UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA

Dottorato di Ricerca in Medicina molecolare e rigenerativa
(nell'ambito della Scuola di Dottorato in Medicina Molecolare e rigenerativa)

Ciclo **XXVIII**

Titolo tesi:
Characterization of human respiratory epithelial stem cells for tracheal replacement via a bioengineered human airway.

Relatore:
Prof. Graziella Pellegrini

Candidato:
Vincenzo Giuseppe Genna

Coordinatore del Dottorato:
Prof. Rossella Tupler

Direttore della Scuola di Dottorato:
Prof. Rossella Tupler

INDEX

SUMMARY 5

INTRODUCTION.....7

PRIMARY TRACHEAL CANCERS 8

Diagnosis 9

Treatment for tracheal cancer 9

TISSUE –ENGINEERED AIRWAY 12

Scaffold selection 12

Type of cells for scaffold colonization 13

Bioreactors..... 13

Pharmacological support to tissue regeneration 14

Human tissue-engineered trachea……..... 14

HUMAN RESPIRATORY SYSTEM 19

Nose..... 20

Pharynx..... 20

Larynx 21

Trachea 21

Bronchi and bronchioles..... 23

Alveoli 24

RESPIRATORY EPITHELIUM 25

Tracheo-bronchial epithelium 26

Bronchiolar epithelium..... 29

Alveolar epithelium..... 30

Respiratory epithelium functions…… ... 31

AIRWAY EPITHELIAL STEM CELLS. 35

Tracheobronchial progenitor cells……..... 36

Basal cells…….. 36

Submucosal gland ducts progenitor cells……..... 38

Bronchiolar progenitor cells……..... 39

Variant Clara cells 39
Alveolar cells…….. 40
Epithelial stem cells identification…… 41

STUDY OBJECTIVES 44

MATERIALS AND METHODS 46

PRECLINICAL STUDIES 47

Porcine airway epithelial cell culture 47
Immunofluorescence analysis…… .... ……47
Air-lift interface cell culture and trans-epithelial electrical resistance measurement……... ……48

HUMAN AIRWAY EPITHELIAL CELLS ANALYSIS 48

Human airway epithelial cell culture..... 48
Colony-forming efficiency assay…… ... 49
Clonal analysis…… ... 49
Western-blot analysis…… . 49
Air-lift interface cell culture……... 50
Immunohistochemistry……... 51
Immuno-cytochemistry analysis……..... 52
Migration and wound-healing assays…… 52
Tracheal clones clonogenic potential analysis 53
Tracheal clones composition analysis 53

RESULTS.....54

PORCINE AIRWAY EPITHELIAL CELLS CHARACTERIZATION.....55

Airway epithelial cel marker localization in invivo porcine airway section..... 55
Porcine tracheal and bronchial epithelial cell cultures…….. 58
Tracheal and bronchial porcine epithelial cells growth and differentiation in air-lift interface (ALI) condition 61

PRELIMINARY STUDIES ON NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS (NHBE).....63

Immunofluorescence analysis of Normal Human Bronchial Epithelial cells cultures 64

CHARACTERIZATION OF HUMAN TRACHEAL EPITHELIAL CELLS.....68

Isolation of airway epithelial cells from human tracheal biopsy and culture conditions selection 68

Migration and wound-healing assays 70

Airway epithelial cell markers expression in invivo human tracheal tissue 72

Nasal epithelium 75

Adenoid respiratory epithelium 75

Bronchial epithelium 77

Human tracheal epithelial cells culture 78

Airway epithelial cells markers analysis during serial passages of human tracheal epithelial cells life-span 79

Tracheal epithelial cells differentiation potential 82

Identification of human tracheal epithelial stem cells 85

Differentiation potential of human tracheal epithelial clones 92

Tracheal epithelial clones cellular composition 94

DISCUSSION.....98

References 105

SUMMARY

Primary tracheal tumors are uncommon neoplastic lesions of the airways with poor prognosis and high mortality rate. Surgical resection is considered as the treatment of choice, however, most patients with primary malignant tracheal cancers cannot undergo surgery due to tracheal involvement over 50% and only transitory treatment of symptoms is possible. Recent clinical experiences have shown that these patients can be treated by transplantation of bioengineered human airway tracts. Donor human tracheas can be decellularized and a bioengineered human trachea can be obtained by scaffold colonization with in vitro expanded autologous somatic epithelial cells and chondrocytes derived from mesenchymal stem cell of the patient. This bioengineered human trachea can be, therefore, implanted to restore respiratory function in a patient with end-stage airway disease. However, on long term evaluation, airway epithelium was shown to be essential to prevent infections, fibrotic reactions and stenosis of transplanted bioengineered human respiratory tract.

The overall aim of this project was to optimize cell culture conditions for adult human airway epithelial cells to maintain airway stem cells population and their proliferative and differentiation potential. To achieve these objectives, characterization of human respiratory epithelial cells was performed in vitro, as well as identification of progenitors cells and their in vitro maintenance. Progenitors cells differentiation in all cell types of tracheal tract, was also studied.

Two culture systems were evaluated for their capability to maintain expression of epithelial stemness/proliferation markers (p63, Bmi1, Ki67), differentiation markers (14-3-3σ, Involucrin, ZO1) and airway epithelial cells markers (AQ3, CK18, MUC5AC). In presence of feeder layer and fetal bovine serum, human airway epithelial cells showed higher proliferation and migration levels, maintenance of stem/progenitor cells markers expression and differentiation of a stratified and polarized epithelium. This culture condition was then selected for further analysis.
Tracheal epithelial cells were isolated from human biopsies and cultured in vitro for several passages to evaluate their growth rate, cell yield, colony forming efficiency and proliferative potential measured as the total number of cell doubling before senescence.

Finally, single cell analysis were performed to characterize stem cells and transient amplifying cells populations. Clonal analysis revealed that human tracheal epithelial cells are able to generate the three clonal types holoclone, meroclone and paraclone previously identified in other human epithelia, suggesting the holoclones as the putative stem cells of tracheal epithelium.

In the selected culture condition, human respiratory epithelial cells are able to maintain stem cells population and differentiate in all cells types required to properly re-populate biological or synthetic scaffolds used to develop a bioengineered human trachea for transplantation.

INTRODUCTION

PRIMARY TRACHEAL CANCERS

Primary tumors of the trachea are uncommon neoplastic lesions of the airway, representing about 0,1% to 0,4% of all malignant tumors, with 2.6 new cases per 1.000.000 people every year (South Thames, UK).

Tracheal cancers origin from the respiratory epithelium, salivary glands and tracheal mesenchyme. According to several reported clinical database analysis such as the 1973-2004 NCI Surveillance, Epidemiology and End Results (SEER) database, squamous cell carcinoma is the predominant histology, representing the 44,8% of tracheal tumors. The second most common tracheal tumors are the adenoid cystic carcinomas (ACCs), diagnosed in 16,3% of patients. Only 24,2% of patients presented with tumors localized to the trachea, while 36,7% of patients presented with regional disease (extension into adjacent blood vessels and bones) and 18,7% with distant disease [1].

Squamous-cell carcinoma is diagnosed mainly in men in their sixth and seventh decades of age and is associated with cigarette smoking. Adenoid cystic carcinoma arise from the minor salivary glands in the tracheal submucosa, is equally diagnosed between the sexes in their fourth and fifth decades and is not associated with cigarette smoking.

Average survival varied widely with different histological type of tracheal neoplasms. Patients affected by adenoid cystic carcinoma had significantly better prognosis than those who suffered from tracheal carcinoma, in particular, 5-year survival were 12,6% and 74,3% for squamous cell carcinoma and adenoid cystic carcinoma respectively.

Stages	Definitions
T-Stage	
T1	Primary tumor confined to trachea; size $<$ 2 cm
T2	Primary tumor confined to trachea; size $>$ 2 cm
T3	Spread outside the trachea but not to adjacent organs or structures
T4	Spread to adjacent organs or structures
Tx	Unknown or cannot be assessed
N-Stage	
N0	No evidence of regional nodal disease
N1	Positive regional nodal disease
Nx	Unknown or cannot be assessed

Fig.1: Proposed primary tracheal cancer staging according to SEER database [2]

Diagnosis

Tracheal tumors do not cause relevant symptoms until they occlude 50-75% of tracheal lumen and even in these case their presence is diagnosed late due to appearance of early non specific obstructive symptoms such as cough (71,6%), dyspnea (65,7%), stridor (38,8%), hoarsness (31,3%) and others [3, 4]. The presence of these symptmos together with haemoptysis (38,8%) generate the suspicion of tracheal cancers. However these difficulties can lead to misdiagnosis of chronic obstructive pulmonary disease and asthma and explain why most patients with malignant tracheal cancers present with local advanced and inoperable disease [5].

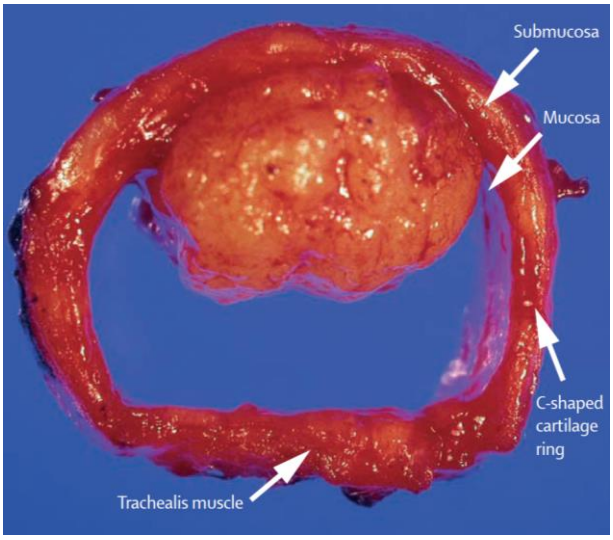


Fig.2: Cross section of intra-luminal tracheal tumor [5]

The definitive diagnosis of tracheal cancers is obtained with computed tomography (CT), the most useful method to evaluate tracheal tumors and their extension. Bronchoscopy is also useful to obtain tissue samples from tumors for tracheal cancers staging and to analyse the location and extention of disease along tracheal length.

Treatment for tracheal cancer

Management of tracheal tumors includes:

- Surgery: is the treatment of choice for all benign and early malignant tracheal tumors. The type of surgical treatment (laryngectomy with resection of the upper airway, trachea or carina) depends especially on tumor histology and location and amount of airway remaining after resection.

With modern techniques, up to 50% of the trachea can be removed in adult and up to 30% in children. Absolute contraindication to surgery include involment of more than 50% of tracheal length, the presence of many positive limphonodes, invasion of unresectable organs and presence of distant metastases [5].

- Endoscopic resection: intra-luminal tracheal tumors can be resected endoscopically to palliate symptoms in inoperable patients or to keep airway open until following definitive resection.

- Radiotherapy: is indicated before resection of otherwise inoperable tumors, as an adjuvant after resection and to palliate severe symptoms.

The better long-term results observed in patients who had resection suggest surgery as the best curative treatment currently available.
Surgical resection alleviate obstruction symptmos, is curative in patients affected by benign or early malignant lesions, and prolong survival in patients with advanced disease. Early diagnosis is the most important factor to obtain a better overall survival and the extent of disease at the time of diagnosis represent an important prognostic indicator. Patients with distant disease had a poor 5-year overall survival (3,9%) when compared with patients affected by regional or localized disease (25,5% and 46,8% respectively). Surgical unresectability usually depends on airway reconstruction limits determined by the location and extention of tumor involment. Advances in reconstruction techniques have allowed for more extensive surgical treatment, however, even for patients with operable cancers, the percentage of 5-years overall survival for all patients remain low (27,1%) suggesting the necessity of new therapeutic strategies.

Primary tracheal cancers represent one of the several causes for the narrowing of the trachea, including [6]:

- **Adjacent neoplasms**: the most common malignant casuse of tracheal narrowing is an adjacent neoplastic disease such as lung cancer or esophageal cancer that compresses the trachea.

- **Iatrogenic tracheal injury**: post-intubation tracheal stenosis is a well recognized complication of endotracheal intubation. The stricture occurs when cuff pressure damages the tracheal mucosa causing a local ischemia that leads to fibrotic narrowing of tracheal lumen.

- **Infections**: the most common infection causing airway abnormalities is mycobacterium tuberculosis. Tracheal stenosis can occur both during active disease and after the treatmen.

- **Tracheomalacia**: this airway morpholocic abnormality is due to the weakness of the cartilaginous component of the tracheobronchial walls as well as hypotonia of fibroelastic elements. The most common etiologies include chronic obstructive pulmonary disease, long-term intubation, tracheal trauma, congenital abnormalities, chronic inflammation and infections.

TISSUE –ENGINEERED AIRWAY

Benign and malignant tracheal diseases can be treated by surgical resection of the affected area and subsequent end-to-end anastomosis. However, this type of treatment is possible only when tracheal disease is restricted to 30% of total tracheal length in children or 50% in adults. The allotransplantation of a long portion of tracheal airway requires lifelong immunosuppression. Moreover almost all patients treated with this technique showed allograft necrosis and infections and some of them died due to inadequate graft revascularization and infections. Artificial prostheses have also been used to treat patients affected by extended tracheal diseases but this approach has been associated with material migration, infections, stenosis, rupture or absence of epithelization of the endoluminal surface [7].

Tissue engineering is a recent alternative approach aiming to restore or replace an irreversible damaged tissue/organ by using a scaffold colonized with autologous cells. This regenerative medicine approach is based on some important components including natural or synthetic scaffolds, autologous cells, a bioreactor and pharmacological support.

Scaffold selection [7]

The ideal scaffold for tracheal replacing should be biocompatible, non-immunogenic, non-toxic and non-tumorigenic, able to support the seeded cells and the neovascularization process, characterized by air-liquid tight seals, longitudinal flexibility and lateral rigidity to avoid collapsing events, and resistance to fibroblast and bacterial invasions. Moreover, the scaffold matrix should maintain the required tissue-specific mechanical properties. Unfortunately, the optimal synthetic tracheal scaffold doesn't exist yet and efforts have been made to optimize available natural and synthetic scaffolds:

- **Natural scaffolds**: decellularized natural scaffolds seem to be the most promising supports for tracheal tissue engineering. The most effective method to obtain a tracheal natural scaffold is a detergent enzymatic approach based on several cycles of deoxycholate and DNase treatments.

This technique produce a biocompatible nonimmunogenic decellularized tracheal matrix characterized by quite native mechanical properties and preserved extracellular matrix (ECM), an important component for tissue regeneration, cell homing and cell differentitation. Moreover, this decellurarization approach allows tracheal matrix to maintain proangiogenic factors such as basic fibroblast growth factor, important to support neovascularization process. The use of natural scaffold is limited by the need of human donor and the relatively long processing time.

- **Synthetic scaffolds**: various synthetic scaffolds have been studied and developed because of some of their advantages such as the rapid availability, the easiness to customize and sterilize, and the absence of donor need. However, several negative characteristics of synthetic scaffolds limited their clinical application. These aspects include vascularization and inflammatory problems, migration of the material, integrity and stiffness problems.

Type of cells for scaffold colonization

The use of autologous cells to repopulate the selected scaffold reduce the risk of immunologic rejection after transplantation and is then considered the most appropriate scaffold colonization approach. Adult mesenchymal stem cells (MSCs) and airway epithelial stem cells (ESCs) represent the cell sources to be used for tracheal tissue engineering.

Bioreactors [7]

A bioreactor is an ex-vivo perfusion system for three dimensional organs used to functionally maintain the regenerating organs for ex-vivo colonization or culture. The bioreactors consist of an organ chamber, fluid-handling hardwares and sensors.
The chamber for the organ must provide a physiological environment, structural support for the organ and in some cases, the arterial and venous vessels perfusion.
The fluid-handling hardware consists of pumps, tubes and valves that regulate nutrient substances perfusion, liquid flow and pressure, and hydrodynamic shear stress, all of which influencing cell organization and differentiation. Finally, the use of specific softwares and sensors allows to measure any structural and metabolic/biochemical changes inside the organ chamber, helping to support the organ regeneration.

Pharmacological support to tissue regeneration [7]

Pharmacological intervention seems to be useful to optimize the regeneration of the transplanted tissue preventing necrosys due to the absence of an efficient vascularization and cell migration.

There are numerous active substances that could be bound onto the scaffold surface or systemically administrated to enhance cell adhesion, cell homing and graft vascularization.

The systemic administration of granulocyte colony stimulating factor (G-CSF) allows to obtain hematopoietic stem cells mobilization from bone marrow to bloodstream; these cells home into the damaged tissue and start to secrete several anti-inflammatory, antiapoptotic and proangiogenic factors supporting tissue repair.

An additional factor used to counteract apoptotic events occurring within the transplanted trachea is erytropoietin (EPO). This renal hormone is a direct antagonist to TNFα pro-apoptotic effects within an inflammatory site, representing a tissue-protective factor for the regenerating organ. However, EPO administration presents also negative side effects because of the increased risk of thromboembolic events and the proangiogenic action of EPO may support tumor progression.

Human tissue-engineered trachea

In June 2008, the world's first bioengineered trachea transplantation was performed into a young woman with end-stage post-tuberculosi collapse of left main bronchus [8]. This patient showed a severe bronchomalacia of the left main bronchus and the conventional treatments adopted such as a stent placement revealed ineffective, leading to the proposal of a complete left main bronchus resection and its replacement with a bioengineered human trachea.

In this process a donor human trachea was decellularized and repopulated with autologous respiratory epithelial cells for the inner surface and autologous mesenchymal stem cells (MSC)-derived chondrocyte to regenerate the outer cartilage layer of the scaffold.

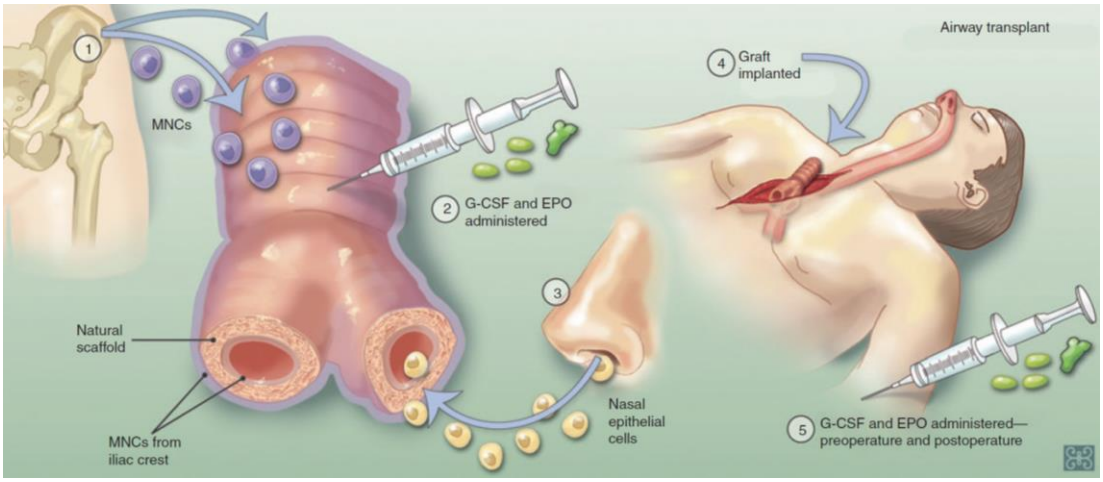


Fig. 3 Phases of human trachea bioengineering process using natural scaffold, respiratory epithelial cells and mesenchymal stem cells [7]

The lung function was restored after transplantation and patient showed no immunological or clinical signs of rejection.

This outcome was achieved through the combination of three important factors:

- **Donor decellularized trachea**: a 7 cm tracheal tract from a death donor was decellularized through 25 cycles of detergent-enzymatic treatment. The main objective of the decellularization protocol is the complete cellular material removal from the organ (to assure loss of antigenicity) without damaging the composition and biomechanical properties of the remaining ECM. The detergent-enzymatic method (DEM) is based on a defined number of cycles of three washing step: a first washing step in distilled water for cellular elements lysis and intracellular contents release; a 4h incubation time within 4% deoxycholate solution to solubilize cellular membranes and intracellular components; a final treatment of 3h with 1M NaCl + DNase I solution to induce DNA degradation. After 25 cycles of DEM, tracheal natural scaffold showed MHC class I and II antigens disappearing from the matrix (only few distorted and anuclear chondrocyte were detectable) preserving ECM structure, biomechanical properties and angiogenic factors such as β-FGF or TGF-β. Human trachea lacks a specific vascular supply and is provided by a complex network of vessels originating from the right inferior thyroid and bronchial arteries. An early revascularization of the transplanted scaffold is then necessary to avoid graft necrosis. Moreover, an increased number of DEM cycles ≥ 26 caused a loss of the mechanical properties of decellularized scaffold [9, 10, 11].

- **Autologous cells**: the importance of a functionally respiratory epiyhelium has been demonstrated by several experimental and clinical studies showing how the lack of an epithelium on the luminal surface is responsible of scars and subsequent stenosis formation. Respiratory epithelium regulates also important airway functions such as lung fluid balance, inflammatory cells modulation, clearance of inhaled particles and regulation of airway smooth muscles. To repopulate the inner surface of the decellularized scaffold, airway epithelial cells were isolated from a bronchial biopsy taken from the right main bronchial mucosa. The extracted epithelial cells were expanded in vitro and then seeded on the scaffold [9]. In order to obtain phenotypically stable chondrocytes, bone marrow MSC were cultured in the presence of β-FGF (to increase cellular proliferation rate and ECM production) and induced to chondrogenic differentiation with TGF-β3, dexamethasone, insulin and recombinant parathyroid hormone-related peptide to prevent terminal hypertrophic differentiation of MSC-derived chondrocyte and calcification events [9].

- **Bioreactor**: the bioreactor is designed to separate cell suspensions in the tracheal lumen from that on the outer surface in order to correctly divide the epithelial compartment from the mesenchymal one. Moreover, the organ chamber rotate the airway construct around its longitudinal axis, moving cells between culture medium and air, and this rotation has been demonstrated to induce the development of a more functional cartilage tissue. The bioreactor dynamic culture system allows also a more uniform cell distribution in the scaffold, nutrient supply and waste removal, generate hydrodynamic shear stress, pressure and stretch that regulate cell metabolic activities, cell differentiation and matrix components secretion, with consequent positive effects on tissue regeneration. The double chamber bioreactor is able to support the two autologous cell populations expansion and migration on the scaffold, their different media requirements, and to guarantee a sufficient scaffold oxygenation despite the thickness of the tracheal walls [9].

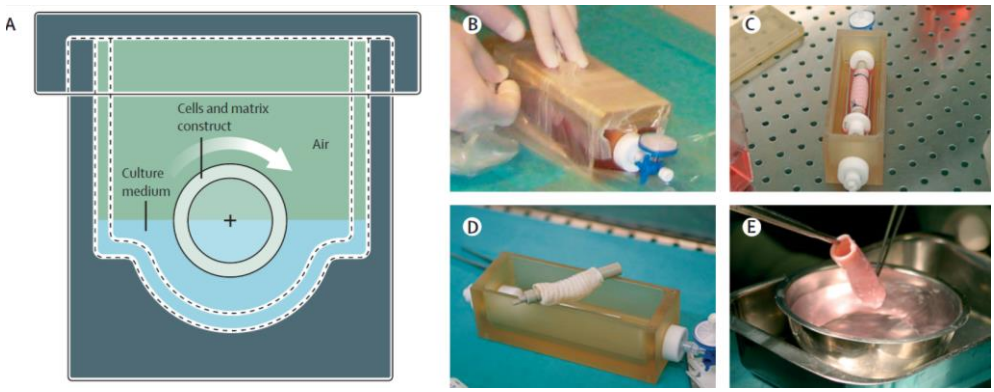


Fig. 4 Bioreactor for bioengineered human trachea [8]

The autologous airway epithelial and chondrocyte cells were cultured onto the matrix within the bioreactor for a period of 96h and the bioengineered trachea was subsequently used to replace the patient collapsed left bronchus, leading to restoration of lung function.

At 1 month, a biopsy sample taking induced a local mucosal bleeding, indicating successful revascularization. However, it is not clear if the observed riepithelization originated from seeded cells or from migration of adjacent healthy airway epithelial cells. From the first year after transplantation, patient was followed up every 3 months with CT scan and bronchoscopy when necessary.

Six months after the surgery, the proximal anastomosis showed a progressive scar formation and once the tracheal lumen reduced more than 50%, the stenosis was bronchoscopically treated and a Dumon bronchial stent placed. In the following years, the patient presented several stent obstructions and the relapsing stenosis were treated with new Dumon stents that were finally replaced because of intolerance, with polydioxanone stent. This stent was well tolerated but reabsorbable, and to prevent a complete anastomosis obstruction, two metallic stents were consecutively placed but both stents were not tolerated. The 30 days reabsorption time of the polydioxanone stent required several bronchoscopy re-interventions to counteract stenosis re-formation [12].

	Subglottic treatment	Proximal anastomosis*	Distal anastomosis*	Graft body*	Stent type	Stent size (mm)	Interval between prostheses (months)
December 2008	..	II	..	..	Dumon	12×40	6
June 2009	..	II	..	..	Dumon	12×40	7
November 2010	..	I (granulation tissue)	..	Granulation tissue	Dumon	12×30	18
January 2011	..	II	..	..	Polydioxanone	13×25	2
April 2011	..	I	..	..	Polydioxanone	13×25	3
June 2011	..	III	..	..	Polydioxanone	13×25	2
September 2011	..	III	..	..	Silmet	12×40	3
November 2011	..	III	Granulation tissue	Granulation tissue	Ultraflex	14×40	2
November 2011	..	..	..	..	..	..	..
December 2011	1	I	Granulation tissue	..	Polydioxanone	14×23	1
February 2012	2 (dilatation)	..	..	..	..	..	..
March 2012	1	I (granulation tissue)	..	..	Polydioxanone	14×23	3
June 2012	1	II	..	..	Polydioxanone	14×23	3
September 2012	2 (dilatation)	I	..	..	Polydioxanone	14×20	3
December 2012	2 (dilatation)	..	..	..	Polydioxanone	14×23	3
March 2013	1	I	..	..	Polydioxanone	14×20	3

*According to the Cotton-Myer grading system.[7]

Fig. 5 Series of bronchoscophic treatments and corresponding used stent [12]

A similar approach was applied in 2010 to replace a 7-cm stenosed segment in a 12-years-old child affected by a congenital tracheal stenosis.

In this case, to reduce the time required for ex-vivo expansion of autologous epithelial and mesenchymal stem cells, a decellularized donor human trachea was intraoperatively repopulated by scaffold saturation with MSCs suspension and several supporting factors including human recombinant erythropoietin, G-CSF and TGFβ. Tracheal epithelial fragments removed from the excised trachea were placed as free grafts within the lumen of the scaffold [13]. Following transplantation, the patient required several post-operative procedures to remove the granulation tissue and to treat the malacic regions with the insertion of bio-absorbable or self-expanding stents. After those treatments, the child recovered, although a biopsy of the proximal transplanted trachea at 42 months revealed the presence of a mix of squamous and respiratory epithelia with few ciliated cells [14].

The use of decellularized natural scaffolds allows to take advantage of the preserved extracellular matrix (ECM), an important component for tissue regeneration, cell homing and cell differentitation. However, this approach is characterized by a long period for the decellularization process and the need of human donors. To overcome these problems, a patient affected by a recurrent tracheobronchial tumor was treated with the transplantation of a bioengineered human airway based on a synthetic scaffold (POSS-PCU; polyhedral oligomeric sil sesqui-oxane [POSS] covalently bonded to poly-[carbonate-urea] urethane [PCU]) repopulated with autologous mononuclear cells and conditionated with TGFβ, G-CSF and epoietin beta before transplantation. After surgery, bronchoscopic examination showed first the presence of necrotic tissue, then large granulation areas with signs of partial epithelization only [15, 16].

These examples of airway tissue engineering showed that a bioengineered human airway represents a promising clinical strategy to restore the lung function in otherwise inoperable patients. However, the recurrence of cicatricial stenosis and the presence of inflammatory tissue, highlighted the necessity of a functional long-term stable re-epithelialization to avoid fibrotic reactions and stenosis of the transplanted respiratory graft.

The formation of a correctly differentiated and self-renewing respiratory epithelium is based on the presence of airway stem cells, requiring several studies to identify them and to maintain them in in-vitro culture conditions and after their seeding onto the selected scaffold.

HUMAN RESPIRATORY SYSTEM

The human respiratory system can be anatomically divided into the upper and lower respiratory tract. The upper respiratory tract includes nose, pharynx and larynx, whereas the lower respiratory tract can be divided into tree regions:

1. the cartilaginous proximal airway, including the trachea and bronchi;
2. the non-cartilaginous distal airway, comprising bronchioles and terminal bronchioles;
3. the gas exchange region, composed by respiratory bronchioles and alveoli.

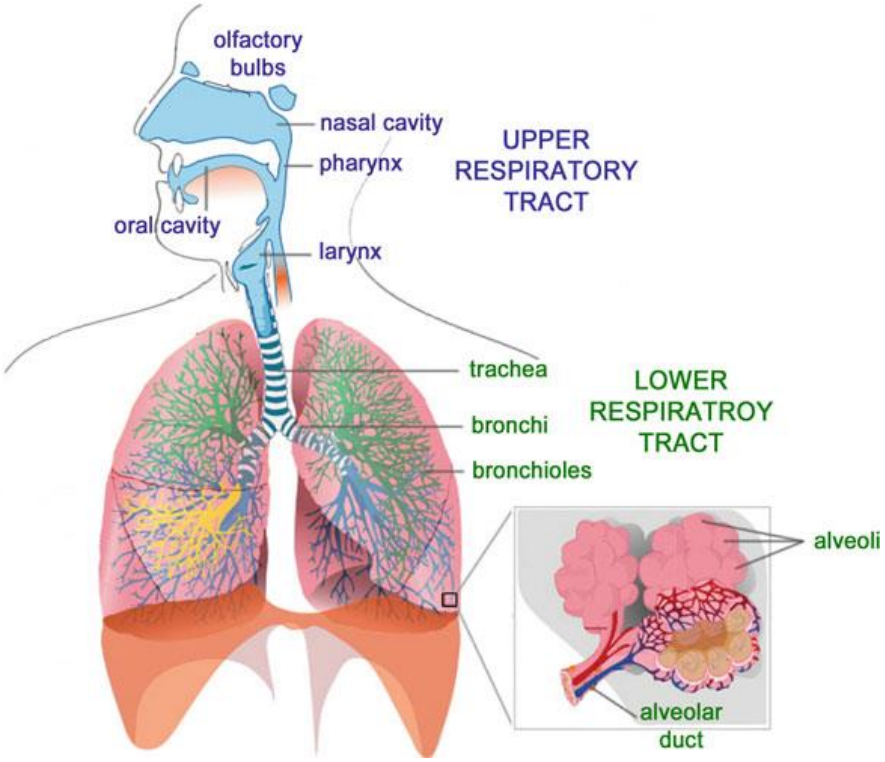


Fig. 6 Schematic representation of human respiratory tree [17]

The entire pulmonary tree is lined by a respiratory epithelium and the epithelial cells composition varies significantly along the different airway regions: a characteristic pseudostratified epithelium in proximal airway, a columnar/cuboidal epithelium in the distal region and a flattend epithelium in alveolar respiratory tract.

The primary function of the respiratory system is gas exchange, however the respiratory epithelium is also involved in several others important functions such as:

- filtering, warming and humidifying the inhaled air;
- acting as a barrier against different type of insults;
- facilitating mucociliary clearance;
- wound healing repair to restore normal airway function;
- modulating airway smooth muscle cells and inflammatory cells;
- secreting important substances such as surfactant proteins, mucins and antimicrobial peptides to protect respiratory surface [17, 18].

Nose

The nose structure includes the external portion termed as the nose and the internal regions being the nasal cavities. Air enters the anterior nasal cavity through the nostrils and from the two consequently vestibules the air passes through the main nasal passages before reaching the choanae, openings that allow air passage from the posterior region of nasal cavities into the nasopharynx.
The nose and the nasal cavities warm, humidifies and filter the inhaled air, and cointain the olfactory epithelium for smell.
The anterior nasal cavities surface is made up of a stratified squamous epithelium similar to that of the external skin, whereas the posterior region of the nasal cavities is lined with a pseudostratified columnar ciliated respiratory epithelium [17].

Pharynx

The pharynx is a tubular structure of about 12.5 cm in length that connects the posterior nasal and oral cavities to the larynx. Structurally the pharynx can be divided into three regions which are the nasopharynx, the oropharynx and the laryngopharynx.
The food and air are directed down to their respectively correct passageway, the oesophagus and the trachea, by the controlling function of the epiglottis. The epiglottis is a flap of elastic cartilage tissue that close off the trachea when food is swallowed to prevent food entering the larynx. The surface of nasopharynx is covered by respiratory epithelium, whereas the oropharynx and laryngopharynx surfaces are lined with non-keratinizing stratified squamous epithelium needed as a protection from food passage [17].

Larynx

The larynx is a short and rigid tube of about 4 cm long and 4 cm width that serves as a passageway from oropharynx to the trachea. Larynx also controls correct food passage through epiglottis movement, contributes to remove foreign particles and to warm and humidify the inhaled air thanks to the respiratory epithelium, and it is responsible of sound generation by controlling vocal cords tension [17].

Trachea

The human trachea is a semiflexible tube of 10-12 cm in length and 1.2-2.3 cm in width, connecting the larynx to the principal bronchi.
The trachea consists of four layers including an inner mucosa, an underlying submucosa, a layer of cartilage and smooth muscles and an outer adventitial lining [6, 19]:

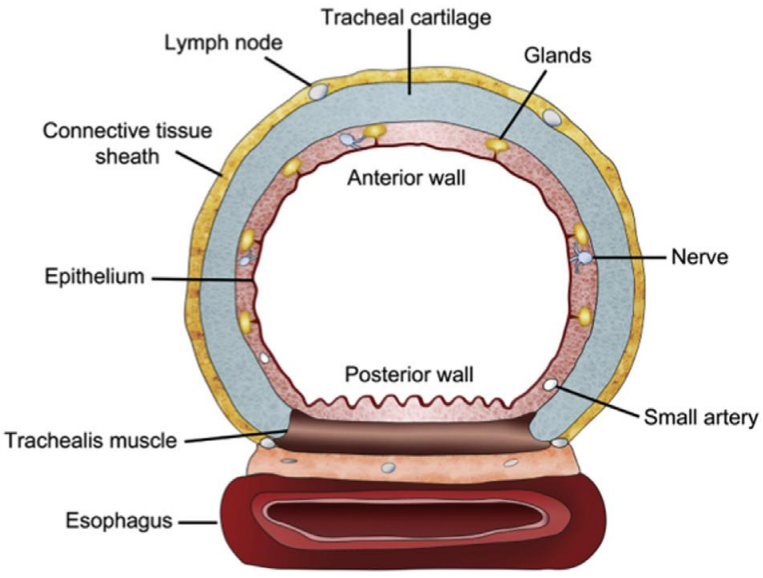


Fig. 7 Tracheal wall anatomy [6]

- **Mucosa**: consists of a pseudostratified columnar ciliated epithelium supported by a sub-epithelial connective tissue (lamina propria) and by an underlying layer of elastic fibers.

- **Sub-mucosa**: consists of dense fibro-elastic connective tissue including several tubulo-alveolar glands containing especially mucus-producing cells but also serous cells.

Ducts from these glands pass through the lamina propria to open onto the epithelial surface to lubricate the inner lining of the trachea. The secretion of the trachea derives mostly from these glands, although the goblet cells of the respiratory epithelium also contribute to the formation of the mucous layer.

- **Fibrocartilage layer**: the tracheal wall consists of up to 20 C-shaped cartilaginous rings forming the anterior and lateral circumference, and smooth muscle at the posterior side. The function of the rings is to support the trachea during expiration preventing it from collapsing in on itself, while the membranous portion is composed of longitudinal and transverse smoth muscle fibers with the last ones (m. trachealis) controlling tracheal lumen diameter. Each cartilaginous rings is surrounded by a layer of dense connective tissue called perichondrium, which is linked to the others by a fibroelastic connective tissue giving flexibility to the trachea and allowing its extension during inspiration.

- **Adventitia**: is a layer of connective tissue including nerves, blood and lymphatic vessels, that binds the trachea to the surrounding tissue.

