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REVIEW

Human embryonic stem cells and lung regeneration

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Human embryonic stem cells are pluripotent cells derived from the inner cell mass of preimplantation stage embryos. Their unique potential to give rise to all differentiated cell types has generated great interest in stem cell research and the potential that it may have in developmental biology, medicine and pharmacology. The main focus of stem cell research has been on cell therapy for pathological conditions with no current methods of treatment, such as neurodegenerative diseases, cardiac pathology, retinal dysfunction and lung and liver disease. The overall aim is to develop methods of application either of pure cell populations or of whole tissue parts to the diseased organ under investigation. In the field of pulmonary research, studies using human embryonic stem cells have succeeded in generating enriched cultures of type II pneumocytes *in vitro*. On account of their potential of indefinite proliferation *in vitro*, embryonic stem cells could be a source of an unlimited supply of cells available for transplantation and for use in gene therapy. Uncovering the ability to generate such cell types will expand our understanding of biological processes to such a degree that disease understanding and management could change dramatically.
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Abbreviations: BM, bone marrow; CCSP, Clara cell secreting protein; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; EB, embryoid body; ES cell, embryonic stem cell; FGF, fibroblast growth factor; GATA6, GATA-binding factor 6; hES cell, human embryonic stem cell; IVF, *in vitro* fertilisation; mES cell, mouse embryonic stem cell; MSC, mesenchymal stem cell; RA, retinoic acid; SAGM, small airway growth medium; SPC, surfactant protein C; TGF-β, transforming growth factor-β; TITF-1, thyroid transcription factor 1

Introduction

Despite extensive progress being made over recent years in both science and medicine, there are a large number of pulmonary diseases with inefficient therapeutic applications. Cystic fibrosis (CF) is one of the most prevalent of such conditions. It is most commonly caused by an autosomal-recessive deletion (ΔF508) in the CF transmembrane regulator (*CFTR*) gene, which results in inefficient ion transport to the apical side of the epithelial cell membrane (Bannykh *et al*., 2000). Diseases with no available therapies, which count only on the management of symptoms or rely on lung transplantation as a means of therapy, have attracted the interest of stem cell research as a means of providing alternative approaches for the correction of malfunctioning or damaged tissues. In the case of pulmonary conditions, the complex structure of the pulmonary epithelium, together with an incomplete understanding of its development and pathology, has made this an area of particular interest for stem cell biology, regenerative medicine, developmental biology and pharmacology.

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Development of the human lung

The human lung has a complex three-dimensional structure featuring a large variety of endoderm-derived epithelial cells along its proximal–distal axis. Human lung development is divided into four stages, they are: embryonic stage, 0–5 weeks post-fertilization; (pseudo)glandular stage, 6–16 weeks; canalicular stage, 17–25 weeks and terminal sac (or saccular) stage, 26 weeks to term (Bishop *et al*., 2006). Each stage is determined by structural changes in the formation of the airway tree. The lung bud, which originates from the embryonic gut, gives rise to the trachea and the two main bronchi (cartilaginous airways) by the end of the embryonic stage. The two main bronchi follow a regulated process, known as branching morphogenesis, to form distal bronchioles and the alveoli as development continues. Distal bronchioles consist of bronchioles, terminal bronchioles and respiratory bronchioles. The terminal structures, the

alveoli, are clusters of air spaces in the shape of saccules, which facilitate gas exchange.

Each domain is characterized by a unique type of airway epithelium. The primordial epithelium found at the embryonic stage gives rise to a pseudostratified epithelium during the glandular stage. This lines the trachea and the bronchi and consists mainly of basal, goblet, ciliated, intermediate, neuroendocrine and submucosal gland epithelial cells. As branching progresses, a simple columnar epithelium forms, consisting mainly of Clara cells, neuroendocrine and ciliated cells, covering the bronchioles. The distal part of the respiratory airways is lined by a thin epithelium consisting of flattened squamous (type I) and cuboidal (type II) pneumocytes (Bishop *et al*., 2006). This highly specialized organization of lung morphogenesis is regulated through a temporal and spatial distribution of a large number of signalling molecules, the interactions of which are mediated by growth factors expressed in the lung mesenchyme and endoderm (reviewed in Kumar *et al*., 2005).

