Towards a Combined Gene and Cell Therapy for Lung Diseases: The Case of Induced Pluripotent Stem Cells

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Respiratory diseases represent the major cause of morbidity and mortality worldwide, and for them a definitive cure is not included in the pharmacopeia. For example, despite improvements in mechanical ventilation, acute lung injury and its severe form, acute respiratory distress syndrome, are the leading cause of death in critical care, with mortality rates of 40 to 60%. In the field of chronic lung diseases, treatment with antibiotics and other medicals has prolonged the life span of individuals with cystic fibrosis, the most lethal diseases of the Caucasian population with autosomal recessive inheritance, but this is still limited to 40 years. There is urgent and desperate need of novel effective therapies for these patients.

A growing body of evidence strongly supports the notion that stem cells can be used to treat different pathologies stemming from the respiratory system [1], including acute [2] and chronic [3] lung diseases. Many of these disorders are characterized by tissue injury due to the inflammatory response and ensuing remodelling of the airways. Thus, at first, the rationale for using stem cells in lung diseases is that their application to the injured lung could allow their engraftment into the airways and replenish a niche with defined progenitor and true stem cell characteristics. Secondly, they might provide the lung microenvironment with paracrine effectors which act on the different cellular structural components of the lung, i.e. epithelial cells, fibroblasts, and endothelial cells.

Diverse approaches have focussed on either endogenous or exogenous stem cells. The respiratory tract contains several sources of endogenous stem cells residing in the many anatomical regions of the lung. However, these progenitor/stem cell niches are poorly known in their functional properties (meaning that their differentiation capacities have not been fully elucidated) and most of this knowledge has been obtained in animal models, such as the mouse which does not perfectly reproduce human anatomy and physiology. However, it is well recognized that the damaged lung epithelium is repaired by resident lung progenitor cells serving as the source of the new epithelial cell population [4], with only a possible minor contribution from circulating or bone marrow-derived stem/progenitor cells. Nevertheless, it is known that the regenerative potential of the lung declines with age and furthermore an extensive damage may not properly be repaired by the endogenous stem/progenitor niches.

Exogenous stem cells can be originated either from the embryo, extra-embryonic fetal tissues, or from adult tissues. Embryonic Stem Cells (ESC) are pluripotent cells derived from the inner cell mass of the blastocyst within the first 5-7 days after an egg is fertilized by sperm. They can produce derivatives of all three embryonic germ layers: endoderm, ectoderm and mesoderm [5]. Although ESC could be used in principle for the treatment of lung diseases arising from the alveolar region, such as acute lung injury [1,6], the enthusiasm about their use has faded away with time, for ethical issues (destruction of embryos), immune rejection [7], and the possibility of tumour

formation [8]. Fetal stem cells are derived from extra-embryonic tissues (amniotic fluid, placenta, umbilical cord blood and Wharton's jelly), exhibit less growth capacities than ESC, demonstrate low immunogenicity *in vivo* [9], and do not give rise to tumours. These cells are still poorly characterized and are being presently evaluated in the field of regenerative medicine. Recent reports show that fetal stem cells from amnion can be used for their anti-inflammatory and anti-fibrotic effects in a bleomycin-induced fibrosis mouse model [10]. Adult stem cells for the purpose of regenerative medicine can be obtained from various sources, including the bone marrow and the adipose tissue. The bone marrow harbors Hematopoietic Stem Cells (HSC), Mesenchymal Stromal Stem Cells (MSC), Multipotent Adult Progenitor Cells (MAPC), and progenitor cells of endothelium (endothelial progenitor cells, EPC) and of fibroblasts/myofibroblasts (fibrocytes), which have been used in the context of pulmonary medicine [11]. The adipose tissue, which exists in various places throughout the human body, contains pluripotent stem cells called Adipose tissue-derived Stromal Cells (ASC) [12]. Autologous ASC have also been considered for the treatment of a rat model of pulmonary emphysema [13]. Although adult stem cells can be directly isolated from the patient and are therefore immunologically compatible with the patient. They are generally hard to isolate and grow in culture; and moreover, transplantation of a sufficient number of cells to adult tissue needs a large-scale cell supply.

More recently, great attention has been given to induced Pluripotent Stem Cells (iPSC), which were first generated from adult somatic cells (mouse fibroblasts) through retroviral-mediated expression of four "stemness" genes (*KLF4, SOX2, OCT4*, and *cMYC*) [14]. Subsequently, *cMYC* was omitted as its contribution concerned only accelerated proliferation and in further works, iPSC were obtained after using LIN28 as the fourth gene [15,16]. These iPSC showed similar function and molecular phenotype characteristics to ESC and many methods employing viral and nonviral vectors have been used to obtain iPSC [17,18]. Since constitutive expression of reprogramming transgenes interferes with iPSC differentiation into lineages of all three primary germ layers [19], and aberrant expression of some or all of the reprogramming factors could lead to tumorigenesis in vivo [20] and may affect global gene expression [21]. Methods to obtain iPSC free of reprogramming transgenes have been
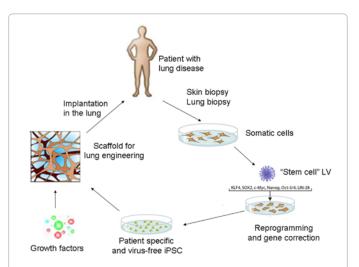
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searched. This could be achievable using non-integrating vectors, or even direct protein delivery, but the efficiencies were exceedingly low that prevent reliable application for reprogramming disease-specific adult human somatic cells [22-25].