The human trachea functions includes:

- **Air transport**: tracheal tube conducts air between larynx and the rest of respiratory tree;
- **Air conditioning**: during inspiration the proximal airway warm and humidify the inspired air up to 37°C and 100% saturation respectively;
- **Protection from injuries and microorganisms**: tracheobronchial submucosal glands produce a mucin-rich secretion covering and protecting the underlying respiratory epithelium. This secretion is composed of two phases: an acqueous sol layer next to the epithelium and a more superficial mucus layer. The sol layer is a water fluid containing anti-bacterial enzymes that makes ciliary beating possible and enables the mucus above to be transported by the cilia of the ciliated cells. The mucus layer is composed of high molecular weight glycoproteins (mucins) that form, together with proteins and lipids, a gel network with high adhesive properties. The mucus traps any inhaled particulates such as dusts and microorganisms, transporting them orally by the mechanically forces of ciliary beating and the airflow during expiration, while the antibacterial enzymes destroy the infectivous agents [19].

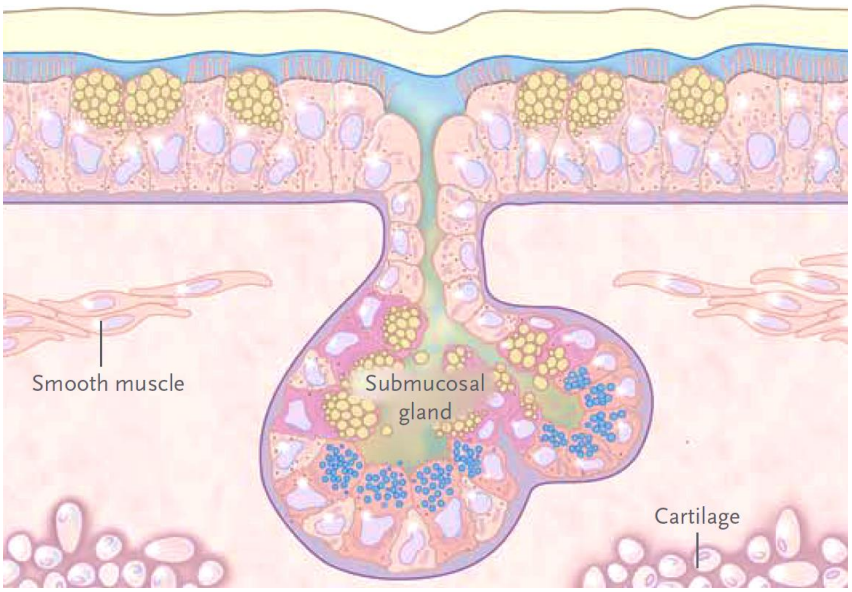


Fig. 8 Submucosal glands of the upper airway and biphasic composition of air surface liquid [20].

Bronchi and bronchioles

The mainstem bronchi are histologically similar to the trachea but have smaller diameter and thinner walls: the right mainstem bronchus is shorter, has a more vertical course and divides into three intra-pulmonary bronchi than the left mainstem bronchus that divides into two lobar bronchi. The bronchi continually divide into smaller bronchi up to 23-24 generations of divisions and this process is associated with some important structural changes:

- The cartilaginous rings turn into irregular plate of cartilage and disappear in bronchiolar branches of about 1 mm in diameter.
- The pseudostratified columnar epithelium change to columnar and then to cuboidal epithelium in the terminal bronchioles where some ciliated cells are still present.
- The amount of smooth muscle in the airway walls increase towards smaller respiratory structures.

The terminal bronchioles (0.5 mm diameter) represent the last conductive structures and divide into respiratory bronchioles, the first respiratory regions. The respiratory bronchioles are characterized by the presence of some alveolar bulges in their walls and further divide into alveolar ducts, short tubes that open into the alveolar sacs.

Alveoli

Alveolar sacs are made up of several alveoli, spherical air sacs with a diameter of about 200µm and surrounded by a rich network of blood capillaires needed for gas exchange.

Alveolar epithelium consists of a simple flattened epithelium including two types of epithelial cells (alveolar epithelial Type I and Type II cells) involved in gas-exchange function and pulmonary surfactants secretion.

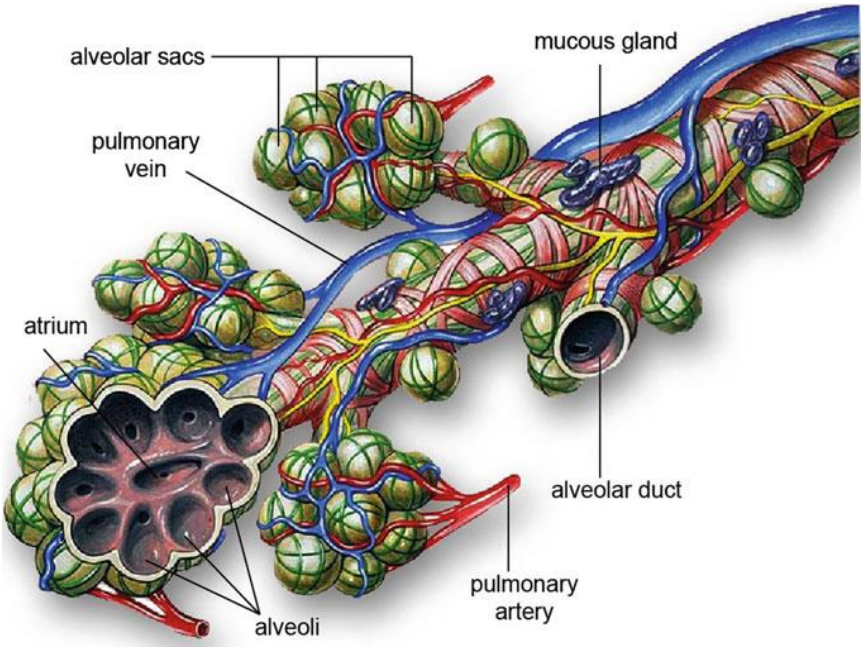


Fig. 9 Gas-exchange region of human airway [17]

RESPIRATORY EPITHELIUM

Human airway epithelium changes along proximal to distal branches reflecting the physiological functions of the different respiratory tracts. According to their functions and localization, airway epithelial cell types are differenty distributed into the three tracheo-bronchial, bronchiolar and alveolar epithelia.

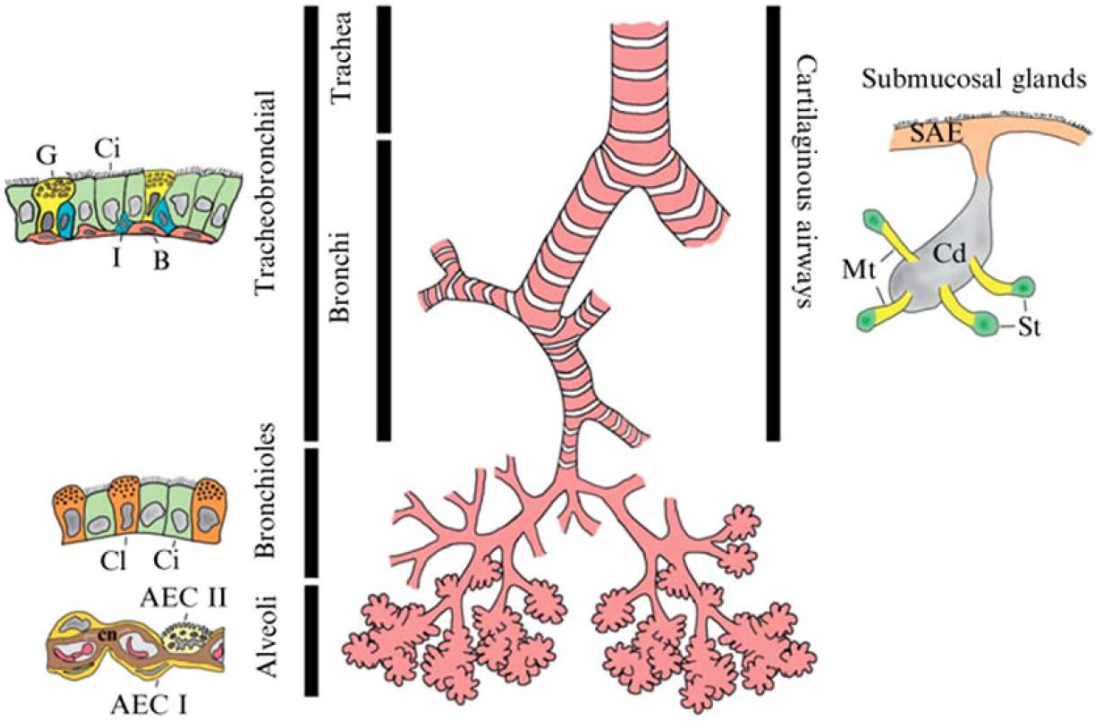


Fig. 10 Epithelial cell types in the human airway different regions [21]. B, basal cells; I, intermediate cells; G, goblet cells; Ci, ciliated cells; Cl, Clara cells; AECI and AECII, alveolar epithelial type I and II cells; SAE, surface airway epithelium; Cd, collecting duct; Mt, mucous tubule; St, serous tubule.

Most of the available informations about airway epithelial cells, their distribution along the respiratory tree and their functions derived from animal studies, although in some cases these data were also confirmed in human airway:

Cell type	Functions	Molecular markers
Basal cells	Considered the tracheobronchial airway stem cells, these cells have the capacity to fully regenerate the respiratory epithelium after ablation.	Trp63; CK14; CK5; NGFR

Clara cells	Secretory cells able to differentiate into ciliated cells and in response to inflammatory cytokines such as IL-13 into goblet cells.	Scgb1a1 (also known as CCSP in mice or uteroglobin in human)
Goblet cells	Mucous cells preferentially localized in proximal airway and submucosal glands.	Spdef; MUC5AC; MUC5B
Serous cells	Secretory cells concentrated in distal regions of submucosal glands.	Lysozyme; lactoferrin
Ciliated cells	Cells with numerous apical motile cilia involved in the mucociliary clearance.	FoxJ1; acetylated α tubulin
Neuroendocrine cells	Solitary cells in proximal airways and grouped in clusters called neuroepithelial bodies (NEB) in distal conductive regions.	CGRP
Brush cells	Rare chemosensory cells with many apical microvilli.	Villin; fimbrin
Type I pneumocyte	Thin flattened cells whose plasma membranes make up 90% of the gas-exchange surface area.	Aquaporin 5
Type II pneumocyte	Cuboidal cells responsible of surfactants secretion.	Sftpc; Aq3

Tracheo-bronchial epithelium

The epithelial cells of the proximal airway include the surface epithelial cells of the tracheo-bronchial region and the submucosal glands cells. Surface epithelial cells of the proximal airway can be further grouped into:

- **Basal cells** [18, 22]: form a monolayer along the base membrane and, although some columnar cells adhere to the basal lamina in the larger airway, the structural role of basal cells is to attach the columnar layer to the basement membrane. Basal cells are also involved in specific functions such as:

  - **Regulation of neurogenic inflammation**: this inflammatory process begins when inhaled foreign particles induce airway epithelium axons to secrete neuropeptides into the lateral intercellular space causing increased vascular permeability, neutrophil adhesion, glands secretion and cough.

Basal cells seem to be involved in neurogenic inflammation because they contain the leukemic inhibitory factor (LIF) that stimulate neuropeptides release and they express the neutral peptidase (NEP) surface enzyme that cleaves neuropeptides in the lateral intercellular spaces.

- **Inflammatory response**: basal cells role in the inflammatory response is linked to their expression of inflammatory cells receptors such as intercellular adhesion molecule-1 (ICAM-1), lymphocyte endothelial-epithelial cell adhesion molecule (LEEP-CAM) that mediate lymphocytes adhesion to epithelial and endothelial cells, 4-1BB receptor associated with T-cell activation, and Fas receptor and its ligand FasL.

- **Transepithelial water movement**: basal cells regulate airway surface liquid through the expression of aquaporin water channels such as AQ3.

- **Oxidant defence**: the airway tissue defence from oxidative stress is mediated by basal cells expression of multidrug resistance-proteins (MRP), transmembrane transporters involved in cell detoxification and defence against oxidant stress via efflux of oxidant agents.

- **Formation of lateral intercellular space**: the lateral intercellular space is a small space between cells containing nerve fibers, dendritic cells and diffusible molecules. In human airway, these intercellular space contains also hyaluronan, long high-molecular polysaccharide chains bound to basal cells transmembrane CD44 receptors and involved in regulation of the hydrated state of intercellular spaces.

These cells are almost ubiquitous in the conducting airway epithelium, although their number decreases with airway size. In human airway, basal cells extend distally to terminal bronchioles of about 0.5 mm in width and only the respiratory bronchioles consist of a simple cuboidal epithelium withouth basal cells [23]. Among the main markers expressed by basal cells there are CK14 and CK5, EGFR, NGFR, and Trp63. The importance of p63 for basal cells development has been showed through Trp63-null mice that lack basal cells in the tracheal respiratory epithelium containing predominantly ciliated cells [23, 24].
Several studies in animal models showed basal cells to repopulate all epithelial cell types found in the trachea, including basal, ciliated, goblet and clara cells, confirming the potential of, at least, some of them to act as a stem cells population in the upper airway [25, 26].

- **Ciliated cells**: are the predominat cell type of human airway, accounting for over 50% of all epithelial cells, and seem to be generated by basal cells or secretory cells [27]. Ciliated cells are columnar epithelial cells characterized by the presence of about 200-300 cilia on the luminal surface of each cells. The cilia are 0.25μm in diameter and 6-3.6μm in length in the proximal and distal airway respectively. The most important function of ciliated cells is mucociliary clearance: cilia are covered by the sol phase of airway secretions and extend into the gel layer, where barb-like structures on the tips of the cilia alternatively grab and release the mucus during cilia beating. A recent lineage tracing experiment showed that ciliated cells are a terminally differentiated population at homeostasis condition: the percentage of lineage-labeled ciliated cells decreased over time, estimating an half-life of about 6 months in the trachea and 17 months for bronchiolar ciliated cells [28]. Others studies, however, have shown ciliated cells undergo to transdifferentiation processes in pathological conditions supporting the theory of plasticity of airway ciliated epithelial cells [18].

- **Goblet cells**: are characterized by the presence of numerous mucous granules that give the cells their typical goblet shape. In normal human trachea, there are up to 6.800 goblet cells/mm2 of surface epithelium, although chronic airway inflammatory disease, mucous cells hyperplasia and metaplasia increase their number [27].

- **Pulmonary neuroendocrine cells (PNECs)**: are specialized epithelial cells found as solitary cells or in cluster called neuroepithelial bodies (NEBs). Recent studies on human airway, reported approximately 40 PNEC/10.000 cells [18].
  Mature PNECs are secretory spindle-shaped cells that produce bioactive amines and peptides including serotonin, calcitonin, gastrin-releasing peptide (GRP) and calcitonin gene-related peptide (CGRP). PNECs seem to play a role as hypoxia sensitive airway chemoreceptors and to provide a niche that regulate localized epithelial cells growth such as the expansion of a variant Clara cells population in mouse distal airway [29].

- **Brush cells**: are a population of epithelial cells characterized by the presence of apical tufts of microvilli and thought to regulate airway surface fluid secretion and breathing. Most of these cells contain the G-protein gustducin, supporting the hypothesis of brush cells chemoreceptorial role [30].

Submucosal glands are concentrated in the upper airway (restricted to the most proximal trachea in the mouse but further extended into the cartilaginous airway of human lungs) and occur at a frequency of about 1 gland/mm^2. Each submucosal gland consists of several tubulo-alveolar structures that feed into a collecting duct opening onto the airway surface. The tubules contain mucous cells in their proximal regions and serous cells in the distal acini. Serous cells are pyramidal-shape cells involved in the secretion of water, electrolytes, anti-microbial, anti-inflammatory and anti-oxidant compounds. All of these secretory products generate the sol phase facilitating cilia beating and mucociliary clearance process. Mucous cells of submucosal glands are columnar cells whose primary function is to secrete mucin MUC5B that generate, together with MUC5AC produced by surface goblet cells, the gel phase on the apical surface of airway epithelium.

Recent animal studies showed the presence of label-retaining cells (LRCs) residing in the submucosal glands ducts in the upper mouse trachea and in specific foci at the cartilage-intercartilage junctions in the lower trachea, suggesting that these glands could provide a protective niche for airway stem cells [31, 32].

Bronchiolar epithelium

The epithelial cell types of distal bronchioles include mainly ciliated cells and Clara cells. In human, Clara cells are located in bronchial and bronchiolar epithelia.

These cells are columnar secretory cells that release some important substances such as Uteroglobin (also named Clara cell secretory protein-**CCSP** or Secretoglobin 1a1-Scgb1a1), proteases, antimicrobial peptides and cytokines.

These cells are also considered an important regulators of inflammatory response, sources of surfactant apoprotein A, B and D [33], and endowed with the capacity to metabolize and inactivate xenobiotic substances through their cytochrome p450 activity.

Clara cells are involved in the normal maintenance of the human distal airway epithelium. Recent injury models studies have shown that in mice whose airway Clara cells were deleted by naphthalene, a population of variant Clara cells lacking cytochrome p450-2F2 survive and regenerate the bronchiolar epithelium.

These cells reside in pools associated with neuroepithelial bodies (NEB) and the associated neuroendocrine cells are tought to provide a niche regulating the expansion of this variant Clara cells population [29].

Another pool of Clara cells secretory protein (CCSP) expressing cells were localized to bronchioalveolar duct junctions (BADJ) and found to include proliferating and label retaining cells in vivo and multipotent cells in vitro, suggesting these bronchioalveolar stem cells (BASCs) as the stem cells that maintain the Clara cells and alveolar cell populations in distal airway [34].

Alveolar epithelium

The alveolar epithelium is composed of large, flat alveolar type I cells that cover 90% of alveolar walls, and cuboidal alveolar type II cells:

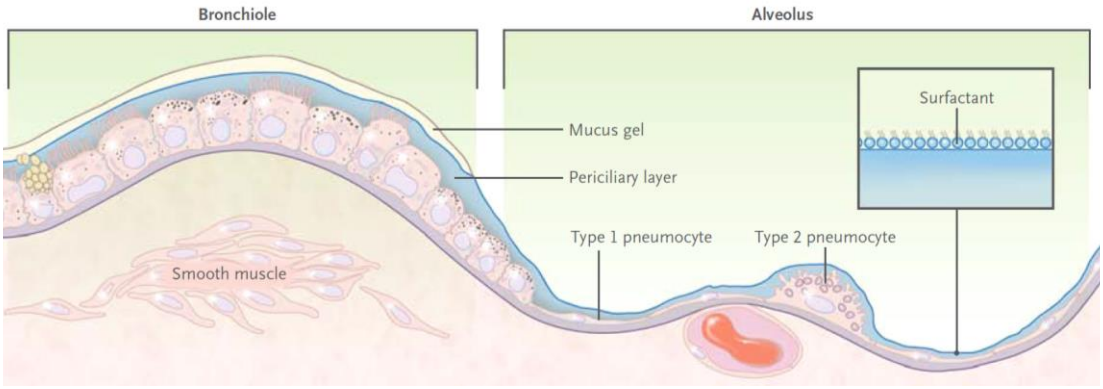


Fig. 11 Epithelial cell types of alveolar region [20]

- Pneumocyte of type I: are large, flat squamous cells that function as a thin, gas-permeable membrane. These cells cannot proliferate and are considered to be terminally differentiated.

- Pneumocyte of type II: are small cuboidal cells whose primary function is surfactants secretion.
  Pulmonary surfactants include proteins (surfactant proteins SP-A, SP-B, SP-C and SP-D) and phospholipids (such as dipalmitoylphosphatidylcholine and phosphatidyglycerol) that lower surface tension at the air-liquid interface and prevent the alveolar structure from collapsing [18]. Alveolar type II cells are considered to be the stem cells of the alveolar epithelium due to their ability to proliferate and differentiate into alveolar type I cells following an injury event.
  Alveolar type I cells are indeed sensitive to several agents such as NO_2, ozone and bleomycin and in in-vivo animal studies where these cells were lethally damaged, alveolar type II cells were observed to proliferate and differentiate in type I cells to repopulate the alveolar epithelium [18].

Respiratory epithelium functions [35]

The barrier function of the airway epithelium is based on three primary components: the mucociliary layer, the tight junctions complexes located between the apices of adjacent epithelial cells and the antimicrobial peptides secreted by the airway epithelial cells.

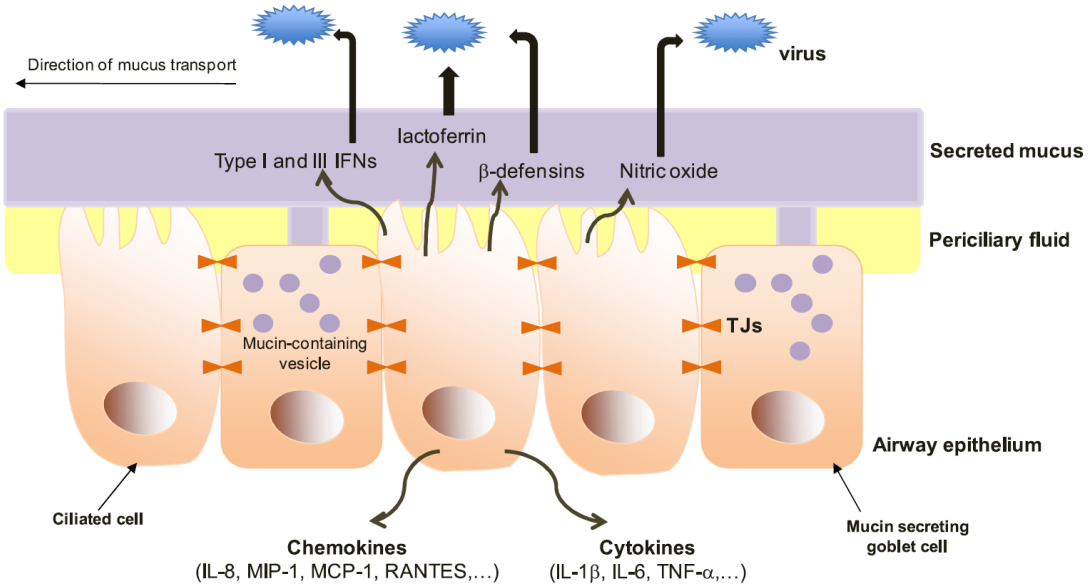


Fig. 12 Schematic representation of airway epithelial cells functions [36]

These three components have the important function of clearing inhaled pathogens, allergens and particulate matter withouth inducing inflammation and maintaining lung tissue homeostasis:

- **Mucociliary clearance**: this function is based on the contributes of both mucus layer that traps inhaled pathogens or foreign particles and cilia beating that sweeps the trapped material toward the pharynx. The airway surface liquid (ASL) is made up of an upper layer of mucins that floats on a lower periciliary layer.
  Normal healthy mucus consists of 97% water and 3% solids (mucins, salts, lipids and cellular debris) and the degree of hydration heavly affects the visco-elastic properties of the mucus layer, which in turn determine how effectively it is cleared by cilia beating and cough [20]. The airway mucus is secreted by both goblet cells and submucosal glands and is constituted mainly by mucins, high molecular weight glycoproteins that cross link to generate an adhesive layer.

Mucins structure consists of a peptide core characterized by the presence of several regions rich in serine and threonine residues linked to oligosaccharide chains: mucins are in fact made up of 50-90% of carbohydrate and they are highly anionic due to the carboxyl and sulfate groups of their terminal sugar chains. These glycan side chains bind large amount of water, allowing mucus layer to act as a liquid reservoir for the periciliary layer [20]. Airway mucins present anti-viral and anti-inflammatory properties and contribute to the innate immune defense through interaction with other mucus components such as IgA and defensins [36].

At least 11 mucins (MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC13 and MUC19) have been identified in humans but the predominat mucins in normal human airways are MUC5AC and MUC5B, with the first produced mainly by the goblet cells and the latter secreted by submucosal glands. The periciliary layer is relatively less viscous, is approximately 7μm in height and acts as a lubricant layer for cilia beating. Liquid volume depends by the amount of sodium chloride in the airway lumen, which in turn is regulated by sodium absorption through the sodium channels and by the chloride extrusion through the cystic fibrosis transmembrane conductance regulator (CFTR). Periciliary layer contains the membrane-bound mucins muc-1, muc-4 and muc-16:

- MUC1 is a small mucin localized on the cell surface and microvilli of both ciliated and secretory cells;
- MUC4 is highly expressed on cilia where it prevent mucus penetration and provide lubrication through bound water;
- MUC16 is the largest mucin, is expressed by both secretory and ciliated cells and it can be cleaved to be integrated into the upper gel layer.

The mucus layer is swept in a proximal direction by ciliary beating. Normal healthy cilia beat 12-15 times per second, propelling the gel layer with a velocity of 1 mm per minute [20].

- **Apico-lateral junctional complexes**: the physical barrier function of conductive airway epithelium is assured by the tight and adherens junctions located at the apicolateral borders of respiratory epithelial cells. These intercellular junctions protect airways from inhaled pathogens and other environmental insults.

Tight junctions consist of several trasmembrane proteins (such as occludins and claudins) and cytoplasmic scaffolding proteins (zonula occludens ZO-1, ZO-2, ZO-3): transmembrane proteins seals the membranes of adjacent cells, while scaffolding proteins anchor transmembrane proteins to the cytoskeleton. Adherent junctions mediate cell-to-cell adhesions and are made up mainly of epithelial cadherin (E-cadherin), β-catenin and α-catenin. The E-cadherins extracellular domains form homotypic adhesion between adjacent cells, while the intracellular domains interact with β-catenin and α-catenin, which in turn associate with cytoskeleton components. E-cadherins are also involved in cells proliferation and differentiation by regulating EGFR and β-catenin activities.

- **Antimicrobial products secretion**: airway epithelial cells produce several antimicrobial substances such as enzymes, protease inhibitors, oxidant and antimicrobial peptides. These substances act as a biochemical barrier against foreign microorganisms:

  - **Enzymes:** lysozyme is an enzyme secreted by respiratory epithelial cells and exerts its antimicrobial action against gram-positive bacteria and also against gram-negative bacteria in presence of lactoferrin. Lactoferrin is an iron-chelator that inhibits bacterial growth by sequestering iron and protect from viruses infections by either preventing virus binding to the host cells or by binding to the virus particles.

  - **Protease inhibitors:** airway epithelial cells secrete some protease inhibitors such as secretory leukoprotease inhibitor (SLPI), antichymotrypsin, α1-antiprotease and elastase inhibitor, that oppose to the effects of pathogens proteases.

  - **Oxidants:** nitric oxide (NO) and hydrogen peroxide are two oxidants produced by respiratory epithelial cells. These cells present two constitutively expressed NO synthases (NOS1 and NOS3) and one inducible NOS2 activated by viral infections and pro-inflammatory cytokines. The dual oxidases 1 and 2 belonging to the family of NADPH oxidases, are located in the cellular membrane and secrete hydrogen peroxide to the extracellular milieu, where it combines with thiocyanate and lactoperoxidase to generate the oxidant hypothiocyanite that kills gram-positive and gram-negative bacteria.

- **Antimicrobial peptides:** the human β-defensins (hBD) are the most abundant antimicrobial peptides, they are expressed on the airway epithelium surface and protect from bacteria and viruses. Four hBDs have been identified in airway epithelial cells and two of these, hBD2 and hBD3, act also against respiratory viruses.

The respiratory epithelium can also produce several active substances such as lipid mediators, growth factors (EGF, TGFβ), cytokines and chemokines.

Through the activities of cyclooxygenase, lipoxygenase and mono-oxygenase enzymes, human airway epithelium is a major source of arachidonic acid metabolites that regulate airway smooth muscle tone, mucus secretion, neurotransmitter release and inflammation. The prostaglandins PGE2, for example, is considered as a bronchoprotective mediator due to its inhibitory effects on mucus secretion, nerve activity and smooth muscles tone [27].

Airway epithelial cells activate an immune response to viral infection by releasing chemokines and cytokines into the submucosa. These substances promote recruitment of immune cells including neutrophils, NK cells and macrophages into infected tissues as well as their activation [36].

AIRWAY EPITHELIAL STEM CELLS

Human surface epithelia are constantly renewed thanks to the presence of stem cells and progenitor cells populations representing the only proliferating cells in a normal adult tissue. For example, human epidermis and corneal epithelia are renewed about every 3-4 weeks and 6-12 months respectively. Stem cells have the capacity to self-renew and to generate committed progenitors cells, named "**transient amplifying (TA) cells**", which in turn terminally differentiate into the cell lineages of the tissue of origin after a limited number of cell divisions. The most important function of TA cells is to increase the number of differentiated cells generated by each stem cell division, allowing stem cells to remain slow-cycling in normal healthy condition [37,38].

Stem cells have been identified in different human organs but a definitive human airway stem cell able to generate multiple epithelial lineages of both the proximal and distal respiratory regions of the lungs has not been found. However, several studies suggested the existence of different airway epithelial cells populations acting as stem/progenitor cells according to their localization within the respiratory tree [21]:

- Basal cells, Clara cells and some submucosal glands epithelial cells have been suggested as the stem/progenitor cells of the proximal conductive airway;
- A variant Clara cells population localized within NEBs and BADJs are thought to function as the stem cells of the bronchioles;
- Alveolar epithelial type II cells (AEC II) are considered the alveolar stem cells due to their ability to differentiate into alveolar epithelial type I cells.

These data derived especially from injury in animal studies due to the slow cellular turnover in airway and the necessity to activate stem cells population and induce their proliferation. There are several injury models according to the type of target cells [21]:

- Oxidant gases: high concentration of oxygen (O_2), nitrogen dioxide (NO_2) or ozone (O_3) have been used to cause hyperoxic lung injury in animal models. Continuous exposure to O_2 resulted in the destruction of alveolar walls, while NO_2 damages alveolar type I cells (AEC I) through oxidation of unsatured fatty acid of the plasma membrane.
- Bleomycin: this compound induce alveolar injury causing oxidative damages to DNA and lipid peroxidation. AEC I are more sensitive to bleomycin than AEC II.

- Sulfur dioxide (SO2): this common air pollutant is water soluble and can be easily absorbed into the mucous layer of the airway leading to severe tracheobronchial damage (most surface basal cells survive), whereas the submucosal glands and bronchioles are spared.
- Polidocanol: it's a surface-active detergent causing widespread denudation of the proximal airway epithelium after intratracheal instillation.
- Naphthalene: this chemical agent is toxic to CYP 450-2F2 positive Clara cells and damages both proximal and distal airway.

The ex-vivo epithelial xenograft models represent another approach used to study the progenitor-progeny relationships between airway epithelial cells. According to this method, isolated airway epithelial cells are seeded onto tracheal grafts where endogenous epithelium has been depleted. These respiratory grafts are then subcutaneously implanted into immunodeficient hosts to follow the regeneration process. This technique allowed to observe the different phases required to properly regenerate a fully differentiated human respiratory epithelium onto a denuded rat trachea and lead to the identification of a multipotent stem/progenitor cells population isolated from human bronchial airway, involved in submucosal glands formation and able to differentiate into all the epithelial cell types of the respiratory surface [39, 40].

Tracheobronchial progenitor cells

Proximal airway progenitor cell populations have been studied through the combination of in-vivo and in-vitro models. These experiments showed basal cells to exhibit stem/progenitor cells potential.

Basal cells

Several in-vivo and in-vitro studies suggest tracheobronchial basal cells as the stem cells population of the proximal airway due to their some important properties:

- A subset of basal cells were observed to be LRCs because of retention of BrdU labeling after SO_2 damage induction.
- CK5-positive basal cells showed a greater ability to proliferate and generate large colonies in vitro than CK5-negative cells.
- In a xenograft regeneration model, rat tracheal basal cells were able to restore the entire respiratory epithelium of a denuded trachea.

Lineage tracing experiments were performed to confirm the stem cell identity of tracheabronchial basal cells population. A transgenic animal model where CCSP-expressing cells were depleted, showed that Clara cells ablation lead to basal cells hyperplasia and the observation that increased basal cells proliferation preceded regeneration of Clara cells population suggested basal cells as the progenitors of the secretory cells. Moreover, some days after injury a small number of CCSP/CK14 double positive cells were observed, supporting the idea that basal cells may be able to differentiate into Clara cells. CK14-expressing basal cells were then genetically labeled (LacZ positive) to analyse their differentiation potential: 4 days after naphthalene treatment and clara cells ablation, all β-gal positive cells showed a basal-cell like morphology and were CK14 positive. However, at the end of regeneration process, there were cluster of β-gal positive cells including labelled basal, ciliated and CCSP-expressing cells derived from the CK14-positive basal cells. These results demonstrated that basal cells represent a multipotent cells population able to restore a normal airway epithelium after an in-vivo injury [25, 26, 41].

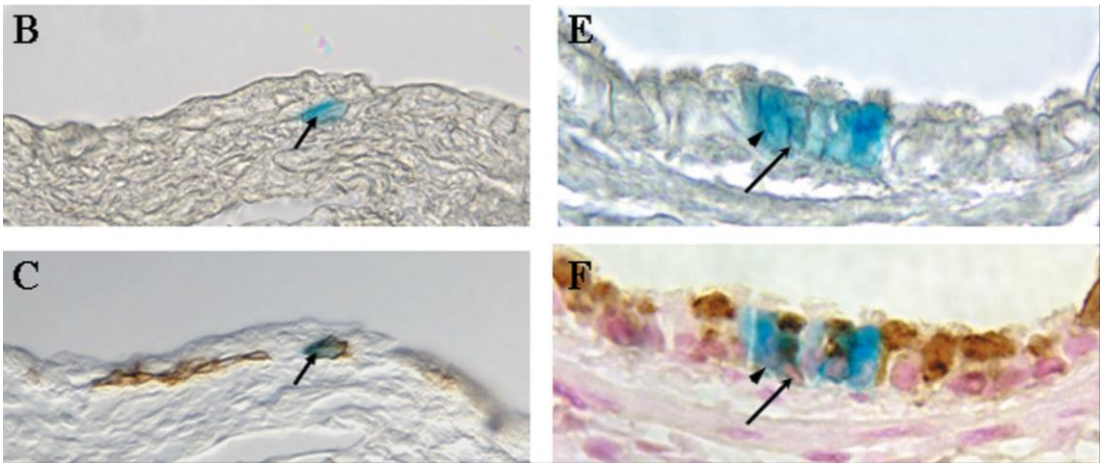


Fig. 13 Cluster of different respiratory epithelial cells derived from a single labeled basal cell [26]

Other studies have used a CK5-CreER transgene to label basal cells and analyse their stem/progenitor cells properties. Even in this case, after SO_2 extensive damage of tracheal epithelium, labeled basal cells proliferate and generate cluster of labeled cells including Clara cells and ciliated cells.

An in-vitro sphere-forming assay was also performed to evaluate basal cells differentiation potential. This experiment revealed that single mouse or human basal cells were able to generate tracheospheres and bronchospheres respectively consisting of a "pseudostratified" epithelium with p63+/CK14+ basal cells peripheral to CK8+/acetylated tubulin+ luminal cells. These results confirmed airway basal cells as multipotent progenitor cells of the tracheobronchial epithelium [42].

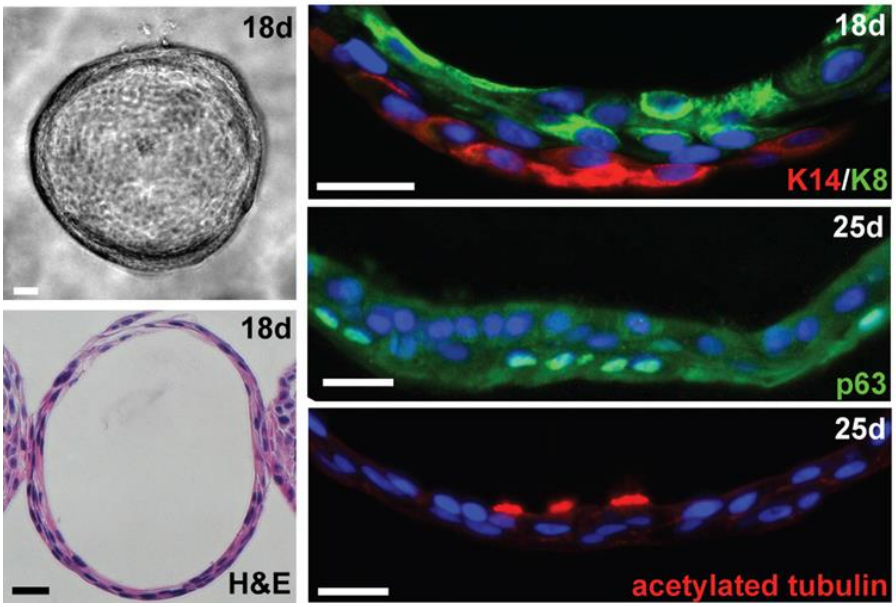


Fig. 14 Bronchosphere generated by single human bronchial epithelial basal cell [42]

Submucosal gland ducts progenitor cells

Label-retaining cells are those cells that maintain, within their genomic DNA, an incorporated nucleotide analog such as BrdU for long periods of time. These cells are considered stem cells due to their slow cycling phenotype.