Transcription factors involved in the development of the airway epithelium

A number of evolutionary conserved transcription factors have been shown to be important for the regulation of the various stages of lung development and epithelial cell differentiation. Morrisey *et al*. (1998) have demonstrated that the transcription factor GATA-binding factor 6 (GATA6), a member of the family of zinc-finger domain-containing nuclear proteins, is implicated in lung endoderm specification, and it was subsequently shown that *GATA6* interacts with genes such as the human thyroid transcription factor 1 (*TITF-1*) and surfactant protein C (*SPC*), thus controlling the late stage of lung branching morphogenesis and distal epithelial cell differentiation (Yang *et al*., 2002). In particular, *GATA6* and *TITF-1* have overlapping temporal and spatial expressions in the peripheral epithelial cells of the developing lung, where *GATA6* activates the transcription of *TITF-1* (Shaw-White *et al*., 1999). *SPC* expression is directly regulated through this synergistic action of the N-terminal and zinc-finger domains of *GATA6* and the homeodomain region of *TITF-1* (Liu *et al*., 2002). Ectopic expression of *GATA6* in mouse embryonic stem (mES) cells has been shown to induce differentiation towards extraembryonic endoderm, a prerequisite for lung organogenesis (Fujikura *et al*., 2002).

Another factor implicated in epithelial cell differentiation is the forkhead box (f-box) transcription factor *FoxJ-1*, which has been shown to be expressed in ciliated cells and is required for the late-stage formation of cilia, by reorganization of the basal bodies within the apical compartment of cells previously committed to a ciliated cell phenotype (You *et al*., 2004). Functional analysis of the regulatory region of lung-specific genes has shown that normal promoter activity often requires the synergistic interaction of multiple cell-specific and inducible transcription factors. A highly complex interaction of the mesenchyme and the epithelium is also required for the coordination of the transcription factor interactions (reviewed in Kumar *et al*., 2005). The complexity of these pathways will need to be carefully considered when creating a system replicating the development of the human lung epithelium.

Investigation of the effect of the *Sox17* (SRY (sex-determining region Y) box 17), a marker of definitive endoderm in mice, has revealed the important function of this factor in the differentiation of respiratory epithelial cells into the various cells of the conducting airways (Park *et al*., 2006a, b). In particular, it was suggested that multiple respiratory epithelial cells display high phenotypic plasticity levels, which can be regulated by different levels of *Sox17*, resulting in cells with multiple characteristics of epithelial cells of the conducting airways (Park *et al*., 2006b).

Investigation of the various molecular pathways that control lung development and epithelial cell differentiation is important for understanding and regulating the mechanisms that result in the pathological phenotypes of CF and chronic obstructive pulmonary disease (Calverley and Walker, 2003).

Endogenous stem cells of the lung

The normal tracheobronchial epithelium is continuously being renewed in a specialized and highly controlled way, which maintains the complex structure of this epithelium. The replacement of the terminally specialized epithelial cells occurs through the specialized differentiation of the endogenous lung stem cell population. These stem cells are characterized by unlimited self-renewal capacity and the ability to differentiate into all required cell types of the pulmonary epithelium upon appropriate stimuli. Identifying the endogenous stem cell population of the pulmonary epithelium will allow for further understanding of the developmental processes of lung formation as well as putative use of the specific cell populations in treating a variety of pulmonary diseases.

It is suggested that there are more than one stem cell population, a hypothesis reinforced by the highly complex and heterogeneous cell composition observed along the proximal–distal axis of the tracheobronchial tree (Wu and Wei, 2004). The determination of the stem cell population of the pulmonary epithelium has been complicated further by the great variation observed in the cell type composition of the lung epithelium from different species (Jeffery, 1983; Plopper *et al*., 1992). Reports of candidate stem cell populations so far include the undifferentiated columnar cell type found in the early developmental stages of the tracheobronchiolar epithelium (Plopper *et al*., 1992), basal cells (Hong *et al*., 2004a, b; Hajj *et al*., 2007), Clara cells (Hong *et al*., 2001, 2004b) as well as CCSP-secreting cells that localize in the bronchoalveolar junctions (Giangreco *et al*., 2002; Kim *et al*., 2005a), type II pneumocytes (Emura, 1997; Uhal, 1997) and side population cells that show both epithelial and mesenchymal differentiation potential (Majka *et al*., 2005; Reynolds *et al*., 2007). All the proposed cell types have extensive self-renewal capacity and some of these populations have been shown to be able to contribute towards the regeneration of damaged lung epithelium upon engraftment. The first report to functionally identify a stem cell population in the human pulmonary epithelium was

carried out by Zepeda *et al*. (1995). Although the morphological and functional characteristics of the stem cell type were not identified, the existence of such cells was proven using retroviral marking of human bronchial epithelial cells that were subsequently used for establishing xenograft models (Zepeda *et al*., 1995).

Further studies into the development of submucosal glands in the lung epithelium have identified more than one cell type having progenitor abilities for gland development (Engelhardt *et al*., 1995). The same study has also demonstrated the presence of multiple endogenous cell populations with stem cell characteristics in the lung epithelium. It is generally acknowledged that both basal and secretory cells have the ability to participate in the renewal of the epithelium but the exact pathways of differentiation are under ongoing investigation. More recently, the investigation of markers specific to human airway epithelial progenitor cells has revealed the presence of aquaporin-3-positive basal cells, which show regeneration potential in both the epithelial cell layer and the submucosal glands (Avril-Delplanque *et al*., 2005).