Lentiviral Vectors (LV), such as those obtained from engineering of Human Immunodeficiency Virus (HIV) type 1, have been considered for pulmonary medicine in the context of gene therapy. They are endowed with interesting properties, including the ability to efficiently transduce dividing and nondividing cells, including stem cells [26]. Since they stably integrate within the host genome, the risk of insertional mutagenesis, as in the case of retroviral vectors, can be envisioned. However, while the Moloney murine leukemia virus and its derived vectors integrate preferentially in transcriptionally active promoters and regulatory regions, HIV and its derived LVs target gene-dense regions and the transcribed portion of expressed genes, away from regulatory elements [27]. Thus, LVs may have a safer profile of retroviruses for clinical applications.

Recently, LVs have been applied to the generation of iPSC. It has been reported the use of a single LV bearing a "stem cell cassette" encoding all four reprogramming factors, OCT4, SOX2, KLF4, and cMYC in a single polycistronic vector [28]. This vector accomplished reprogramming of postnatal mouse fibroblasts with high efficiency and allowed the derivation of mouse iPSC containing a single viral integration. An excisable version of the LV based on Cre/loxP technology was generated and allowed the derivation of murine iPSC free of exogenous transgenes [19]. The same technology was used to generate a humanized version of the single LV flanked by loxP sites to achieve reprogramming of normal or diseased postnatal human skin fibroblasts [29]. This vector efficiently reprogrammed fibroblasts obtained from humans with either of the two most common inherited lung diseases: cystic fibrosis, which affects the airway epithelium, or alpha1-antitrypsin deficiency-related emphysema, which affects the lung interstitium and epithelium. The generation of patient-derived iPSC clones was independent of the age of individuals from which the cells originated. Moreover, reliable and robust reprogramming was obtained from either fresh or banked clinical samples, a finding of particular importance if iPSC are to be used to generate progenitors from historical specimens. Finally, iPSC obtained from patients were differentiated in serum-free culture conditions into definitive endoderm, the developmental precursor lineage of lung and liver epithelia. Although the iPSC were free of exogenous transgenes, a 200 bp of the inactive viral LTR (Long Terminal Repeat, i.e. the viral promoter) remained in the host genome after excision, and hence, the theoretical risk of insertional mutagenesis is not completely eliminated. This risk could be further reduced by targeting of LV into a safe genomic locus, as has been described [30].

Once safe transgene-free iPSC have been generated, they should be delivered to the lung. This is possible either via the intravenous route or the direct intratracheal administration. Previous studies demonstrated that systemic administration of stem cells resulted mainly in the alveolar region with levels of engraftment ranging from 0.01 to 0.1% [31,32]. Upon intratracheal dosing, stem cell engraftment either remained very low (maximum 1%) [33] or was enhanced as compared to the intravenous route, leading to 5-10% level, depending on the animal model of disease used. This low level of engraftment is not likely useful to obtain meaningful therapeutic outcomes. In alternative to delivery of naked stem cells, increasing expectation is given to the possibility of bioengineering lung tissue [6,34]. In this case, three components are necessary: 1) the stem cells; 2) the scaffold supporting formation of tissue architecture and cell function; 3) the

Figure 1: Schematic drawing illustrating the ideal clinical setting employing induced Pluripotent Stem Cells (iPSC) for patients with acute and chronic lung diseases. To obtain clinically useful iPSC, somatic cells obtained from skin or lung biopsies of the patient should be transduced with a lentiviral vector (LV) bearing an excisable "stem cell" cassette which, through the cre/lox strategy, generates transgene-free iPSC. In case of genetically determined lung diseases, such as cystic fibrosis, the LV should also correct the gene defect (see text for details). Once iPSC have been obtained by this procedure, they should be embedded in an appropriate scaffold matrix together with morphogens and other factors derived from mesenchymal cells and such engineered lung should be implanted back in the patient.

appropriate cocktail of growth factors and other molecules with trophic, surviving and pro-angiogenic properties [35,36]. Recently, some progresses in the lung tissue engineering have been done, although much work has to be performed as to the biomaterials used for scaffolds and interaction of soluble factors with the extracellular matrix. Moreover, although the trachea was successfully engineered by Macchiarini et al. [37] producing the first treatment of a patient with bronchomalacia secondary to tuberculosis, the lung is a much more complex organ than trachea. In the latter years, nevertheless, whole lung decellularization and formation of a new epithelium has been reported [38,39].

Ultimately, in the most ideal clinical setting, patient specific and transgene free-iPSC should be embedded in a biomimetic scaffold, containing the appropriate factors and allowing angiogenesis, and delivered to the patient's lung (Figure 1). In case of genetic lung disease, such as cystic fibrosis, iPSC should be also corrected in their gene defect. This can be achieved with the same LV used to reprogram them and, in principle, there are different methods. One is gene adding, i.e. transduction of iPSC with the wild-type gene, but this could bring to undesired over-expression and subsequent abnormal function. Another is genome engineering, i.e. *in situ* correction of the defect through the use of TALE or zinc-finger nucleases, with the obvious advantage of not altering the physiological gene expression [40]. The disadvantage of using this technique is the induction of off-target DNA-cleavage activity and ensuing genotoxicity, and this side effect should be worked out before introducing this novel technology in the clinic.

The combination of gene therapy vectors and iPSC may result in a novel source of stem cells for the treatment of lung diseases such as cystic fibrosis and alpha1-antitrypsin. However, it will be imperative to identify the correct lung stem cell niche for each disease, to be able to purify stem cells sufficiently for transplantation studies, to

find the safer vector to be applied to, and last, but not least, to tissue engineering this novel therapeutic tool for delivery to the patient's airways.

Author Disclosure Statement

No competing financial interests exist.

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