LRCs have been identified in mouse tracheal submucosal gland ducts through two injury animal studies: intratracheal instillation of polidocanol or inhalation of SO2 were used to stimulate cell proliferation in an otherwise mitotically quiescent tracheal epithelium and the subsequents repeated injections of BrdU labeled all proliferating cells. During the following chase period, transient amplifying cells diluted BrdU labeling due to their faster proliferation, while airway epithelial stem cells appeared as LRCs and were localized to submucosal gland ducts in the upper mouse trachea and in systematically distributed foci along the surface epithelium of the lower trachea, in particular at cartilage-intercatilage junctions. To verify if gland ducts cells were able to regenerate the airway surface epithelium, mouse tracheas were treated with a protease to remove the surface epithelium and then sub-cutaneously transplantated in T-cell deficient mice. Approximately one month later the respiratory epithelium was regenerated supporting the idea that submucosal glands may represent airway stem cell niches [31, 32].

Bronchiolar progenitor cells

Secretory cells of both proximal and distal airway function as a progenitor cells pool involved in respiratory epithelium regeneration after oxidant gases exposure. Using oxidant gases to selectively injury airway ciliated cells, it was demonstrated that Clara cells lose typical features of quiescent cells and generate transitional state cells, named type A Clara cells, characterized by the absence of secretory granules and the ability to incorporate labeled DNA analogues. These type A cells participate to damage repair by actively proliferating and differentiating into ciliated cells and mature Clara cells, suggesting Clara cells as bipotent transient-amplifying progenitor cells [43].
Recent lineage-tracing experiments used an Scgb1a1-CreER transgene to label and follow Clara cells in postnatal growth and it was observed that in bronchioles labeled Clara cells percentage remained stable for a long time period (up to one year) while the percentage of labeled ciliated cells increased, showing the Scgb1a+ cells to act as stem cells due to their ability to self-renew and differentiate into ciliated cells. During postnatal growth, tracheal labeled Scgb1a+ cell percentage decreased over time, while the percentage of tracheal labeled ciliated cells initially increased but was not maintained. These results confirmed the ability of Clara cells to differentiate into ciliated cells but in this airway region Scgb1a1+ cells behave as a TA population and both Clara and ciliated cell types are replenished by an unlabeled cells acting as the main progenitor cells population [44, 45]. CCSP-expressing cells can also be subdivided into naphthalene-sensitive and naphthalene resistant Clara cells: the first represent the classical numerous secretory Clara cells, the latter a rare subpopulation localized in distinct airway microenvironments.

Variant Clara cells

Clara cells are the most common secretory cells of distal airway and are also involved in the detoxification of both endogenous and xenobiotic lipophilic compound through the oxidation cytochrome P450-mediated. Clara cells are sensitive to naphthalene administration because this substance is metabolized by the cytochrome P450-2F2 isoenzyme and converted into an highly toxic naphthalene 1R, 2S epoxide. In the more distal airways, where there are no basal cells, Clara cells regeneration after naphthalene treatment is mediated by a variant Clara cells population cytochrome P450-2F2-negative preferentially localized adjacent to NEBs and to BADJs. These cells showed multipotential differentiation and are then thought to act as progenitor cells of the bronchiolar airway.

Naphthalene-mediated depletion of Clara cells induced the proliferation of both CCSP-expressing cells (vClara cells) and CGRP-expressing cells (PNECs) within NEBs, however the complete ablation of all Clara cells through a transgenic animal model showed that the remaining PNECs proliferate but were unable to regenerate the respiratory epithelium. These results supported the idea that PNECs may regulate the microenvironment necessary for vClara cells expansion and that this variant of Clara cells may represent the progenitor cells pool of the distal airway [32, 46].

Another pool of vClara cells were identified in bronchioalveolar duct junctions and were considered as bronchioalveolar stem cells (BASCs) due to their some properties [34, 47]:

- resistence to bronchiolar and alveolar damage;
- role in the regeneration process after injury: BASCs number increased after Clara cells damaging with naphthalene treatment and contribute to the regeneration of bronchiolar epithelium;
- self-renewal and multipotent capabilities in in-vitro assays: bronchioalveolar stem cells seem to differentiate into Clara-like cells, alveolar type two-like and type one-like cells in in-vitro cultures, leading to the conclusion that BASCs have the capacity for both bronchiolar and alveolar differentiation.

However, no in-vivo data have demonstrated yet that CCSP-expressing cells associated to BADJs have the ability to differentiate into both bronchiolar and alveolar cell types [43].

Alveolar cells

Pneumocyte type II are considered the progenitor cells of the gas-exchanging regions based on their ability to activate after injury and differentiate in both alveolar type I and type II cells. Recently, a lineage tracing experiment using a SftpC-CreER transgene to label SftpC+ alveolar type II cells, showed definitively that AECs type I are generated by AECs type II cells [44].
Another study of lineage tracing, showed that BASCs cells identified in the BADJs are not the only cell population to co-express Scgb1a1 and SftpC markers. A small percentage of pneumocyte type II express low levels of Scgb1a1 together with high levels of SftpC, and these cells can generate pneumocyte type I , confirming type II cells as the major alveolar stem/progenitor cells population [45].

Epithelial stem cells identification

In contrast to rapidly renewing tissues such as gut, blood and skin, that maintain themselves through an undifferentiated stem cells pool, other organs that turn over more slowly, such as pancreas, liver and airway, use alternative strategies to self-renew, including involment of differentiated cells. For example, liver regeneration after hepatectomy involves differentiated hepatocyte proliferation and if this process is inhibited, interlobular bile duct cells can regenerate the hepatocyte population [41].
Similarly, if airway ciliated cells are damaged, only Clara cells, not basal cells, proliferate and differentiate into ciliated cells, whereas after Clara cells injury, basal cells act as the stem/progenitor cells population that activate and repair the damaged respiratory epithelium.

Adult human stem cells have been identified in several tissues/organs and showed some specific properties such as multipotency, sel-renewal capability, mitotic quiescence in vivo and high proliferative potential in vitro, telomerase activity, high clonogenicity and relatively indifferentiated state. However human airway epithelial stem cells have not been yet definitively identified due to the complexity of the respiratory tree epithelial composition and the absence of specific stem cells markers.
Stem cells identification is based on either in vitro proliferative capacity or in vivo localization of slow-cycling label-retaining cells (LRCs). However, some adult stem cells are not slow-cycling and the "label-retaining" experiments can be performed only on laboratory animals with subsequent species differences.
To solve these problems, the in-vitro analysis of the proliferative potential of human airway epithelial stem cells can be obtained on generated clones. Clones resulting from single cells can be subcultured, producing daughter colonies with different growth potentials. The original clones can be classified depending on the type of generated progeny, as follows [48]:

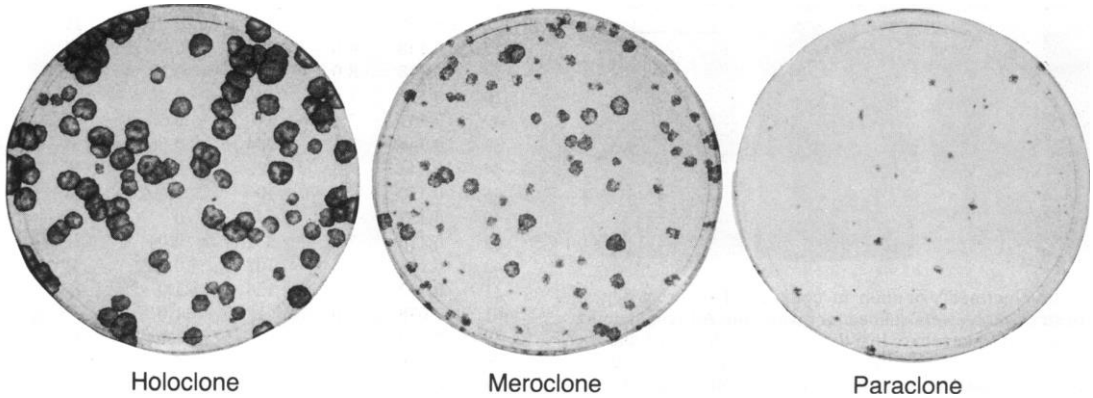


Fig. 15 Colonies generated by three different clonal types [48]

- **Holoclones:** form large with smooth circular perimeter colonies containing mainly small cells concentrated near the perimeter. Holoclones forming cells are considered the epithelial stem cells because holoclones produce 100% clonogenicity, are endowed with self-renewal capability, telomerase activity, the ability to differentiate into distinct cellular lineages, the highest proliferative potential and fewer than 5% of holoclones deriving colonies are terminally differentiated (aborted colonies).

- **Paraclones:** contain cells with a short proliferative lifespan (no more than 15 cell generations) and generate small, highly irregular and terminal colonies made up of large and flattened cells. These old transient amplyfing cells generate more than 95% of aborted colonies or no daughter colonies.

- **Meroclones:** contain a mixture of cells of different growth potential and generate both growing and terminal colonies, with a percentage of aborted colonies between 5% and 95% of the total colonies. Meroclones cells generate large colonies with a wrinkled perimeter and represent a transitional stage between holoclones and paraclones.

The clonal conversion from holoclones to meroclones to paraclones is unidirectional and results in a progressively reduced growth potential. Even if meroclones are endowed with high clonogenic capacity as well as holoclones, regenerative potential is possessed only by holoclones wich can be considered the stem cells of virtually all human epithelia. Epithelial cell cultures containing holoclones can, in fact, permanently restore epithelial defects and holoclones can be isolated from the regenerated tissue years after engrafmnet, confirming the ability of these clones to self-renew and sustain the maintenance of the restored tissue [49].
The identification of specific markers preferentially expressed in holoclones rather than meroclones/paraclone, represent an useful method to isolate and characterize stem cell populations through stem cell markers analysis. Human respiratory epithelial stem cells markers have been not yet identified, however some transcription factors, already considered stem cells markers in others human epithelia, have important functions in airway epithelial cells also:

- **p63** [49, 50, 51]: the gene encoding p63 generate full-lenght (TAp63) and N-terminally truncated (ΔNp63) transcripts by un upstream and a downstream promoter respectively. For both transcripts, alternative splicing generate different α, β, γ, δ, ε isoforms. P63 is involved in generation and regeneration of stratified epithelia and

is essential to maintain the proliferative potential of epithelial stem cells. P63 null mice are characterized by almost complete absence of stratified epithelia, most notably in the epidermis, with some regions covered by terminally differentiated keratinocyte, suggesting p63 as an essential factor to maintain the proliferative potential of epithelial stem cells. Moreover, in contrast to the normal pseudostratified epithelium of the upper airway, the tracheobronchial epithelium of p63-/- mice consists of only columnar ciliated cells withouth any basal cells, confirming the idea that p63 play an important role in maintaining stem cells population. Finally, the higher p63 expression in holoclones of some self-renewing human epithelia such as skin and cornea rather than meroclones/paraclones, confirmed p63 as an epithelial stem cell markers.

- **Bmi1** [38, 52, 53]: is a polycomb group member involved in maintanance of stem cells self-renewal and cell cycle regulation through the inhibition of proteins such as p16 and p19 that suppress cells proliferation and induce apoptosis. Recent studies showed that Bmi1 is required to sustain self-renewal of hematopoietic and neural stem cells and is also expressed, together with C/EBPδ and ΔNp63α, in human limbal stem cells in-vivo and in limbal holoclones in vitro, suggesting Bmi1 important role in maintenance of epithelial stem cells quiescence and self-renewal properties. Finally, it was observed that Bmi1 expression is required for expansion of BASCs cells after naphthalene lung injury in vivo and for proliferative and sel-renewal capability of these putative bronchioalveolar stem cells in in-vitro cell cultures.

- **Sox2** [54, 55]: this Sry-related HMG box protein is required, together with Oct3/4 and Nanog, for maintenance of embryonic stem cells pluripotency. Later in development, this transcription factor is involved in specific functions such as maintenance of neural stem cells during neurogenesis and regulation of tracheal and esophageal morphogenesis. Sox2 downregulation at branching regions is required to allow airway epithelial cells to respond to branching-inducing signals and overexpression of Sox2 in respiratory epithelium leads to an increased number of p63 positive cells, suggesting a role in maintenance of basal stem cells. Another study showed that Sox2 is also required for proliferation and maintenance of mouse bronchiolar Clara cells and their differentiation in ciliated and goblet cells. However, tracheal Clara cell number was not reduced after Sox2 deletion, suggesting a compensatory role of airway basal cells and the necessity of further studies to better understand the role of Sox2 transcription factor in airway epithelial cells.

STUDY OBJECTIVES

Different animal studies as well as in-vitro cell culture analysis showed that respiratory epithelium consists of several specialized epithelial cell types differently organized according to their location along the respiratory tree. Each of the three principal airway regions (tracheaobrochial, bronchiolar and alveolar tracts) seems to be endowed with a regional stem/progenitor cell population. However the definitive identification of epithelial stem cells in human airway remain unclear due to the difficulties in performing lineage tracing experiments and the absence of a optimized cell culture system required to maintain stem cells population in vitro and analyse their differentiation potential.

The isolation of human airway epithelial stem cells and their maintenance in in-vitro cell culture conditions is necessary to generate a bioengineered human airway suitable for clinical transplantation as well as the identification of respiratory stem cells is required to assure that the implanted graft contains a sufficient number of stem cells able to sustain the tissue self-renewal.

This study aims to optimize cell culture conditions for adult human airway epithelial cells in order to maintain respiratory stem cells and their proliferation and differentiation capabilities.
Preclinical studies based on a porcine animal model were first performed to localize airway epithelial cells markers expression in in-vivo sections, and for a preliminary characterization of respiratory epithelial cells proliferative and differentiation potential in in-vitro cell culture conditions.
Then the characterization of human respiratory epithelial cells was performed in vitro, as well as identification of progenitors cells and the analysis of their differentiation potential toward all human tracheal cell types.
Two culture systems were compared for their ability to maintain expression of epithelial stemness/proliferation markers, differentiation markers and respiratory epithelial cell markers. The culture condition characterized by the presence of feeder layer and fetal bovine serum was selected for higher proliferation and migration levels showed by cultured airway epithelial cells, maintenance of stem/progenitor cells markers expression and differentiation potential.

Tracheal epithelial cells were isolated from human biopsies, cultured in vitro for several passages and their proliferative and differentiation potential were analized until senescence. Subsequent single cell analysis were performed to characterize stem cells and transient amplifying cells populations and these experiments revealed that human tracheal epithelium contains the three clonal types holoclone, meroclone and paraclone previously identified in other human epithelia. Tracheal holoclones were finally analized to verify their differentiation potential.

MATERIALS AND METHODS

PRECLINICAL STUDIES

Porcine airway epithelial cell culture

Tracheal and bronchial epithelial cells were isolated from porcine biopsies and cultivated on plastic cell culture plate using the commercially available defined medium Bronchial Epithelial Growth Medium (BEGM, from Lonza). Briefly, tracheal and bronchial porcine biopsies were cut in small pieces and airway epithelial cells isolated through subsequent cycles of enzymatic digestion with trypsin 1X (TrypLE, from Life Technologies) at 37°C for a total time length of about 2h. Isolated cells were cultured in BEGM medium containing Bronchial Epithelial Basal Medium (BEBM) supplemented with bovine pituitary extract, insulin, hydrocortisone, gentamicin/amphotericin-B, retinoic acid, transferrin, triiodothyronine, epinephrine and human epidermal growth factor. Cultures were then fed every other day and for serial propagation assay, cells were passaged when they reached sub-confluent condition.

Immunofluorescence analysis

Tracheal porcine tissue was embedded in an optimal cutting temperature compound (OCT) and stored at -20°C. Next, 10µm-thick sections were cut on a cryostat, mounted onto glass slides and fixed with 4% formaldehyde (5min at RT) or cold methanol (10min at -20°C). Tracheal and bronchial cell cultures grown on chamber slides were washed with phosphate buffered saline (PBS 1X) and fixed with 4% formaldehyde (5min at RT) or cold methanol (10min at -20°C). Glass slides and chamber-slides were permeabilised with 0.3% Triton X100 in PBS 1X (10min at RT) and incubated with primary antibody (Table 1) for 30min at 37°C and later with the corresponding secondary antibody for 30min at 37°C. The nuclei were stained with 4',6 diamidino-2-phenylindole (DAPI)-mounting medium.

Antibodies	Source	Antibodies	Source
CK-5	Abcam	ZO1	Invitrogen
CK-7	Millipore	TrkA	Santa Cruz
CK-8	Progen	P75	Millipore
CK-14	Covance	Ki67	Thermo Scientific
MUC5AC	Progen	Collagen II	Abcam
Acetylated Tubulin	Sigma Aldrich	p63α	Primm

Table 1. Primary antibody used for porcine samples analysis

Air-lift interface cell culture and trans-epithelial electrical resistance measurement

Tracheal and bronchial porcine epithelial cells were cultured in air-lift interface (ALI) condition for about 30 days to allow complete differentiation of airway epithelial cells. Briefly, millicell cell culture inserts (from Merk Millipore) were coated with a layer of human collagen IV (from Sigma Aldrich) at 60µg/ml [21], incubated over night at room temperature and air dried. Next, airway porcine epithelial cells were seeded onto Millipore inserts and cultivated in submerged condition with BEGM culture medium until cells reached confluence. Therefore, BEGM culture medium was removed from the apical and basal chambers and B-ALI differentiation medium (from Lonza) was added to the basal chamber only to start the air-liquid interface cell cultures. The culture medium was changed every other day and the trans-epithelial electrical resistance measured with an electrode until complete differentiation process. Some of these air-lift cultured epithelia were embedded in an optimal cutting temperature compound (OCT) and stored at -20°C until their staining according to the previously described immunofluorescence protocol.

HUMAN AIRWAY EPITHELIAL CELLS ANALYSIS

Human airway epithelial cell culture

Tracheal epithelial cells were extracted from human biopsy and cultivated on a feeder layer (FL) of murine lethally irradiated 3T3-J2 cells. In particular, tracheal biopsy was cut in small pieces and treated with trypsin (0.05% trypsin and 0.01% EDTA) at 37°C for 80 min. Cells were collected every 20 min and cultured in 5% CO_2 and humidified atmosphere. Human tracheal epithelial cells and Normal Human Bronchial Epithelial Cells (NHBE, from Lonza) were cultured in two different cell culture systems: in the first cell culture condition, airway epithelial cells were grown on plastic cell culture plate with BEGM culture medium; in the second culture system, respiratory epithelial cells were cultivated on lethally irradiated 3T3-J2 cells with a keratinocyte growth medium (KC) composed by DME and Ham's F12 media (2:1 mixture), FBS (10%), insulin (5 mg/ml), adenine (0.18mM), hydrocortisone (0.4 mg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), glutamine (4 mM), and penicillin/streptomycin (50 IU/ml).

Epidermal growth factor (10ng/ml) was added at 10 ng/ml 3 days after plating. Cultures were then fed every other day and for serial propagation assay, cells were passaged when they reached sub-confluent condition until replicative senescence.

Colony-Forming Efficiency Assay

A defined number of cells (200-1000) from biopsy processing and from each serial passage was plated into both of two indicator dishes containing lethally irradiated 3T3-J2 cells. Colonies were fixed after 12 days, stained with rhodamine B, and counted under a dissecting microscope. Colonies were classified as progressively growing or aborted, as previously described [48]. Colony-forming efficiency (CFE) values were calculated as the ratio of the number of grown colonies to the number of plated cells, whereas the percentage of aborted colonies is obtained from the ratio between abortive colonies and the number of all colonies grown in the indicator dish. Colony forming efficiency assay provides also important information such as adhesion and migration capability of clonogenic cells, and allows the distinction among clonal types according to the number of aborted colonies.

Clonal analysis

Holoclones, meroclones and paraclones identification was performed through clonal analysis. After limiting dilution, single cells were inoculated into 96-multiwell plates containing a feeder-layer of 3T3-J2 cells. After seven days of culture, single clones were selected under an inverted microscope, photographed and dissociated with trypsin digestion to obtain a single cells suspension. Each clone was then divided into two cell culture plate. One-quarter of the clone was plated into an indicator dish, cultivated for 12 days and then fixed and stained with rhodamine B to define the clonal type. Three-quarters of the clone were cultured in another vessel for 7–12 days to obtain a cellular extract useful for Western blot and immunocytochemistry analysis.

Western Blot analysis

Proteins from cell culture serial passages and from clonal analysis were extracted by RIPA buffer at 0–4°C. Equal proteins amounts were electrophoresed either on 4-12% Bis-Tris Protein gels (from Life Technoligies) or 8% sodium dodecyl sulphate–polyacrylamide gels and transferred to nitrocellulose membranes.

Protein bands immunoreactions were performed with different primary antibodies (Table 2) and the corresponding HRP-coniugated secondary antibody (from Santa Cruz Biotechnologies). Proteins signals were developed using a chemiluminescent labeling reagent (Super Signal West Pico Chemiluminescent Substrate, from Thermo Scientific) and bands quantification was performed with Image J software.

Air-lift interface cell culture

Normal human bronchial epithelial cells (NHBE) and some human tracheal clones were cultured in ALI condition with BEGM/B-ALI and KC respectively for about 30 days to obtain complete differentiated airway epithelia. Briefly, a coating of human collagen IV (from Sigma Aldrich) at 60µg/ml [21], was prepared onto millicell cell culture inserts (from Merk Millipore), incubated over night at room temperature and air dried. Next, human airway epithelial cells were seeded onto coated Millipore membrane and cultivated in submerged condition with BEGM or KC culture medium until cells reached confluence. Therefore, BEGM and KC culture media were removed from the apical and basal chambers and B-ALI differentiation medium (from Lonza) or KC were added only into the basal chambers to start the air-liquid interface cell cultures. The culture media were changed every other day until 30th day of culture. Airway differentiated epithelia were embedded in OCT compound and stored at -80°C until their immunofluorescence staining.

Tracheal epithelial clones derived from another clonal analysis were cultured onto a human dermis after epidermis removal. To perform this experiment, human dermis was incubated for few seconds in heated PBS 1X (30-60 sec at 70°C), then plunged into cold PBS 1X (4°C) and the epidermis removed with sterile tweezers [56]. A feeder-layer of 3T3 cells and then the three-quarters of different human tracheal clones were plated onto several human dermis scaffolds. Human airway epithelial cells were cultured in submerged condition with KC culture medium for about seven days. Therefore, KC culture medium was removed from the apical and basal chamber of millicell cell culture inserts and new KC medium added into the basal chamber only to start the air-liquid interface cell culture. The culture medium was changed every other day until complete differentiation of respiratory epithelial cells. Air-lift cultured clones were fixed with 3% paraformaldehyde (20min at RT), embedded in OCT compound and stored at -80°C until their use.

Immunohistochemistry

Human biopsies taken from different airway regions (nose, adenoids, trachea and bronchi) were embedded in OCT compound (Killik; Bio-Optica), frozen at -80°C and therefore cut on a cryostat to obtain 10-20µm-thick sections that were thaw-mounted onto glass slides. In-vivo sections, airway epithelial cells cultured on glass coverslips (NHBE and tracheal epithelial cells) as weel as sections of respiratory cells grown in air-lift interface condition, were fixed with 3% paraformaldehyde (5min at RT) or cold methanol (10min at -20°C).
Airway epithelial cells were then permeabilised with 0.5-1% Triton X100 in PBS 1X (10min at RT) and incubated with primary antibody (Table 2) for 30min at 37°C and later with the appropriate secondary antibody for 30min at 37°C (Alexa-Fluor 488 and 568). The nuclei were stained with 4',6 diamidino-2-phenylindole for 5 min and the samples mounted with Dako mounting medium. Images were acquired with laser-scanning confocal microscope (LSM 510, Zeiss).

Antibodies	Source	Antibodies	Source
CK-4	Progen	TrkA	Santa Cruz
CK-6	Abcam	P75	Millipore
CK-7	Millipore	Ki67	Leica
CK-8	Progen	p63α	Primm
CK-13	Progen	P63 4A4	Ventana (IHC), Santa Cruz (WB)
CK-14	Covance	Bmi1	Cell Signaling
CK-17	Progen	EGFR	Cell Signaling
CK-18	Abcam	Involucrin	Leica
CK-19	Novus Biologicals	TTF1	Abcam
Pankeratin	Abcam	AQ3	Abcam
MUC5AC	Progen	14-3-3σ	Abcam
Acetylated Tubulin	Sigma Aldrich	Laminin	Abcam
Uteroglobin	R&D Systems	CD104	Thermo Scientific
ZO1	Invitrogen	CD151	Abcam

Table 2. Primary antibody used for human samples analysis. IHC, immunohistochemistry; WB, western-blot assay.

Immuno-cytochemistry analysis

A defined number of human airway epithelial cells (10.000-15.000 per slide) obtained from serial passages of tracheal life-span or tracheal clones amplification, were placed via Cytospin and Single Cytofunnel (from Thermo Scientific) onto glass slide and fixed in methanol (10min at -20°C). For goblet cells number quantification, glass slides were incubated with the primary antibodies against MUC5AC (Progen) and Pankeratin (Abcam) for 1h at 37°C, whereas for p63 positive cells quantification, slides were incubated with the antibody anti-human p63 (4A4, from Ventana). Fluorescent secondary antibody (Alexa-Fluor 488 and 568) were incubated for 1h at RT. The nuclei were stained with DAPI for 5 min and coverslips were mounted onto the slides with Dako mounting medium. MUC5AC fluorescence signals were acquired using the Cell Observer Z.1 Microscope (Zeiss), while p63 4A4 fluorescence signals were acquired with Imager A1 Fluorescence Microscope (Zeiss). Image analysis was performed with AxioVision software v.4.8. The most reliable method to quantify p63 is by Quantitative Fluorescent Immunohistochemistry (Q-FIHC) based on the use of fluorescence microscope (Zeiss) and an advanced image analysis software (Axio Vision) [57]. Compared with others quantification techniques, Q-FIHC allows fluorescence signals measurement from a lower number of cells. After images acquisition and analysis, a two columned matrix can be obtained containing cells size (diameter) and the corresponding signal intensity. These data were then processed to characterize airway epithelial cells distribution according to their size and p63 intensity levels.

Migration and wound healing assays

Six double-chamber cell culture inserts (from Ibidi) were placed into a 6-well plate and a feeder layer of 3T3 cells was plated into three of them. Human tracheal epithelial cells were seeded into both chambers of the six inserts and cultured with BEGM or KC medium until confluence condition was reached. Cell culture inserts were then removed using sterile tweezers and airway epithelial cells migration was recorded by Cell Observer Z.1 Microscope (Zeiss) until gaps closure. Human airway epithelial cells grown in BEGM and KC media were also compared for their wound healing capabilities. Briefly, human tracheal epithelial cells were plated into two rectangular cell culture plates (from Cell Comb Scratch Assay-Millipore) one of them containing a feeder layer of 3T3 cells.

Airway epithelial cells directly seeded on plastic were cultured with BEGM whereas those plated on 3T3 feeder-layer were cultivated with KC medium. When confluence condition was reached, cell culture media were removed and Cell Combs (from Cell Comb Scratch Assay-Millipore) used to create scratches in two directions across the monolayer. The scratched monolayers were then washed and incubated with appropriate culture medium inside the incubator of Cell Observer Z.1 Microscope (Zeiss) to observe airway epithelial cells damaging reaction.

Tracheal clones clonogenic potential analysis

Each of 19 human tracheal clones obtained from single cells analysis were divided into two cell culture plates. One-quarter of the clone was seeded into an indicator dish for clone's classification while the three-quarters of the clone were in turn divided into two wells of a 24-well plate and cultured with BEGM or KC medium (the latter in presence of feeder-layer). When clones cultures reached confluence, BEGM was replaced with B-ALI differentiation medium whereas clones on feeder layer continued to be cultivated with KC medium. Both cell culture media were changed every other day until 30^{th} day of culture. Therefore, each clone was detached using trypsin digestion and re-plated (5.000-10.000 cells) into an indicator dish to perform colony forming efficiency (CFE) assay. The remaining cells were used for proteins extraction.

Tracheal clones composition analysis

Each of 22 human tracheal clones at 7^{th} day of culture were divided into two cell culture plate. One-quarter of the clone was seeded into an indicator dish to define clonal type while the three-quarters of the clone were in turn divided into two chamber-slide and fixed with 3% paraformaldehyde (10 min at RT) or cold methanol (10min at -20°C) 3h after plating.
Basal cells, goblet cells and Clara cells staining was perfomed incubating primary antibodies against CK14, MUC5AC and Uteroglobin for 1h at 37°C and the corresponding fluorescent secondary antibody (Alexa-Fluor 488 and 568) for 1h at RT. The nuclei were stained with DAPI for 5 min and the samples mounted with Dako mounting medium. Images were acquired with Cell Observer Z.1 Microscope (Zeiss) and images analysis was performed with AxioVision software v.4.8.

RESULTS

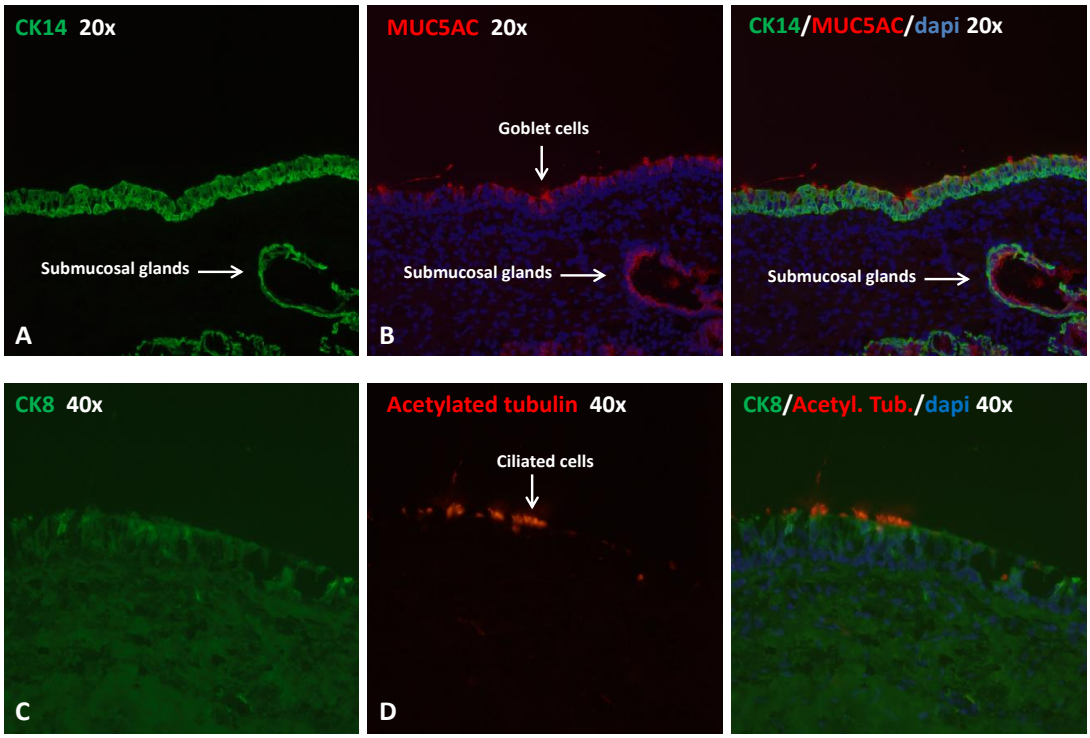
PORCINE AIRWAY EPITHELIAL CELLS CHARACTERIZATION

Porcine airway biopsies were processed for evaluation of stem cell maintenance, expression of differentiation markers and potential for long term proliferation. To achieve these objectives, different experiments were performed:

- Comparative analysis of markers in pig airway whole mount sections.
- Development of primary cultures from pig tracheal and bronchial biopsies.
- Life span of porcine tracheal and bronchial epithelial cell cultures and characterization of markers expression during in-vitro serial passages.
- Evaluation of epithelial cells growth and differentiation in air-lift interface (ALI) condition.
- Trans Epithelial Electric Resistance (TEER) measurement in porcine tracheal and bronchial regenerated epithelia for evaluation of airway epithelial cells ability to recreate a functional and differentiated respiratory epithelium.

Airway epithelial cells markers localization in in-vivo porcine airway sections.

Sections from porcine tracheal tissue were stained with primary antibodies against airway epithelial cells proliferation and differentiation markers to reveal their in-vivo localization and way of expression as well as porcine tracheal tissue structure (Fig.16).



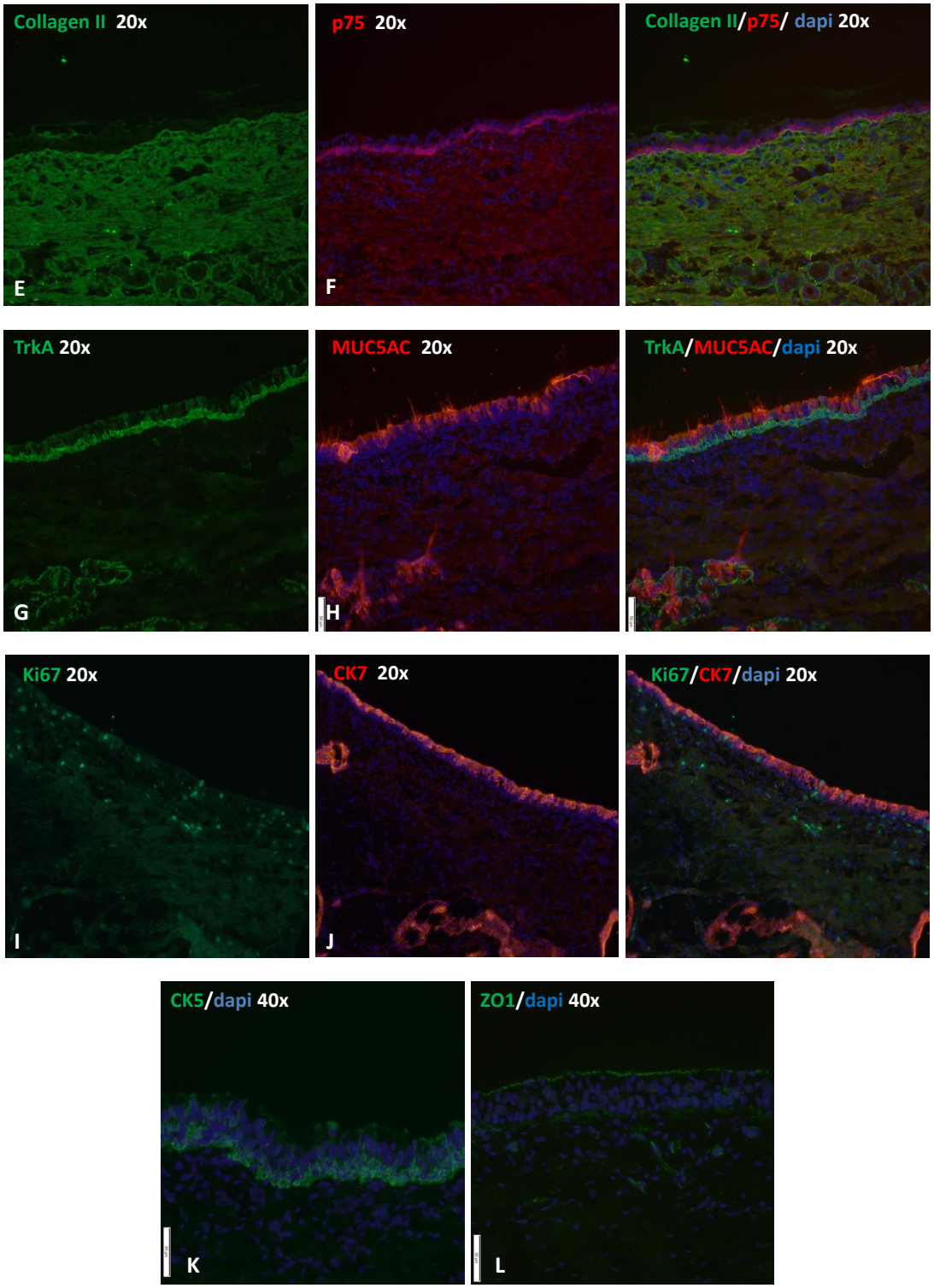


Fig. 16 Characterization of airway epithelial cells in in-vivo sections of tracheal porcine tissue. Airway differentiated goblet cells (B, H) and ciliated cells (D) were identified, as well as basal cells markers (F, G, K) and sopra-basal epithelial cell markers (J, L). Sub-mucosal glands (A, B) containing goblet cells are indicated by arrows.