Cell–cell and cell–matrix interactions of this complex epithelium appear to determine a large part of the regeneration mechanism of the pulmonary epithelium through paracrine, autocrine and endocrine pathways (reviewed in Kumar *et al*., 2005). The type of stimuli used to observe lung epithelial regeneration has also been found to have an important function in the determination of the particular cell type that contributes to the renewal of the lung epithelium. In particular, the nature and the degree of injury induced in the lung, whether mechanical or toxic, determine the cell type that participates in regeneration and the extent of repopulation in each case (Beckett *et al*., 2005; Herzog *et al*., 2006). In the case of ciliated cells, Rawlins *et al*. (2007) have shown that although the ciliated cell type of the lung shows a transient morphological change after lung epithelium injury, this terminally differentiated cell type does not contribute to the regeneration of the pulmonary epithelium.

Clarifying the regeneration properties of the various cell types of the lung epithelium will form the basis of deciding on the target cell population in engraftment studies, especially when attempting to replicate this cell type using human embryonic stem (hES) cells.

hES cells

hES cells are pluripotent cells derived from the inner cell mass of the preimplantation embryos (Thomson *et al*., 1998). These immortal cells can be maintained in a culture in an undifferentiated state indefinitely, maintaining their proliferating capacity and have the unique potential to give rise to cells and tissues of all three embryonic germ layers (Thomson *et al*., 1998) as well as trophoblast cells (Gerami-Naini *et al*., 2004). These characteristics make embryonic stem (ES) cells an invaluable tool in the area of developmental biology, allowing the in-depth study and understanding of the mechanisms of cell differentiation as well as normal developmental processes. Moreover, they can be potentially used as a renewable source of cell populations in the area of regenerative medicine for the treatment of degenerative human diseases.

hES cells are morphologically distinct, as they exhibit a high nucleus-to-cytoplasmic ratio, with prominent nucleoli and form compact colonies with well-defined borders (Thomson *et al*., 1998) (Figure 1). They express high levels of telomerase activity, which is consistent with their high proliferative potential in culture (Thomson *et al*., 1998). Surface markers characteristic of undifferentiated hES cells are stage-specific embryonic antigen-3, -4, TRA (Trafalgar antigen)-1-60, TRA-1-81, Octamer-binding transcription factor 4 and alkaline phosphatase. hES cells can differentiate *in vitro* when grown in suspension and form embryoid bodies (EBs), which express markers specific to the three embryonic germ layers (Itskovitz-Eldor *et al*., 2000; Reubinoff *et al*., 2000). Further molecular characterization of differentiating hES cells and EBs demonstrated a temporal but not spatial gene expression pattern that resembles early human embryogenesis (Dvash and Benvenisty, 2004; Dvash *et al*., 2004). When injected in SCID (severe combined immunodeficient) mice, hES cells form teratomas, which include cells from all three embryonic germ layers (Thomson *et al*., 1998). Clonal hES lines have been derived that exhibited markers of undifferentiated hES cells and maintained the ability to differentiate into cells from all three germ layers (Amit *et al*., 2000). This demonstrated the pluripotency of single hES cell.

An important growth factor that has been shown to be able to maintain the undifferentiated state of hES cells when added to the culture medium is basic fibroblast growth factor (FGF) also known as FGF2. FGF2 is a member of the FGF

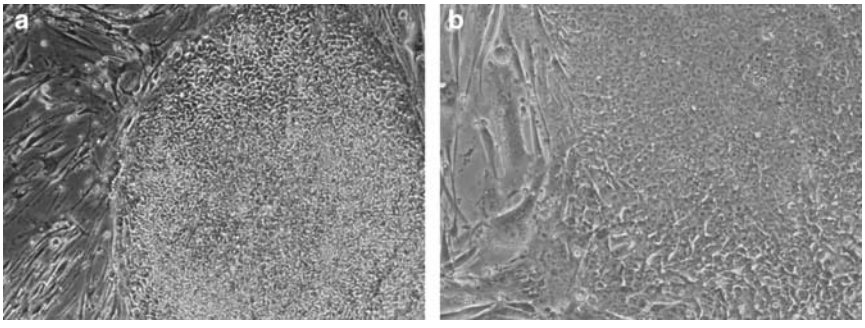

Figure 1 hES cell lines (**a**) KCL-003-CF1 passage 38 on MEFs, × 10 (**b**) KCL-002 passage 59 on MEFs, × 20. The KCL-003-CF1 hES cell line is homozygous for the ΔF508 mutation, a 3 bp deletion in the coding region of the CFTR gene.

family of ligands that signal through binding and subsequent dimerization to four different FGF receptors (FGFR1–4) on the cell surface inducing intrinsic tyrosine kinase activity. In particular, FGF binding to FGF receptor is followed by the activation of the Ras-mitogen associated protein kinase, PI3 kinase and phospholipase C-γ pathways (Reviewed in (Dailey *et al*., 2005; Eswarakumar *et al*., 2005; Mohammadi *et al*., 2005)). The activation of the PI3 kinase pathway by FGF2 has also been connected with the expression of extracellular matrix molecules that support hES cell proliferation (Kim *et al*., 2005b).

