UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Medicina Sperimentale e Molecolare

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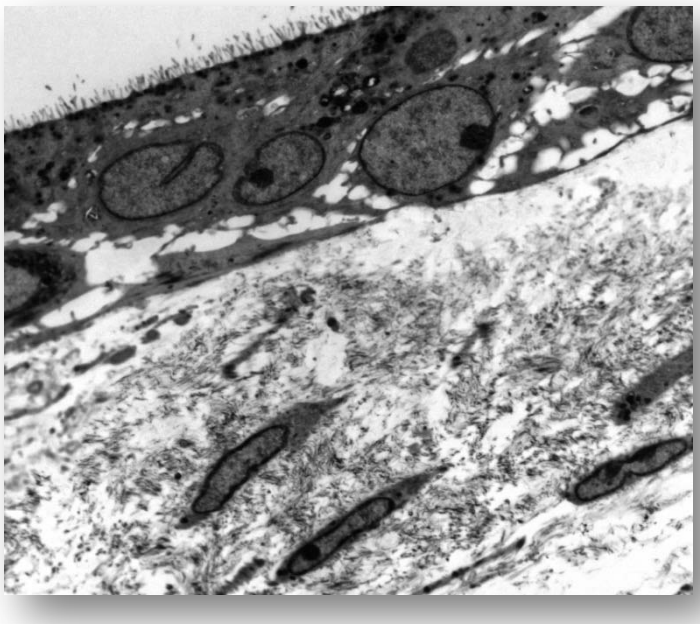
Effects of cigarette smoke in tissue-engineered human bronchial mucosa: new insights on COPD pathogenesis.



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1. INTRODUCTION

1.1 The Human Respiratory System

The Human respiratory system includes the lungs, several structures in the chest involved with moving air in and out of the lungs and pathways connecting them to the outside environment.

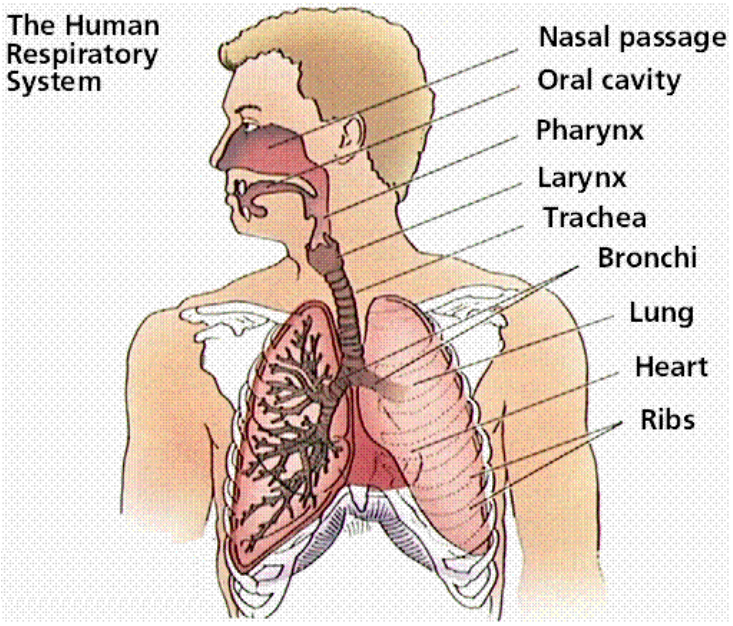
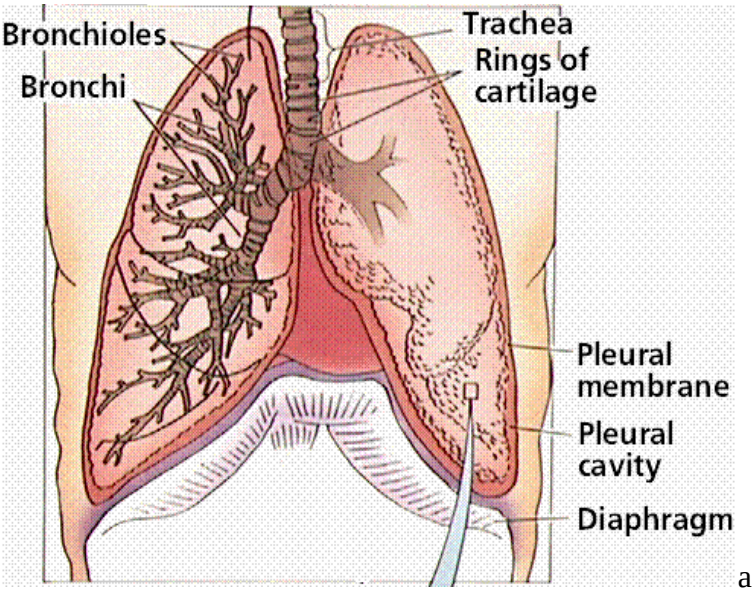


Figure 1.1. The human respiratory system

During the breathing process, air enters the body through the nose, is warmed, filtered, and passed through the nasal cavity. Air then passes the pharynx which has the epiglottis that prevents food from entering the trachea. The upper part of the trachea contains the larynx. The vocal cords are two bands of tissue that extend across the opening of the larynx. After passing the larynx, the air moves into the bronchi that carry air in and out of the lungs.



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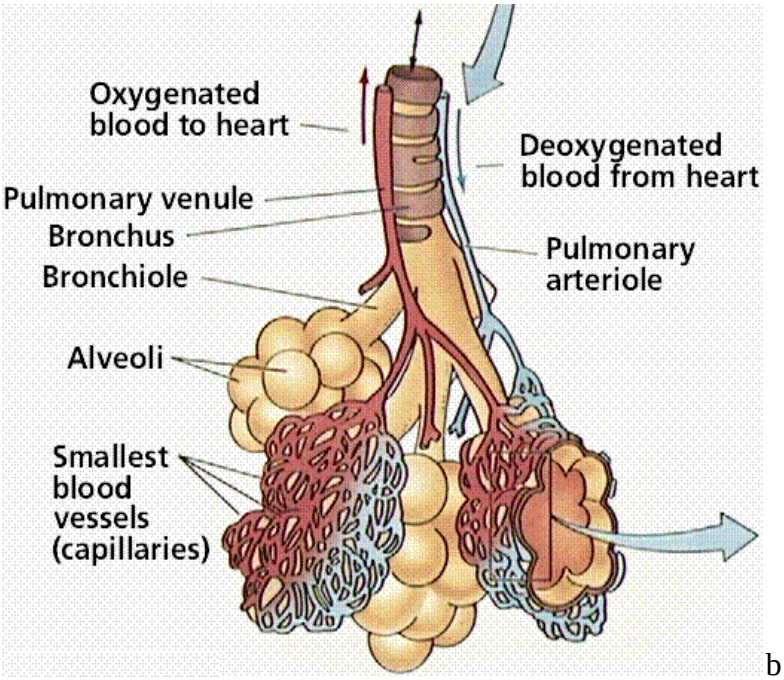

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Figure 1.2. Lungs (a) and alveoli (b), and their relationship with the diaphragm muscle and capillaries.

Bronchi are reinforced to prevent their collapse, and lined with ciliated epithelium and mucus-producing cells. Bronchi branch into smaller and smaller tubes known as bronchioles. Bronchioles terminate in grape-like sac clusters known as alveoli. These are surrounded by a network of thin-walled capillaries. Only about 0.2 μm separate the alveoli from the capillaries due to the extremely thin walls of both structures.