Immunofluorescence staining of porcine tracheal sections revealed basal cells selectively labeled by Cytokeratin 5 (CK5) and p75/TrkA receptors (Fig.16 F, G, K). Epithelial differentiation markers included CK7 for sopra-basal epithelial cells and ZO1 preferentially localized at the apical tight junctions of respiratory epithelium (Fig.16 J, L).

Finally, goblet cells and ciliated were identified by the expression of MUC5AC and acetylated tubulin respectively (Fig.16 B, D). Sub-mucosal glands were also observed in tracheal sections and characterized by the presence of numerous mucus producing cells (Fig.16 B, H).

Comparative analysis of some tracheal markers was also performed in pig nasal whole mount sections, revealing a skin-like epithelial organization. Cytokeratins 5 and 14 expression appeared in all epithelial layers of nasal sections (Fig. 17 A, B), ZO1 showed an intercellular localization (Fig. 17C), whereas basal cells were characterized by the expression of the proliferation marker ki67 (Fig. 17 D) and NGF receptors p75 and TrkA (Fig. 17 E,F).

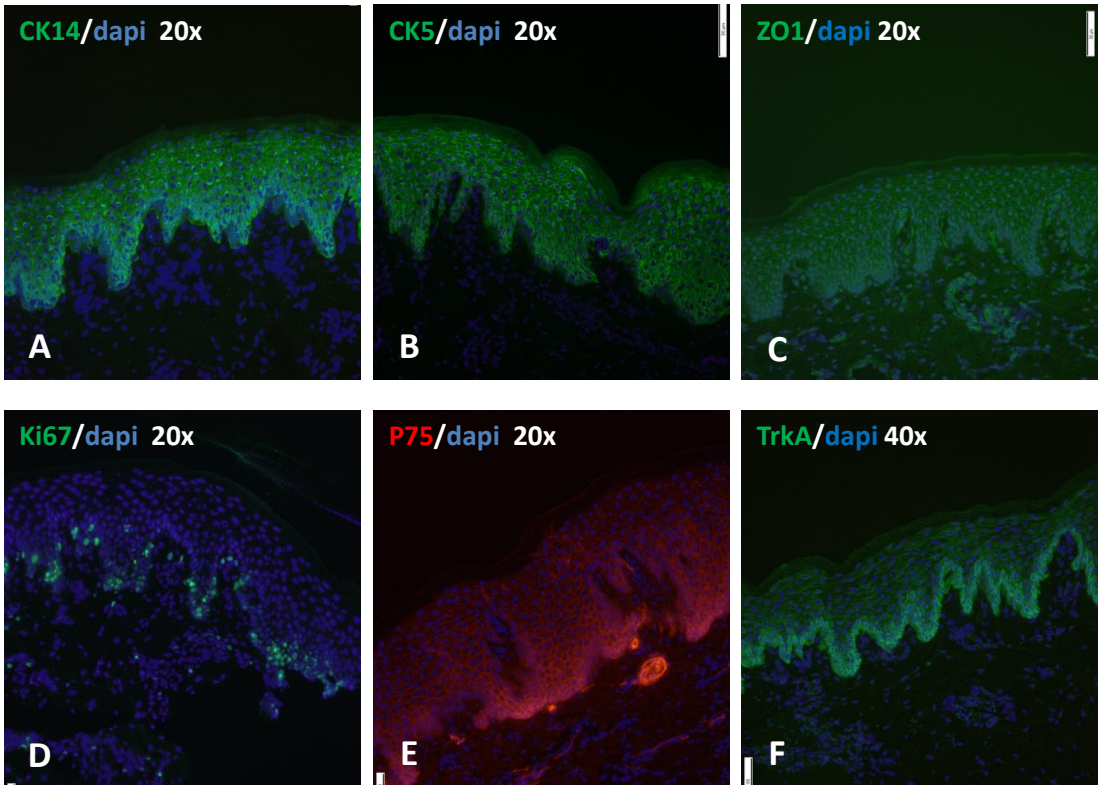


Fig. 17 Immunofluorescence analysis of airway epithelial cells in in-vivo sections of porcine nasal tissue. Porcine nasal tissue showed a skin-like structure with a stratified epithelium rather than a pseudo-stratified respiratory epithelium. Basal layer contained proliferating ki67 positive cells and was characterized by p75 and TrkA receptors expression.

Porcine tracheal and bronchial epithelial cell cultures.

Tracheal and bronchial epithelial cells were isolated from porcine biopsies through trypsin digestion and plated into plastic cell culture plates with BEGM culture medium. Serial passages were performed to analyze proliferative potential of both porcine airway epithelial cells.

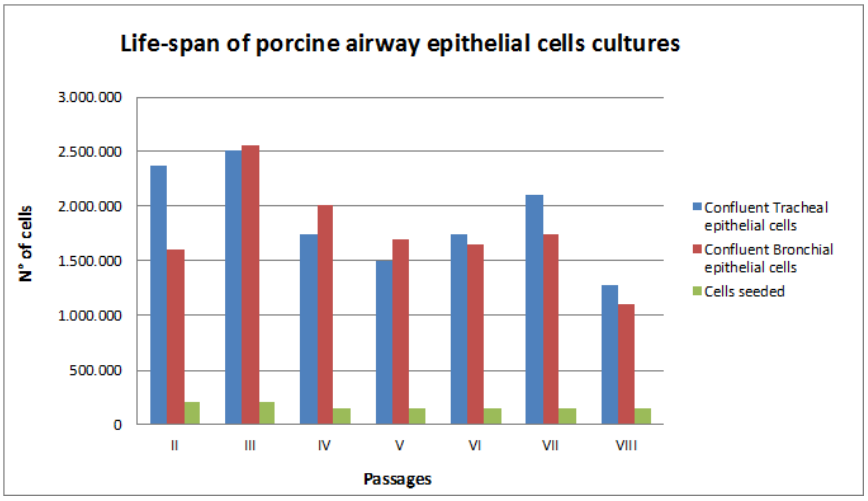


Fig. 18 Life-span of porcine airway epithelial cells. Respiratory epithelial cells were serially cultivated in BEGM defined medium and the number of tracheal epithelial cells at the end of each passage was compared to that of bronchial cultures.

Tracheal and bronchial porcine epithelial cells were sub-cultured for at least eight passages and comparative life span analysis showed no significant differences between bronchial and tracheal proliferative potential (Fig. 18, 19).

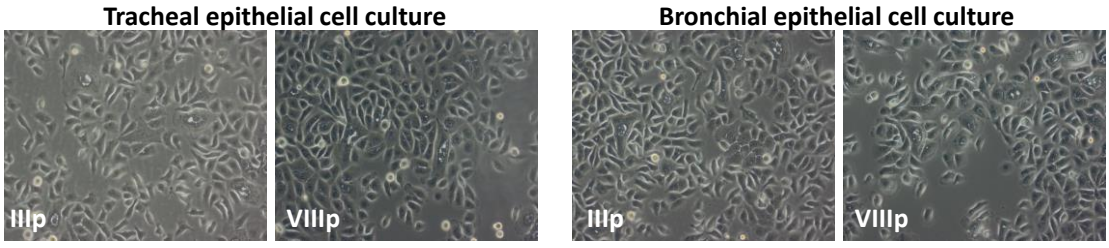


Fig. 19 Porcine respiratory epithelial cells cultures pictures at different passages. Tracheal and bronchial epithelial cells showed no morphological or proliferative differences during serial passages.

Tracheal and bronchial porcine epithelial cells from each passage were also cultured on chamber-slide and fixed at sub-confluence condition.

Comparative immunofluorescence analysis of markers expression, confirmed no significantly differences between the two type of airway epithelial cells even at the last cell culture passage (Fig. 20).

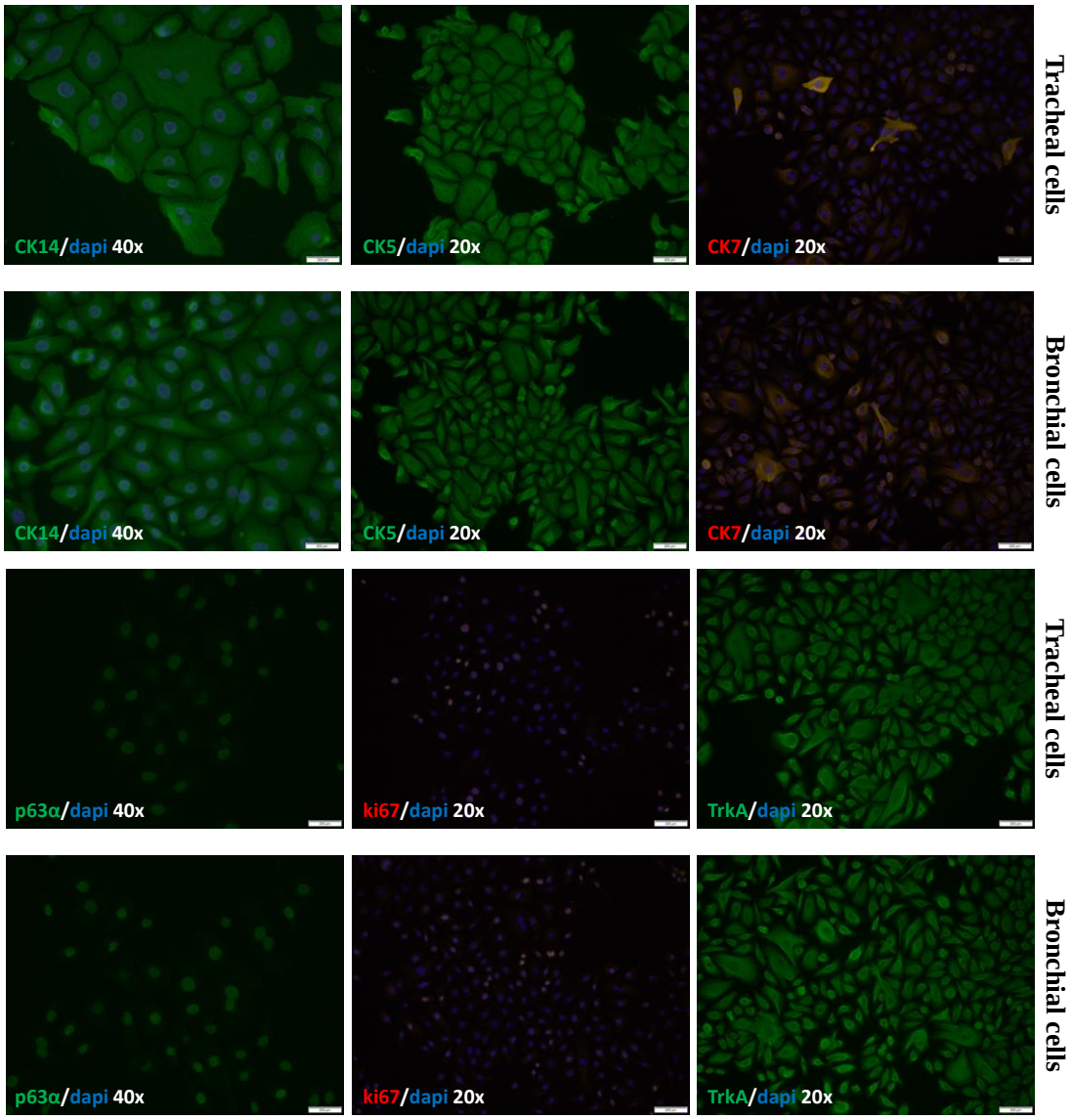


Fig. 20 Markers expression comparison at the end (IXp) of tracheal and bronchial epithelial cells cultures. Basal cells markers (CK14, CK5, TrkA), proliferative (p63α, ki67) and epithelial differentiation markers (CK7) were analyzed.

Porcine airway epithelial cells were then cultured for 30 days in submerged condition to analyze markers modulation during epithelial cell replicative senescence.

As shown in Fig. 21, at 30th day of culture there were a reduced expression of proliferation markers (such as p63α and ki67), an increased number of airway differentiated cells (identified by MUC5AC, acetylated tubulin and CK7 markers), whereas CK14 and TrkA were differentially expressed among the confluent respiratory epithelial cells. The tight junctions marker ZO1 appeared to be de-localized after 30 days in submerged culture condition, suggesting that the air-contact is required to allow airway epithelial cells cultured in BEGM to organize in a well polarized respiratory epithelium.

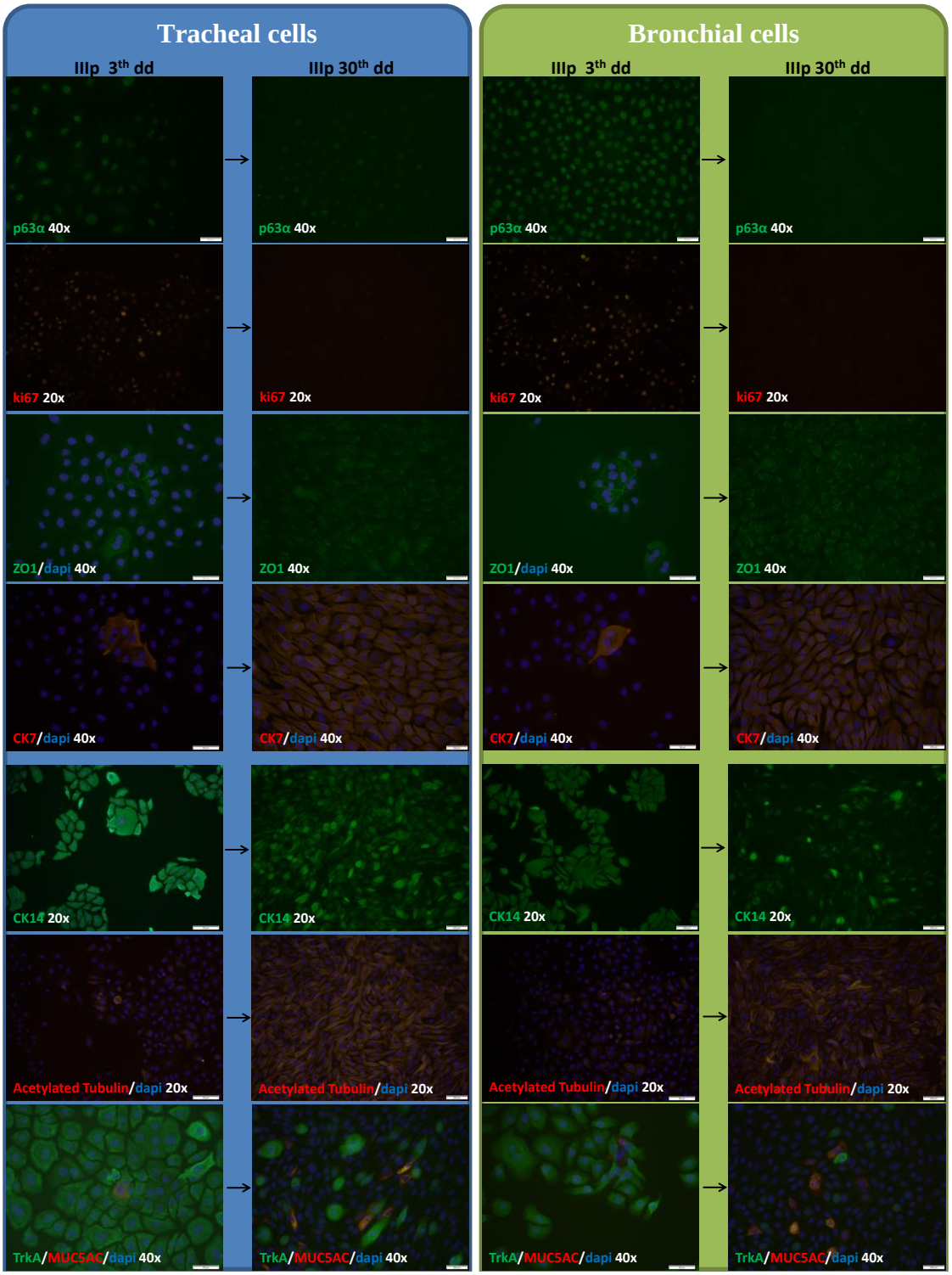


Fig. 21 Tracheal and bronchial epithelial cells comparison between 3th and 30th day of submerged culture. Both tracheal and bronchial porcine epithelial cells showed a reduction of proliferation markers (p63, ki67) expression after 30 days of submerged culture and an increase of airway epithelial cells differentiation markers (MUC5AC for goblet cells and acetylated tubulin for ciliated cells).

Tracheal and bronchial porcine epithelial cells growth and differentiation in air-lift interface (ALI) condition.

The differentiation potential of airway epithelial cells was evaluated by cultivating tracheal and bronchial epithelial cells in air-lift condition for about 30 days. These cells were seeded onto collagen IV coated polycarbonate membrane (Millicell Cell Culture Inserts, from Millipore) and received B-ALI differentiation culture medium only from the basal compartment, leaving the apical region of respiratory epithelial cells exposed to the air. The resulting epithelia were characterized by evaluation of differentiation markers expression and measurement of Trans-Epithelial Electrical Resistance (TEER). The increased resistance observed during air-lift cultures, suggested that porcine tracheal and bronchial epithelial cells had generated a complete differentiated respiratory epithelium (Fig. 22).

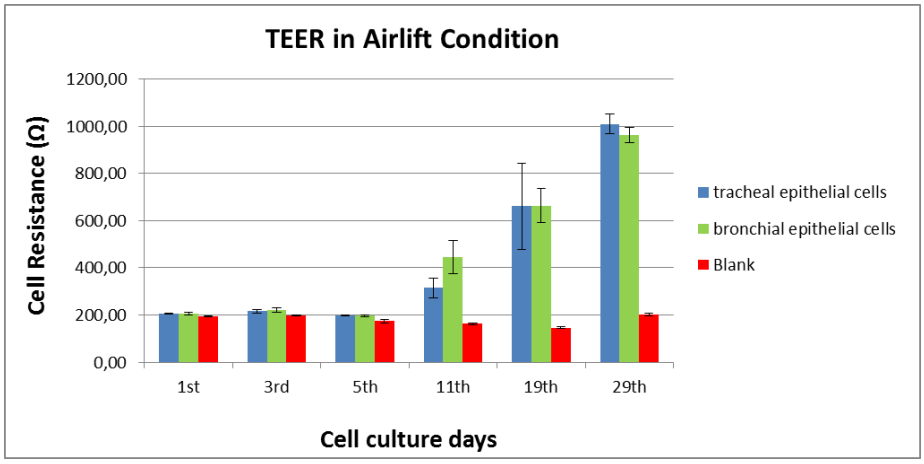
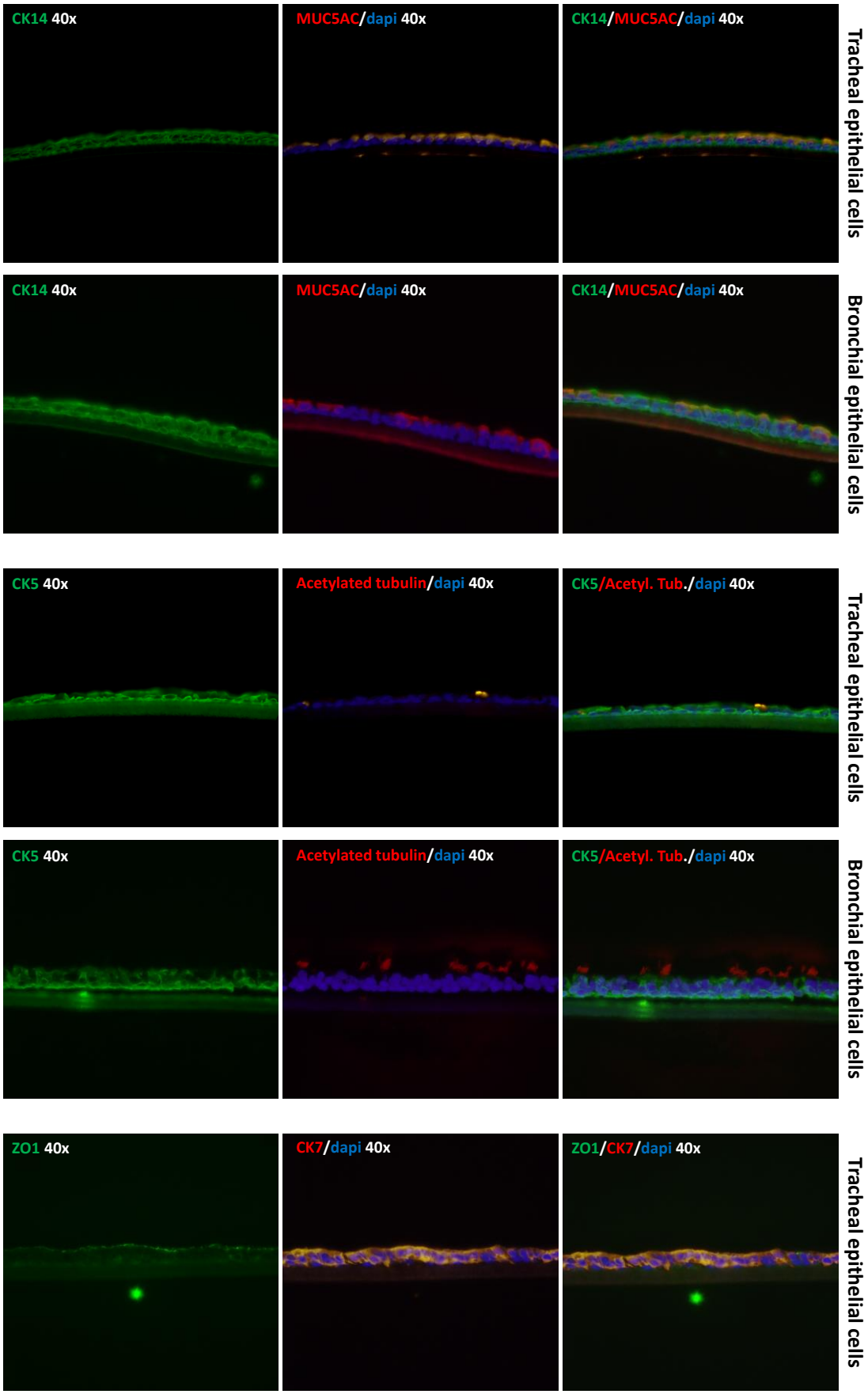


Fig. 22 Trans-epithelial electrical resistance (TEER) measurement of tracheal and bronchial epithelia during 30 days of air-lift cultures. During airway epithelial cells air-lift cultures, trans-epithelial electric resistance was measured in triplicate by an electrode and similar increasing values were revealed for both tracheal and bronchial epithelium.

At the end of the air-lift cultures, tracheal and bronchial epithelia were embedded in OCT compound and the subsequent sections were analyzed by immunofluorescence staining. As shown in Fig. 23, both tracheal and bronchial epithelial cells were able to generate a fully differentiated respiratory epithelium characterized by the expression of epithelial cells specific markers (CK5, CK14 and CK7) and by the presence of mature airway epithelial cells such as goblet cells (MUC5AC) and ciliated cells (acetylated tubulin). The airway epithelial cells ability to recreate a functional and differentiated epithelium was confirmed by the expression of the tight-junctions marker ZO1 at the apical region of epithelial cells and the increase of tracheal and bronchial TEERs in air-lift condition.


CK14 40x
MUC5AC/dapi 40x
CK14/MUC5AC/dapi 40x
Tracheal epithelial cells
CK14 40x
MUC5AC/dapi 40x
CK14/MUC5AC/dapi 40x
Bronchial epithelial cells
CK5 40x
Acetylated tubulin/dapi 40x
CK5/Acetyl. Tub./dapi 40x
Tracheal epithelial cells
CK5 40x
Acetylated tubulin/dapi 40x
CK5/Acetyl. Tub./dapi 40x
Bronchial epithelial cells
ZO1 40x
CK7/dapi 40x
ZO1/CK7/dapi 40x
Tracheal epithelial cells

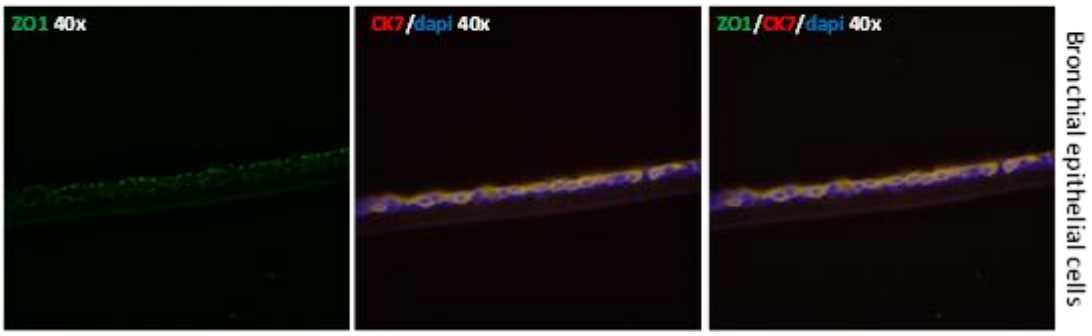


Fig. 23 Tracheal and bronchial porcine epithelia differentiated in air-lift interface culture condition. Immunofluorescence analysis showed that both tracheal and bronchial epithelial cells generated goblet cells (MUC5AC), ciliated cells (acetylated tubulin) and that respiratory epithelia were tightly sealed by apical tight junctions (ZO1).

The preclinical studies performed on porcine samples showed that airway epithelial cells can be easily isolated from respiratory tissues, cultured in vitro for several passages maintaining their differentiation potential and the trans epithelial electrical resistance, and used to obtain a fully differentiated epithelium when cultivated in air-lift condition. Moreover, tracheal and bronchial epithelial cells showed neither proliferative or differentiation potential differences, suggesting that both respiratory regions may contain a similar progenitor cells population and may be indifferently used for further analysis. This observation lead to investigation of human airway behavior, since the described tracheo-bronchial similarity can allow to use both samples for bioengineered human airway generation.

PRELIMINARY STUDIES ON NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS (NHBE)

Two culture systems, described in Fig. 24, were selected for preliminary tests on commercially available Normal Human Bronchial Epithelial cells (NHBE, Lonza) to evaluate stem cell maintenance and proliferative/differentiation markers expression.

Kc culture medium	BEGM/B-ALI
Presence of feeder-layer	Absence of feeder-layer
High calcium concentration	Defined medium
Fetal bovine serum (10%)	Bovine pituitary extract
Presence of hormones and growth factors	Presence of hormones and growth factors
Epithelial clinical grade culture conditions	Research culture conditions

Fig. 24 Comparison between two culture conditions selected for human airway epithelia cells cultures.

NHBE cells were cultured for several passages in both culture conditions (KC/BEGM), revealing similar clonogenic and proliferative potential (Fig. 25 A, B) for up to four passages, but a different epithelial cells organization (Fig. 25 C, D). In KC culture condition, airway epithelial cells showed a 3D tissue-like behavior, whereas in absence of feeder-layer and fetal bovine serum, these cells seemed to be a 2D culture, unable to produce a proper tissue architecture.

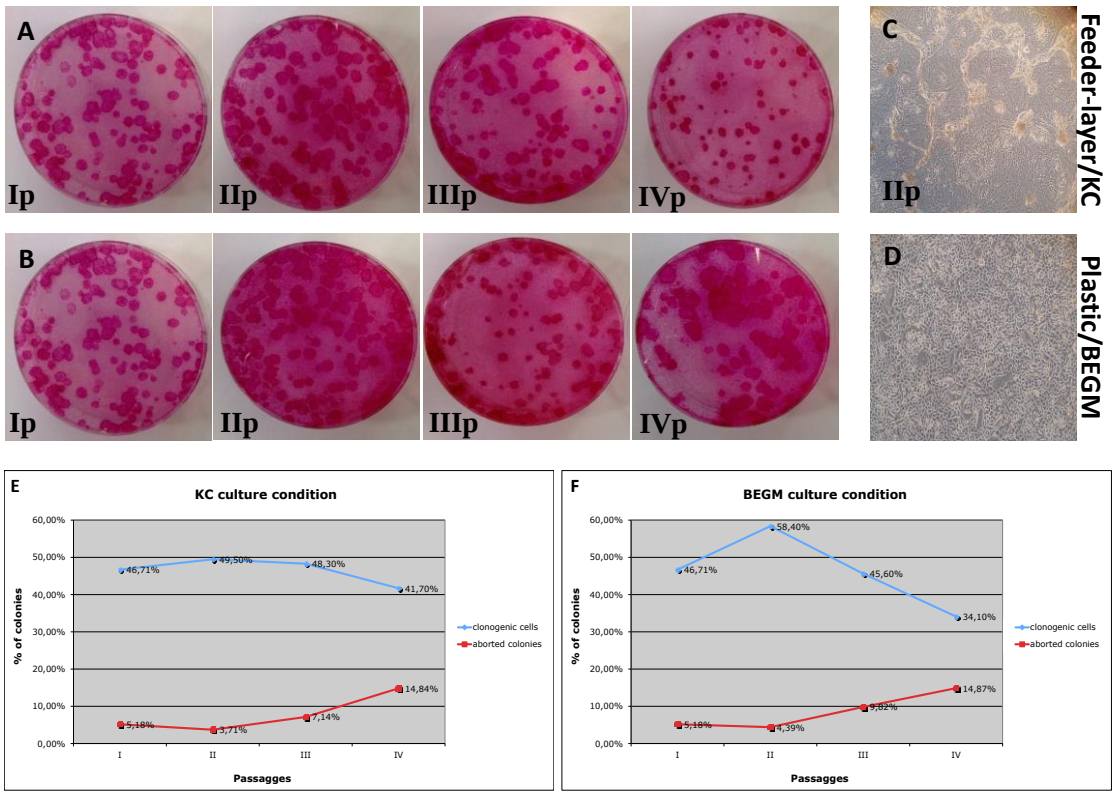


Fig. 25 Serial passages of NHBE cells in KC and BEGM culture media. Colony forming efficiency assay (A, B) showed similar clonogenic potential of NHBE cells grown in KC or BEGM culture medium, however cell cultures pictures (C, D) revealed a different cell-cell interaction among the two culture systems. Line graphs (E, F) represent clonogenic cells and aborted colonies during serial passages.

Immunofluorescence analysis of Normal Human Bronchial Epithelial cells cultures.

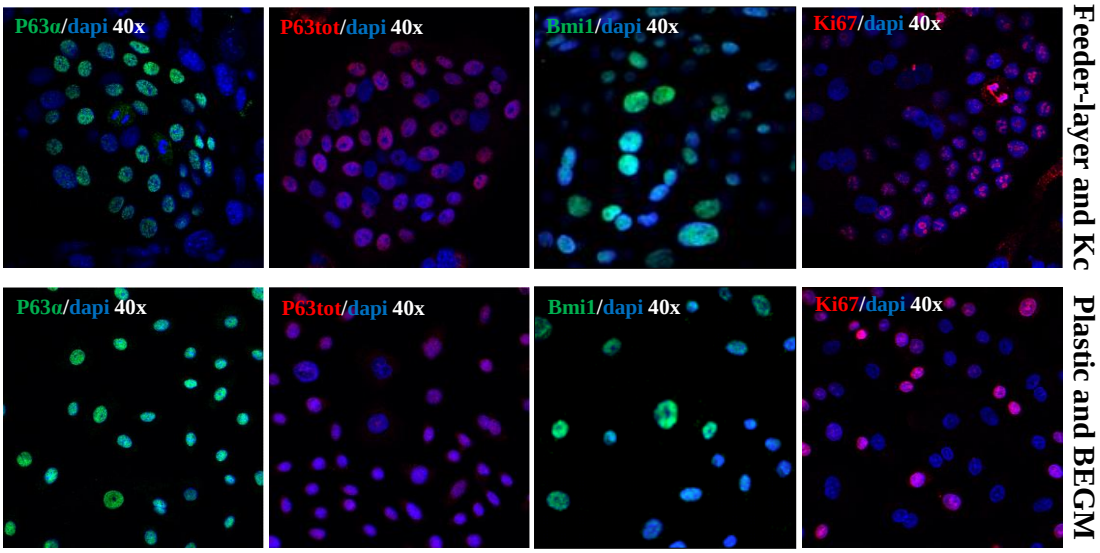
NHBE cells were cultivated onto glass slide coverslip and used for immuno-fluorescence staining to analyze the expression of different markers including:

- Proliferation and epithelial stem/progenitor cell markers (p63 4A4, p63α, Bmi1, Ki67);

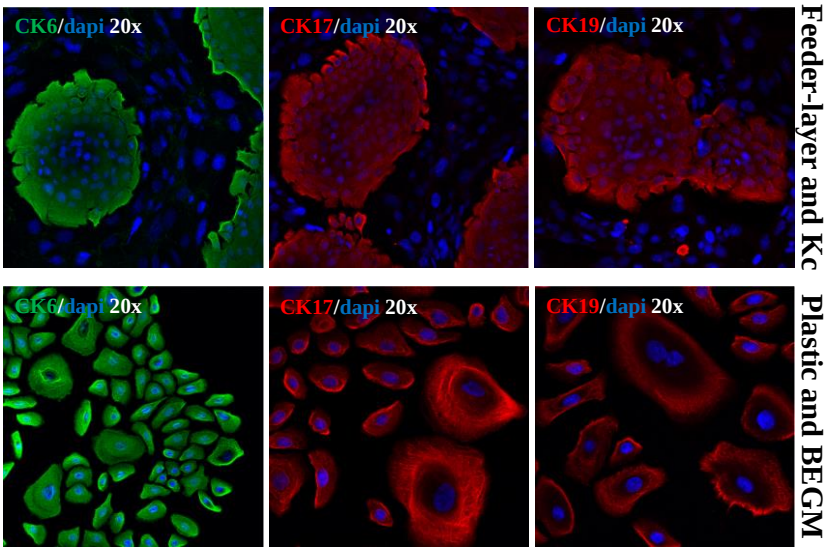
- Intermediate filaments (CK6, CK17, CK19);
- Epithelial cells differentiation markers (CK4, Involucrin, ZO1);
- Airway epithelial cells markers (AQ3, CK7, CK18, MUC5AC).

As shown in Fig.26 A, human bronchial epithelial cells cultured in both culture systems expressed the stem/progenitor cells markers p63/Bmi1 and the proliferation marker ki67. The analysis of some airway epithelial cells specific markers (CK7, CK18, AQ3) and others intermediate filaments (CK6, CK17, CK19), showed similar expression levels among the two culture conditions. However in presence of feeder-layer and KC culture medium, bronchial epithelial cells were able to better interact each others generating well organized colonies (Fig. 26 B, C).