Another family of ligands that has been shown to mediate signalling involved in the regulation of the undifferentiated growth of hES cells is the transforming growth factor-β (TGF-β) superfamily (reviewed in Massague, 2003; Shi and Massague, 2003). More recently, TGF-β1 and Activin A (both members of the TGF-β superfamily) have been shown to be able to support the undifferentiated growth of hES cells both in the absence and in the presence of feeders (Amit *et al*., 2004; Beattie *et al*., 2005; Xiao *et al*., 2006). Nevertheless, the growth rate of hES cells appears to be reduced as compared with cultures where FGF2 is added to the growth medium, indicating a putative mitogenic action of FGF2 on hES cells (Amit *et al*., 2004). The exact signalling pathways that these growth factors act through are under ongoing investigation and new possible pathways are often suggested. An example is the recent report of a suggested mechanism of FGF2 initiating the release of insulin growth factor II and TGF-β factors from the microenvironment of hES cells, which prove sufficient for the maintenance of the undifferentiated state of hES cells (Bendall *et al*., 2007). A precise understanding of the molecular pathways involved will further aid in the establishment of a medium able to sustain more effectively the undifferentiated growth of hES cells. In addition to the molecular aspects and exogenous factors that control the undifferentiated hES cell growth, there is research into the effects of the microenvironment, such as cell density, in hES cultures (Peerani *et al*., 2007).

hES cell culture

There are a number of technical and methodological issues with the long-term culturing of human ES cells that currently are under intense investigation. These include derivation and efficient propagation of hES cells in defined culture conditions and the establishment of universal protocols for the reproducible differentiation of hES cells into enriched populations of cell types of interest. Traditionally, hES cells have been propagated on a substrate of inactivated mouse embryonic fibroblasts (Thomson *et al*., 1998), either by mechanical or by enzymatic means. Nevertheless, there have been reports that prolonged passaging by enzymatic dissociation encourages the appearance of chromosomal abnormalities (Buzzard *et al*., 2004; Draper *et al*., 2004; Mitalipova *et al*., 2005; Thomson *et al*., 2008). Hence, new alternatives have been developed, such as recombinant trypsin and Accutase, that show increased cell survival upon passaging, while maintaining the undifferentiated phenotype (Bajpai *et al*., 2008).

hES cells show low survival rate in single cell suspension (~1%) (Thomson *et al*., 1998; Amit *et al*., 2000). This has been a great obstacle throughout cell culture maintenance techniques and gene transfer studies where clonal selection is of great importance. The recently discovered Y-27632 Rho-associated kinase inhibitor appears to increase the survival rate of single cell-dissociated hES cells to ~27% (Watanabe *et al*., 2007). Also it has been reported that ES cells exhibit decreased growth rate and reduced differentiation efficiency when cultured with antibiotics such as gentamicin and combined penicillin–streptomycin (Cohen *et al*., 2006). Although the latter study was on mES cells, it provides further evidence that culture conditions of hES cells are still partly unexplored.

One of the most high-profile future uses of hES cells is in therapeutic applications, which dictates the need for well-defined derivation and culture systems devoid of animal sera and animal-derived components and substrates (that is, xeno-free conditions). There is ongoing research to create a well-defined culture media with a combination of extracellular matrices, such as laminin and fibonectin (Xu *et al*., 2001; Amit *et al*., 2004), growth factors (Beattie *et al*., 2005; Wang *et al*., 2005b; Xu *et al*., 2005) as well as autogeneic feeder systems (Stojkovic *et al*., 2005). Even though newly developed cell maintenance techniques and experimental assay setups are reported frequently, their validity remains to be confirmed and their potential long-term effects on hES cell characteristics are still to be examined.

In the recent years, numerous hES cell lines have been generated, most of them genetically normal. Nevertheless, through the donation of embryos shown to harbour known genetic disorders after preimplantation genetic diagnosis, many hES cell lines that carry mutations for disorders, such as Myotonic dystrophy, Huntington's disease (Mateizel *et al*., 2006) and CF (KCL-003-CF1) (Pickering *et al*., 2005; Mateizel *et al*., 2006) (Figure 1), have been established. hES cell lines that carry common mutations of monogenic diseases can be used as an *in vitro* model of the disease, bypassing the need for animal models and providing new tools for analysing and understanding the molecular mechanisms of the disease as well as for drug screening.