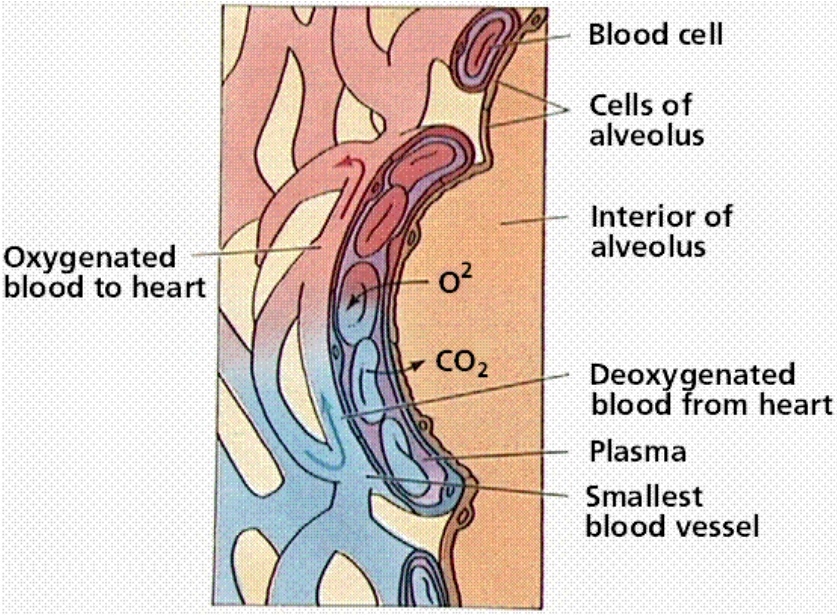
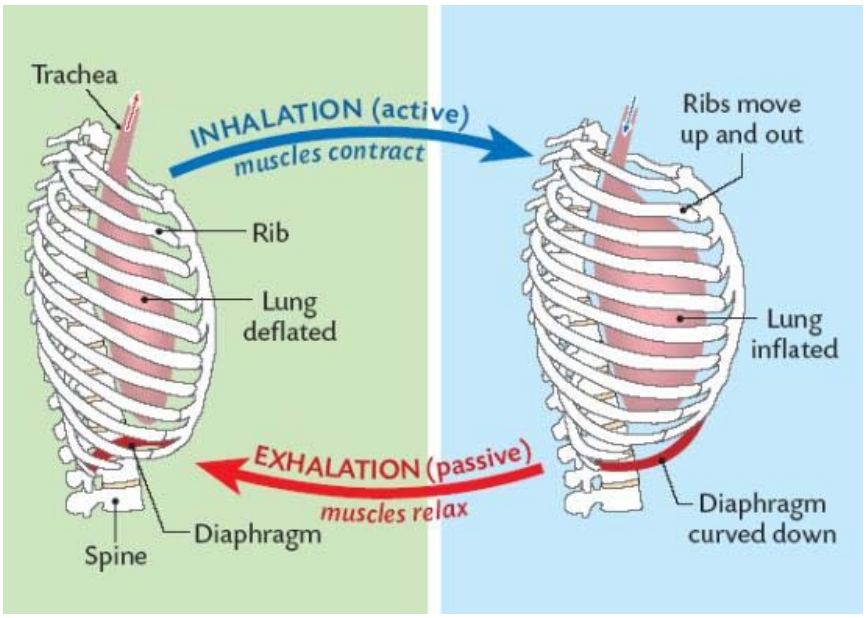


Figure 1.3. Gas exchange across capillary and alveolus walls.

The lungs are large, lobed, paired organs in the chest, essential for the respiration. Their main function is to carry atmospheric oxygen to the blood, and to expel carbon dioxide to the atmosphere. Thin sheets of epithelial pleura separate the inside of the chest cavity from the outer surface of the lungs. The lower part of the thoracic cavity is formed by the diaphragm. The term ventilation is used to describe the mechanics of breathing in and out. When you inhale, muscles in the chest wall contract, lifting the ribs and pulling them

outward, while the diaphragm moves downward, enlarging the chest cavity. Reduced air pressure in the lungs causes air to enter the lungs. Exhaling reverses these steps.

Figure 1.4. Inhalation and exhalation mechanics.



1.2. Microscopic anatomy of the lungs

1.2.1. Airways

The trachea bifurcates at the level of the carina giving rise to two branches; these are the main bronchi, one for the right and one for the left lung, that, in turn, are divided into two secondary or lobar bronchi in the left lung and three secondary bronchi in the right lung. Lobar bronchi divide into several tertiary or segmental bronchi, and bronchioles are further divided into primary and then in the terminal bronchioles, and, finally, into respiratory bronchioles. The primary structure of the bronchi is similar to that of the trachea: their walls are formed by the superposition, proceeding from the inside towards the outside, of a

thin tunica submucosa, a muscular coat and a fibrous tunic. The epithelium of the bronchus is pseudostratified; this type of epithelium linens most of the respiratory tract. It is supported by a basement membrane with different types of cells, including ciliated columnar cells, muciparous goblet cells, serous cells, basal cells that are part of the diffuse neuroendocrine system, and stem cells that are able to divide asymmetrically to give rise to cells capable of differentiating into other cell types of the epithelial lining. While the bronchial diameter decreases, the structure of the respiratory tree changes: the epithelium becomes more and more cubic, with loss of cilia and scarce goblet cells, and then the alveoli become flattened.

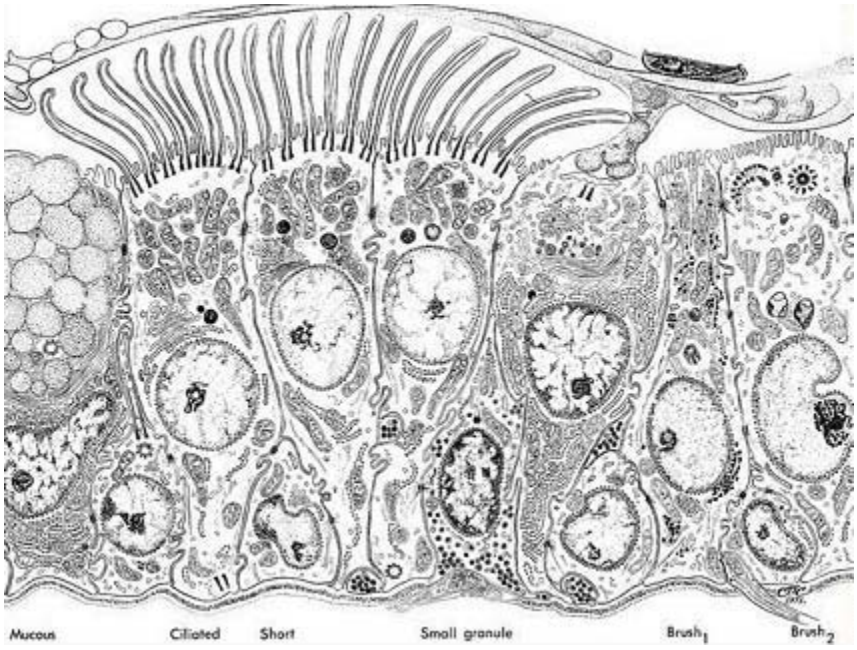


Figure 1.5 Illustration of normal human bronchial epithelium

1.2.2. Parenchyma

The human lung is a parenchymal organ that derives from the branching of a hollow structure, the main bronchus. The morphofunctional units of the lung from an anatomical point of view are the pulmonary lobules. These lobules are bound by septa of connective tissue (interlobular septa) which are in continuity with the sub-mesothelial connective layer of the visceral pleura, and are macroscopically visible on the external surface of the organ, as well as on the inner surface of the parenchyma (1). Pulmonary lobules derive from the smallest parts of conducting airways, called bronchioles, characterised by the absence of cartilage in their walls and a diameter smaller than 1 mm. Pulmonary lobules are constituted by: (a) one terminal bronchiole, from which derive (b) 3-6 respiratory bronchioles, each one supplying (c)have a diameter of 2 cm and 3-5 alveolar sacs (2) Each respiratory bronchiole with its alveolar sacs is described as an *acinus*, the functional subunit of the pulmonary lobules. Indeed, the terminal bronchioles are not involved in hematosis, because of their lack of alveoli that, *vice versa*, delimit the respiratory bronchioles, alveolar ducts and alveolar sacs. Terminal bronchioles, respiratory bronchioles, alveolar ducts and alveolar sacs develop around the 16th to 28th week of gestation from the expansion and branching of major airways (3). As a consequence, their structure resembles that of the other parts of the lower airways: they have a wall comprised of three layers: (a) an internal mucosa; (b) an intermediate submucosa; and (c) an external adventitia (4). The epithelium is composed of several different cytotypes, whose structural and functional features are described in Table 1. Between the epithelium and the *lamina propria* there is a thin basal membrane, formed by a *lamina basalis* and a *lamina reticularis*; these two *laminae* have a different proteins composition, the *basalis* being synthesised from epithelial elements and the *reticularis* from the connective ones (1). The *lamina propria* is composed of loose connective tissue, and it contains smooth muscle

