JF, Fradette J (2009) Adipose-tissue engineering: taking advantage of the properties of human adipose-derived stem/stromal cells. *Pathol Biol (Paris)* **57**: 309-317.
- Van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, Rey-Parra GJ, Galipeau J, Haromy A, Eaton F, Chen M, Hashimoto K, Abley D, Korbitt G, Archer SL, Thebaud B (2009) Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. *Am J Respir Crit Care Med*, in press.
- Voynow JA, Fischer BM, Roberts BC, Proia AD (2005) Basal-like cells constitute the proliferating cell population in cystic fibrosis airways. *Am J Respir Crit Care Med* **172**: 1013-1018.
- Walles T, Giere B, Hofmann M, Schanz J, Hofmann F, Mertsching H, Macchiarini P (2004) Experimental generation of a tissue-engineered functional and vascularized trachea. *J Thorac Cardiovasc Surg* **128**: 900-906.
- Wang D, Haviland DL, Burns AR, Zsigmond E, Wetsel RA (2007) A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* **104**: 4449-4454.
- Wang X, Zhang Y, Amberson A, Engelhardt JF (2001) New models of the tracheal airway define the glandular contribution to airway surface fluid and electrolyte composition. *Am J Respir Cell Mol Biol* **24**: 195-202.
- Warburton D, Perin L, DeFilippo R, Bellusci S, Shi W, Driscoll B (2008) Stem/progenitor cells in lung development, injury repair, and regeneration. *Proc Am Thorac Soc* **5**: 703-706.
- Ward JE, Harris T, Bamford T, Mast A, Pain MC, Robertson C, Smallwood D, Tran T, Wilson J, Stewart AG (2008) Proliferation is not increased in airway myofibroblasts isolated from asthmatics. *Eur Respir J* **32**: 362-371.
- Watkins DN, Berman DM, Burkholder SG, Wang B, Beachey PA, Baylin SB (2003) Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* **422**: 313-317.
- Weidenbecher M, Henderson JH, Tucker HM, Baskin JZ, Awadallah A, Dennis JE (2007) Hyaluronan-based scaffolds to tissue-engineer cartilage implants for laryngotracheal reconstruction. *Laryngoscope* **117**: 1745-1749.
- Weidenbecher M, Tucker HM, Awadallah A, Dennis JE (2008) Fabrication of a neotrachea using engineered cartilage. *Laryngoscope* **118**: 593-598.
- Weidenbecher M, Tucker HM, Gilpin DA, Dennis JE (2009) Tissue-engineered trachea for airway reconstruction. *Laryngoscope* **119**: 2118-2123.
- Weiss DJ (2008) Stem cells and cell therapies for cystic fibrosis and other lung diseases. *Pulm Pharmacol Ther* **21**: 588-594.
- Williams KM, Chien JW, Gladwin MT, Pavletic SZ (2009) Bronchiolitis obliterans after allogeneic hematopoietic stem cell transplantation. *JAMA* **302**: 306-314.
- Winkler ME, Mauritz C, Groos S, Kispert A, Menke S, Hoffmann A, Gruh I, Schwanke K, Haverich A, Martin

U (2008) Serum-free differentiation of murine embryonic stem cells into alveolar type II epithelial cells. *Cloning Stem Cells* **10**: 49-64.

Wong AP, Keating A, Lu WY, Duchesneau P, Wang X, Sacher A, Hu J, Waddell TK (2009) Identification of a bone marrow-derived epithelial-like population capable of repopulating injured mouse airway epithelium. *J Clin Invest* **119**: 336-348.

Xu J, Torres E, Mora AL, Shim H, Ramirez A, Neujahr D, Brigham KL, Rojas M (2008) Attenuation of obliterative bronchiolitis by a CXCR4 antagonist in the murine heterotopic tracheal transplant model. *J Heart Lung Transplant* **27**: 1302-1310.

Yamashita M, Kanemaru S, Hirano S, Magruffov A, Tamaki H, Tamura Y, Kishimoto M, Omori K, Nakamura T, Ito J (2007) Tracheal regeneration after partial resection: a tissue engineering approach. *Laryngoscope* **117**: 497-502.

Yanagi S, Kishimoto H, Kawahara K, Sasaki T, Sasaki M, Nishio M, Yajima N, Hamada K, Horie Y, Kubo H, Whitsett JA, Mak TW, Nakano T, Nakazato M, Suzuki A (2007) Pten controls lung morphogenesis, bronchioalveolar stem cells, and onset of lung adenocarcinomas in mice. *J Clin Invest* **117**: 2929-2940.

Yang J, Yamato M, Nishida K, Ohki T, Kanzaki M, Sekine H, Shimizu T, Okano T (2006) Cell delivery in regenerative medicine: the cell sheet engineering approach. *J Control Release* **116**: 193-203.

Yeung AH, Cowan MJ, Horn B, Rosbe KW (2009) Airway management in children with mucopolysaccharidoses. *Arch Otolaryngol Head Neck Surg* **135**: 73-79.

Yoshida M, Nakayama K, Yasuda H, Kubo H, Kuwano K, Arai H, Yamaya M (2009) Carbocysteine inhibits oxidant-induced apoptosis in cultured human airway epithelial cells. *Respirology* **14**: 1027-1034.

Zani BG, Kojima K, Vacanti CA, Edelman ER (2008) Tissue-engineered endothelial and epithelial implants differentially and synergistically regulate airway repair. *Proc Natl Acad Sci USA* **105**: 7046-7051.