Current progress in lung regeneration

Endoderm differentiation

The great differentiation potential of hES cells is a very important factor for their use in therapeutic applications. Current research is directed towards the investigation of the various differentiation pathways of hES cells. Of particular interest is the direction of hES cells towards definitive endoderm, which in turn gives rise to organs, such as the thyroid, thymus, liver, pancreas and lung, as well as the epithelial lining of the digestive and respiratory tract. Studies so far have demonstrated that Nodal, a member of the TGF superfamily, is one of the main pathways essential for the specification of endoderm, whereas lower levels of Nodal result in the mesoderm formation (Vincent *et al*., 2003). On the basis of these observations, it has been shown that using activin A, another member of the TGFβ family, in a

combination with low serum levels in cultures of ES cells, results in enriched cultures of hES-derived definitive endoderm (D'Amour *et al*., 2005). Two of the hES cell lines used for the assays were cultured on Matrigel and the rest on fibroblast feeders. The developmental competency of these hES-derived definitive endoderm cells was accessed *in vivo* by transplantation into SCID mice, followed by histological examination of the resulting grafts. This revealed that these cells have the ability to progress towards further endodermal differentiation *in vivo* (D'Amour *et al*., 2005). The production of definitive endoderm cells from hES cells would be the first step towards generating cells of the definitive endoderm lineage, such as pancreatic and lung epithelial cells.

Differentiation into lung cells

So far, investigation into the mechanisms that control the formation of the various epithelial cell types of the lung has included the use of mouse and human ES cells. The first report of derivation of a lung-specific cell type from stem cells was through studies of the effect of small airway growth medium (SAGM) in differentiating mES cells (Ali *et al*., 2002). The cultures obtained were heterogeneous, the differentiation efficiency was not quantified and the need for further investigation into the factors that direct differentiation was highlighted. Further investigation into the identification of a defined medium for the differentiation of ES cells into alveolar epithelium revealed that retinoic acid (RA) and triiodothyronine may have an inhibitory role in the formation of type II pneumocytes (Rippon *et al*., 2004). When compared with real-time (RT) PCR, differentiating mES cells transferred in SAGM showed a 20-fold increase in the expression of type II pneumocyte marker SPC to mES cells grown in basic Dulbecco's modified Eagle's medium (differentiation medium) (Rippon *et al*., 2004). The same study suggests that RA may promote maturation of proximal cell lineages during the final stages of mES cell differentiation. The main disadvantages of directing ES cell differentiation using a defined medium are the extensive assay time required and the low yields of target cells obtained.

More extensive studies into the effects of each of the growth factors found in SAGM (bovine serum albumin, insulin, transferring, bovine pituitary extract, epinephrine, triiodothyronine, RA, hydrocortisone and human epidermal growth factor) in the differentiation of mES cells into type II pneumocytes indicated that the initial effects observed were due to a serum-free environment rather than the growth factor combination in the SAGM. In particular, it was found that each growth factor individually reduced the efficiency of ES cell differentiation when compared with the results obtained in assays using the SAGM with the complete range of growth factors included. From these results, it is concluded that the effects of growth factors on ES cell differentiation, such as EGF, RA and triiodothyronine, cannot be predicted according to their role in lung development (Samadikuchaksaraei and Bishop, 2007).

At the same time, it was shown that it is possible to direct cell differentiation towards alternative phenotypes through ES cell exposure to differentiated cell extracts (Hakelien *et al*., 2002; Qin *et al*., 2005). This led to new attempts of directing mES cells towards a respiratory epithelial phenotype using murine lung epithelial cell extract (Wikenheiser *et al*., 1993). It was demonstrated that the exposure of mES cells to an alveolar epithelial cell extract can result in cell types positive for type II pneumocyte-specific markers (Qin *et al*., 2005). In particular, cells were monitored for signs of differentiation by prior stable transfection of mES with a 4.8-kb murine SPC promoter/green fluorescent protein construct, followed by immunocytochemistry and electron microscopy. The maintenance of hES characteristics after their transfection with an enhanced (E) green fluorescent protein transgene and the advantages of such a system for hES studies were demonstrated prior to this study (Liu *et al*., 2004). The transfected cells were used for the production of EBs, which were then exposed to murine lung epithelial-12 cell extract and cultured under differentiation-inducing conditions for a further 14 days. The resulting type II pneumocytes were shown to subsequently differentiate into type I pneumocytes. This study draws attention to the putative use of cell extract-mediated differentiation in generating lung epithelial cells from ES cells, despite the lack of understanding of the underlying regulatory mechanisms of this process. It is suggested that transcription regulators from the cell extract contribute in the differentiation process and the need for further investigation into the many variables of the extract-based ES cell culture is emphasized.