cells, myofibroblasts, fibrocytes, macrophages, lymphoid cells, mast cells, endothelial cells of hematic and lymphatic capillaries and nerve fibres; a pool of spindle cells are indicated as fibroblasts, but a part of them are probably a heterogeneous population of otherwise non-specifiable cells (4) Smooth muscle cells are intimately associated with numerous elastic fibers that, together with a small amount of reticular and collagenous fibres, form the feltwork of *lamina propria*. The structure of the alveoli greatly differs from that of the bronchioles. They are delimited by fibroelastic septa (interalveolar septa), that derive from the interlobular septa; nevertheless, they communicate among themselves *via* pores of Kohn (Fig. **1.5**). The alveoli are bordered by both squamous (Type I pneumocytes, TIPs) and cuboidal (Type II pneumocytes, TIIPs) epithelial cells (5) The former are large and flat cells, functionally involved in gas exchange, that cover more than 90% of the alveolar surface; they contribute to form the so-called “air/blood barrier” together with capillary endothelial cells and each own LB (6).

Cytotype	Ultrastructural Features	Functions	Other Putative Roles	Comments
Ciliated cells	Cuboidal, each cell has approximately 250 cilia; each cilium is approximately 6 um long	Transport of mucus stream	Unknown	The most prevalent cytotype; they decrease during chronic inflammation
Clara' cells	Cuboidal/columnar non ciliated, non-mucus secreting cells. Granules are present in apical cytoplasm	Secretory function contributing to the cleaning of smallest airways	Progenitors of other cells (Type II Pneumocytes ?); role in surfactant production	The second most prevalent cytotype; they augment during chronic inflammation
Basal cells	Small round cells with scarce cytoplasm, close to the basal membrane	Precursors of other cytotypes	Stem cells	They are rare in bronchioles; we do not know yet which cytotypes they originate; they are also involved in carcinogenesis
Neuroendocrine (Kulchitsky) cells	Small round cells with numerous secretory granules	Part of diffuse neuroendocrine system	Unknown	They are rare in bronchioles; they may be present single or in small groups; they may originate microcytoma
Goblet cells	Cuboidal/columnar mucus secreting cells	Secretory function contributing to the cleaningof smallest airways	Unknown	They are rare in bronchioles; they augment during chronic inflammation
Lymphocytes	Small round cells with scarce cytoplasm, scattered among the other cytotypes or adjacent to the luminal surface of epithelium	Immune surveillance	Unknown	They are rare in bronchioles; they augment during chronic inflammation

Table 1. Main morphofunctional features of the bronchiolar epithelial cell types and their pathophysiologic roles (Bucchieri et al., 2009)

1.3. Embryogenesis of the lung

The development of the lungs, in comparison with the development of other organs during the prenatal period, occupies a special position. The reason for this is because breathing organs are unnecessary for intrauterine existence. Nevertheless they must develop and be ready to function immediately following birth. This explains why the entire development of this organ extends from the embryonic period through the fetal period up to birth. However, during the intrauterine life, the lungs are an important source of amniotic fluid, which is “inhaled” and “exhaled” by the fetus. It is essential for this fluid to be breathed into the lungs in order for them to develop normally. This fluid is also very important for several other reasons such as mechanic protection of the fetus and as a source of proteins, carbohydrates, lipid and phospholipids, urea and electrolytes which all contribute to the growth of fetus. The lung development is divided in five phases: the embryonic phase (3^{rd} week-8^{th} week), the pseudoglandular phase (8^{th} week-16^{th} week), the canalicular phase(16^{th} week-24^{th} week), the saccular phase (24^{th}week-36^{th} week) and the alveolar phase (36^{th} week- 1,5 years after birth). The embryonic phase starts with the formation of a groove in the ventral lower pharynx, the sulcus laryngotrachealis. From the lower part of this sulcus, the true lung primordium will form, and its further division will form the main bronchi with the asymmetry they present in adults. This will start the subdivision of lobes and it will also form the pulmonary vessels. During the pseudoglandular phase, the development of the entire bronchial tree up to the terminal bronchiole occurs, which means that at this point the respiratory ducts will have already been formed. The ducts are coated by cuboidal epithelial cells which are the precursors cell of ciliated epithelium and secretory cells. After the 10 week, cartilage and smooth muscle cells as well as bronchial glands can be found in the wall of bronchi. Also during this phase, the lung begins cyclical contraction at a rate of approximately 1 contraction for second. The canalicular phase is characterized by

the formation of the lung acinus, the invasion of capillaries into the mesenchyme, the differentiation of the epithelial cells that start the production of the amniotic fluid and surfactant. Between the 20th week and 22nd week the epithelial cells start the differentiation into type I and type II pneumocytes. The saccular and alveolar phases are characterized by the final development of the alveolus, which is now able to perform the gas exchange, and by the specialization of the II type of pneumocytes which are able to produce the mature surfactant. The surfactant consist of glycerophospholipids, specific proteins, neutral fats and cholesterol. It covers the alveolar surface and reduces the surface tension, meaning that it prevents collapse of the alveoli during the expiration

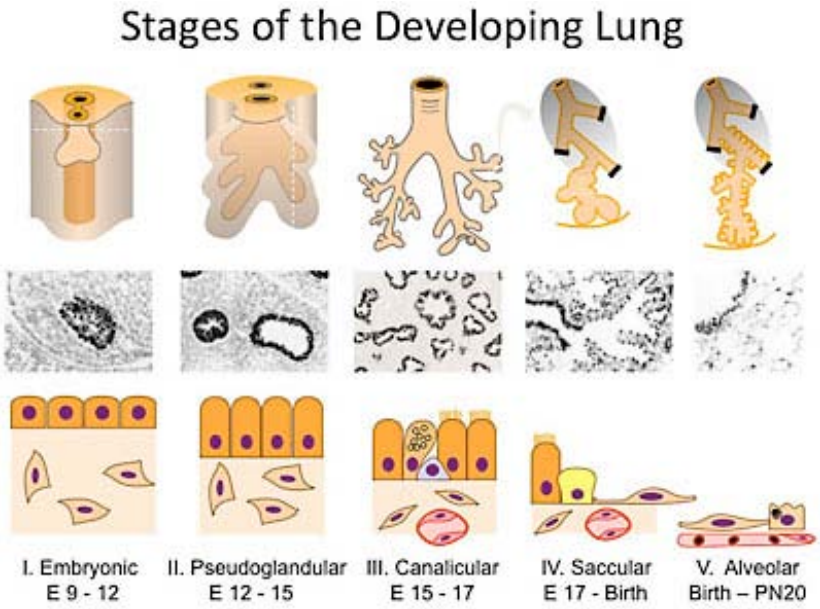


Figure 1.6: Human lungs development.