A. Proliferation and epithelial stem cells markers



B. Intermediate filaments



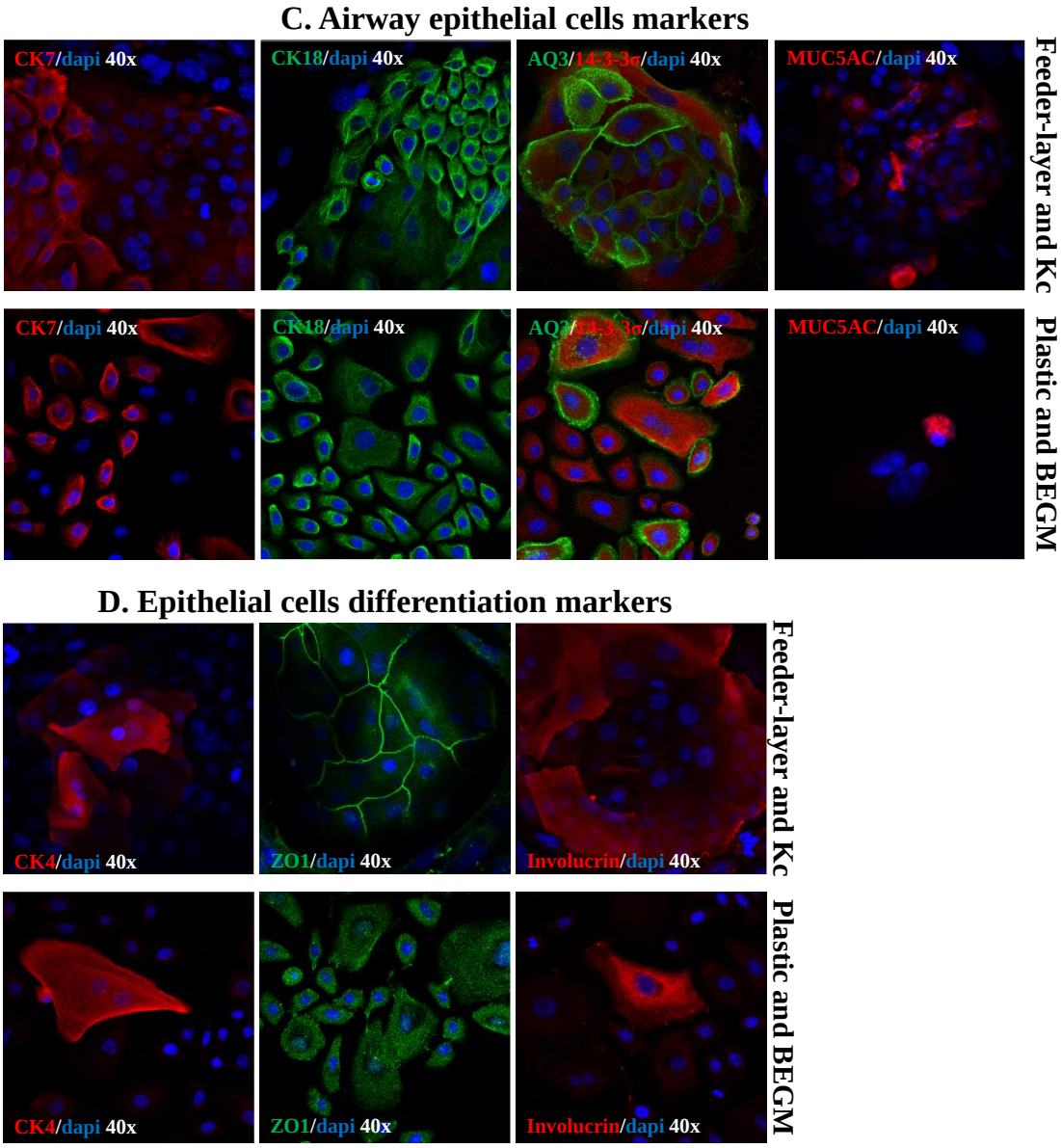


Fig. 26 Selected culture conditions comparison. Culture systems effects on cultured NHBE cells were evaluated by analyzing the expression of proliferation/stemness markers (A), intermediate filaments (B,C), airway epithelial specific markers (C) and epithelial cells differentiation markers (D).

The main differences between the two culture systems concerned with differentiation markers expression. Some epithelial cells differentiation markers (such as CK4, ZO1, Involucrin) and airway epithelial cells specific markers (AQ3, MUC5AC) were differentially expressed among the two culture conditions, suggesting a capability to correctly differentiate and form tight junctions in presence of feeder-layer and KC culture medium (Fig. 26 C, D).

Only when seeded on polycarbonate membrane (Millicell cell culture inserts, from Millipore) and cultured for 30 days in air-lift interface condition with B-ALI culture medium, NHBE cells revealed their ability to generate a fully mature respiratory epithelium correctly polarized, containing goblet and ciliated cells (Fig. 27).

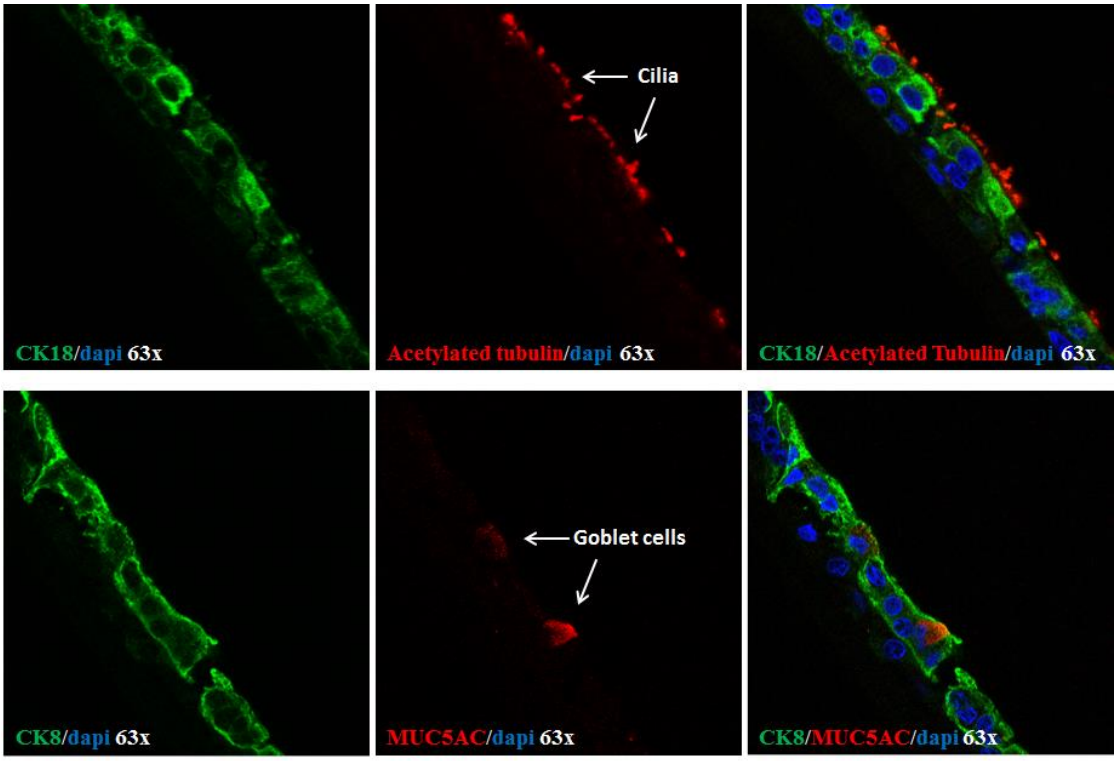


Fig. 27 Normal human bronchial epithelial cells differentiation in air-lift culture condition. After 30 days of air-lift culture, NHBE generated a differentiated respiratory epithelium containing goblet cells and ciliated cells (as indicated by arrows).

CHARACTERIZATION OF HUMAN TRACHEAL EPITHELIAL CELLS

Isolation of airway epithelial cells from human tracheal biopsy and culture conditions selection.

The comparison between the two culture conditions was performed also on human primary tracheal epithelial cells to confirm the differences observed in the previous studies on NHBE cells.

Tracheal biopsies were taken from death donor, were cut in small pieces and processed with two different enzymatic treatments to isolate airway epithelial cells and culture them in the two culture systems previously described.

Airway epithelial cells extractions by different enzymes were compared. The use of trypsin enzyme was more efficient for isolation of an epithelial cells population with higher clonogenic potential (as shown by the indicator dishes of Colony Forming Efficiency assay) and it reduced the fibroblast contamination in comparison with dispase digestion (Fig. 28). Indeed, clonogenic cells percentage after trypsin treatment was 11,2% compared to 1% of growing colonies obtained by dispase digestion. Moreover, primary culture resulting from trypsin tracheal epithelial cells extraction showed no human fibroblasts contamination.

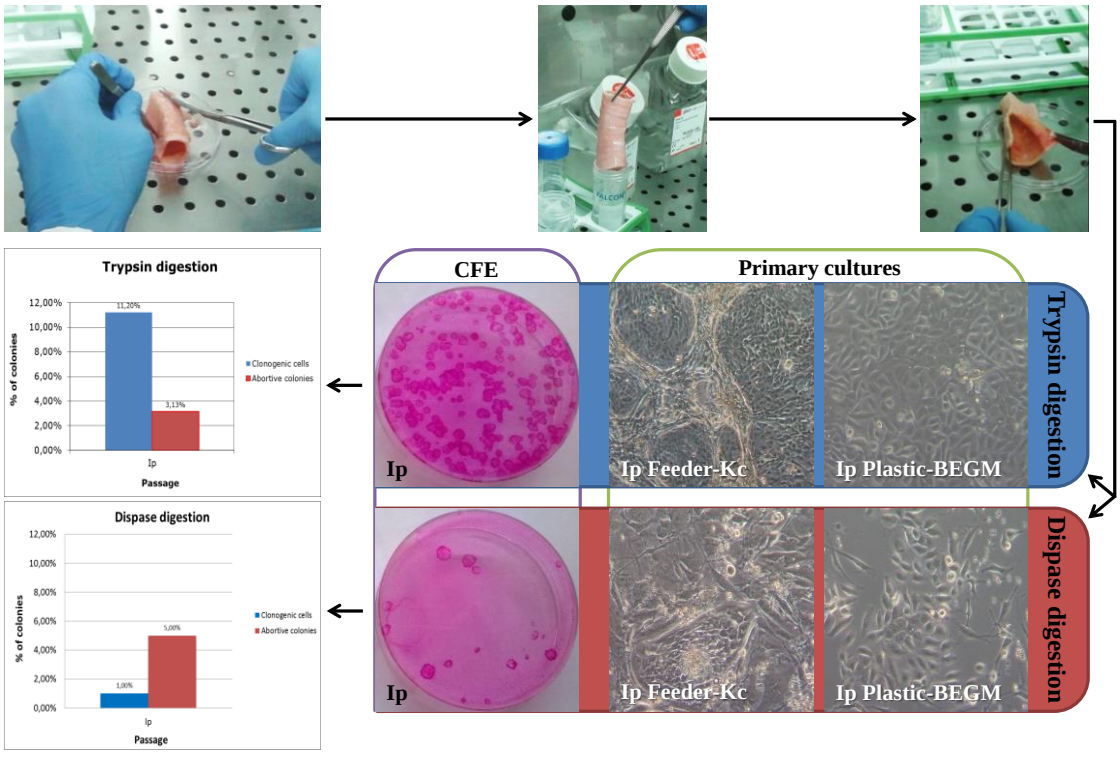


Fig. 28 Isolation of human tracheal epithelial cells and colony forming efficiency assay. A human trachea was washed several time in PBS 1X, cleaned from connective tissue and finally cut in small pieces. Tracheal epithelia cells were extracted with trypsin or dispase digestions and cultivated with KC or BEGM culture medium. Colony forming efficiency assay showed an higher percentage of clonogenic cells obtained with trypsin treatment rather than dispase digestion.

Tracheal epithelial cells cultured with KC or BEGM culture medium, were sub-cultivated for six passages to compare airway epithelial cells proliferative and clonogenic potential in the two culture conditions. The percentages of aborted colonies were similar in both culture systems, however clonogenic cells number decreased faster in absence of feeder-layer/KC than in presence of BEGM defined medium (Fig. 29).

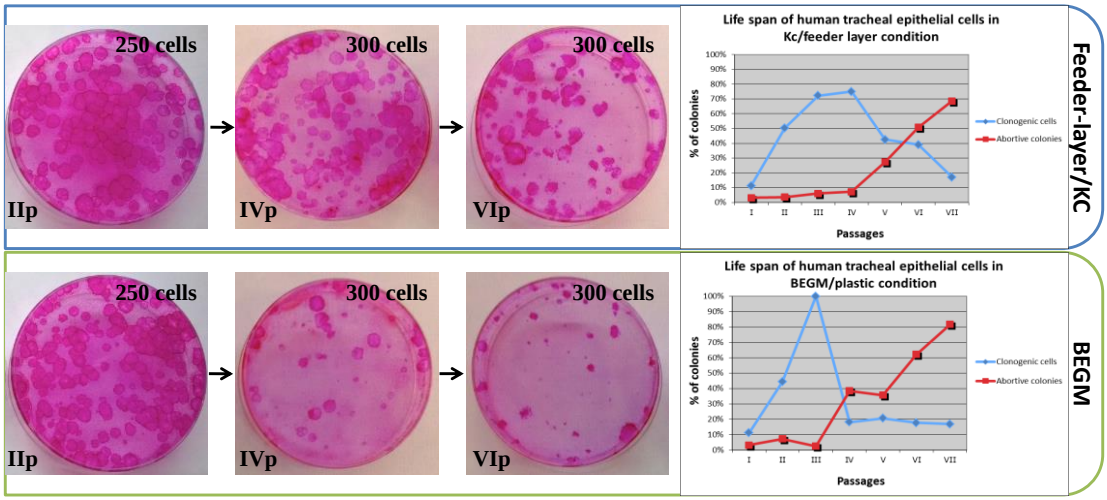


Fig. 29 Serial passages of tracheal epithelial cells in two selected culture conditions. Airway epithelial cells cultivated in presence of BEGM showed a drop of clonogenic cells starting from fourth passage.

Moreover, tracheal epithelial cells grown in presence of feeder-layer and KC growth medium showed a lower doubling time (29h) and an higher cell doubling value (30,9) than airway epithelial cells cultivated in BEGM (48,3h and 20,7 cell doubling respectively), suggesting a greater growth rate in the first culture condition (Fig. 30).

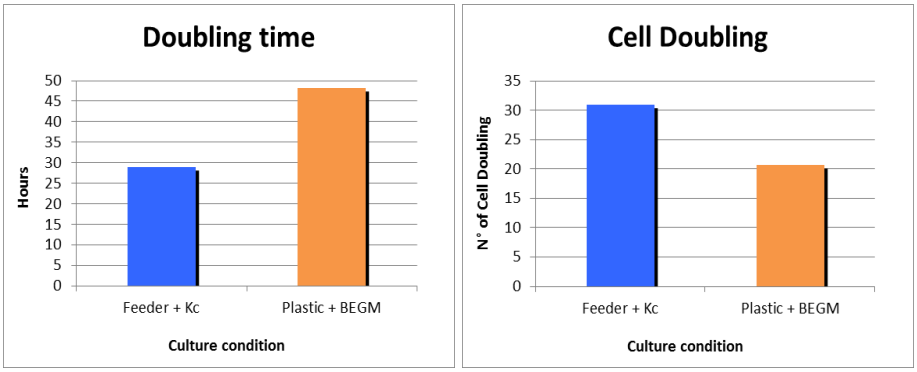


Fig. 30 Doubling time and cell doublings of human tracheal epithelial cells grown in KC or BEGM culture condition. In the first culture system, airway epithelial cells proliferated more and faster than in presence of defined BEGM.

Migration and wound-healing assays

Culture conditions effects on human tracheal epithelial cells migration and proliferation capabilities were further investigated performing a migration assay and a wound healing assay.

As shown in Fig. 31, migration assay required tracheal epithelial cells to be plated into dual-chamber silicon inserts and grown until reaching confluence condition.

Thereafter, cell culture inserts were removed and airway epithelia cells allowed to close the resulting gaps.

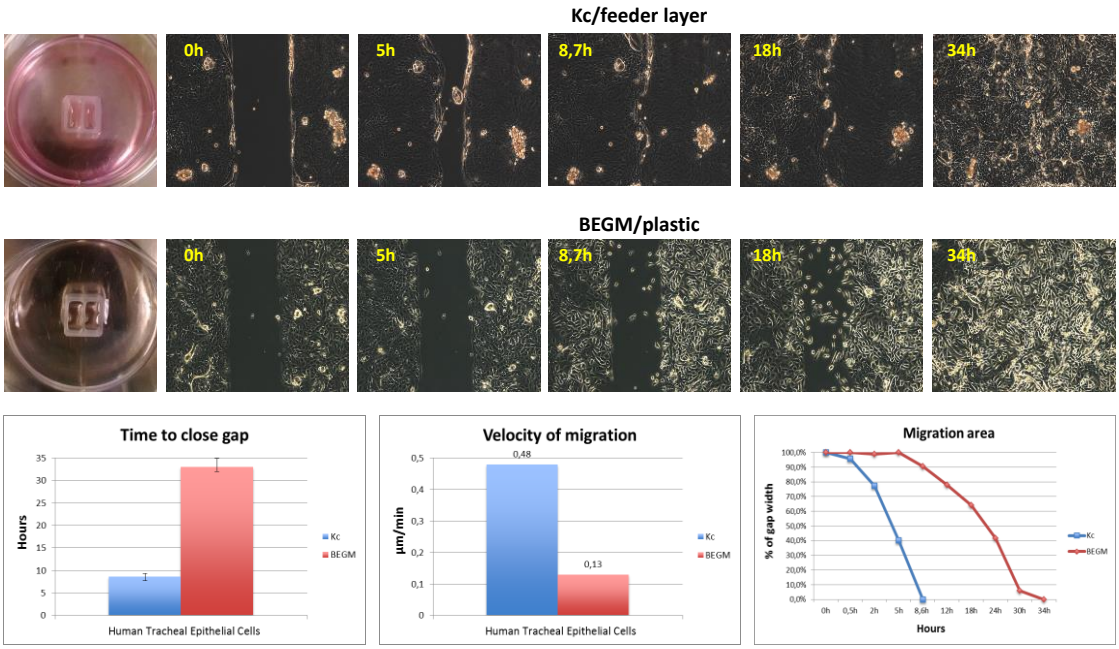


Fig. 31 Migration assay. Analyzing the time needed to close a gap of 500µm, airway epithelial cells grown with feeder-layer/KC were four times faster than BEGM cultured cells, closing the gap in 8,7h rather than in 34h.

Tracheal epithelial cells were able to close the gap in about 9h when cultivated in presence of feeder-layer and KC whereas they required more than 30h in BEGM culture condition. This assay confirmed airway epithelial cells higher migration capability in the first culture system, revealing a migration velocity of 0,48 µm/min compared to 0,13µm/min of respiratory epithelial cells grown with BEGM defined medium.

A scratch assay was then performed to evaluate airway epithelial cells wound reaction. Tracheal epithelial cells cultured on feeder-layer and with KC culture medium were able to repair the damage in about 14h, whereas those cells cultivated with BEGM did not close the gap even 90h after the scratch (Fig. 32).

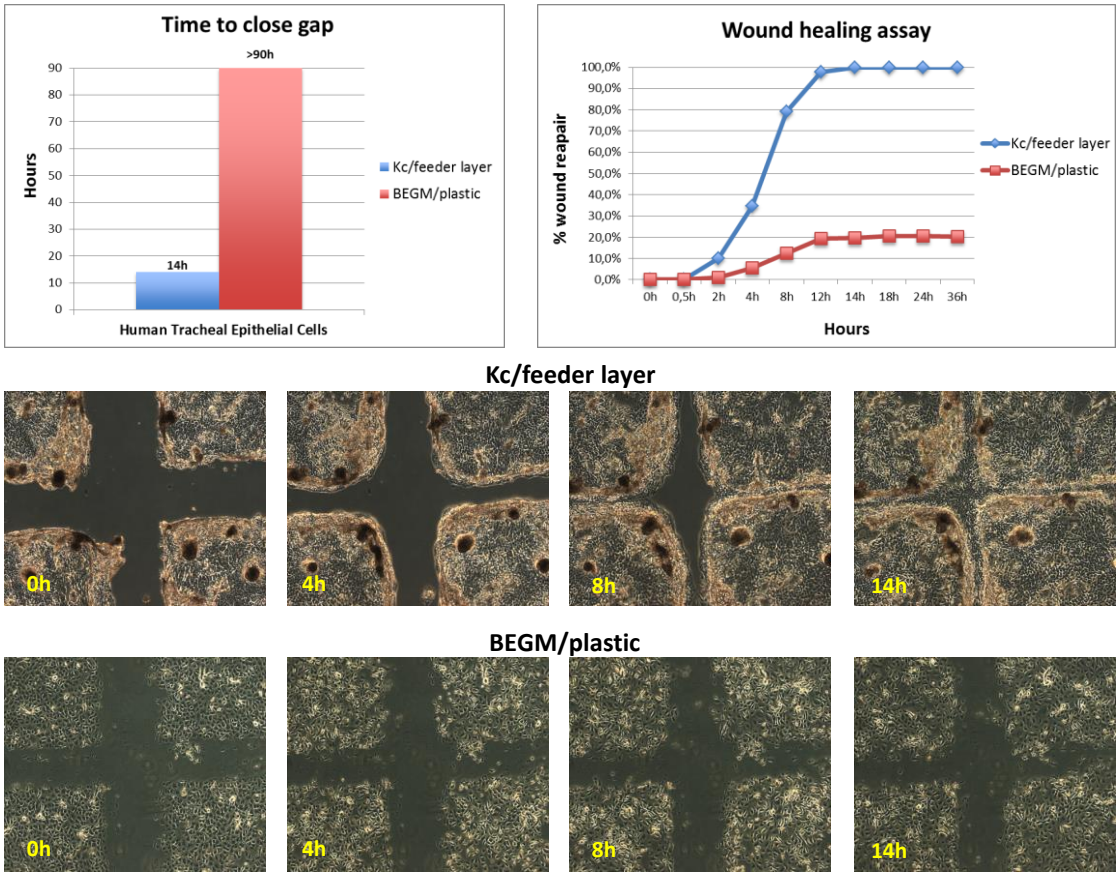


Fig. 32 Wound-healing assay. Tracheal epithelial cells were cultured with feeder-layer/KC or BEGM until reaching confluent condition. Therefore, a double scratch was performed and the time required to repair damaged epithelia was measured. Airway epithelia cells grown in BEGM were not able to close the gap, suggesting their poor capability to properly repair an induced injury.

The wound-healing experiment revealed that airway epithelia cells cultivated in presence of feeder layer and with KC growth medium were able to correctly interact each other and quickly repair the damaged monolayer.

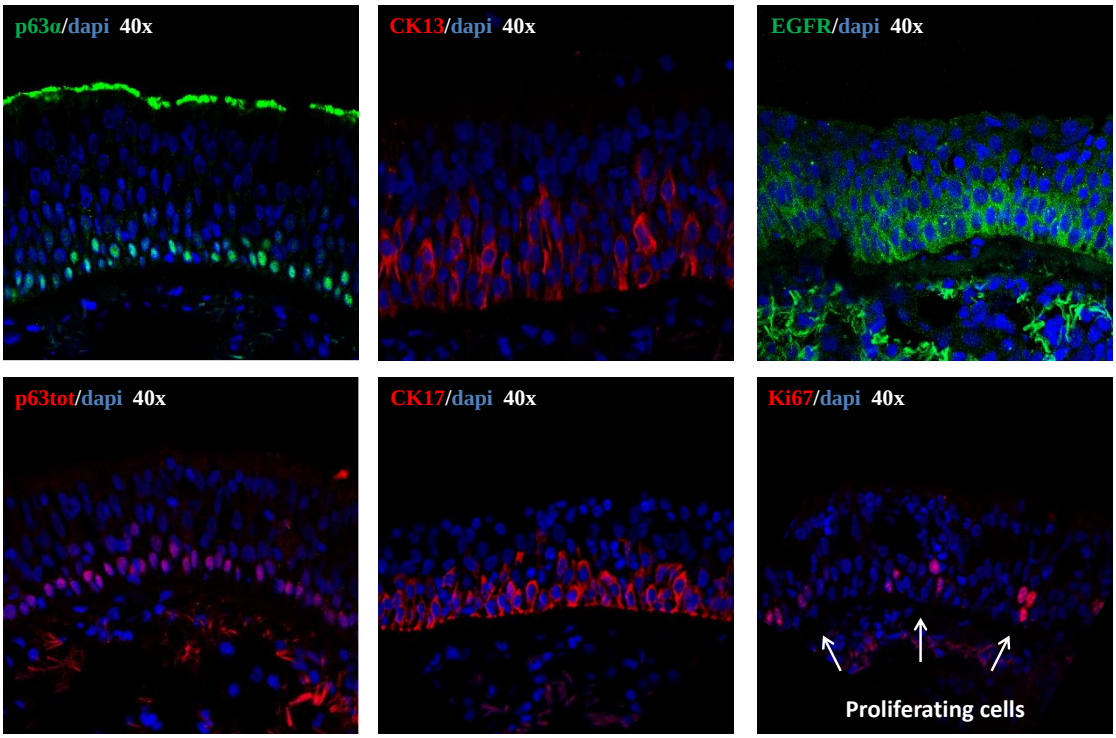
These migration/repairing capabilities are very important when considering scaffold colonization process required to repopulate the natural/synthetic matrix used to generate a bioengineered human airway for clinical transplantation.
Therefore, the culture condition based on feeder-layer/KC appeared to be the most suitable method to cultivate airway epithelial cells and it was then selected for further subsequent analyses.

Airway epithelial cells markers expression in in-vivo human tracheal tissue.

The localization and the level of expression of some important epithelial cells markers and airway epithelial differentiation markers were investigated in in-vivo sections of human tracheal sample.

As shown in Fig.33, the epithelial stem/progenitor cells marker p63 is almost exclusively expressed in tracheal basal layer, characterized also by the presence of few proliferating ki67 positive cells, confirming proximal airway epithelium as a slow turn-over tissue.

Tracheal basal cells were also identified by the expression of some specific cytokeratins such as CK13, CK17 and CK14, and by the trans-membrane receptors EGFR, TrkA and p75. Moreover, as expected basal layer showed positive immunoreaction for hemi-desmosome components such as CD104 and CD151.



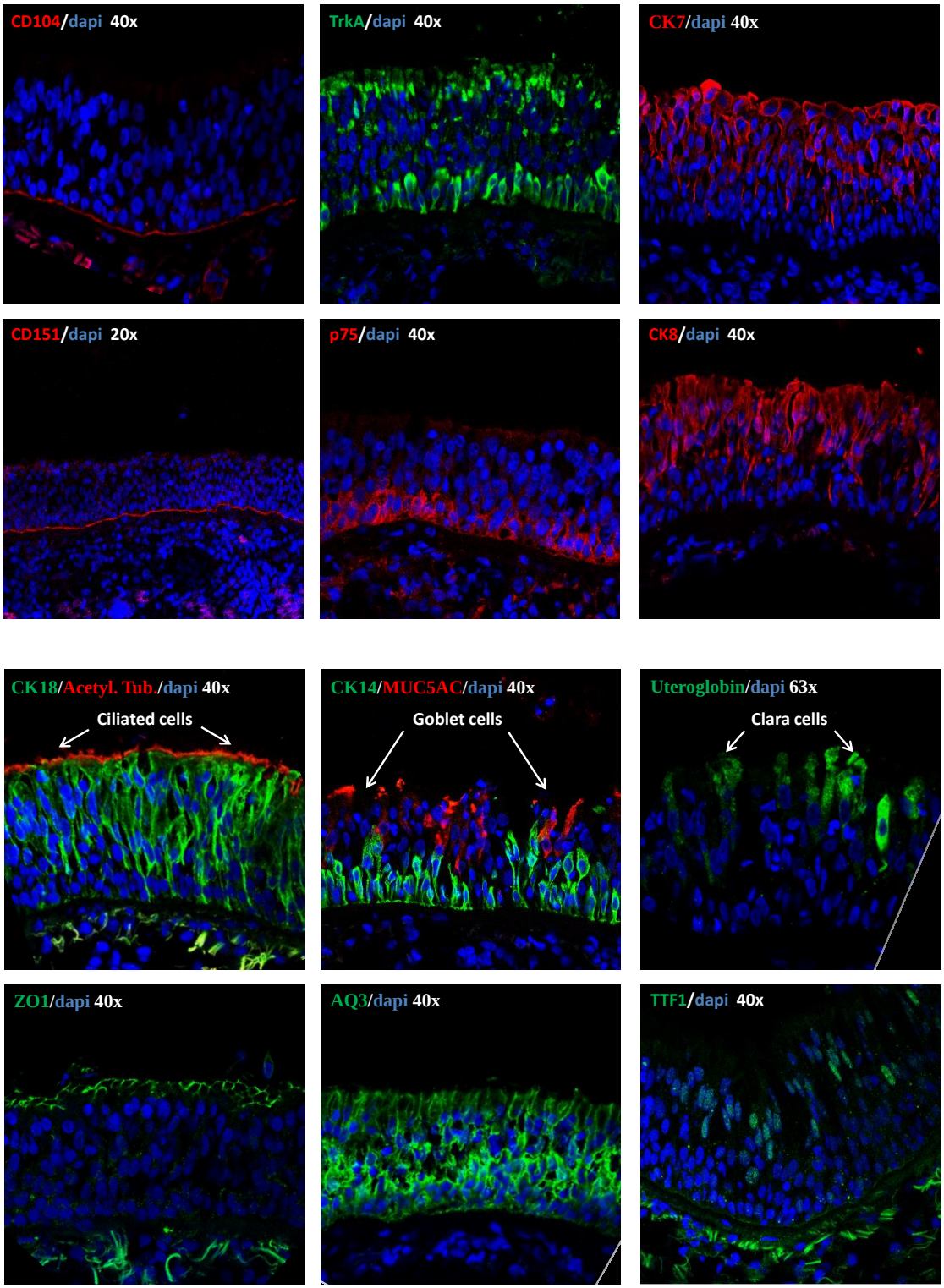


Fig. 33 Immunofluorescence analysis of in-vivo tracheal sections. Stemness/proliferation markers (p63, ki67), basal cells markers (CK13, CK14, CK17, EGFR, p75, TrkA), and columnar epithelial cells markers (CK7, CK8, CK18) were evaluated. Airway specific differentiated cells (indicated by arrows) included ciliated cells, goblet cells and Clara cells (identified respectively as acetylated tubulin, MUC5AC or uteroglobin positive cells). Barrier function of human respiratory epithelium was revealed by the expression of ZO1 positive apical tight junctions.

Human tracheal in-vivo sections immunofluorescence analysis showed sopra-basal cells characterized by the expression of columnar epithelial cells specific cytokeratins CK7, CK8 and CK18, some of them including ciliated cells (acetylated tubulin positive cells), goblet cells and Clara cells (identified respectively by MUC5AC and Uteroglobin/TTF1). Finally, tracheal epithelium revealed sealed by the presence of tight-junctions located at the upper regions of airway epithelial cells, as shown by ZO1 positive staining (Fig. 33).

Several sub-mucosal glands were also observed in human tracheal sections, containing a great number of goblet cells (identified by MUC5AC) and characterized by a weak positive staining for uteroglobin, likely suggesting the presence of few Clara cells (Fig. 34).

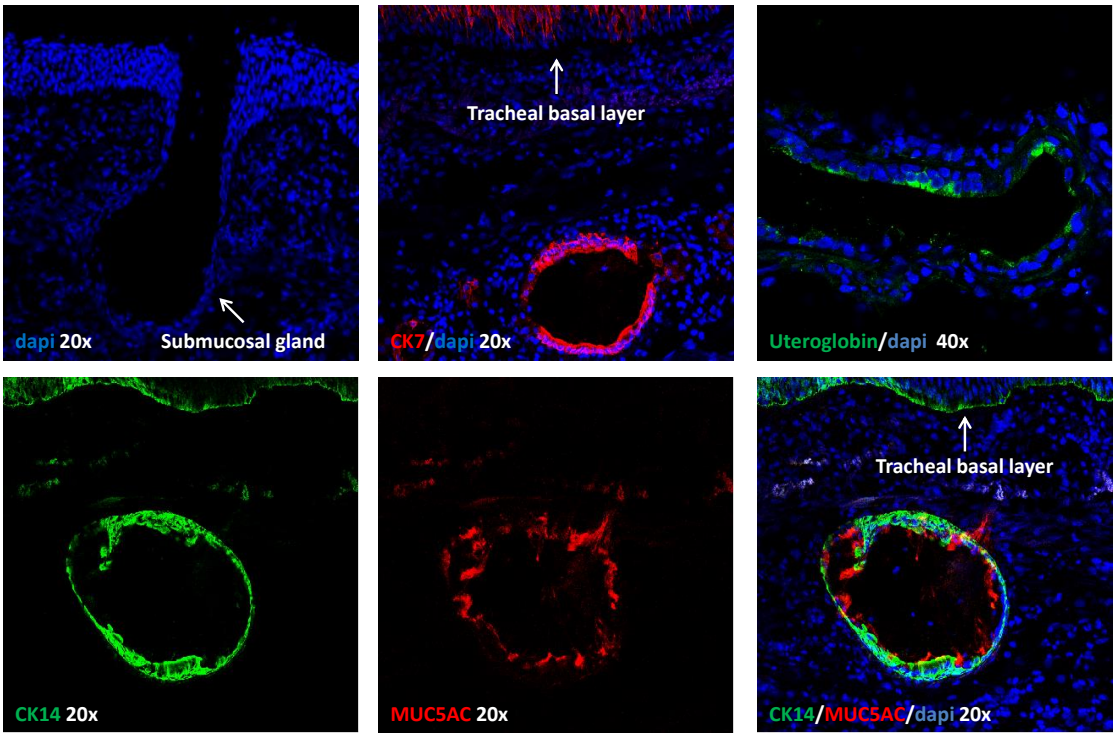


Fig. 34 Sub-mucosal glands structure. Sub-mucosal glands appeared connected to tracheal lumen by epithelial ducts and are characterized by the presence of numerous secretory cells including goblet and Clara cells.

A comparison of markers expression was also performed among tracheal sections and other human airway regions to ascertain similarity of the epithelial cell composition aimed at using alternative cell sources for airway epithelial cells isolation and culture.

Nasal epithelium

As shown in Fig. 35, anterior nasal epithelium is characterized by a skin-like structure with the upper layers positive for the terminal epithelial differentiation marker Involucrin.

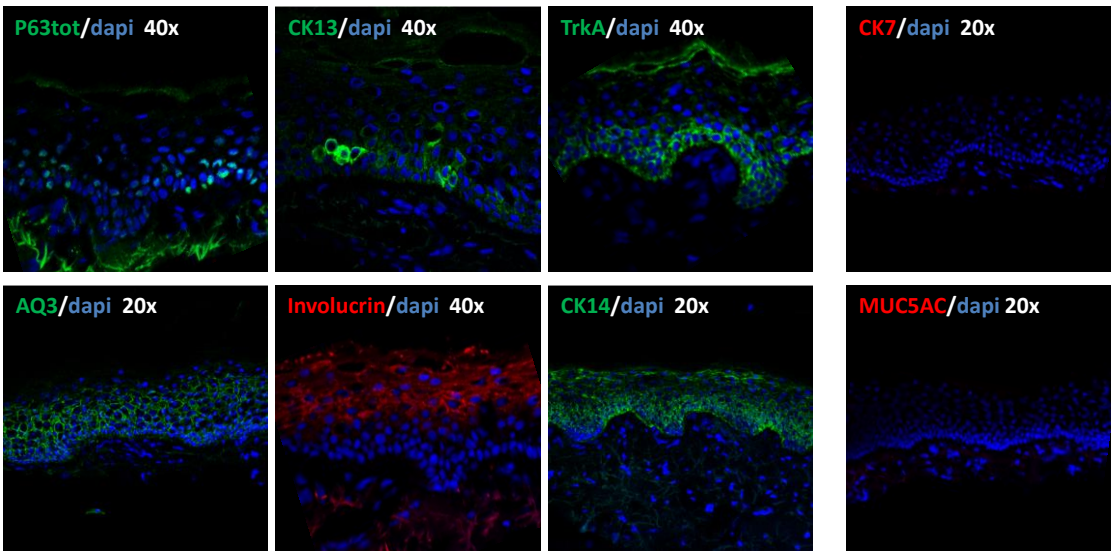


Fig. 35 Markers localization and expression in human anterior nasal epithelium. Basal cells and some sopra-basal cells expressed p63, CK13 and TrkA receptor. Involucrin positive staining revealed a skin-like structure of this epithelium and the differences with the rest of respiratory epithelium were confirmed by the absence of goblet cells and CK7 positive cells.

All nasal epithelial layers appeared positive for CK14 expression and the airway epithelial marker AQ3, whereas basal cells expressed TrkA receptor and some of them showed positive staining for p63 and cytokeratin CK13. The absence of CK7 positive cells and Goblet cells confirmed the different epithelial cell composition of this respiratory tract from the lower human airways.

Adenoid respiratory epithelium

The second respiratory areas analyzed were human adenoids, a lymphoid tissue located posterior to nasal cavity and covered by a pseudo-stratified respiratory epithelium. The analysis of epithelial cells markers expression in this region, revealed basal cells characterized by the expression of stem/progenitor cells transcription factor p63, cytokeratin CK14, and TrkA/p75/EGFR receptors (Fig. 36).