On the basis of the observations that ES cell fate is affected by the microenvironment surrounding the cells as well as the well-known property of mammalian cells to adapt and remodel in response to their environmental stimuli, Van Vranken *et al*. (2005) examined the effects of pulmonary mesenchyme on the differentiation capacities of mES cells. Previous studies on the effect of mesenchyme type on pulmonary epithelium differentiation had shown the significant plasticity of this epithelium, which depends on the type of mesenchyme used in each assay (Shannon *et al*., 1998). When murine EBs were grown in direct or indirect co-cultures with lung mesenchyme, there was evidence of the formation of lung epithelial cells originating from mES cells (Van Vranken *et al*., 2005).

In 2005, Coraux *et al*. demonstrated the generation of Clara cells as well as a fully differentiated airway epithelium from mES cells. The differentiation pathways that were examined included the differentiation of mES cells either by initial formation of EBs or by direct culture of undifferentiated mES cells on various substrates, such as collagen type I, collagen type IV, collagen type VI and gelatine. Both experimental routes examined the effects of the different substrates, keratinocyte growth factor and RA on the differentiation potential of mES cells, in both submerged cultures and air–liquid interface. Mouse ES cells grown on collagen type I show differentiation into Clara cells as early as day 8 in culture, with or without keratinocyte growth factor and RA. Ciliated cells can also be obtained after air–liquid interface culture of the type I collagen-induced mES cells. When other substrates were used, Clara cells were obtained from day 15 in culture. Differentiation of mES cells induced by the EB formation appeared not to lead to ciliated cells (Coraux *et al*., 2005).

Lung cell generation from adult stem cells

In parallel to research on the differentiation abilities of murine and human ES cells, there has been considerable investigation on the contribution of adult stem cells to the repair of airway epithelium as well as the possibility of generating a pulmonary epithelium cell population from adult stem cells. Bone marrow (BM)-derived stem cells are the best characterized adult stem cell population (Bonnet, 2003). They include haematopoietic stem cells and mesenchymal stem cells (MSCs). It has been reported that human MSCs (hMSCs) have the ability to differentiate into epithelial-like cells when co-cultured with small airway epithelial cells (Spees *et al*., 2003). Nevertheless, it was observed that up to 1% of the hMSCs obtained the epithelial phenotype after fusion with the small airway epithelial cells, which accounts for approximately 25% of all hMSCs that obtained an epithelial phenotype and an overall $\sim 10^{-2}$ frequency of cell fusion per hMSC plated in co-culture (Spees *et al*., 2003). Further studies on cell fusion as a mechanism of developmental plasticity of stem cells partially contradicted previous data and concluded that epithelia from BM-derived cells are not the result of cell–cell fusion, with the exception of severe tissue injury that appears to promote cell fusion (Harris *et al*., 2004). Despite conflicting data on the mechanism through which BM-derived stem cells obtain an airway epithelial phenotype, MSCs carrying the CFTR mutation have been subjected to gene correction and their ability to correct CFTR function was subsequently tested in co-cultures with airway epithelial cells (Wang *et al*., 2005a). It was found that gene-corrected MSCs from CF patients are able to contribute to apical chloride secretion in response to cyclic AMP stimulation (Wang *et al*., 2005a). Cell fusion was also observed in this study, although it was a rare event. There have been reports of cell fate alteration through spontaneous fusion and the concern for higher malignancy potential (Ying *et al*., 2002). Nevertheless, the resulting cells can display some functional correctness of the damaged epithelium, hence making this path an important one for clinical applications, despite the overall low efficiency of re-epithelization. *In vivo* studies using mouse models of CF and transplantation of MSCs carrying the wild-type CFTR gene have confirmed this observation (Loi *et al*., 2006). As a more efficient alternative to BM-derived MSCs, the regeneration and repair of injured airway epithelium were investigated using umbilical cord blood-derived MSCs (Sueblinvong *et al*., 2008). It was found that like BM-derived MSCs, cord blood-derived MSCs also have the ability to contribute to the airway epithelial regeneration *in vivo*.

An additional factor that appears to have an important role in the degree of epithelial repair is the type and the extent of injury induced to the epithelium. The correlation between injury threshold and BM-derived epithelia engraftment was examined in the case of irradiation-induced injury (Herzog *et al*., 2006). The results showed that there is a critical relationship between the extent of lung injury and the differentiation of BM cells into lung epithelia, drawing attention to the possibility of taking into account the type and extent of lung injury when considering the clinical applications of stem cells. Additional investigations into the effects the microenvironment may have on the differentiation abilities of stem cells have shown a direct correlation of the matrix elasticity to the acquired phenotype (Engler *et al*., 2006).