Zhao F, Zhang YF, Liu YG, Zhou JJ, Li ZK, Wu CG, Qi HW (2008) Therapeutic effects of bone marrow-derived mesenchymal stem cells engraftment on bleomycin-induced lung injury in rats. *Transplant Proc* **40**: 1700-1705.

### Discussion with Reviewers

**Reviewer I:** Of the various airway diseases, which one is the closest to being treated through the use of stem or progenitor cells? Which of the airway diseases does the author think are best approached by a cellular therapy as opposed to surgical or pharmaceutical approaches?

**Author:** It will be technically easiest to repair lesions in the largest airways (larynx, trachea, principal bronchi). An example is the successful replacement of the left main bronchus in a patient with bronchomalacia (Macchiarini *et al.*, 2008). Other possible conditions that would be more suitable for repair by tissue engineering than by surgical or pharmaceutical approaches would be the repair of

defects due to obstructing tracheal tumors, trauma, or post-intubation tissue reactions that necessitate replacement of tracheal tissue. It is likely that these conditions will be the first to be successfully treated by tissue engineering. However, these conditions are relatively rare in comparison with “common” airway diseases such as asthma and COPD or even in comparison with cystic fibrosis, which has a frequency of about 1 in 3000 newborns, but is (eventually) lethal. For these diseases, the only realistic surgical approach would be (heart-)lung transplantation, which is limited, in addition to immunological problems, mainly by the availability of donor tissue. In the tissue engineering approach, the problem is that to cure the disease, the (epithelium of the) small, intrapulmonary, airways has to be repaired. Although cystic fibrosis is often named as a disease that would benefit from stem cell techniques, to my knowledge, no proposals have (yet) been made how this could be done by tissue engineering. If one would be allowed to speculate, growing an entire lung *ab initio* and *in vitro* would be a theoretical possibility. It is, however, reasonable to assume that a (partly) successful pharmacological approach will come earlier.

**Reviewer II:** Based on your review, which source of stem cells do you envision being the closest to being used effectively? What would you envision being the primary obstacles to overcome in bringing this to the clinic?

**Author:** The experimental evidence necessary to answer this question is yet incomplete. The little data that have been obtained would suggest that, in particular for the cases where bioengineered airway tissue is used for repair of localized defects, the situation is simpler than maybe thought. What is required is a cartilage/connective tissue base, for which heterologous human tissue or even animal tissue can be used, after proper pretreatment, and (homologous) epithelium, which can either grow spontaneously to cover the cartilage/connective tissue base, or be grown in culture based on a biopsy from the eventual recipient of the transplant. The other extreme would be replacement of the entire epithelium, which would, e.g., be necessary in cystic fibrosis. Would it be possible to grow an entire lung *ab initio*, or possibly on the basis of a cartilage/connective tissue skeleton? The epithelial cells might have to be heterologous, since cells from the donor him/herself would carry the mutation causing the disease. However, this would be equivalent to a lung transplant, which is already used with some success today. The only advantage would be that one wouldn't be limited by access to suitable donors. Another possibility would be to use autologous epithelial cells in which the genetic defect has been corrected by gene therapy in such a way that the correction is stable in the course of many cell divisions. There is hence no dearth in obstacles bringing this to the clinic.

**H. Plenk:** Would it not be logical to use the term “progenitor cells” for the endogenous cells and the term “stem cells” for the exogenous cells?

**Author:** That would indeed be logical, but a practice has already developed where the terms “stem cells” and

progenitor cells” are used interchangeably, and confusingly. Giangreco *et al.* (2009) use the term “stem cells” to denote bronchiolar stem cells, which do not contribute significantly to tissue homeostasis, and “progenitor cells” to denote Clara cells that maintain airway homeostasis. However, in your nomenclature system, both cell types would be called “progenitor cells”.

**Reviewer III:** You suggest that the ferret may be a good animal model for airway studies. Are there reagents (cDNAs, antibodies etc.) for studies using ferrets?

**Author:** Yes, anti-ferret antibodies can be obtained, e.g., from Lifespan Biosciences ([www.lsbio.com](http://www.lsbio.com)). Methods for synthesis of ferret cDNA have been described by Sbarra *et al.* (1998) and more recently by Nakata *et al.* (2008) (see Additional References). In addition, there are numerous studies published on ferret airways, e.g., those cited in the body of the paper (Sehgal *et al.*, 1996; Wang *et al.*, 2001; Liu and Engelhardt, 2008; Abanses *et al.*, 2009).

**H. Plenk:** How is the border between the mucosa and the submucosa defined in the mouse and the human trachea, respectively? To my knowledge there is nothing like a muscularis mucosae, as found in the digestive tract, thus

no submucosa. It would therefore be better to write “seromucous glands” instead of “submucosal glands”. Please comment.

**Author:** The reviewer is correct in stating that there is no muscularis mucosae in the respiratory tract. Nevertheless, use of the word “submucosa” and “submucosal glands” is extremely common, both in histology textbooks and in published papers. A check in MEDLINE shows that the term “airway submucosal gland” is much more common than “airway seromucous gland”. I have therefore chosen to follow practice, rather than theory, in the choice of terminology.

#### Additional References

Nakata M, Itou T, Sakai T (2008) Molecular cloning and phylogenetic analysis of inflammatory cytokines of the ferret (*Mustela putorius furo*) J Vet Med Sci **70**: 543-550.

Sbarra V, Bruneau N, Mas E, Hamosh M, Lombardo D, Hamosh P (1998) Molecular cloning of the bile salt-dependent lipase of ferret lactating mammary gland: an overview of functional residues. Biochim. Biophys Acta **1393**: 80-89.