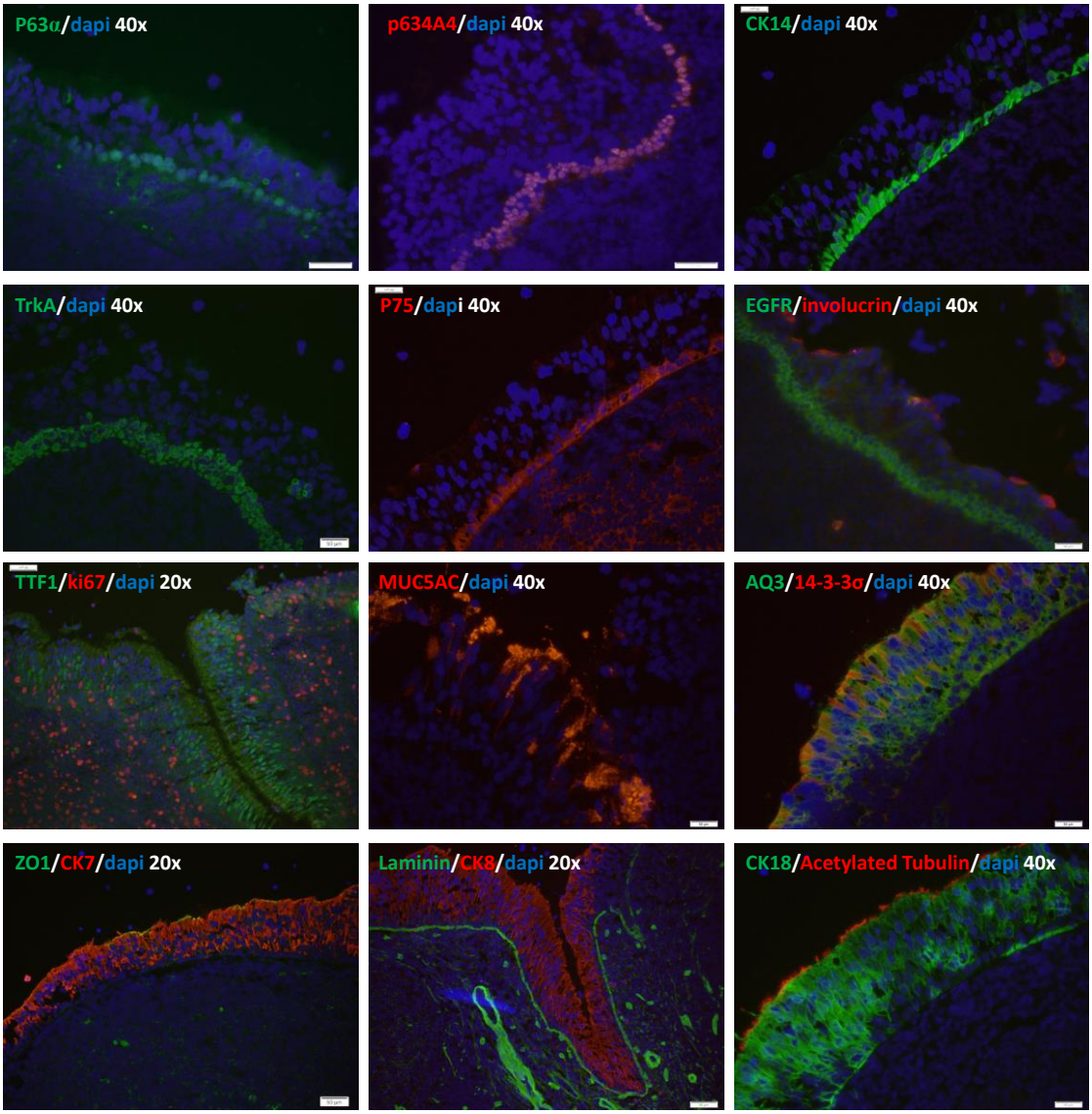


Fig. 36 Characterization of airway epithelia cells markers in human adenoids. Adenoids epithelium appeared to be composed by p63α/CK14/p75 positive basal cells, and columnar CK7/CK8/CK18 positive cells including Goblet cells and ciliated cells.

Human adenoids respiratory epithelium contained also Goblet cells (MUC5AC positive cells), ciliated cells (identified by acetylated tubulin) and probably Clara cells (due to the presence of numerous cells expressing TTF1 marker). As in other human airway regions, adenoids epithelial cells expressed also the water channel AQ3 and the three cytokeratins CK7, CK8, CK18 specific for columnar epithelial cells. The apical region of this respiratory epithelium was protected and sealed by the presence of tight junctions, identified by ZO1 marker (Fig. 36).

Bronchial epithelium

Human bronchial epithelium was finally evaluated for airway epithelial cells markers expression, revealing an high similarity with tracheal tissue.

Immunofluorescence analysis of bronchial sections showed the presence of ciliated cells, goblet cells and Clara cells (identified by acetylated tubulin, MUC5AC and uteroglobin respectively) in sopra-basal layers, whereas basal cells were positive for p63, CK14, CK17 and p75 receptor. This respiratory epithelium expressed also the water channel AQ3 and the apical region was characterized by the presence of ZO1, an important component of sealing tight junctions (Fig. 37).

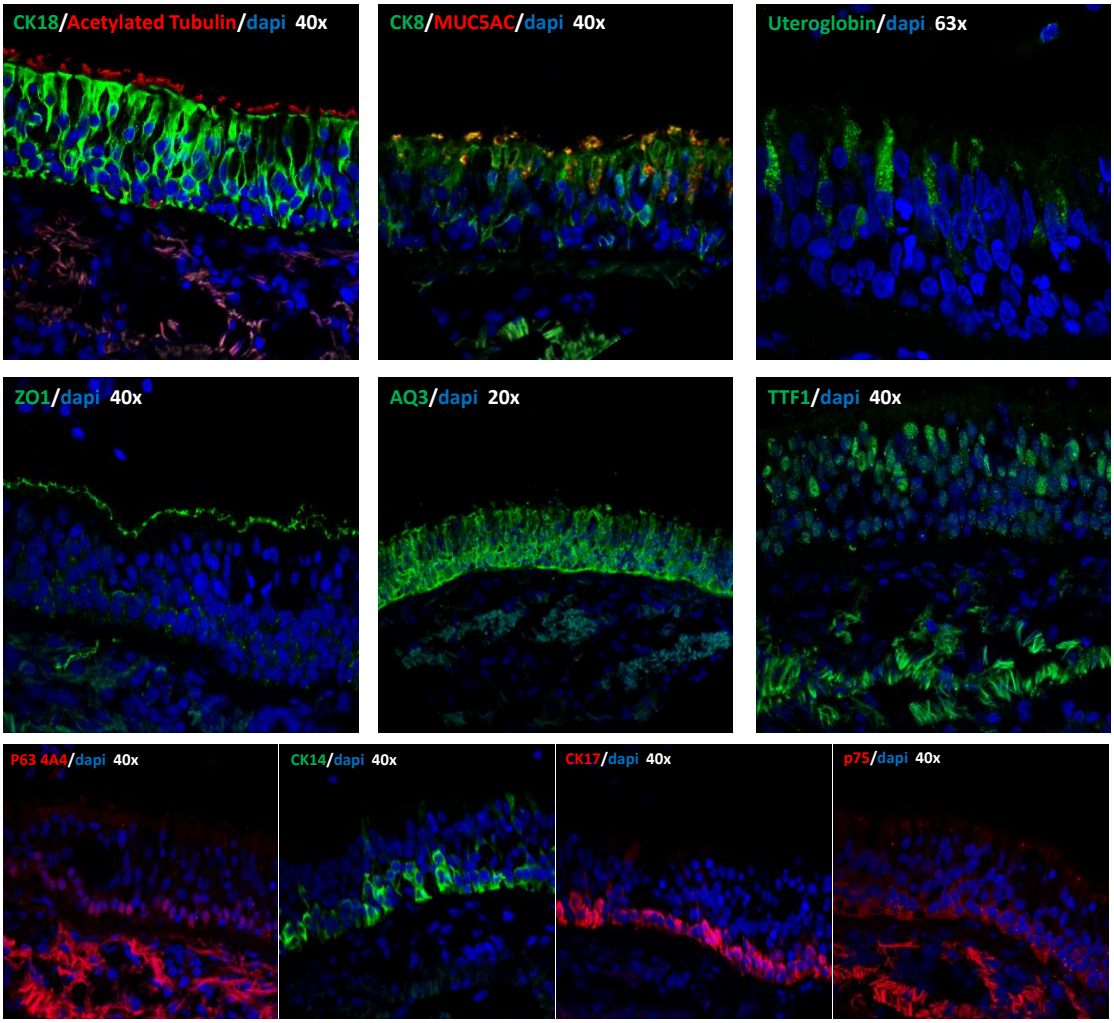


Fig. 37 Analysis of human bronchial respiratory epithelium. Immunofluorescence analysis of bronchial sections revealed an very similar cellular composition to tracheal epithelium. Bronchial columnar epithelial cells included ciliated cells, goblet cells and Clara cells, whereas basal cells were identified by the expression of p63, CK14, Ck17 and p75 receptor.

The first bronchial epithelium showed the same structure and cellular composition of tracheal tissue, suggesting the possibility to isolate the airway epithelial cells needed for natural/synthetic scaffolds colonization from the entire tracheo-bronchial respiratory tract.

Human tracheal epithelial cells culture

Tracheal epithelial cells were cultured for several passages on feeder-layer with KC culture medium to evaluate growth rate, colony forming efficiency and proliferative potential measured as total cell doubling number before senescence.
As shown in Fig. 38, we observed a progressive decrease of clonogenic cells as well as an increase of aborted colonies during serial passages, suggesting that airway epithelial cells do not proliferate indefinitely but undergo to physiological replicative senescence, within this culture system.

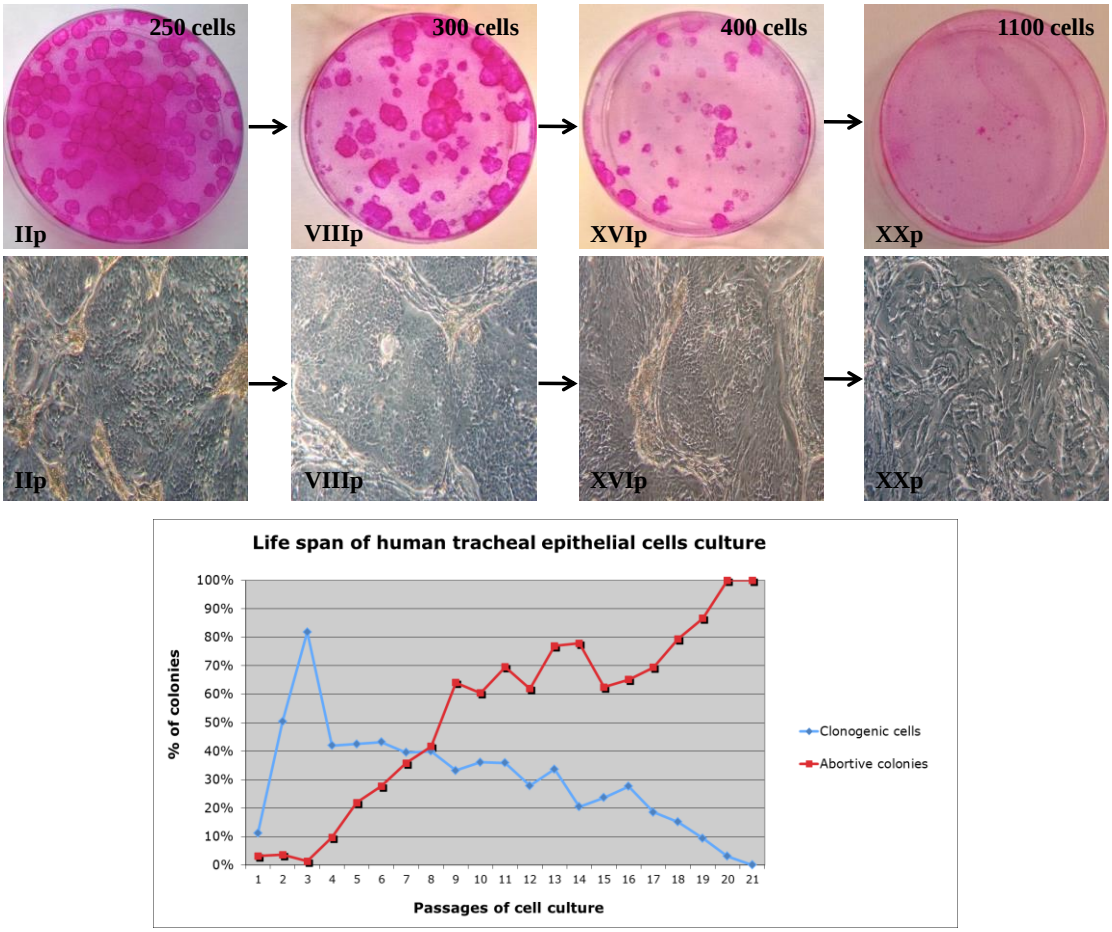


Fig. 38 Colony forming efficiency analysis of human tracheal epithelial cells life-span. The analysis of the indicator dishes revealed an inverse relationship between growing colonies and aborted colonies during serial passages, as represented in line graph. Tracheal epithelial cell culture pictures showed also the cell sizes increasing during replicative senescence.

In the selected culture condition, human tracheal epithelial cells showed a proliferative potential of approximately 100 cell doubling, suggesting the maintenance of the stem cells population and their ability to self-renew during several passages.

Airway epithelial cells markers analysis during serial passages of human tracheal epithelial cells life-span.

An aliquot of tracheal epithelial cells from each of serial passages was used for proteins extraction to analyze the expression of specific markers. The stem/progenitor cells markers p63 and Bmi1 decreased their expression levels during the life-span of tracheal epithelial cells, suggesting their involvement in stem/progenitor cells maintenance. The expression of basal cytokeratin CK14 remained stable in all passages, whereas the differentiation markers 14-3-3σ, CK7 and Uteroglobin showed an increase during serial sub-cultivations, confirming their bound to differentiated and specialized airway epithelial cells such as Clara cells (Fig. 39).

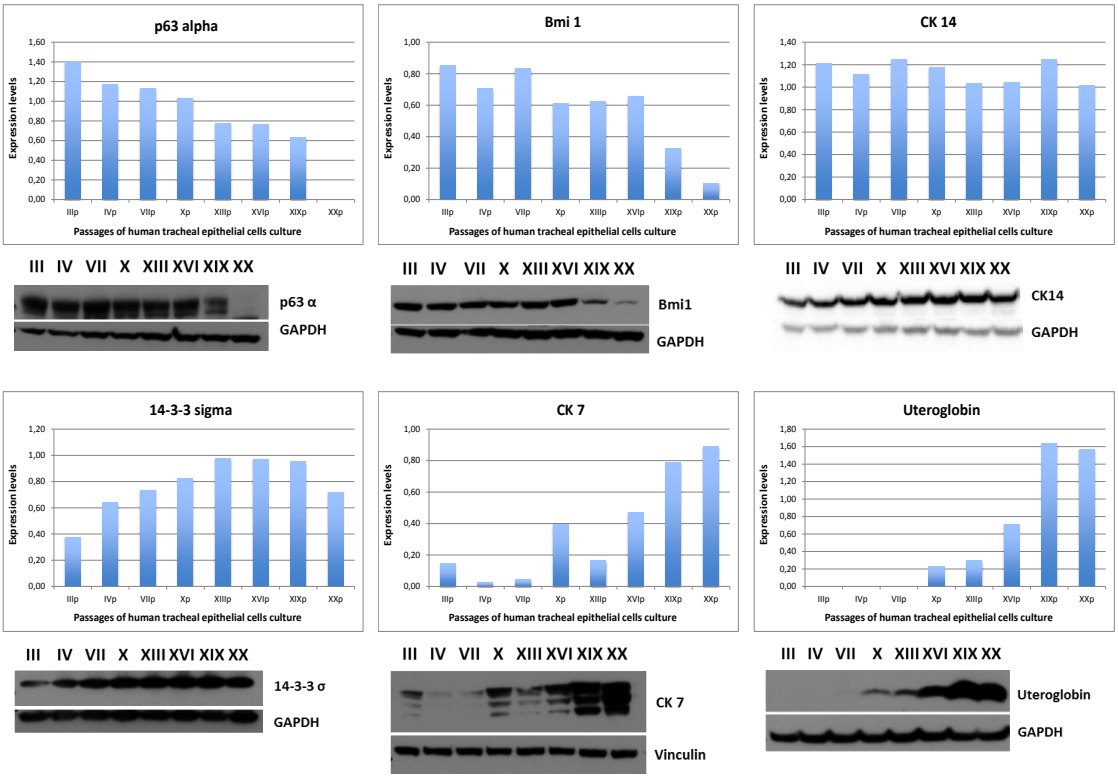


Fig. 39 Western-blot analysis of markers expression during serial passages of human tracheal epithelial cells. Protein bands of stem/progenitor cells markers and differentiation markers are reported as well as their quantification by Image J software.

To evaluate goblet cells differentiation process during tracheal epithelial cells life-span, a defined number (10.000-15.000) of cells from each passage was placed onto a glass slide and the quantification of respiratory mucous cells was performed by immunofluorescence staining for MUC5AC marker.

This experiment revealed that goblet cells increase their number during tracheal epithelial cells serial cultivation with the last passage including more than 30% of mucous cells (Fig. 40).

Therefore, goblet cells, as well as Clara cells, represent a specialized cell population that increase their number during the replicative senescence process.

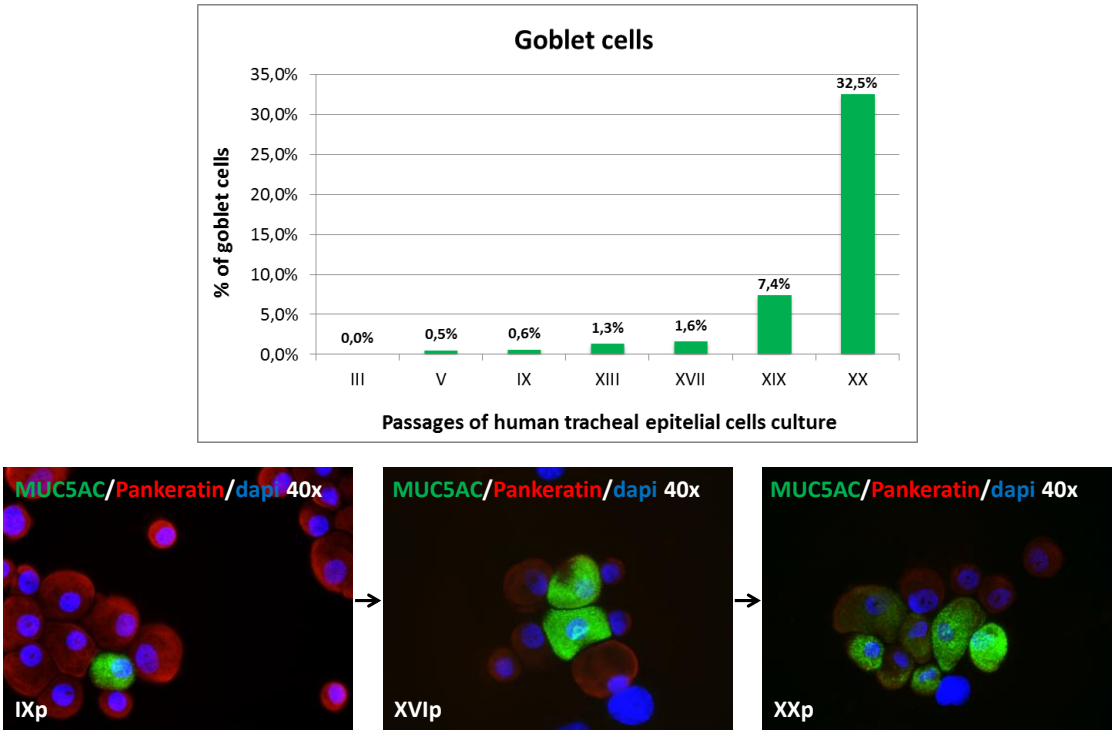
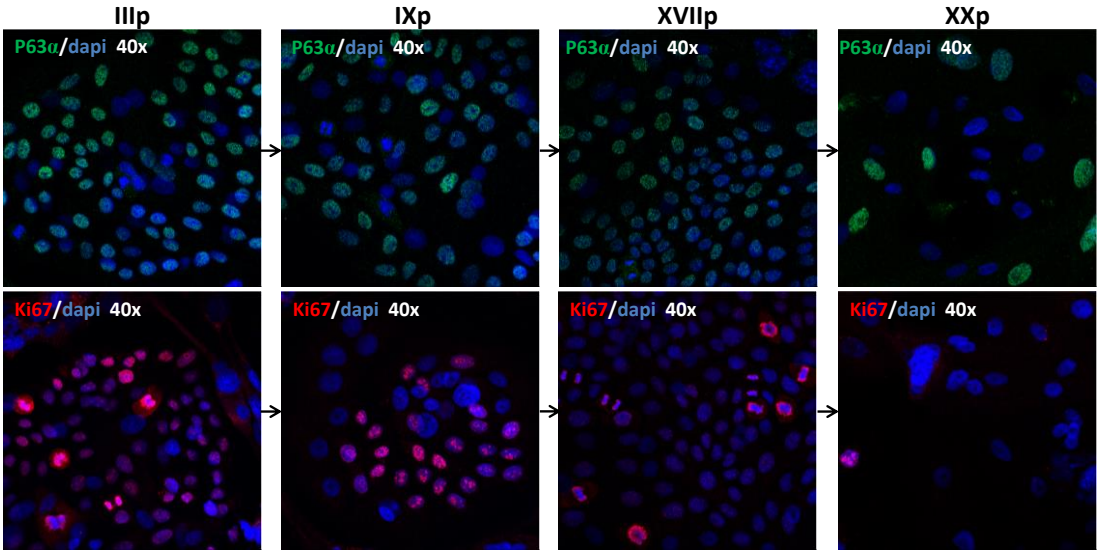


Fig. 40 Goblet cells quantification during tracheal epithelial cells life-span. All tracheal epithelial cells plated on glass slide were stained by anti-pankeratin antibody (red), whereas goblet cells were identified by anti-MUC5AC antibody (green).

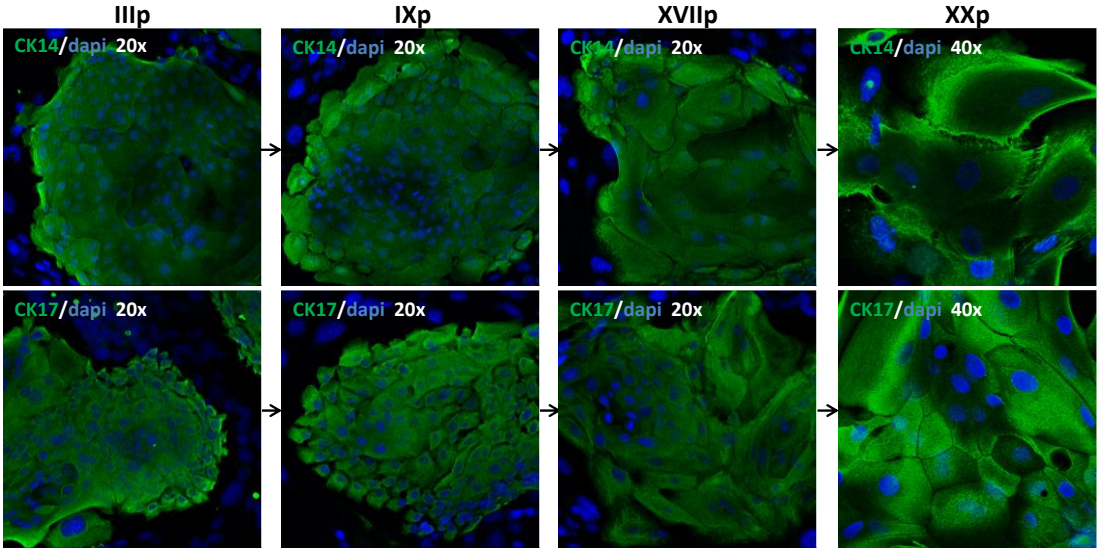
These data were confirmed by immunofluorescence analysis on tracheal epithelial cells cultivated onto coverslip glass slide at different cell culture passages.

As shown in Fig. 41, during tracheal epithelial cells life-span there was an expression decrease of proliferation and stem/progenitor cells markers ki67 and p63 (A), whereas basal epithelial cells cytokeratins CK14 and CK17 remained stable (B).

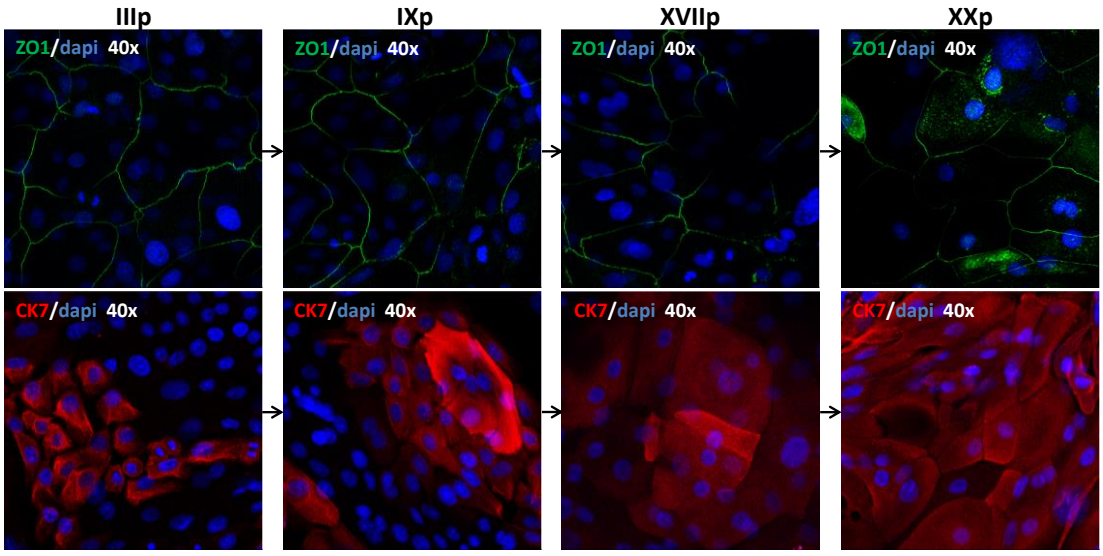
A. Proliferation and epithelial stem cells markers



B. Basal epithelial cells markers



C. Epitelial cells differentiation markers



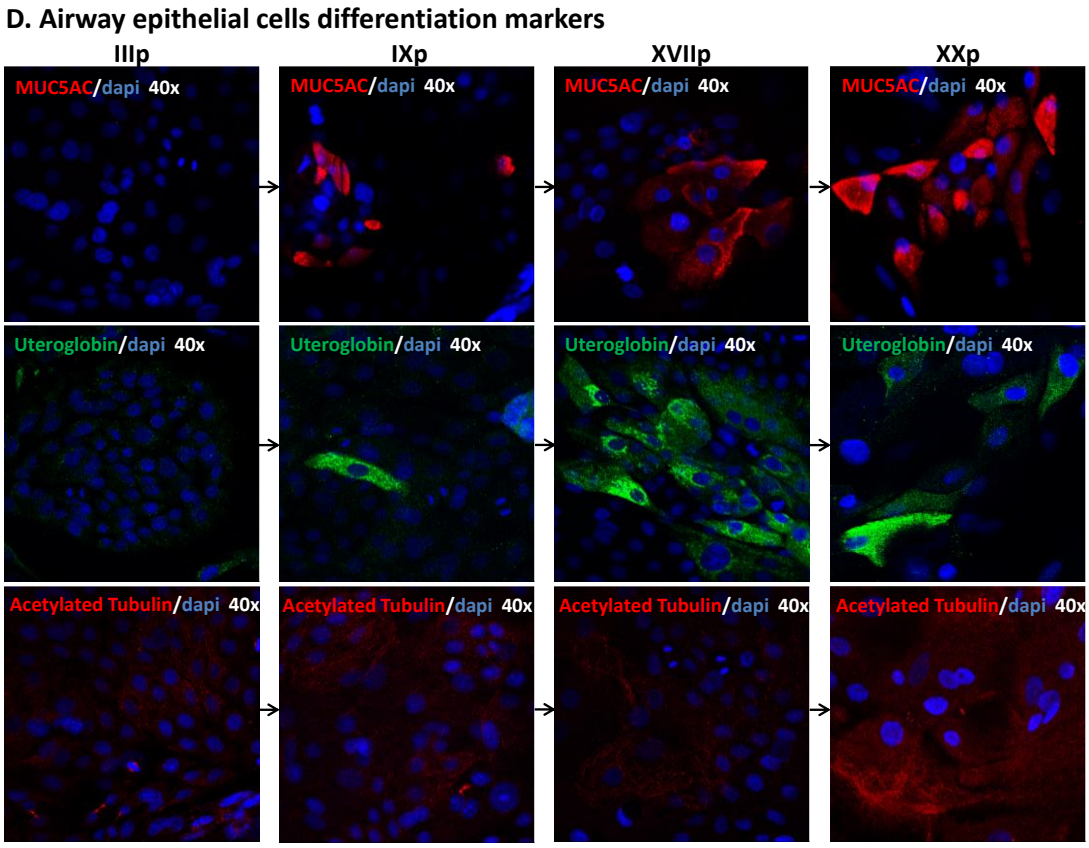


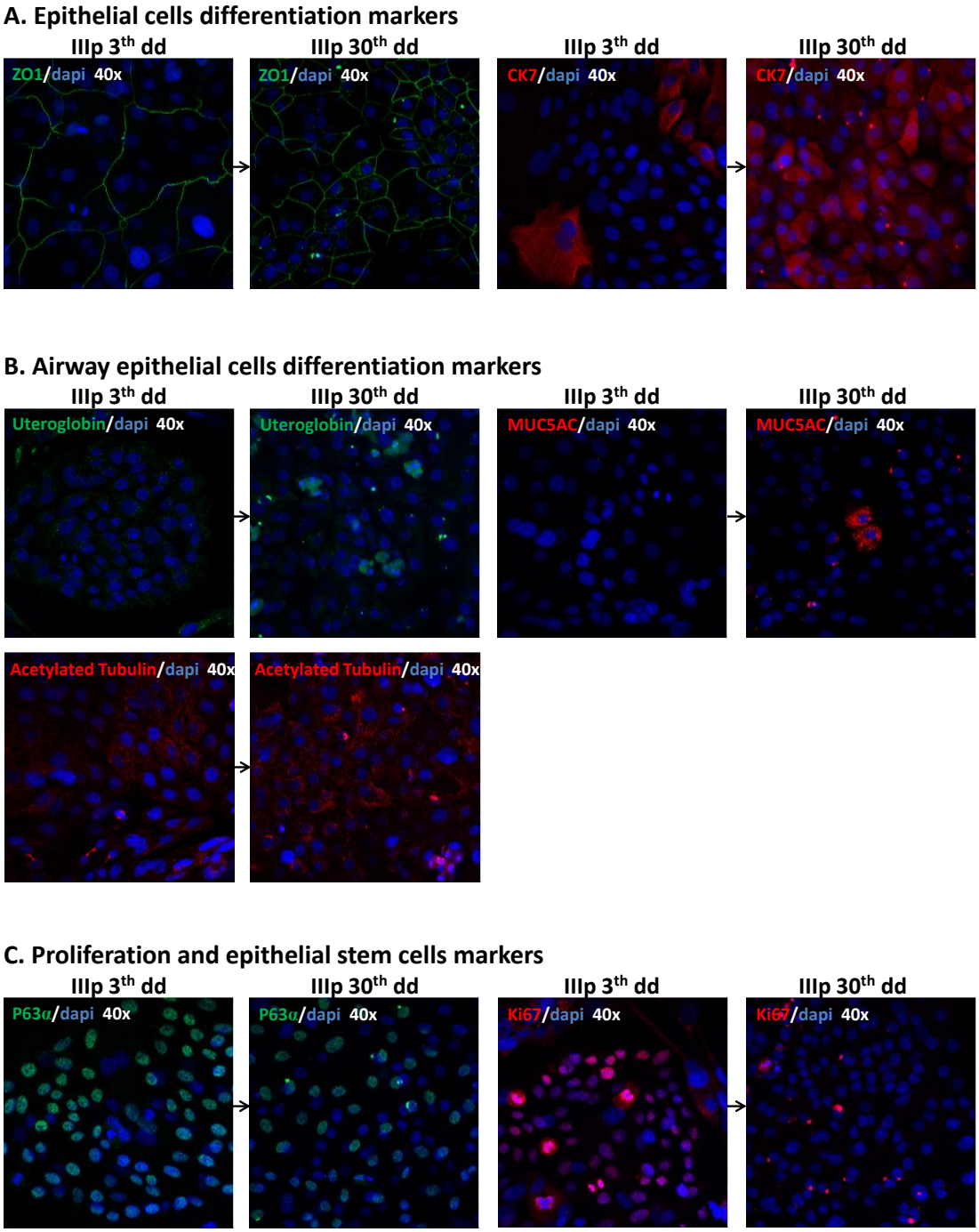
Fig. 41 Immunofluorescence analysis of tracheal epithelial cells serially cultivated. During tracheal epithelial cells life-span, stemness/proliferation markers p63α and ki67 decreased (A), whereas basal epithelial cell cytokeratins CK14 and CK17 were equally expressed among early and late passages (B). The late passages of tracheal epithelial cell life-span were characterized by an increased expression of epithelial differentiation markers (C) and an increased number of airway differentiated cells, as shown by Goblet, Clara and Ciliated cells markers expression (D).

Finally, during tracheal epithelial cells serial passages, the epithelial cells differentiation marker CK7 (Fig. 41 C) and the airway epithelial cells differentiation markers MUC5AC, Uteroglobin and acetylated tubulin (Fig. 41 D) appeared to be up-regulated. These results suggested that, as observed for others human epithelia [58], airway differentiated cells generation from tracheal epithelial cells are triggered after a number of cell doublings, needed to begin transient amplifying cells differentiation process.

Tracheal epithelial cells differentiation potential

When cultivated in submerged culture for about 30 days, tracheal epithelial cells underwent preferentially epithelial stratification process rather than airway differentiation process.

In this culture condition, we observed an increased expression of epithelial differentiation markers ZO1 and CK7 (Fig. 42A), and low number of airway differentiated cells (Fig. 42B), suggesting that especially Goblet cells and Clara cells differentiation processes could be bound to a defined number of cell doublings of tracheal epithelial cells.



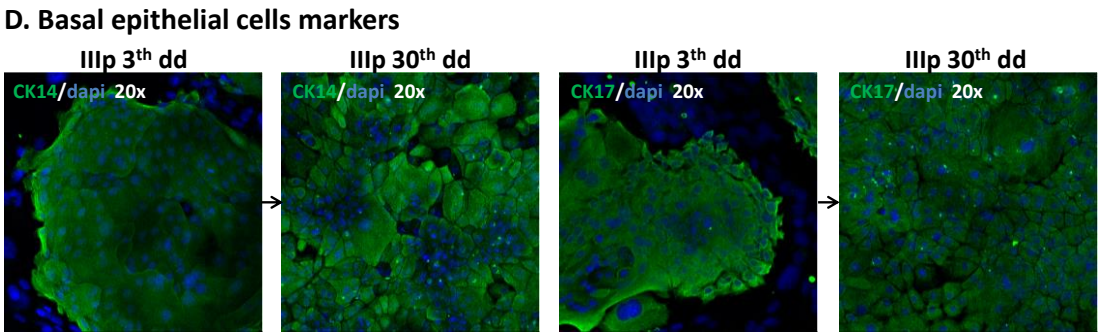


Fig. 42 Tracheal epithelial cells cultured for 30 days in submerged conditions. In this culture condition, tracheal epithelial cells underwent epithelial differentiation process (A, B), reducing the expression of stemness/proliferation markers (C), but maintaining basal cell markers expression (D).

Moreover, the stratified epithelium obtained in this experiment still expressed the basal epithelial cells markers CK14/CK17 (Fig.42 D), with very few proliferating cells (Ki67+) but many p63 positive cells (Fig. 42 C). Therefore, after 30 days of submerged culture, tracheal epithelial cells stop to proliferate and underwent epithelial differentiation process, maintaining some p63α positive cells endowed with high proliferative potential.

Human tracheal epithelial cells capability to generate a mature respiratory ciliated epithelium was finally proved by air lift culture, plating cells on human scaffold (dermis devoid of endogenous epidermis) and culturing the tissue at air-lift interface. After thirty days of culture, the immunofluorescence analysis of human dermis repopulated with airway epithelial cells, revealed the presence of a fully differentiated epithelium characterized by the presence of numerous ciliated cells, identified by anti-acetylated tubulin antibody (Fig. 43).