Differentiation into lung progenitor cells

Despite the progress made in identifying specific lung cell types arising from ES cell differentiation in culture, it would be very useful to be able to generate an airway epithelial progenitor cell population from ES cells. This would facilitate the study of development and cell type differentiation of the human lung as well as generate the source of various lung cell types depending on individual requirement. There are numerous studies in this field, with many different cell types being identified as putative airway progenitor cells (Wu and Wei, 2004). This is mainly due to the complex structural organization of the lung in combination with its large variety of cell types. Thus, the lung has been subdivided into regions with their own progenitor cell type (Otto, 2002). Recently, progenitor cells of the mouse lung epithelium have been generated in culture (Rippon *et al*., 2006). This was achieved by a combination of factors for directed endoderm differentiation, such as Activin A and knockout serum replacement and small airway basal medium.

Differentiation assays using hES cells

Studies using hES cells have succeeded in directing them towards type II pneumocytes, utilizing the same techniques used with mES cells (Samadikuchaksaraei *et al*., 2006). Despite the fact that the differentiation efficiency of this first attempt was low, $\sim 2\%$ of the total cell population, a 99% pure population of type II pneumocytes from hES cultures was later obtained (Wang *et al*., 2007a). This was achieved by using Matrigel as a substrate to plate hES cells without the intermediate step of EB formation and enriching the resulting cultures using antibiotic selection.

Current problems and future applications

The potential of stem cells to generate such a diverse number of cell types is of interest to scientists in development biology, medicine and pharmacology. A fundamental question at the core of stem cell biology is whether it will be possible to fully understand cell cycle control pathways from simple survival to the development of an alternative phenotypic state. Numerous factors that are implicated in these basic biological principles are being continuously uncovered, and the complex network of interactions between the microenvironment surrounding the stem cells as well as the one created by them is under extensive investigation. The nature of stem cell research is such that technical obstacles are encountered far more frequently than in the culture of other cell lines. This forms a large part of the coordinated efforts to establish efficient, reproducible and standard protocols for their undifferentiated propagation.

The genetic makeup of these pluripotent cells is also under extensive investigation. A better understanding of the transcriptional pathways involved for the maintenance of their undifferentiated state will allow the development of

more efficient culture conditions as well as differentiation protocols. The latter currently differ according to the stem cell line used, the differentiated cell type that is sought and the previous reports on the field. A typical example of such variations observed in differentiation assays is the intermediate step of EB formation that is often omitted from the protocols (Wang *et al*., 2007a) and the diversity of growth factors and substrates used to direct differentiation.

In addition to the technical challenges that surround the propagation of and experimentation with stem cells, particularly in the case of hES cells, there is an additional factor of assessing the biology of the resulting cells. Although techniques, such as immunocytochemistry and real-time PCR, are routinely used for the analysis of resulting differentiated cells, they are often proven to be incomplete or even misleading. An example of reported inconsistencies regarding the stem cell contribution to the regenerated tissue is described in the case of MSC-mediated lung epithelium regeneration (Spees *et al*., 2003; Harris *et al*., 2004). Although this could simply be an example specific to MSCs, it points out the possibility of false speculations that can be easily made in this emerging field of research.

Developmental biology of the lung

Of particular interest is the on-going debate about the determination of a stem cell population in the pulmonary epithelium, despite the fact that over the years a large number of cell types have been identified that display the core properties of stem cells. The main problem is that currently there is no system that allows for a continuous investigation of the developmental pathways during the formation of the lung epithelium. Hence, the information obtained is fractioned and the resulting image incomplete, allowing for speculations as to the exact type of endogenous stem cell population. A potential use of hES cells would be in expanding our understanding of airway epithelial development. It has been shown that mES cells can generate a fully differentiated and functional airway epithelial tissue (Coraux *et al*., 2005). Using such a model from hES cells will allow for the *in vitro* observation of the developmental pathways and cell lineage hierarchy in the human lung, which would in turn assist current investigations of potential endogenous lung epithelial stem cells.

Regenerative medicine and gene therapy in the lung

Owing to their potential of indefinite proliferation *in vitro*, hES cells could be a source of an unlimited supply of cells available for transplantation studies and for use in gene therapy. hES cells could be appropriately manipulated *in vitro* by directed differentiation towards the cell type of interest, which could be subsequently grafted to the appropriate tissue and contribute to its regeneration. This can be of great importance in the development of therapies for pulmonary diseases that currently rely on lung transplantation as the only means of treatment. The generation of lung cell types from hES cells has already been documented (Samadikuchaksaraei *et al*., 2006; Wang *et al*., 2007a), and a more extensive range of proximal and distal lung epithelium cell types have been generated from mES cells (Rippon *et al*., 2004, 2006; Coraux *et al*., 2005).