1.4. Epithelial-Mesenchymal Trophic Unit

The anatomic and functional relationship between the attenuated fibroblast sheath and epithelial tissue is called Epithelial-Mesenchymal Tropic Unit (EMTU). The area between these two cell layers, the basement membrane zone, contains extracellular matrix and a network of nerve fibres. The concept of EMTU describes the signalling between epithelial cells and the underlying fibroblasts which are in close physical contact with the epithelial layer. This interaction is necessary to initiate numerous cellular functions of the lung, such as differentiation during lung growth, repair of damaged tissue and regulation of the inflammatory response (7). These changes alter the function of the epithelium and its ability to communicate with the underlying mesenchymal cells to provide an appropriate microenvironment for promoting tissue remodeling and for sustaining the persistent inflammatory responses characteristic of chronic COPD. Cytokines and chemokines are produced by Fibroblasts in response to various stimuli and their fixed position in the tissue suggests that they can respond in a local manner to bacterial products, tissue injury, or other environmental factors. Under normal conditions, the epithelium of the airway that has lost cuboidal/columnar cells may repair itself with remarkable speed. Indeed, the basal cells, which are more firmly attached to the underlying basal membrane, *via* hemidesmosomes, progressively restore the normal epithelium by a process of cell proliferation and differentiation (8). Although it is commonly believed that frequent episodes of disepithelisation and re-epithelisation, i.e. following chronic infective/inflammatory diseases, are the basis of carcinogenesis in some anatomical regions (like uterine exocervix, stomach, liver, etc), since they increase the possibility of DNA mutations, we want to state that bronchial carcinogenesis is an extremely rare complication of COPD. This fact may indicate that 1) other events besides basal cell mutation are necessary for cancer development in airway epithelium and/or that 2) the DNA repair mechanisms are more efficient in airway epithelium. In addition, we do not

have sufficient knowledge on EMTU biology, specifically regarding stem cell (SC) niches. For example, basal cells of the epithelium are commonly considered as a population of SCs, but the underlying connective tissue lacks the well-recognized stem phenotype. It is widely recognised that the epithelium and the mesenchyme cooperate in foetal lung development through exchange of soluble mediators; this cooperation plays a pivotal role in airway growth and branching. (9). Furthermore, it is well known that epithelial elements and subepithelial mesenchymal cells also interact in adult tissue *via* autacoid mediators, cytokines and growth factors (9). The epithelial-mesenchymal tropic unit functions during development and is crucial to the process of branching morphogenesis. It is suggested that similar processes are involved in the structural alterations of airways in asthma(10) .

1.5 Chronic Ostructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a heterogeneous group of slowly progressive diseases characterized by airflow limitation and gradual loss of lung function that is not fully reversible.(11). Also known as chronic obstructive lung disease (COLD), chronic obstructive airway disease (COAD), chronic airflow limitation (CAL) and chronic obstructive respiratory disease (CORD), is the co-occurrence of chronic bronchitis and emphysema, a pair of commonly co-existing diseases of the lungs in which the airways become narrowed.(12) This leads to a limitation of the flow of air to and from the lungs, causing shortness of breath. In clinical practice, COPD is defined by its characteristically low airflow on lung function tests.(13) In contrast to asthma, this limitation is poorly reversible and usually gets progressively worse over time. In England, an estimated 842,100 out of 50 million people have a diagnosis of COPD; thus, approximately 1 person in 59 is diagnosed with COPD at some point in their lives.(14) The COPD average attributable excess health care expenditures are nearly US$6,300 per Medicare patient.(15-17) A broader view of the causes of population health disparities including race, ethnicity,

socioeconomic status, and geography is necessary for better solutions to complex population health problems. The main cause for development of COPD is noxious particles or gas, most commonly from tobacco smoking, which triggers an abnormal inflammatory response in the lung.(4-5) The inflammatory response in the larger airways is known as chronic bronchitis, which is diagnosed clinically when people regularly cough up sputum. In the alveoli, the inflammatory response causes destruction of the tissues of the lung, a process known as emphysema. The natural course of COPD is characterized by occasional sudden worsenings of symptoms called acute exacerbations, most of which are caused by infections or air pollution.

1.5.1 Chronic bronchitis

Lung damage and inflammation in the large airways results in chronic bronchitis. Chronic bronchitis is defined in clinical terms as a cough with sputum production on most days for 3 months of a year, for 2 consecutive years.(18) In the airways of the lung, the hallmark of chronic bronchitis is an increased number (hyperplasia) and increased size (hypertrophy) of the goblet cells and mucous glands of the airway. As a result, there is more mucus than usual in the airways, contributing to narrowing of the airways and causing a cough with sputum. Microscopically there is infiltration of the airway walls with inflammatory cells. Inflammation is followed by scarring and remodeling that thickens the walls and also results in narrowing of the airways. As chronic bronchitis progresses, there is squamous metaplasia (an abnormal change in the tissue lining the inside of the airway) and fibrosis (further thickening and scarring of the airway wall). The consequence of these changes is a limitation of airflow.(19) Patients with advanced COPD that have primarily chronic bronchitis rather than emphysema were commonly referred to as "Blue Bloaters" because of the bluish color of the skin and lips (cyanosis) seen in them(20). The hypoxia and fluid retention leads to them being called "Blue Bloaters".

1.5.2 Emphysema

Lung damage and inflammation of the air sacs (alveoli) results in emphysema. Emphysema is defined as enlargement of the air spaces distal to the terminal bronchioles, with destruction of their walls(18). The destruction of air space walls reduces the surface area available for the exchange of oxygen and carbon dioxide during breathing. It also reduces the elasticity of the lung itself, which results in a loss of support for the airways that are embedded in the lung. These airways are more likely to collapse causing further limitation to airflow. The effort made by patients suffering from emphysema during exhalation, causes a pink color in their faces, hence the term commonly used to refer to them, "Pink Puffers". There are two types of emphysema: centrilobular (characterized by focal enlargement of air spaces around the bronchioles); panlobular (enlargement of all air spaces, around bronchioles and in the periphery)

1.1.1 Signs and symptoms

Essentials signs for diagnosis include: history of cigarette smoking; chronic cough and sputum production (in chronic bronchitis); dyspnea; rhonchi, decreased intensity of breath sounds, and prolonged expiration on physical examination; airflow limitation on pulmonary function testing that is not fully reversible and most often progressive.

One of the most common symptoms of COPD is shortness of breath (dyspnea). People with COPD commonly describe this as: "My breathing requires effort," "I feel out of breath," or "I can't get enough air in".(21) .People with COPD typically first notice dyspnea during vigorous exercise when the demands on the lungs are greatest. Over the years, dyspnea tends to get gradually worse so that it can occur during milder, everyday activities such as housework. In the advanced stages of COPD, dyspnea can become so bad that it occurs during rest and is constantly present.

Other symptoms of COPD are a persistent cough, sputum or mucus production, wheezing, chest tightness, and tiredness.(22-23) People with advanced (very severe) COPD sometimes develop respiratory failure. When this happens, cyanosis, a bluish discoloration of the lips caused by a lack of oxygen in the blood, can occur. An excess of carbon dioxide in the blood can cause headaches, drowsiness or twitching (asterixis). A complication of advanced COPD is cor pulmonale, a strain on the heart due to the extra work required by the heart to pump blood through the affected lungs.(24) Symptoms of cor pulmonale are peripheral edema, seen as swelling of the ankles, and dyspnea.

There are a few signs of COPD that a healthcare worker may detect although they can be seen in other diseases. Some people have COPD and have none of these signs. Common signs are: tachypnea, a rapid breathing rate; wheezing sounds or crackles in the lungs heard through a stethoscope; breathing out taking a longer time than breathing in; enlargement of the chest, particularly the front-to-back distance (hyperaeration); active use of muscles in the neck to help with breathing; breathing through pursed lips; and increased anteroposterior to lateral ratio of the chest (i.e. barrel chest).