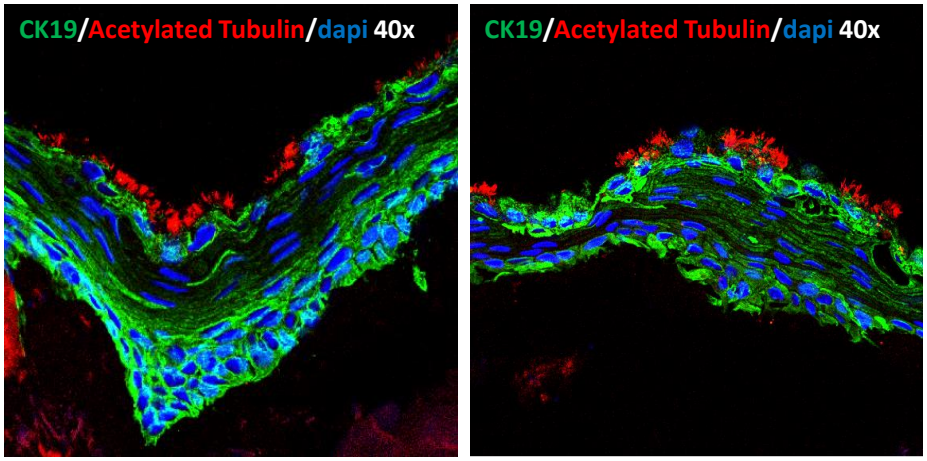


Fig. 43 Tracheal epithelial cells cultured on human dermis in air-lift condition. In the selected culture condition, tracheal epithelial cells generated a ciliated epithelium when exposed to air-contact for about 30 day of culture.

This experiment confirmed cultured tracheal epithelial cells multipotency and the suitability of the selected culture system maintaining cell capability to generate all epithelial cells types of human tracheal region including basal cells, goblet cells, Clara cells and ciliated cells.

Identification of human tracheal epithelial stem cells.

Tracheal epithelial cells culture analysis proved that culture system based on feeder-layer and KC culture medium was able to maintain stem cells population and their proliferative and differentiation potentials. However, further studies will be required for clinical application of human epithelial cells. The identification of epithelial stem cells, the analysis of their proliferative and differentiation potential as well as the percentage of stem cells contained in epithelial cells cultures used for clinical transplantation, are important informations required to guarantee the implanted graft capability to self-renew and maintain itself on patient lifetime.

The identification of human epithelial stem cells can be performed by clonal analysis.
Clonogenic keratinocytes generate in in-vitro cell cultures, three types of clones defined as paraclones, meroclones and holoclones, with the latter considered as the epithelial stem cells for some their important properties such as the highest proliferative potential and long term regenerative capability.
In the selected culture condition, tracheal epithelial cells generated the three clonal types holoclones, meroclones and paraclones (Fig. 44), previously identified in others human epithelia such as skin, oral mucosa and cornea [50, 59].

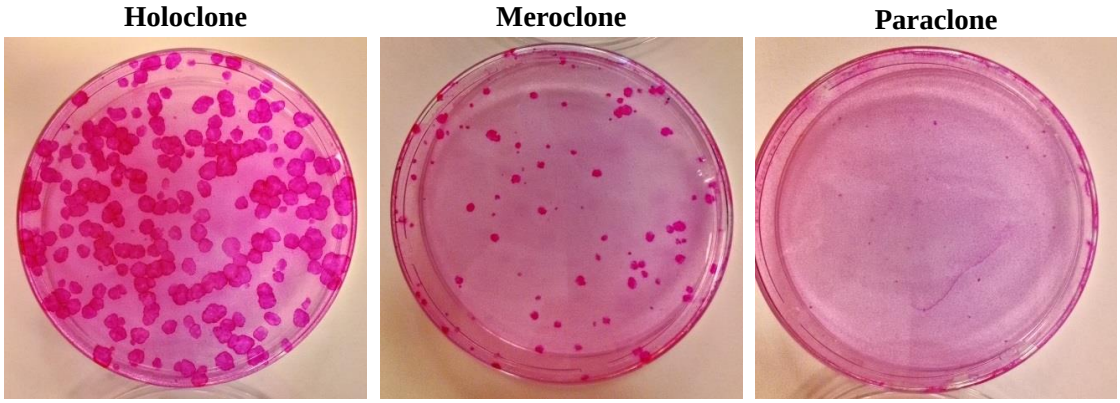


Fig. 44 Holoclone, meroclone and paraclone from human tracheal epithelial cells culture. Holoclones generate less than 5% of aborted colonies, paraclones produced more than 95% of aborted colonies, meroclones represent an intermediate state.

To confirm stem cells identity of human tracheal holoclones, several experiments were performed to evaluate their proliferative and differentiation potential.

Cultures from tracheal holoclones, meroclones and paraclones were evaluated to compare epithelial cell size distribution and p63 expression levels.

The analysis of holoclones, meroclones and paraclones derived cells revealed that tracheal holoclones contained the highest percentage of small cells (more than 80% of holoclones epithelial cells had a diameter < 20µm) compared to meroclones and paraclones cells (the latter containing less than 20% of epithelial cells < 20µm) (Fig. 45).

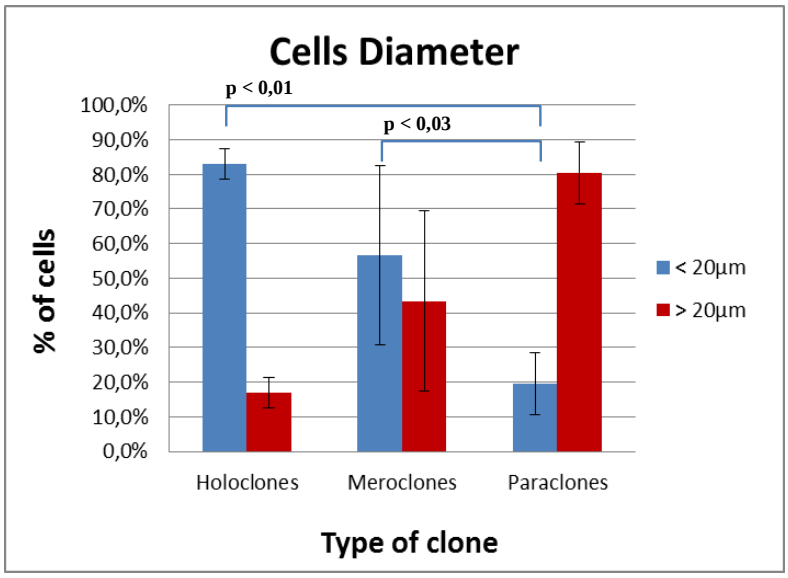


Fig. 45 Holoclones, meroclones and paraclones cell size analysis. A total number of about 3500 holoclones, 8800 meroclones cells and 800 paraclones derived cells were analyzed. Epithelial cell size evaluation revealed a decrease of small cells percentage and an increase of large cell number during clonal conversion from holoclones to paraclones.

In depth analysis of tracheal epithelial cells sizes showed that holoclones contain cells of 12-15µm and 16-19µm in diameter with a frequency of 48,5% and 32,1% respectively.

These percentages were very similar in early meroclones (50,3%-30,3%) but decreased in meroclones (45,9%-27,4%) and late meroclones (16,7%-18,7%), reaching the final values of 11,2% and 11,5% in paraclones (Fig. 46).

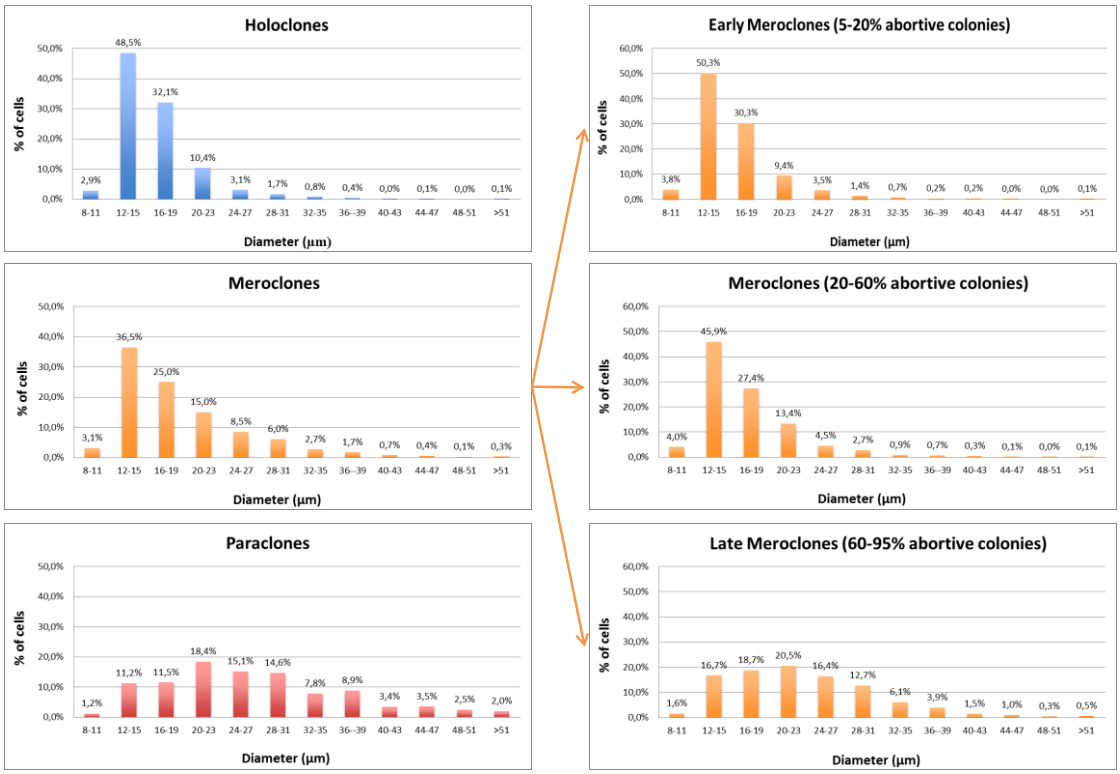


Fig. 46 Holoclones, meroclones and paraclones cell distribution according to defined size ranges. Tracheal epithelial cells derived from holoclones, meroclones and paraclones were divided into several size ranges to evaluate the distribution of small and large airway epithelial cells. Holoclones and meroclones contained the highest percentages of small cells (12-19µm in diameter), whereas paraclones were composed by epithelial cells of different size.

The immunofluorescence quantification of p63 intensity levels in holoclones, meroclones and paraclones, showed that holoclones cells were characterized by the highest expression levels of p63 transcription factor and that these stem-cells derived clones contained the highest percentage of p63 bright cells, identifiable as those cells endowed with the greatest proliferative potential.

Conversely, p63 was poorly detected in paraclones derived cells, whose cultures contained a lower percentage of p63 bright cells.

Moreover, using both cellular diameter and p63 intensity level as selection parameters, a pool of putative tracheal epithelial stem cells composed by the smallest p63 bright cells can be identified (Fig. 47).

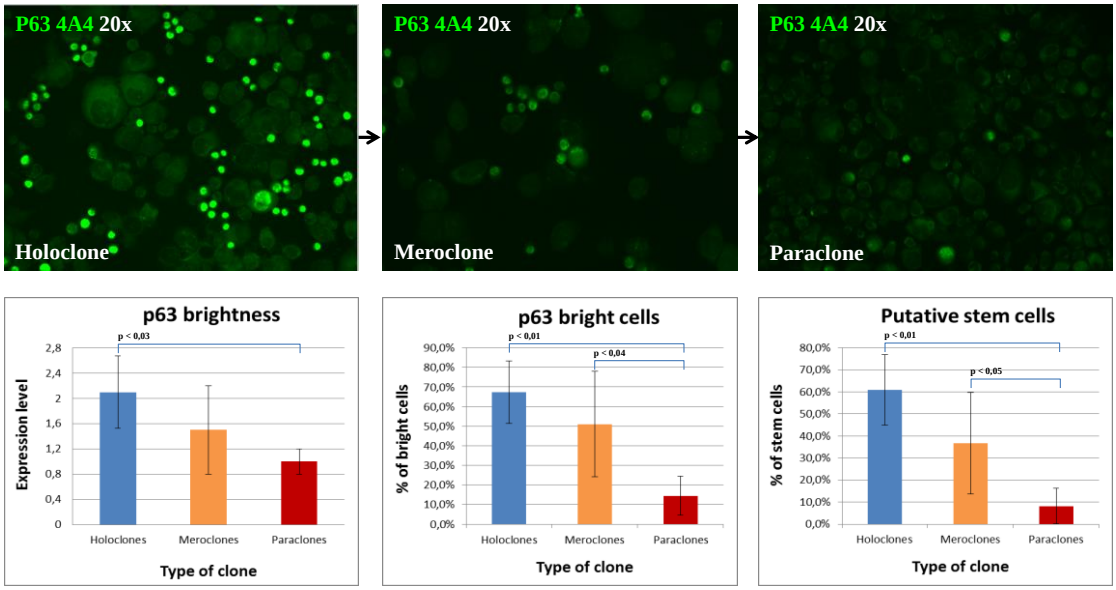


Fig. 47 Quantitative immunofluorescence analysis of p63 expression in human trachea holoclones, meroclones and paraclones cells. Immunofluorescence staining (p63 in green) showed the differences among the three clonal types. P63 intensity levels, p63 bright cells and putative tracheal epithelial stem cells were quantified as reported in bar graphs.

This experiment showed p63 transcription factor to be more expressed in holoclones epithelial cells, suggesting that they can be an important indicator for presence of tracheal epithelial stem cells.

Further comparative evaluations about progenitor/stem cells markers expression in holoclones, meroclones and paraclones, were performed by western-blot analysis of proteins extracts obtained from tracheal epithelial clone progenies.

Western-blot assay revealed that the expression of stem cells markers p63, Bmi1 and Sox2 decreased during clonal conversion from holoclones to paraclones. In particular, Bmi1 level reduction occurred gradually, whereas p63 and Sox2 appeared to be preferentially expressed in holoclones rather than meroclones/paraclones (Fig. 48).

The expression of these two transcription factors remained relatively high in early meroclones but their presence was very poor from intermediate meroclones to paraclones, suggesting that both p63 and Sox2 could be used as potential tracheal epithelial stem cells markers.

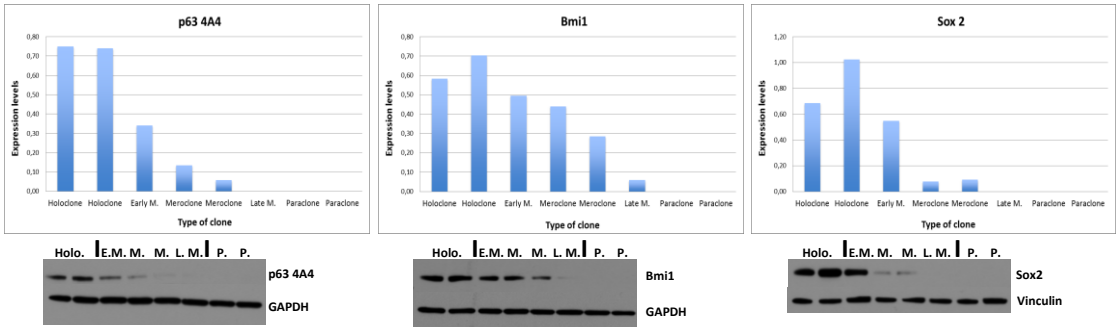


Fig. 48 Western blot analysis of stem cell markers expression in human tracheal epithelial holoclones, meroclones and paraclones. P63, Bmi1 and Sox2 protein bands quantification by Image J software are reported in the above bar graphs.

Stem cells differentiation potential was evaluated by differentiating the three tracheal clonal types holoclones, meroclones and paraclones, and by analysis of the differentiation state in the progeny of each clone.

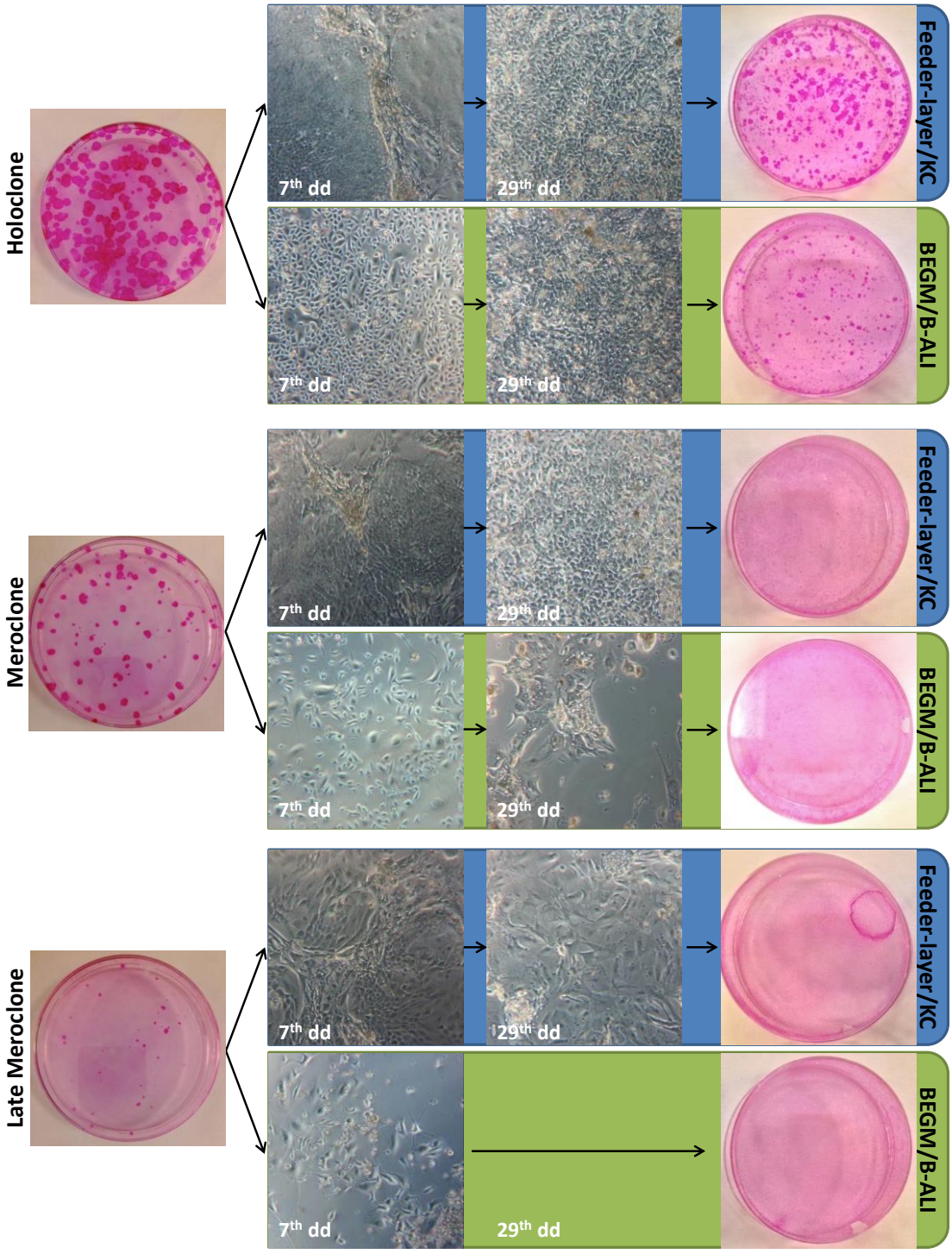
After cloning, one quarter of each tracheal clone (XX in total) was plated into an indicator dish for clones classification, whereas the remaining three quarters were plated in two well of a 24wells-plate. The isolated clones were cultured with KC culture medium (one well) or BEGM/B-ALI defined medium (second well) for about 30 days to induce their differentiation and analyze the residual proliferative/clonogenic potential.

Only holoclones, characterized by the presence of tracheal epithelial stem cells, were able to maintain proliferative/clonogenic capabilities, whereas tracheal epithelial meroclones and paraclones, composed by transient amplifying cells only, completely differentiated.

This experiment showed that in BEGM/B-ALI condition also, the only clone found at the end of differentiating culture condition was the holoclone (Fig. 49). Re-plating holoclones derived-cells in an indicator dish, an average of 4,62% of clonogenic cells was obtained, whereas none of others clone types were able to generate growing colonies (Fig. 50).

Conversely, when cultured in KC culture medium, tracheal holoclone and few meroclones were able to reach thirty days in culture (Fig. 49), however when replating in a new indicator dish, only holoclone generated growing colonies with maintenance of a percentage of clonogenic cells of approximately 8% (Fig. 50).

These results further confirmed the suitability of culture condition based on feeder-layer and KC to preserve tracheal epithelia stem cells clonogenic/proliferative potential.



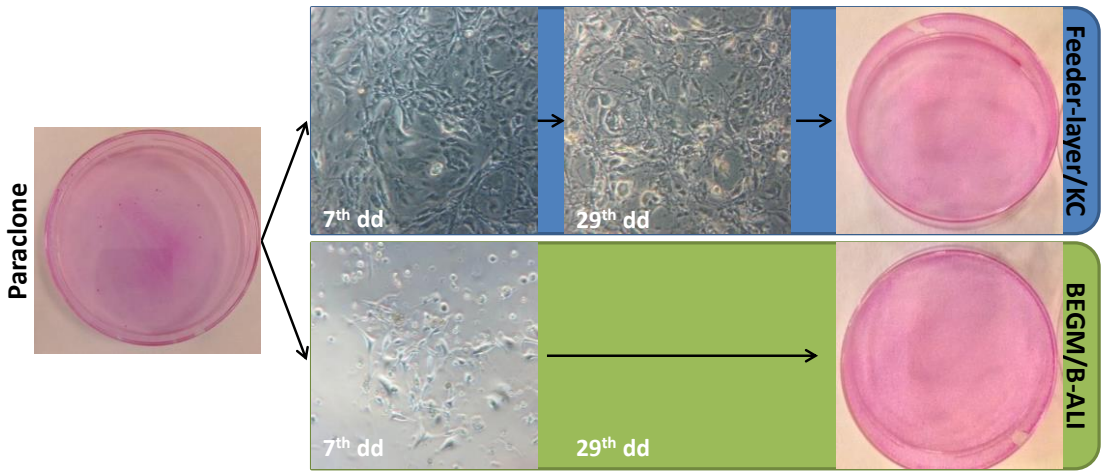


Fig. 49 Colony forming efficiency assay of tracheal epithelial clones after 30 days of submerged cultures. Two different tracheal epithelial clone halves were plated into two cell culture plates and cultured with KC or BEGM/B-ALI medium respectively. Tracheal clones cultures photos showed the differences among the two culture condition and among the three clonal type. Colony forming efficiency assay at the end of the experiment revealed the residual clonogenic/proliferative potential of each clone.

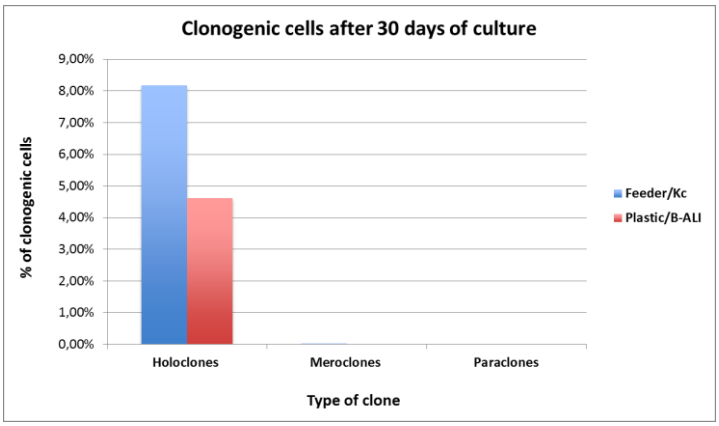


Fig. 50 Comparison of tracheal epithelial clones clonogenicity. Colony forming efficiency of holoclone, meroclones and paraclones derived-cultures showed the highest clonogenic/proliferative potential of human tracheal epithelial holoclone.

This experiment confirmed human tracheal epithelial holoclones as clones endowed with the highest proliferative/clonogenic potential and therefore containing airway epithelial stem cells able to preserve the regeneration properties.

The analysis of some basal/stem cell markers of the holoclone and meroclones after the thirty days of submerged culture, showed that p63α expression levels were similar among the two clonal types. On the other hand, holoclone derived-cells showed the highest expression of CK14 and Bmi1 (Fig. 51), highlighting the importance of basal cells expressing Bmi1 for tracheal epithelial stem cells function.

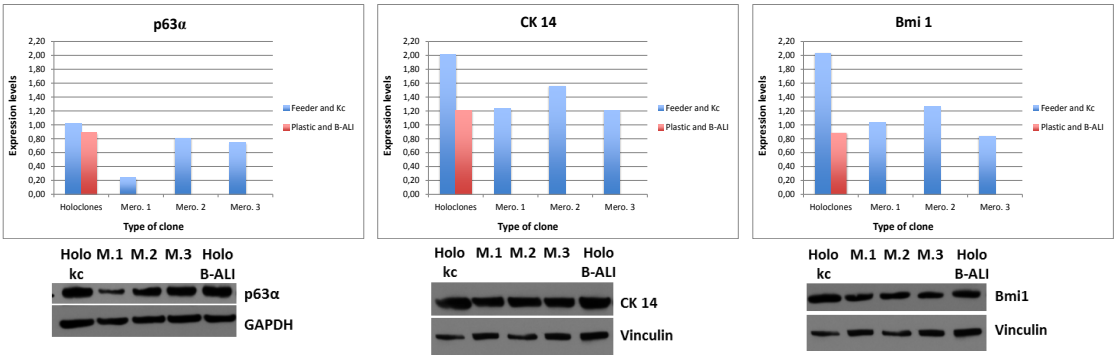


Fig. 51 Basal/stem cells markers expression comparison among holoclone and meroclones cultured for thirty days in submerged condition. Protein quantification showed similar p63 expression levels among the two clonal types but higher expression of CK14 and Bmi1 in holoclones rather than meroclones derived cells. Bmi1 higher expression in holoclones derived cells is consistent with its role as a repressor of replicative senescence. This BMI-1 role would explain the maintenance of proliferative/clonogenic potential at the end of submerged culture of holoclones.

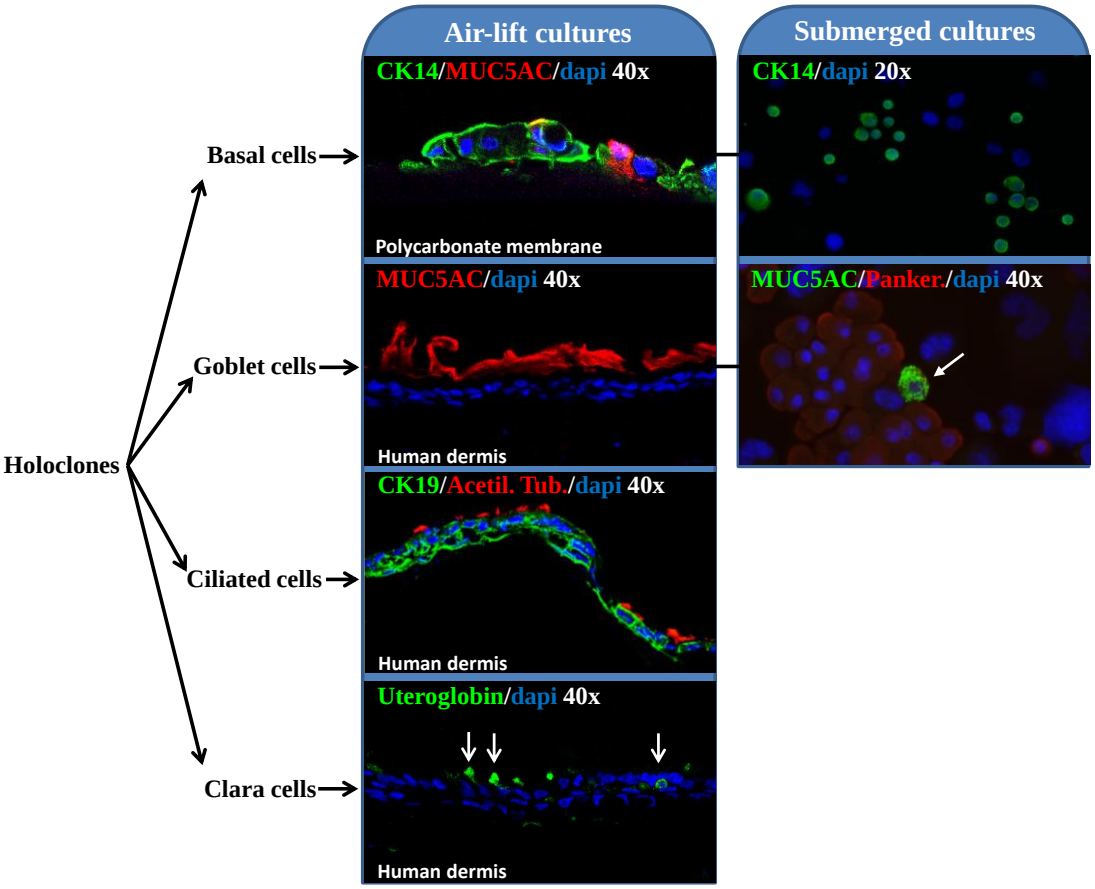
Differentiation potential of human tracheal epithelial clones.

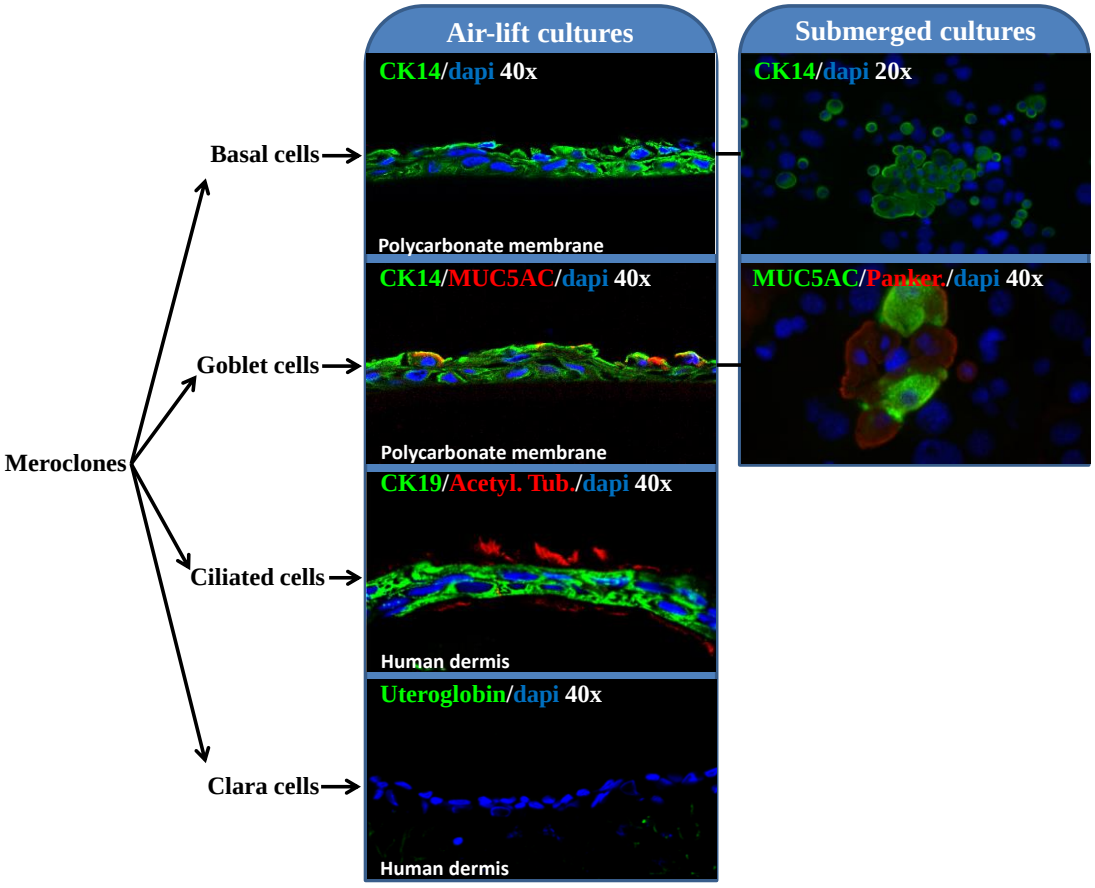
One of the most important characteristics of stem cells is their ability to differentiate in all epithelial lineages of the original tissue.

To confirm tracheal epithelial holoclones as the stem cells of this airway region, their differentiation capabilities were evaluated by immunofluorescence analysis of tracheal epithelial clones cultivated in submerged and air-lift conditions. The analysis of tracheal clones was performed on cytospins of their respective cultures, in addition two different supports were used to perform the air-lift interface cultures: polycarbonate membrane (millicell cell culture inserts, from Millipore) and human dermis after epidermis removal. At the end of cultures, the differentiation potential of holoclones, meroclones and paraclones were compared.

As shown in Fig.52, human tracheal clones showed distinct differentiation potentials:

- holoclones generated basal cells (CK14+), goblet cells (MUC5AC+), ciliated cells (acetylated tubulin +) and Clara cells (uteroglobin+);
- meroclones were able to differentiate into basal cells, goblet cells and ciliated cells but not into Clara cells;
- paraclones had no enough proliferative potential to grow in air-lift condition for 30 days of culture, however their submerged cultures showed a residual presence of basal and goblet cells.


Air-lift cultures
Submerged cultures
CK14/MUC5AC/dapi 40x
CK14/dapi 20x
Basal cells
Polycarbonate membrane
MUC5AC/dapi 40x
MUC5AC/Panker./dapi 40x
Goblet cells
Holoclones
Human dermis
CK19/Acetil. Tub./dapi 40x
Ciliated cells
Human dermis
Uteroglobin/dapi 40x
Clara cells
Human dermis


Air-lift cultures
Submerged cultures
CK14/dapi 40x
CK14/dapi 20x
Basal cells
Polycarbonate membrane
CK14/MUC5AC/dapi 40x
MUC5AC/Panker./dapi 40x
Goblet cells
Meroclones
Polycarbonate membrane
CK19/Acetyl. Tub./dapi 40x
Ciliated cells
Human dermis
Uteroglobin/dapi 40x
Clara cells
Human dermis

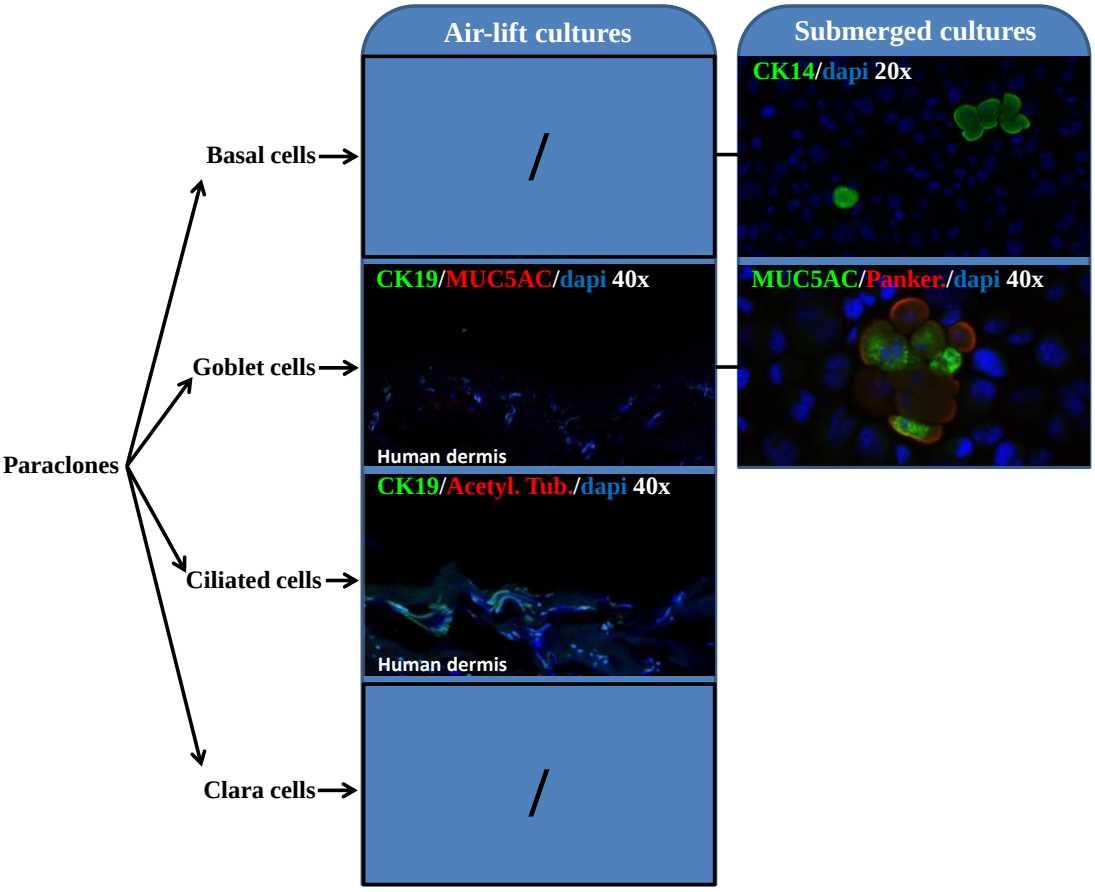


Fig. 52 Differentiation potential analysis of tracheal epithelial clones. Human dermis was used as growing supports after epidermis removal. The three quarters of each clones were plated onto dermis and cultured in air-lift condition for about 30 days. Subsequent immunofluorescence analysis showed the distinct differentiation potentials of the three clonal types.