In addition to hES cells providing a treatment by direct transplantation and repopulation of the pathological or damaged tissue, they could be used as vectors for gene therapies. Of particular interest is the possible therapeutic use of hES in CF patients. Although there has been extensive research on the correction of *CTFR* gene function through gene therapy, there has been a number of obstacles, such as delivery failure of the gene carrier vector, immune reaction as well as cases of insertional mutagenesis (Davies *et al*., 2001; Davies, 2006). MSCs have already been shown to be possible carriers of the corrected *CFTR* gene (Wang *et al*., 2005a) that can engraft in the malfunctioning pulmonary epithelium. hES cells show greater plasticity, hence they could be a more efficient model for replicating the gene transfer with higher possibilities of integration.

Current advances towards clinical applications

The nature of ES cells is such that their potential applications in medicine were recognized very early on. The developmental pathways underlying the differentiation abilities of hES cells are under continuous and extensive study and new aspects of these mechanisms are rapidly being uncovered. The differentiation protocols published result in more enriched populations of the differentiated cell type of interest and the characterization of the molecular identity of the resulting cells becomes more extensive and accurate over the years (Synnergren *et al*., 2008).

The main interest has focused on pathological conditions with no current methods of treatment, such as neurodegenerative diseases, cardiac pathology, retinal dysfunction, lung and liver pathology. The overall aim is to develop methods of application either of pure cell populations or of whole tissue parts to the diseased organ under investigation, thus resulting in a reversal of the pathological condition and treatment of the cause of the disease rather than the management of symptoms. Progress of various degrees has been noted in most of the areas of interest for regenerative medicine. The common advancement is the generation of the desired cell population and at the same time the common hindrance has been the isolation of the pure progenitor population capable of regenerating the organ of interest and its expansion and survival *in vitro*. Despite those problems, there has been considerable progress in the field of heart regeneration (reviewed in Laflamme and Murry, 2005), retinal regeneration (Osakada *et al*., 2008) and neurodegenerative diseases such as Parkinson's disease (Wang *et al*., 2007b; Newman and Bakay, 2008).

An example of recent advances that demonstrate the putative therapeutic use of hES cells is the field of retinal regeneration. Over the last few years, retinal progenitor cells produced *in vitro* from hES cells and that are able to differentiate into cells that express the desired photoreceptor markers have been reported (Lamba *et al*., 2006). Nevertheless, they still required culture conditions that were not ideal for the widespread application of this technique, such as their co-culture with adult retinal cells. More recently, hES cells have been differentiated into putative photoreceptors

in a stepwise protocol focusing on the initial production of retinal progenitor cells that are subsequently subjected to a specific cocktail of factors and over long time in culture they are able to produce cells that express many of the genes involved in phototransduction (Osakada *et al*., 2008). Similar positive results are being reported in studies into Parkinson's disease, where hES cells have been successfully differentiated towards dopaminergic neurons both *in vitro* and *in vivo* (Iacovitti *et al*., 2007). A more efficient method for large-scale production of functional dopaminergic neurons has been recently reported and their therapeutic potential in animal models has also been demonstrated (Cho *et al*., 2008).

Even though there are many examples of the great potential in utilizing the physiological properties of hES cells in regenerative medicine, there are still aspects that need to be examined and thoroughly understood. The most typical one is overcoming the risks of tumour formation. This is an area under current investigation, and markers that can be helpful in minimizing the number of pluripotent and hence putative carcinogenous cells are being identified (Choo *et al*., 2008). Other challenges to the clinical application of hES cells are the method of cell delivery, which often proves to be inefficient, the survival of the transplanted cell population and, perhaps more importantly, the control of proliferation and maintenance of karyotypic stability of the engrafted stem cells (Maitra *et al*., 2005). With time, a more detailed understanding of hES cell biology will allow for their safe, efficient and widespread use in medicine.

Drug testing/pharmacology

The creation of a fully differentiated and functional airway epithelium from mES cells (Coraux *et al*., 2005) shows the potential of creating such a system from hES cells. This would allow for the detailed study of the developmental pathways and cell lineage correlations during the formation of the human epithelium. Such an understanding could provide valuable information on the series of events preceding the formation of a pathological state on the lung, hence increasing the possibilities of finding efficient targets for the development of treatments.

Another very appealing aspect of developing a pulmonary epithelium system from hES cells is reducing the need for animal models of human pulmonary disease. This would be of great benefit as hES cells could be derived from pathological blastocysts or subsequently manipulated to replicate the disease phenotype. This would create a study system that is more accurate than the animal models due to the large variations observed among the lung epithelia of the various species.

Conclusions

Overall, the future applications of knowledge acquired in the field of hES cells can be of great benefit to both science and medicine. Uncovering the secrets of such a cell type will expand our understanding of biological processes to such a degree that disease understanding and management could change dramatically. Technical advances remain to be made for the full benefits of hES cell biology to be revealed and applied effectively.

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Conflict of interest

The authors state no conflict of interest.

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