1.5.4 Ethiopathogenesis

Most cases of COPD occur as a result of long-term exposure to lung irritants that damage the lungs and the airways. The most common irritant that causes COPD is cigarette smoke. Pipe, cigar, and other types of tobacco smoke also can cause COPD, especially if the smoke is inhaled. Breathing in secondhand smoke, air pollution, and chemical fumes or dust from the environment or workplace also can contribute to COPD. In rare cases, a genetic condition called alpha-1 antitrypsin deficiency may play a role in causing COPD. People who have this condition have low levels of alpha-1 antitrypsin (AAT)—a protein made in the liver. Having a low level of the AAT protein can lead to lung damage and

COPD if you're exposed to smoke or other lung irritants. If you have this condition and smoke, COPD can worsen very quickly.

1.5.4.1 Cigarette smoke

Cigarette smoking is considered to cause serious damage to health and a factor favoring the occurrence of pathologies of the respiratory system, the cardio-vascular and tumor development.

Cigarette smoke is the major risk factor associated with the development of chronic obstructive pulmonary disease (COPD). Recent studies propose a link between endoplasmic reticulum (ER) stress and emphysema, demonstrated by increased ER stress markers under smoking conditions. COPD is the third largest cause of death in the USA2 and fourth worldwide,(25). Smoking is responsible for 90% of COPD in the United States. Although not all cigarette smokers will develop COPD, it is estimated that 15% will. Smokers with COPD have higher death rates than nonsmokers with COPD. They also have more frequent respiratory symptoms (coughing, shortness of breath, etc.) and a more rapid deterioration in lung function than non-smokers. It is important to note that when a COPD patient stops smoking, their decline in lung function slows to the same rate as a nonsmoker. Effects of passive smoking or "second-hand smoke" on the lungs are not well-known; however, evidence suggests that respiratory infections, asthma, and symptoms are more common in children who live in households where adults smoke. Cigarette smoking damages the lungs in many ways. For example, the irritating effect of cigarette smoke attracts cells to the lungs that promote inflammation. Cigarette smoke also stimulates these inflammatory cells to release elastase, an enzyme that breaks down the elastic fibers in lung tissue.

1.5.4.2 Air pollution

Air pollution can cause problems for persons with lung disease, but it is unclear whether outdoor air pollution contributes to the development of COPD. However, in the non-industrialized world, the most common cause of COPD is indoor air pollution. This is usually due to indoor stoves used for cooking.

1.5.4.3 Occupational pollutants

Some occupational pollutants such as cadmium and silica do increase the risk of COPD. Persons at risk for this type of occupational pollution include coal miners, construction workers, metal workers, cotton workers, etc. (Most of this risk is associated with cigarette smoking and these occupations, an issue not well controlled for. These occupations are more often associated with interstitial lung diseases, especially the pneumoconioses) Nevertheless, the adverse effects of smoking cigarettes on lung function are far greater than occupational exposure.

1.5.4.4 Alpha-1 antitrypsin deficiency

Another well-established cause of COPD is a deficiency of alpha-1 antitrypsin (AAT). AAT deficiency is a rare genetic (inherited) disorder that accounts for less than 1% of the COPD in the United States. As discussed previously, normal function of the lung is dependent on elastic fibers surrounding the airways and in the alveolar walls. Elastic fibers are composed of a protein called elastin. An enzyme called elastase that is found even in normal lungs (and is increased in cigarette smokers) can break down the elastin and damage the airways and alveoli. Another protein called alpha-1 antitrypsin (AAT) (produced by the liver and released into the blood) is present in normal lungs and can block the damaging effects of elastase on elastin. The manufacture of AAT by the liver is

controlled by genes which are contained in DNA-containing chromosomes that are inherited. Each person has two AAT genes, one inherited from each parent. Individuals who inherit two defective AAT genes (one from each parent) have either low amounts of AAT in the blood or AAT that does not function properly. The reduced action of AAT in these individuals allows the destruction of tissue in the lungs by elastase to continue unopposed. This causes emphysema by age 30 or 40. Cigarette smoking accelerates the destruction and results in an even earlier onset of COPD. Individuals with one normal and one defective AAT gene have AAT levels that are lower than normal but higher than individuals with two defective genes. These individuals MAY have an increased risk of developing COPD if they do not smoke cigarettes; however, their risk of COPD probably is higher than normal if they smoke. Though their Alpha-1 antitrypsin blood levels may be in the normal range, the function of this enzyme is impaired relative to normal patient. Some may even develop bronchiectasis instead of emphysema.

1.5.5 COPD treatment

While many medications are available to treat COPD, no drug has demonstrated effectiveness in halting the progression of the disease. Rather, the goal of drug therapy at this time is to maintain control of symptoms and prevent COPD exacerbation. The current treatment strategies include: quitting cigarette smoking; taking medications to dilate airways (bronchodilators) and decrease airway inflammation; vaccination against flu influenza and pneumonia; regular oxygen supplementation; and pulmonary rehabilitation.

1.6. Cigarette smoke and airways inflammation.

The principal sources of oxidants in the bronchial airways are represented by cigarette smoke, environmental pollution and local inflammation; exposure to cigarette smoke (CS) is certainly one of the primary stimuli of airway inflammation (26).

Exposure to cigarette smoke (CS) represents a considerable oxidant burden on the respiratory epithelium, which is the first line of defense to inhaled substances. CS, which is one of the most important indoor air pollutants, is a complex mixture of over 4,000 different compounds, and high levels of oxidants and ROS have been detected in both mainstream and sidestream smoke (27) It has been estimated that there are 1014 free radicals in each puff of cigarette smoke (28).

A high toxicity has been observed for at least 52 components of CS: 18 phenols, 14 aldehydes, eight *N*-heterocyclics, seven alcohols, and five hydrocarbons (29). Most of these compounds are capable of generating ROS during their metabolism. Thus, the mechanism of cigarette smoke toxicity is thought to incorporate oxidative stress, which mediates cell death via necrosis and apoptosis, due to the fact that cigarette smoke has been shown to cause oxidative DNA damage and cell death (30).

Some lipophilic components of CS can enter airway epithelial cells increasing intracellular ROS production by disturbing mitochondrial activity (30) The oxidative damage to cellular components occurs when the increase in ROS production causes oxidative damage in cellular components by overwhelming their antioxidant defense mechanisms; the presence of apoptosis confirms that this causes damage to DNA, nuclear DNA being one of the targets of ROS (31) However, CS-mediated DNA damage can result in uncontrolled cell proliferation and transformation (27) if the cells fail to undergo apoptosis. CS also facilitates allergen penetration across respiratory epithelium (32) and is a potent source of

oxidative stress, DNA damage and apoptosis in alveolar epithelial cells by the upregulation of Fas/APO-1 receptor and activation of caspase-3 (33). Moreover, CS is considered a major risk factor for chronic obstructive pulmonary disease (COPD) development as demonstrated in animal models (33)

The link between CS and lung inflammation is therefore quite strong; however, relatively little is still known on the effects of CS on human bronchial mucosa cell differentiation and survival.

1.7. Inflammation and oxidative stress in COPD

Increased inflammation, oxidative stress, cell death, impaired cell repair, protease/antiprotease imbalance, and destruction of the extracellular matrix are all believed to contribute to disease progression in COPD. Several inflammatory cells and their mediators, both of the innate and adaptive immune system, participate in the inflammatory response., Macrophages, neutrophils and CD8+ T cells are the cells usually considered the prime effector cells in pathogenesis of COPD (17), but recently DCs have been suggested to be a potentially important new player/orchestrator of the pattern of inflammation that characterizes this disease (18).

cDCs might play a central role in bridging innate and adaptive immunity via direct cell-cell interactions and/or cytokine production (19-20). These interactions may influence the activation status of cells from the adaptive immune system such as $CD4^+$T cells and $CD8^+$T cells (18,20,24,34) $CD8^+$T cells could be essential for the development of cigarette smoke-induced COPD (35).