These experiments prove the multipotency of human tracheal epithelial holoclones. Moreover, considering the previously described tracheal epithelial holoclones properties such as the highest proliferative/clonogenic potential, the percentages of p63 bright cells and the highest expression levels of stem cells markers (p63, Bmi1 and Sox2), these data support the stem cells property of human tracheal epithelial holoclones.

Tracheal epithelial clones cellular composition.

According to several studies on human and animal airway epithelial stem cells, basal cells of respiratory pseudostratified epithelium are considered as the stem cells of tracheobronchial region due to their regenerative and differentiation potential showed after airway epithelium injury.

However, also Clara cells are able to regenerate damaged respiratory epithelium and are considered as another pool of progenitor cells.

To better understand the role of the different respiratory cell progenitors, 10 tracheal clones were analyzed for their cellular composition. Three quarters of each clone were divided into two chamber-slide, were fixed 3h later to allow cells adhesion and were stained with antibodies against CK14, MUC5AC or Uteroglobin to evaluate the presence of basal cells, goblet cells and Clara cells in holoclones, meroclones and paraclones.

This experiment showed that all tracheal clone types were originally composed by basal cells only, highlighting the regenerative properties, typical of stem cells, in basal cells of the upper respiratory tract (Fig. 53).

Clones type	Basal cells	Clara cells	Goblet cells
Holoclones	OK	-	-
Meroclones	OK	-	-
Paraclones	Ok	-	-

Fig. 53 Early cellular composition of human tracheal epithelial clones. Holoclone, meroclones and paraclones contained basal CK14 positive cells and no one goblet cells or Clara cells.

To characterize basal cells of the different tracheal clonal types, additional 12 tracheal clones were analyzed for their basal cells composition (evaluating a total number of 22 clones) and the observed differences between holoclone, meroclones and paraclones concerned with basal cell number and size within each clonal type (Fig. 54).

Holoclone was the type of clone containing the greater number of basal cells compared to meroclones and paraclones (Fig. 54).

Moreover, basal cell size analysis showed that holoclone and meroclones were composed by basal cells with similar cell diameter (within 18µm) whereas paraclones basal cells were larger (over 22µm).

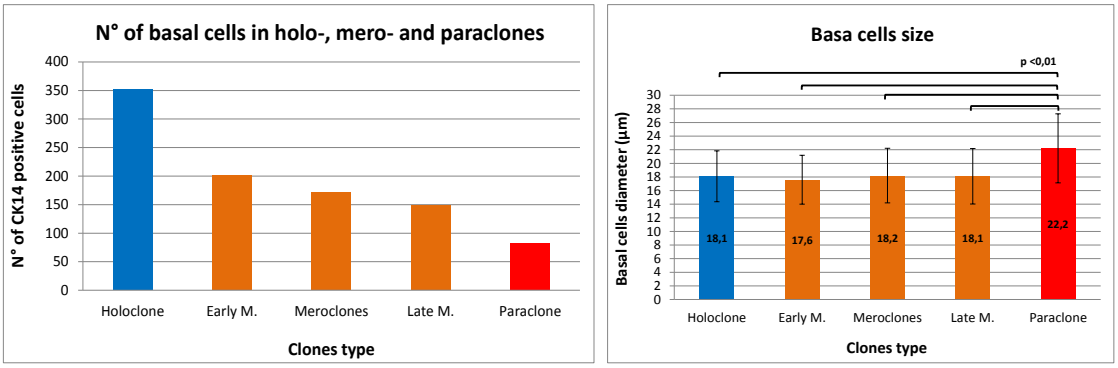


Fig. 54 Basal cells number and size characterization in tracheal holoclone, meroclones and paraclones. Basal cells diameter was similar in holoclone and meroclones, whereas paraclones CK14 positive cells were larger.

In depth analysis showed that holoclone and meroclones were composed preferentially by small basal cells (diameter < 20µm), whereas paraclones contained an higher percentage of large CK14 positive basal cells (Fig. 55).

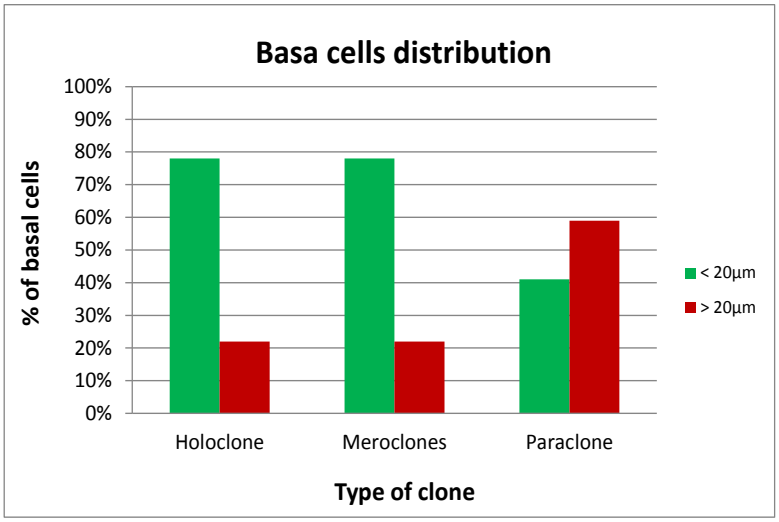


Fig. 55 Holoclone, meroclones and paraclones basal cells distribution according to cell diameter. Holoclone and meroclones were characterized by the similar high percentage of small basal cells (almost 80% of basal cells had a diameter < 20µm), whereas paraclones contained more large basal cells (59%) than small CK14 positive cells (41%).

Further experiments are needed to confirm the role of basal cells as the stem cells of human tracheal epithelium, however these preliminary data show that basal cells are the primary cell type of human tracheal holoclone.

The presence of some double positive CK14/MUC5AC and CK14/Uteroglobin cells suggests that basal cells may be able to undergo a transitional differentiation stage before generation of goblet cells and Clara cells (Fig. 56).

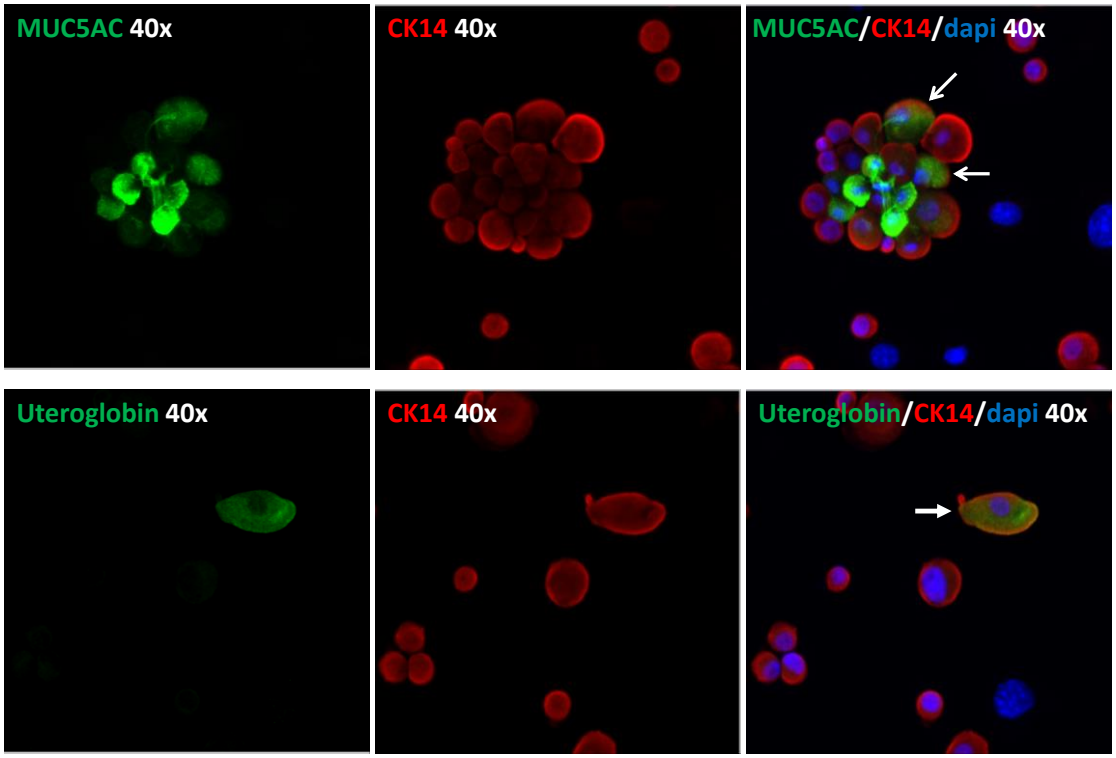


Fig. 56 Differentiation capability of tracheal epithelial basal cells. A defined number of cultured tracheal epithelial cells was placed by cytospin onto glass slide and stained with antibody against CK14, MUC5AC and Uteroglobin markers. As indicated by arrows, immunofluorescence analysis revealed the presence of some double positive CK14/MUC5AC and CK14/Uteroglobin cells identifiable as transitional states of basal cells differentiation process.

DISCUSSION

This study showed that airway epithelial cells can be cultured in vitro, maintaining their stem cells and producing all differentiation lineages. These findings suggest that a stem cells based tissue engineered approach is feasible for the treatment of some specific human airway diseases.

Human tracheal narrowing is a pathological condition caused by several factors including chronic obstructive pulmonary disease, tracheal trauma after intubation, congenital stenosis, chronic inflammation, infections and tracheal tumors.
Tracheal cancers are uncommon neoplastic lesions with an high mortality rate due to their early non-specific symptoms that lead to a delayed diagnosis so that most patients present with local inoperable tracheal disease and are, therefore, treated palliatively. The prognosis of these patients is very poor, with a reported 5-year survival rate of about 5% [15].

To overcome these problems and guarantee a safe reconstruction of human trachea, several tissue engineering strategies have been clinically applied. These tissue engineering approaches used both natural and synthetic scaffolds to replace pathological or congenital stenotic regions of human proximal airway, and different scaffold re-population protocols were applied. However, the observed airway epithelium regeneration was slow and ineffective, compromising the functional recovery of the transplanted bioengineered airway.

The follow-up results of recent clinical transplantations of bioengineered human airway [12, 14, 15, 16] confirmed the extremely important role of a functional respiratory epithelium in preventing infections, fibrotic reactions and stenosis of the transplanted bioengineered airway.

Airway epithelium function and maintenance depend, in turn, by the presence of a stem cells population able to self-renew and differentiate in all specialized epithelial cell types of the respiratory region of origin. Therefore, human airway epithelial stem cells characterization and the identification of a culture system able to preserve their proliferative and differentiation potential appear to be essential to ensure the presence of respiratory epithelial stem cells in the bioengineered human airway and its long-term maintenance after clinical transplantation.

Current knowledge on human airway stem cells is based on in-vitro studies of human cell cultures and indirectly on in-vivo studies of airway animal models.

These studies have demonstrated that in-vitro cultured human airway basal cells are endowed with multipotent differentiation potential [42] and similar results were reported by lineage tracing experiments on murine models [26]. However, animal injury studies have also showed Clara cells as an alternative progenitor cells population and considering the differences among the species, even the role of basal cells as human airway stem cells remain unclear.

This study showed the identification of human airway epithelial stem cells and the characterization of their proliferative and differentiation potential.

The preliminary studies were performed on a porcine animal model and on commercially available human bronchial epithelial cells (NHBE).

The analysis of porcine in-vivo tracheal sections allowed the localization of some airway basal (CK5, p75, TrkA) and sopra-basal differentiation markers (MUC5AC, acetylated tubulin, CK7, ZO1), and the differences with other respiratory tracts such as nasal epithelium were evaluated. The subsequent isolation of tracheal and bronchial epithelial cells from porcine biopsies and their in-vitro cultivation, revealed that both tissues contained airway epithelial cells endowed with similar proliferative and multipotent differentiating potential. In air-lift culture condition, tracheal and bronchial epithelial cells generated basal cells, goblet cells and ciliated cells, suggesting the existence of a common progenitor cells population. These data showed that airway epithelial cells can be easily isolated from respiratory biopsies and cultured in-vitro maintaining their proliferative and differentiation potential.

The next studies on NHBE cells compared two culture conditions on airway epithelial stem cells maintenance as well as their proliferative and differentiation potential. The expression of proliferation and stem/progenitor cell markers (p63, Bmi1 and ki67) was similar among the two culture systems, however in presence of a 3T3-J2 cells feeder-layer and KC culture medium, bronchial epithelial cells generated large well organized colonies and expressed differentiation markers (ZO1, involucrin, MUC5AC), suggesting that airway epithelial cells cultivated with BEGM were unable to form a normal epithelial tissue architecture.

The observed differences were confirmed when human tracheal epithelial cells were cultured in the two culture conditions and their clonogenic and proliferative potentials were evaluated. Indeed the presence of feeder-layer and KC culture medium preserved clonogenic and proliferative potential of tracheal epithelial cells. In this culture system, airway epithelial cells had high growth rate and were activated by a monolayer injury, leading to a rapid wound-healing process. This experiment confirmed that in presence of BEGM defined medium, human respiratory epithelial cells did not properly interact and were unable to repair the damaged epithelium.

Only the epithelial clinical grade culture condition represents, therefore, a physiological-like system where airway epithelial cells maintain the requested proliferation and migration properties. Both of these capabilities are an essential prerequisite to correctly repopulate natural/synthetic scaffolds used for bioengineered human airway generation and to repair tracheal graft epithelium damages occurring in the course of surgical procedures.

After culture condition selection, human tracheal epithelium was analyzed to evaluate cellular composition and to identify specific markers for basal cells and sopra-basal differentiated cells. Immunofluorescence analysis showed the presence of four principal cell types including basal cells, ciliated cells and secretory cells such as goblet cells and Clara cells. Moreover, basal cells were characterized by the expression of several markers including transcription factors (p63), cytokeratins (CK13, CK14, CK17) and transmembrane receptors (EGFR, p75, TrkA). The analysis of other human respiratory regions as alternative cells sources, revealed bronchial epithelium as the most similar to the tracheal one, suggesting the possibility to indifferently isolate and cultivate tracheal or bronchial epithelial cells for the upper airway tissue engineering purposes.

Tracheal epithelial cells cultures were then evaluated for their proliferative and differentiation potential. Human tracheal epithelial cells showed a proliferative potential of about 100 cell doublings in 20 consecutive cell culture passages, suggesting the presence of a self-renewing stem cells population that underwent to a physiological replicative senescence process during serial passages. Therefore, the culture system based on feeder-layer and KC allowed the maintenance of airway epithelial cell proliferative potential withouth inducing any immortalization event as indicated by the progressive reduction of clonogenic cells (CFE assay) until tracheal epithelial cells complete senescence.

Moreover, proteins analysis of serial passages confirmed the reduction of stem/progenitor cell markers (p63, Bmi1) and the increase of differentiation markers (Ck7, MUC5AC, uteroglobin, acetylated tubulin), suggesting that airway differentiated cells generation may be related to the number of cell doublings for each proliferating stem cell or transient amplifying cell.

The multipotent differentiation potential of cultured tracheal epithelial cells was finally demonstrated by cultivating them on human dermis in air-lift condition.

After 30 days of culture, the presence of a ciliated respiratory epithelium was observed, demonstrating that cultured tracheal epithelial cells had the potential to generate all the differentiated respiratory cells types previously observed in in-vivo tracheal sections including basal cells, goblet cells, ciliated cells and Clara cells.

The identification and characterization of human tracheal epithelial stem cells was then performed by clonal analysis. As previously reported for others human epithelia [50, 59], also tracheal epithelial cells generated the three clonal types referred to as holoclones, meroclones and paraclones. Holoclones stem-cells identity was defined by evaluation of several parameters including proliferation potential, clonogenicity, stem cell markers expression and multipotent differentiation potential.

Holoclones, meroclones and paraclones derived-cells analysis, revealed holoclones cells as those characterized by the highest percentage of small p63 bright cells, which in turn may be considered as putative tracheal stem cells for their small size and higher expression levels of the transcription factor p63, a well-known marker of proliferation potential.

The comparison of stem cells markers expression between the three clonal types, showed p63 (4A4) and Sox2 preferentially expressed in holoclones cells with average intensity levels in early meroclones, whereas these two transcription factors were poorly detectable in meroclones and absent in late meroclones and paraclones. The higher expression of these stem cells markers in holoclones rather than meroclones and paraclones, reinforced the hypothesis of tracheal epithelial holoclones as the stem cells population of this airway region and suggested the possibility to use p63 and Sox2 markers to identify the human tracheal epithelial stem cells.

The expression of the stem cells marker Bmi1 was also decreasing during clonal conversion from holoclones to paraclones. However, the expression of this polycomb group member remained fairly high in meroclones whereas was extremely poor in late meroclones and undetectable in paraclones. This behavior seems to confirm Bmi1 role in stem/progenitor cells maintenance by repressing replicative senescence in tracheal epithelial stem cells and youg transient amplifying cells, whereas Bmi1 expression levels drastically reduce in late meroclones and paraclones where replicative senescence process slowdown is no more needed.

Holoclones highest clonogenic/proliferative potential was further demonstrated by cultivating the three clonal types for about 30 days in submerged condition. At the end of the experiment, tracheal epithelial holoclone was the only one endowed with clonogenic/proliferative potential compared to meroclones and paraclones, confirming its stem cells content. Even in this case, Bmi1 showed to be more expressed in holoclones derived-cells, supporting its role in human tracheal epithelial stem cells maintenance. Moreover, tracheal epithelial holoclones were proven able to generate all epithelial lineages of the tissue of origin including basal cells, goblet cells, ciliated cells and Clara cells, confirming their multipotent differentiation potential.

All of these tracheal epithelial holoclones properties such as the the highest proliferative/clonogenic potential, the expression of the stem cells markers p63, Bmi1 and Sox2, and the potential to differentiate in all epithelial cell types of this respiratory region, confirmed tracheal holoclones as the stem cells of human trachea and probably of the entire tracheo-bronchial respiratory tract.

Finally, to better characterize tracheal clones forming cells identity, the three clonal types were evaluated for the presence of basal cells, goblet cells and Clara cells, revealing that tracheal epithelial holoclones, meroclones and paraclones are composed by basal cells originally. Therefore, tracheal epithelial basal cells generating holoclones in in-vitro cell culture conditions represent the stem cells population of this human respiratory region.

According to these data, it is possible to describe a lineage model where tracheal basal stem cells (endowed with the potential to differentiate into basal cells, goblet cells, Clara cells and ciliated cells) generate young transient amplifying basal cells (still able to differentiate into basal cells, goblet cells and ciliated cells), which in turn produce old transient amplifying basal cells with a limited proliferative and differentiation potential (Fig. 57).

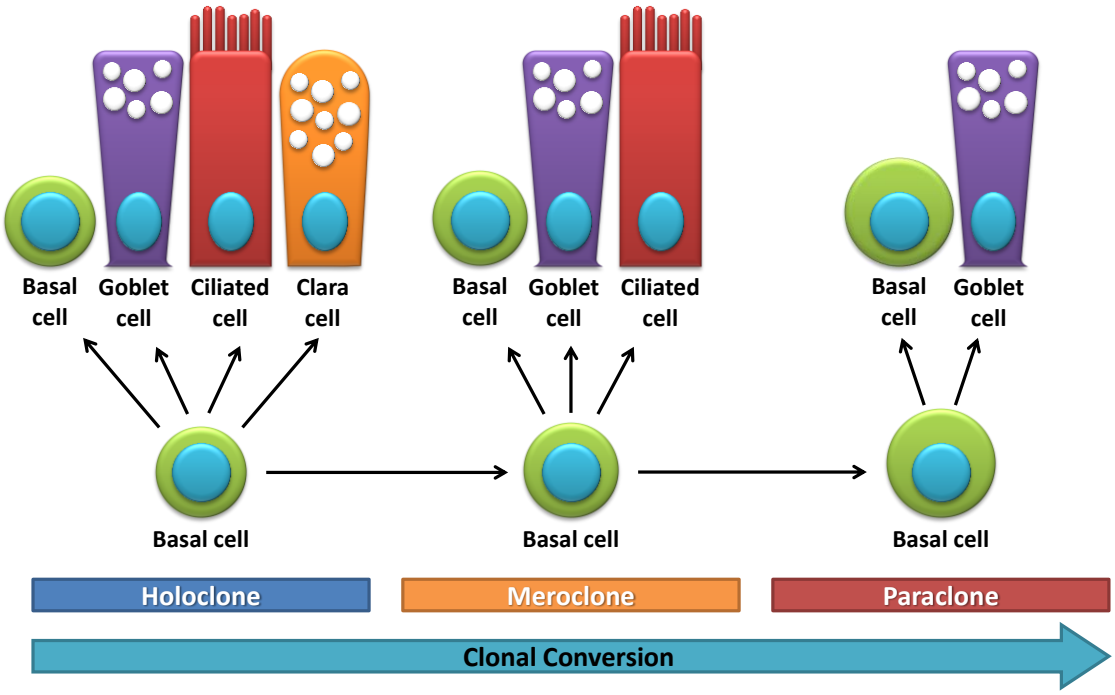


Fig. 57 Schematic representation of human tracheal epithelial cells lineage model. The three tracheal epithelial clonal types are composed by basal cells endowed with different proliferative and differentiation potential. Holoclone-forming basal cells are the stem cells characterized by the highest proliferative/clonogenic and differentiation potential. During in-vitro cell cultures or in-vivo homeostasis, stem cells generate young transient amplifying cells (referred to as meroclones), which in turn proliferate becoming old transient amplifying cells (indicated as paraclones).

Human tracheal epithelial stem cells identification in the selected culture conditions showed that airway epithelial cells can be isolated from human biopsies and ex-vivo expanded maintaining the stem cells population and their proliferative/differentiation potential. Therefore, these cells can be used to repopulate a natural/synthetic scaffold and generate a bioengineered human airway suitable for clinical transplantation in patients affected by otherwise inoperable respiratory diseases.

However, the presence of a sufficient number of stem cells represents a fundamental criteria to guarantee the long-term maintenance of the implanted graft and this explains the necessity to identify, characterize and properly quantify airway epithelial stem cells before clinical application.

Further studies are, then, needed to better characterize airway epithelial stem cells (such as the identification of airway specific stem cells markers) and to identify the more suitable scaffold to preserve the stem cells population and their proliferative and differentiation capabilities.

References

[1] Urdaneta AI, Yu JB, Wilson LD. **Population based cancer registry analysis of primary tracheal carcinoma**. Am J Clin Oncol. 2011 Feb;34(1):32-7

[2] Bhattacharyya N. **Contemporary staging and prognosis for primary tracheal malignancies: a population-based analysis.** Otolaryngol Head Neck Surg. 2004 Nov;131(5):639-42.

[3] Yang KY, Chen YM, Huang MH, Perng RP. **Revisit of primary malignant neoplasms of the trachea: clinical characteristics and survival analysis.** Jpn J Clin Oncol. 1997 Oct;27(5):305-9.

[4] Webb BD, Walsh GL, Roberts DB, Sturgis EM. **Primary tracheal malignant neoplasms: the University of Texas MD Anderson Cancer Center experience.** J Am Coll Surg. 2006 Feb;202(2):237-46. Epub 2005 Nov 21.

[5] Macchiarini P. **Primary tracheal tumours.** Lancet Oncol. 2006 Jan;7(1):83-91.

[6] Lawrence DA, Branson B, Oliva I, Rubinowitz A. **The wonderful world of the windpipe: a review of central airway anatomy and pathology.** Can Assoc Radiol J. 2015 Feb;66(1):30-43

[7] Jungebluth P, Moll G, Baiguera S, Macchiarini P. **Tissue-engineered airway: a regenerative solution.** Clin Pharmacol Ther. 2012 Jan;91(1):81-93

[8] 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. **Clinical transplantation of a tissue-engineered airway.** Lancet. 2008 Dec 13;372(9655):2023-30

[9] Bader A, Macchiarini P. **Moving towards in situ tracheal regeneration: the bionic tissue engineered transplantation approach.** J Cell Mol Med. 2010 Jul;14(7):1877-89

[10] Baiguera S, Jungebluth P, Burns A, Mavilia C, Haag J, De Coppi P, Macchiarini P. **Tissue engineered human tracheas for in vivo implantation.** Biomaterials. 2010 Dec;31(34):8931-8

[11] Haag JC, Jungebluth P, Macchiarini P. **Tracheal replacement for primary tracheal cancer.** Curr Opin Otolaryngol Head Neck Surg. 2013 Apr;21(2):171-7

[12] Gonfiotti A, Jaus MO, Barale D, Baiguera S, Comin C, Lavorini F, Fontana G, Sibila O, Rombolà G, Jungebluth P, Macchiarini P. **The first tissue-engineered airway transplantation: 5-year follow-up results.** Lancet. 2014 Jan 18; 383(9913):238-44.

[13] Elliott MJ, De Coppi P, Speggiorin S, Roebuck D, Butler CR, Samuel E, Crowley C, McLaren C, Fierens A, Vondrys D, Cochrane L, Jephson C, Janes S, Beaumont NJ, Cogan T, Bader A, Seifalian AM, Hsuan JJ, Lowdell MW, Birchall MA. **Stem-cell-based, tissue engineered tracheal replacement in a child: a 2-year follow-up study.** Lancet. 2012 Sep 15;380(9846):994-1000

[14] Hamilton NJ, Kanani M, Roebuck DJ, Hewitt RJ, Cetto R, Culme-Seymour EJ, Toll E, Bates AJ, Comerford AP, McLaren CA, Butler CR, Crowley C, McIntyre D, Sebire NJ, Janes SM, O'Callaghan C, Mason C, De Coppi P, Lowdell MW, Elliott MJ, Birchall MA. **Tissue-Engineered Tracheal Replacement in a Child: A 4-Year Follow-Up Study.** Am J Transplant. 2015 Oct;15(10):2750-7

[15] Jungebluth P, Alici E, Baiguera S, Le Blanc K, Blomberg P, Bozóky B, Crowley C, Einarsson O, Grinnemo KH, Gudbjartsson T, Le Guyader S, Henriksson G, Hermanson O, Juto JE, Leidner B, Lilja T, Liska J, Luedde T, Lundin V, Moll G, Nilsson B, Roderburg C, Strömblad S, Sutlu T, Teixeira AI, Watz E, Seifalian A, Macchiarini P. **Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study.** Lancet. 2011 Dec 10;378(9808):1997-2004

[16] Hamilton N, Bullock AJ, Macneil S, Janes SM, Birchall M. **Tissue engineering airway mucosa: a systematic review.** Laryngoscope. 2014 Apr;124(4):961-8.

[17] Jiyuan Tu, Kiao Inthavong, Goodarz Ahmadi. **The Human Respiratory System in the Computational Fluid and Particle Dynamics in the Human Respiratory System** Springer Netherlands 2013. DOI: 10.1007/978-94-007-4488-2_2

[18] Chang, M. M.-J., Shih, L. and Wu, R. **Pulmonary Epithelium: Cell Types and Functions, in The Pulmonary Epithelium in Health and Disease** (ed D. Proud), John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/9780470727010.ch1

[19] Brand-Saberi BE, Schäfer T. **Trachea: anatomy and physiology.** Thorac Surg Clin. 2014 Feb;24(1):1-5

[20] Fahy JV, Dickey BF. **Airway mucus function and dysfunction.** N Engl J Med. 2010 Dec 2;363(23):2233-47

[21] Liu X, Driskell RR, Engelhardt JF. **Stem cells in the lung.** Methods Enzymol. 2006;419:285-321.

[22] Evans MJ, Van Winkle LS, Fanucchi MV, Plopper CG. **Cellular and molecular characteristics of basal cells in airway epithelium.** Exp Lung Res. 2001 Jul-Aug;27(5):401-15.

[23] Rock JR, Randell SH, Hogan BL. **Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling.** Dis Model Mech. 2010 Sep-Oct;3(9-10):545-56

[24] Daniely Y, Liao G, Dixon D, Linnoila RI, Lori A, Randell SH, Oren M, Jetten AM. **Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium.** Am J Physiol Cell Physiol. 2004 Jul;287(1):C171-81.

[25] Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. **In vivo differentiation potential of tracheal basal cells: evidence for multipotent and unipotent subpopulations.** Am J Physiol Lung Cell Mol Physiol. 2004 Apr;286(4):L643-9. Epub 2003 Jul 18.

[26] Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. **Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium.** Am J Pathol. 2004 Feb;164(2):577-88.

[27] Knight DA, Holgate ST. **The airway epithelium: structural and functional properties in health and disease.** Respirology. 2003 Dec;8(4):432-46.

[28] Rawlins EL, Hogan BL. **Ciliated epithelial cell lifespan in the mouse trachea and lung.** Am J Physiol Lung Cell Mol Physiol. 2008 Jul;295(1):L231-4

[29] Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR. **Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion.** Am J Respir Cell Mol Biol. 2001 Jun;24(6):671-81.

[30] Sbarbati A, Osculati F. **A new fate for old cells: brush cells and related elements.** J Anat. 2005 Apr;206(4):349-58.

[31] Borthwick DW, Shahbazian M, Krantz QT, Dorin JR, Randell SH. **Evidence for stem-cell niches in the tracheal epithelium.** Am J Respir Cell Mol Biol. 2001 Jun;24(6):662-70.

[32] Engelhardt JF. **Stem cell niches in the mouse airway.** Am J Respir Cell Mol Biol. 2001 Jun;24(6):649-52.

[33] Reynolds SD, Malkinson AM. **Clara cell: progenitor for the bronchiolar epithelium.** Int J Biochem Cell Biol. 2010 Jan;42(1):1-4

[34] Giangreco A, Reynolds SD, Stripp BR. **Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction.** Am J Pathol. 2002 Jul;161(1):173-82.

[35] Ganesan S, Comstock AT, Sajjan US. **Barrier function of airway tract epithelium.** Tissue Barriers. 2013 Oct 1;1(4):e24997

[36] Vareille M, Kieninger E, Edwards MR, Regamey N. **The airway epithelium: soldier in the fight against respiratory viruses.** Clin Microbiol Rev. 2011 Jan;24(1):210-29

[37] Pellegrini G, Rama P, Mavilio F, De Luca M. **Epithelial stem cells in corneal regeneration and epidermal gene therapy.** J Pathol. 2009 Jan;217(2):217-28

[38] Barbaro V, Testa A, Di Iorio E, Mavilio F, Pellegrini G, De Luca M. **C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells.** J Cell Biol. 2007 Jun 18;177(6):1037-49. Epub 2007 Jun 11.

[39] Engelhardt JF, Schlossberg H, Yankaskas JR, Dudus L. **Progenitor cells of the adult human airway involved in submucosal gland development.** Development. 1995 Jul;121(7):2031-46.

[40] Dupuit F, Gaillard D, Hinnrasky J, Mongodin E, de Bentzmann S, Copreni E, Puchelle E. **Differentiated and functional human airway epithelium regeneration in tracheal xenografts.** Am J Physiol Lung Cell Mol Physiol. 2000 Jan;278(1):L165-76.

[41] Rawlins EL, Hogan BL. **Epithelial stem cells of the lung: privileged few or opportunities for many?** Development. 2006 Jul;133(13):2455-65. Epub 2006 May 30.

[42] Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, Hogan BL. **Basal cells as stem cells of the mouse trachea and human airway epithelium.** Proc Natl Acad Sci U S A. 2009 Aug 4;106(31):12771-5.

[43] Snyder JC, Teisanu RM, Stripp BR. **Endogenous lung stem cells and contribution to disease.** J Pathol. 2009 Jan;217(2):254-64

[44] Wansleeben C, Barkauskas CE, Rock JR, Hogan BL. **Stem cells of the adult lung: their development and role in homeostasis, regeneration, and disease.** Wiley Interdiscip Rev Dev Biol. 2013 Jan-Feb;2(1):131-48

[45] Rawlins EL, Okubo T, Xue Y, Brass DM, Auten RL, Hasegawa H, Wang F, Hogan BL. **The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium.** Cell Stem Cell. 2009 Jun 5;4(6):525-34

[46] Reynolds SD, Giangreco A, Power JH, Stripp BR. **Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration.** Am J Pathol. 2000 Jan;156(1):269-78.

[47] Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. **Identification of bronchioalveolar stem cells in normal lung and lung cancer.** Cell. 2005 Jun 17;121(6):823-35.

[48] Barrandon Y, Green H. **Three clonal types of keratinocyte with different capacities for multiplication.** Proc Natl Acad Sci U S A. 1987 Apr;84(8):2302-6.

[49] Pellegrini G, Rama P, De Luca M. **Vision from the right stem.** Trends Mol Med. 2011 Jan;17(1):1-7

[50] Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M. **p63 identifies keratinocyte stem cells.** Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3156-61.

[51] Daniely Y, Liao G, Dixon D, Linnoila RI, Lori A, Randell SH, Oren M, Jetten AM. **Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium.** Am J Physiol Cell Physiol. 2004 Jul;287(1):C171-81.

[52] Dovey JS, Zacharek SJ, Kim CF, Lees JA. **Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion.** Proc Natl Acad Sci U S A. 2008 Aug 19;105(33):11857-62

[53] Park IK, Morrison SJ, Clarke MF. **Bmi1, stem cells, and senescence regulation.** J Clin Invest. 2004 Jan;113(2):175-9.

[54] Gontan C, de Munck A, Vermeij M, Grosveld F, Tibboel D, Rottier R. **Sox2 is important for two crucial processes in lung development: branching morphogenesis and epithelial cell differentiation.** Dev Biol. 2008 May 1;317(1):296-309

[55] Tompkins DH, Besnard V, Lange AW, Wert SE, Keiser AR, Smith AN, Lang R, Whitsett JA. **Sox2 is required for maintenance and differentiation of bronchiolar Clara, ciliated, and goblet cells.** PLoS One. 2009 Dec 14;4(12):e8248

[56] Scribner JD, Slaga TJ. **Multiple effects of dexamethasone on protein synthesis and hyperplasia caused by a tumor promoter.** Cancer Res. 1973 Mar;33(3):542-6.

[57] Di Iorio E, Barbaro V, Ferrari S, Ortolani C, De Luca M, Pellegrini G. **Q-FIHC: quantification of fluorescence immunohistochemistry to analyse p63 isoforms and cell cycle phases in human limbal stem cells.** Microsc Res Tech. 2006 Dec;69(12):983-91.

[58] Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M. **Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface.** J Cell Biol. 1999 May 17;145(4):769-82.

[59] Corradini F, Zattoni M, Barbagli G, Bianchi G, Giovanardi M, Serafini C, Genna V, Ribbene A, Balò S, Fidanza F, Lazzeri M, De Luca M, Pellegrini G. **Comparative Assessment Of Cultures From Oral And Urethral Stem Cells For Urethral Regeneration.** Curr Stem Cell Res Ther. 2015 Sep 1

RINGRAZIAMENTI

Un ringraziamento speciale va alla Prof.ssa Graziella Pellegrini che mi ha dato la possibilità di partecipare ad un progetto estremamente interessante e che insieme al Prof. Michele De Luca mi ha consentito di lavorare in un ambiente scientificamente stimolante quale è il Centro di Medicina Rigenerativa S. Ferrari.

Desidero ringraziare Sonia ed Eustachio per aver partecipato al progetto.
Francesca, Michela, Laura e Athanasios per i consigli che hanno saputo darmi in questi anni.
I dottorandi che mi hanno affiancato in questo percorso tra i quali la mia collega Roza.
Chiara C. che, anche se arrivata da poco in laboratorio, si è subito dimostrata di grande aiuto.
Il mio vicino di scrivania Michael e tutti i colleghi che mi hanno supportato durante questo percorso.

Infine, ringrazio i miei genitori che hanno sempre creduto in me, mia moglie Viviana per l'amore e la pazienza con cui mi ha sostenuto in questi anni e la mia bambina Sofia che con i suoi sorrisi riempie di gioia le mie giornate.