COPD, like asthma, is a complex inflammatory disease that involves several types of inflammatory cells and multiple inflammatory mediators. However, the pattern of inflammation and the spectrum of mediators differ between these two airway diseases, at least in the stable state of the disease. Although abnormal numbers of inflammatory cells

have been documented in COPD, the relationship between these cell types and the sequence of their appearance and their persistence are largely unknown. Most studies have been cross-sectional based on selection of patients with different stages of the disease and comparisons have been made between smokers without airflow limitation (normal smokers) and those with COPD who have smoked a similar amount. There are no serial studies and selection biases (such as selecting tissue from patients suitable for lung volume reduction surgery) may give misleading results. Analysis of the cell profile in alveoli and small airways shows an increase in all ofthe cell types implicated in COPD, including macrophages, T-lymphocytes, B-lymphocytes and neutrophils (36)

1.8. The epithelial mesenchymal trophic unit and airway remodeling.

Under normal conditions, the epithelium releases primarily factors that suppress mesenchymal cells, such as prostaglandin (PG) E2 and 15-HETE. The production of PGE2 and 15-HETE is diminished after the epithelium sustains injury or damage, and the ensuing repair responses could promote airway remodeling by activating the fibroblasts/myofibroblasts that lie directly under the epithelial layer in the *lamina reticularis* (37). The inflamed airways of COPD patients contain several inflammatory cells including neutrophils, macrophages, T lymphocytes, and dendritic cells (DCs). The relative contributions of these various inflammatory cells to airway injury and remodeling are not well documented. In particular, the potential role of DCs as mediators of inflammation in the smoker's airways and COPD patients is poorly understood. This signaling between the epithelium and fibroblasts involves the provision of growth factors and survival of mesenchymal cells likely to contribute to the component of COPD that is unresponsive to corticosteroids. In vitro studies have shown that injury to epithelial monolayers results in increased release of fibroproliferative and profibrogenic growth factors including fibroblast growth factor (FGF-2), insulin growth factor (IGF-1), platelet-

derived growth factor (PDGF), endothelin (ET-1), and transforming growth factor (TGF)-β2. In chronic lung inflammatory diseases, the epithelium has increased susceptibility to oxidant injury through the activation of the caspase-3/apoptosis pathway. This feature also carries over into cultured epithelial cells in vitro (38). Impaired epithelial repair is another feature of chronic lung inflammatory diseases, and it is linked to the increased production of profibrotic growth factors such as TGF-β/or FGF-2, as evidenced by low expression of cell markers of proliferation (e.g., proliferating cell nuclear antigen [PCNA]). This reaction can be reproduced in vitro by slowing epithelial repair in the presence of a selective tyrphostin inhibitor of the EGFR tyrosine kinase. The downstream consequences of EMTU activation affect the remodelling events of altered airway structure and function more directly.

1.9. Lungs and tissue engineering

Tissue engineering has greatly progressed in the last twenty years or so, especially with regard to replacing function in specific tissues such as exocrine pancreas, cartilage and bone, skin and blood vessels. However, it is still lacking, proper design models for engineering complex 3D ECM microenvironments to understand disease progression.

In fact, the main objectives of modern tissue engineering comprise repeating specific tissue functions for regenerative medicine and developing in vitro models of human tissues to investigate disease pathogenesis and for testing and screening new medications before expensive clinical trials. For these objectives to be successfully achieved, it is fundamental that the engineered models must repeat *in vitro* the complex interactions existing *in vivo* between cells and their microenvironments.

The lungs are extraordinarily complex organs that evolved for the adaptation of vertebrates to terrestrial life. While intrauterine development can proceed usually in the absence of lung tissue, life after birth depends completely upon respiration, which it is in turn

dependent upon the normal architecture of the lung. The respiratory system consists of separate anatomic regions originating in the external nose and continuing into the nasal passages, pharynx, larynx, trachea, bronchi, lobar bronchi, bronchioles and peripheral airways that conduct respiratory gases to the alveoli. Gas exchanges occur across alveolar epithelial and capillary endothelial cells. Ventilation is driven by mechanical forces dependent upon neuromuscular activity that is precisely controlled by neurosensory inputs to maintain normal pCO2, pO2, and pH. Lung function is completely reliant on its extraordinary structure that exchanges millions of litres of environmental gases throughout our lifetime.

Chronic inflammatory diseases of the lungs, such as COPD, involve complex interactions between different cell types as well as extracellular matrix remodelling, which, together with the mechanical environment that affects cell-cell and cell-matrix interactions cause the pathology.

Diseases such as asthma and COPD are difficult to study *in vivo* using animal models because there are too many important differences at various levels between animal and human cells and also it is virtually impossible to draw out mechanisms or have a great deal of control over these complex interactions.

2. AIMS

Our understanding of the importance of the extracellular microenvironments in regulating cell behaviour is critical to try and comprehend the role of the EMTU in the pathogenesis of COPD. Thus, the availability of a good model of human bronchial mucosa becomes paramount.

One of the main limitations of traditional cell culture models is not being able to follow cell treatments for long exposures due to the fact that after a few days at most any cell culture would have reached confluency and therefore the effects of whatever we want to test will be marred/biased by physiologically occurring cell growth inhibition phenomena. The bronchial 3D outgrowth that was developed by my Tutor, Prof. Fabio Bucchieri, instead is apparently free from this limitation given its ability to grow almost indefinitely. Moreover, the contemporary presence of bronchial epithelial and fibroblast cells in their natural microenvironment puts us in the position of being able to study the effects of medium/long term exposures on a model that closely mimic the human bronchial mucosa and where it is actually fairly easy to highlight eventual alteration of the EMTU.

Although we now know that the link between cigarette smoke (CS) and lung inflammation is quite strong, relatively little is still known on the effects of long-term CS exposure on human bronchial mucosa cell survival and differentiation.

For these reasons our aims were to: characterise, structurally and ultra-structurally, the morphological features of the 3D outgrowths, by means of transmission electron microscopy analysis, to verify the degree of similarity of the 3D bronchial outgrowths with the normal human bronchial mucosa; to immunologically characterise these outgrowths by using immunofluorescence and immunogold techniques and a panel of antibodies directed towards the antigens usually expressed by the adult differentiated human bronchial mucosa; and finally, in view of the scarcity of studies examining the effects of CS exposure on human bronchial mucosa cell survival and differentiation, the current study was designed to address this particular issue, evaluating the responses of the 3D outgrowths to a long-term exposure with cigarette smoke extracts (CSE).

3. Materials and Methods

3.1 Cell cultures

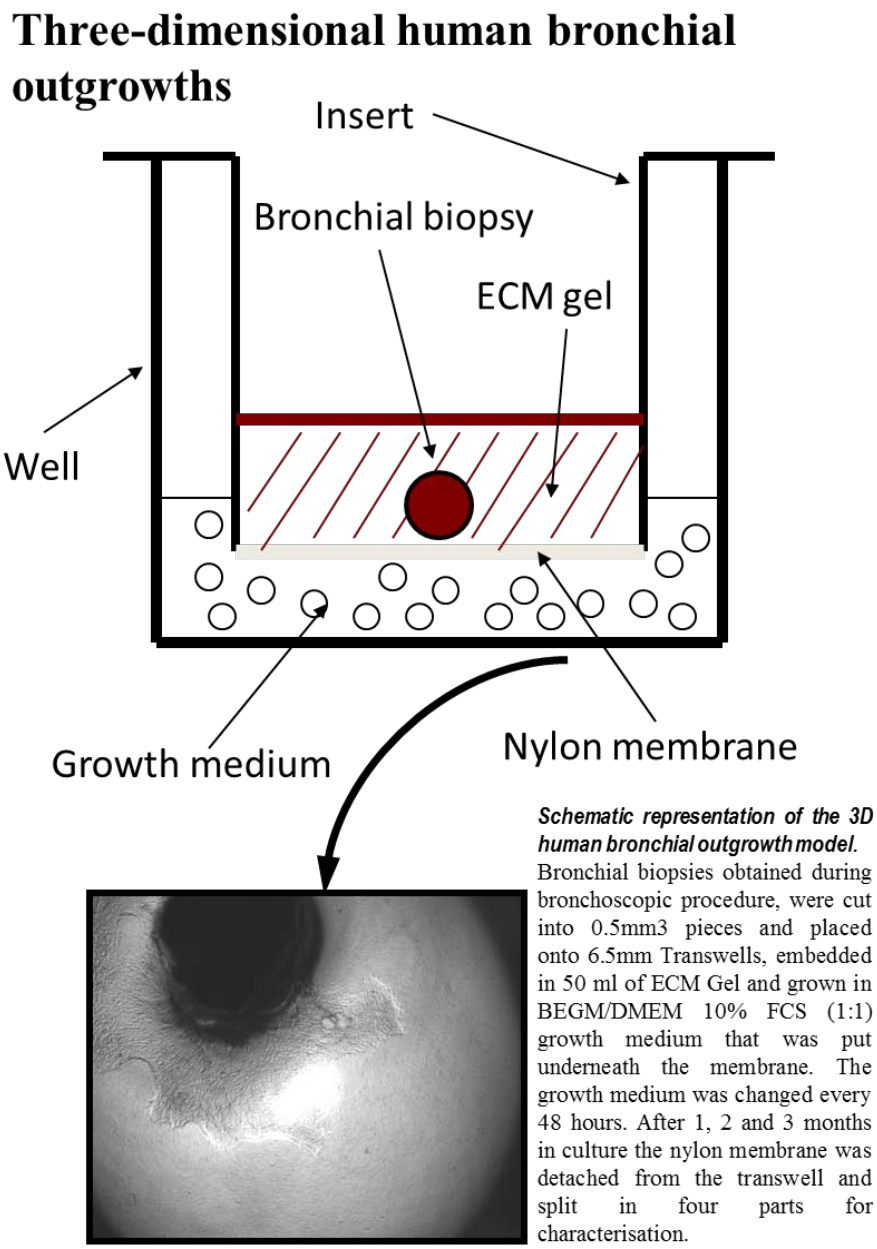
All media and supplements were from Invitrogen unless otherwise specified. Cells, explants and outgrowths were grown in a humidified Heraeus incubator at 37°C, 5% CO2.

3.1.1 Three-dimensional Outgrowth Model.

Bronchial biopsies, were obtained from patients referred to the Unit of Thoracic Medicine of the University of Palermo. The following adopted procedures, conforming to the relevant ethical guidelines for human research, were in agreement with the Helsinki Declaration of 1975 as revised in 1983 and were approved by the Ethic Council of the Policlinico-Hospital of the University of Palermo, Italy, were cut into 0.5mm^3 fragments and placed onto 6.5mm Transwells, embedded in 60 µl of Matrigel^{TM} . Matrigel is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells and marketed by BD Biosciences and by Trevigen Inc This mixture resembles the complex extracellular environment found in many tissues and is considered as a good substrate for cell culture for its heterogeneous composition. The major components of Matrigel are basement membrane proteins such as laminin, entactin, and collagen IV which present cultured cells with the adhesive peptide sequences that they would encounter in their natural environment (51). Matrigel constitutes a uniform and controllable structure very important for accuracy and reproducibility of an *in vitro* model usable for to test compounds (52). The biopsies were washed several times in sterile phosphate-buffered saline (PBS), subsequently they were cut using a sterile scalpel in 0,5 mm^3 pieces and placed onto 6.5 mm Transwells in middle position upon the nylon membrane (Becton Dickinson, Franklin Lakes, NJ, USA) embedded in 60 µl of Matrigel (Becton Dickinson). The Transwells were put on 24 wells culture plates (Corning Life Sciences) Then plates were put at 37°C for 5 minutes to facilitate the matrigel jellification and 330 µl of growth medium mix was added to each well after these 5 minutes This mix was constituted of (Bronchial Epithelium Growth Medium/ Dulbecco's modified Minimum Essential Medium) BEGM/DMEM 10% foetal bovine serum FBS (1:1) The growth medium was replaced every 48hours. The outgrowths were cultured at 37°C in a 5% CO2 atmosphere An inverted light microscope equipped with phase contrast rings (LEICA DM-IRB, Leica Microsystems Srl, Milan, Italy) was used to monitor the outgrowths. The expansion of the outgrowths was monitored with a contrast phase microscope. At specific time points, the

membranes with the outgrowths were prepared for Transmission Electron Microscopy (TEM) and Immunofluorescence.

Figure 3.1



Schematic representation of the 3D human bronchial outgrowth model. Bronchial biopsies obtained during bronchoscopic procedure, were cut into 0.5mm3 pieces and placed onto 6.5mm Transwells, embedded in 50 ml of ECM Gel and grown in BEGM/DMEM 10% FCS (1:1) growth medium that was put underneath the membrane. The growth medium was changed every 48 hours. After 1, 2 and 3 months in culture the nylon membrane was detached from the transwell and split in four parts for characterisation.

3.2 TREATMENTS

3.2.1. Cigarette smoke extracts

Cigarette Smoke Extracts (CSE) were prepared by a modification of the method of Carp and Janoff. (39). Briefly, smoke from two Kentucky 1R4F research cigarettes (University of Kentucky, Lexington, KY) whose filters were removed was bubbled through 50 mL of BEBM for 60-70s. The resulting suspension was adjusted to pH 7.4 with concentrated NaOH, filtered through a 0.22-μM Millex-GS (Millipore, Watford, UK) filter and used immediately on 3D outgrowths at a concentration range of 10, 15 and 20%.

3.3 Transmission electron microscopy.

TEM analysis enabled us to have a better understanding of the structural characteristics of the 3D bronchial outgrowth.
All reagents were ordered from Electron Microscopy Sciences (EMS, PA) unless otherwise specified.

3.3.1 Epon 812

Immediately after excision of the nylon membrane from the Transwells using a scalpel, the outgrowth were cut in pieces and fixed in a solution of 2,5% glutaraldehyde in 0.2 phosphate buffer, pH 7.4, for 20 minute at room temperature. After the outgrowths were fixed the glutaraldehyde was removed and they were stored in Milloing Buffer. After 3 consequently washing in Milloing Buffer the pieces were post-fixed in 1% OsO_4 for 2 h, dehydrated in an ascending graded series of ethanol, treated for 30’ in propylene oxide, infiltrated with epoxy resin (Epon812, Electron Microscopy Science, Hatfield, PA, USA) in propylene oxide (1:3, 1:2, 1:1 for 30 minute at room temperature respectively) and finally embedded in Epon812 with DMP30. The resin was then polymerized at 60°C for 48h. Ultrathin and semithin sections were cut with an ultramicrotome (Ultracut E, Reichert-Jung, Depew, NY, USA) at different thickness and mounted on copper and golden grids and on glass slides for further use.

3.3.2 LR-White

After excision of the nylon membrane from the Transwells using a scalpel, the outgrowth were fixed for 1 hour in paraformaldehyde 4%, after 1 hour the samples were washed in Milloning buffer and they were stored in buffer until they were processed. The samples were dehydrated with ascending methanol series (30, 50, 70, and 100%): each step repeated two times for 20 minutes, and then they were treated with a solution of LR-white methanol one time for 30 minutes, after this step 24 hours in pure resin, next day add the accelerator in the LR-WHITE resin and the specimens were then put in embedding containers in LR- WHITE resin for 24 hours at 60°C. The nylon membranes of the Transwells were excised using a sterile scalpel and the outgrowths were prepared to be embedded in LR-White resin The outgrowths were fixed in paraformaldehyde 4%, for 1 hour, washed in Milloning's phosphate buffer, pH 7.4, and dehydrated with ascending methanol series (30, 50, 70, and 100%) repeating each dehydrating step two times for 20 minutes. Then, the specimens were treated with a solution of Lr-white/methanol for 30 minutes and in pure resin over night at room temperature. The day after, the outgrowths were embedded in LR-white resin in which accelerator was added and were left to polymerize in embedding containers for 24 hours at 60°C.

3.4 IMMUNOSTAINING.

3.4.1 Immunofluorescence.

3D outgrowths were stained in situ after the appropriate treatments and time points. At the end of the treatment outgrowths were washed once with 1ml/well of HBSS and fixed *in situ* in 500□l/well of ice-cold absolute methanol for 20 minutes at –20°C. Outgrowths, inside their plastic supports, were then left to dry in a laminar flow cabinet for 30 minutes and stored at –20°C. Trays were defrosted at room temperature, and washed twice with 1ml/well of phosphate buffered saline (PBS), permeabilised with 500µl/well of Triton X-100 (Sigma, UK) 0.1% in PBS for 3 minutes on ice and washed once with 1ml/well of PBS. Unspecific binding sites were blocked with 250µl/well of DMEM 10% FBS for 15 minutes. During this period the primary Abs were diluted in incubation buffer (DMEM 10%, Tween-20 0.1% and Sodium Azide 0.1% in PBS). The blocking buffer was then removed and without washing the diluted Abs were added to the wells for 45 minutes. Wells were then washed twice with 1ml/well of incubation buffer and when needed

secondary Abs were diluted in incubation buffer and applied to the wells for 45 minutes. Secondary Abs were conjugated with different fluorochromes.

The following primary Abs were used:

- anti-cytokeratin13 (CK13) monoclonal IgG1 Ab (clone 1C7, working dilution 1:100) purchased from Abnova, USA.
- anti-cytokeratin18 (CK18) monoclonal IgG Ab (clone KRT18, working dilution 1:200) purchased from Abnova, USA.
- Anti-Collagen I monoclonal IgG1 Ab (clone 5D8-G9, working dilution 1:50) purchased from Millipore, UK.
- Anti-Laminin monoclonal IgG1 Ab (clone 2G6/A2, working dilution 1:200) purchased from Millipore, UK.

The secondary Abs were:

A secondary FITC-conjugated goat anti-mouse IgG Ab (working dilution 1:400, purchased from Sigma, UK) was used to reveal CK13 and Collagen I positivity.

A secondary TRITC-conjugated goat anti-mouse IgG Ab (working dilution 1:500, purchased from Sigma, UK) was used to reveal CK18 and Laminin positivity.

At the end of the 45 minutes incubation step with the secondary Abs, wells were washed twice with 1 ml of PBS and coverslips mounted with MOVIOL® (DABCO) mounting medium. The trays were then ready to be observed under the fluorescent light of a LEICA inverted fluorescent microscope.

3.4.2 Immunogold

Ultrathin sections were mounted on gold grids to prepare them for the immunogold assay. The outgrowths were included in epoxy resin that notoriously covers antigenic sites, making the execution of immunological investigation techniques considerably more difficult. A pre-treatment to unmask the sites with sodium citrate was performed to ensure better results. Gold grids were placed in a baker filled with a sodium citrate solution and subsequently microwaved for 4 minutes at 850W.

The grids were then washed twice in PBS, and subsequently incubated in a serum blocking solution for 30 minutes and, without washing, the incubation proceeded with primary antibodies diluted in dilution buffer for 1 hour and half at room temperature. Primary antibodies used were against cytokeratin 18, MUC 5AC, Collagen type I and alpha-SMA and they were all diluted 1:15. Grids were rinsed five times with PBS for 3 min and incubated with a secondary antibody conjugated with 10nm colloidal gold particles for 30 minutes at room temperature, and washed again for five times with PBS for 3 min. The grids were then fixed in a 2.5% glutaraldehyde solution in PBS for 15 minutes and finally washed five times in distilled water for 3 minutes. The grids were then prepared for contrast staining by treating them with uranyl acetate for 5 minutes, followed by eight washes with methanol for 2 minutes, treated with Reynolds' solution for 5 minutes and finally rinsed eight times in distilled water for 2 minutes. After this procedure, the grids were ready for electron microscopy.

4. Results

4.1 Morphological characterization of the human bronchial outgrowths.

4.1.1 Phase Contrast Microscopy

3d outgrowths were routinely photographed before and after treatment to record any morphological changes occurring in the cells. An inverted light microscope equipped with phase contrast rings (LEICA DM-IRB) was used to visualize changes in cell size, shape and orientations and this was recorded by digital photography.

Phase contrast microscopy (PhaCo) revealed that the 3D outgrowths were initially formed as a network of spindly cells (likely fibroblasts) and rounder cells (possibly epithelial cells) that were growing out into the Matrigel from the biopsy which was initially placed in the middle of the transwell system (Figure 4.1). After 10-12 days, the nylon membrane covering the bottom of the insert was completely covered with the newly grown tissue and from that time the culture had a three-dimensional structure. After 30 days of culture the morphological features of the outgrowths (as observed with the PhaCo) did not change significantly. In our experience, unless specific damaging events (eg. contamination with infectious organisms), occurred during the culture period, and providing proper culture conditions were maintained, we were able to grow these 3D outgrowths consistently for more than a year.

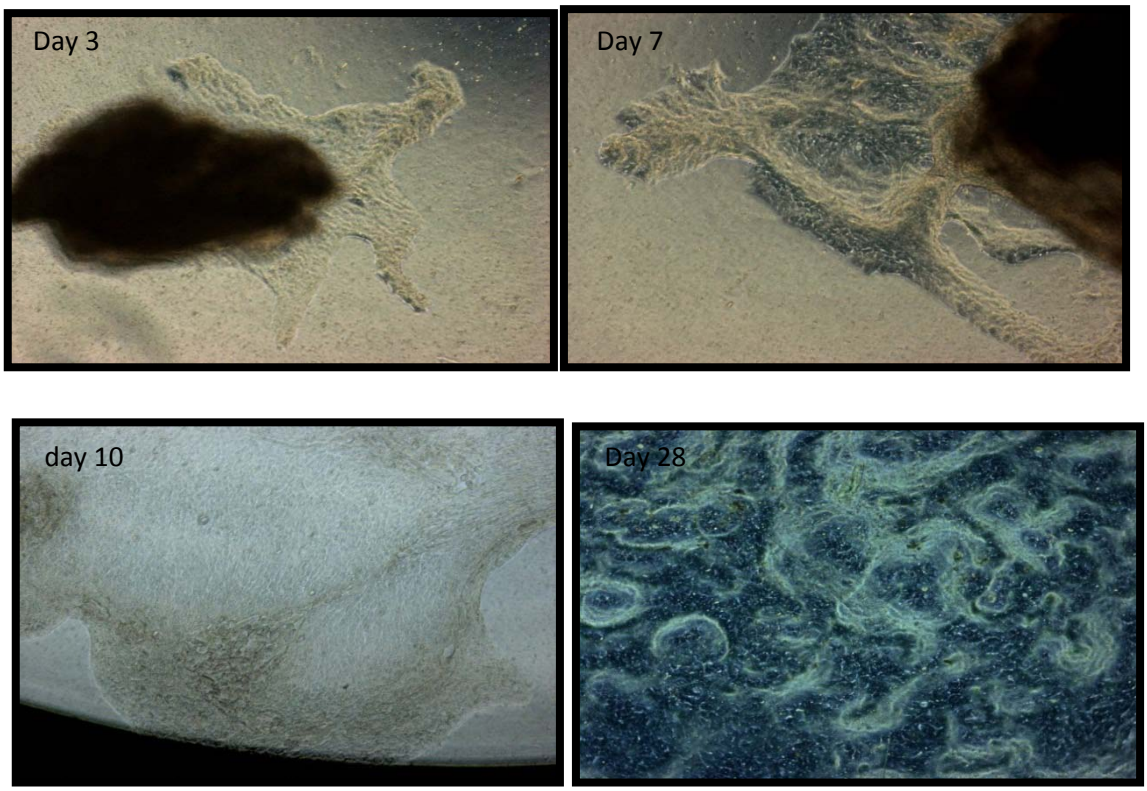
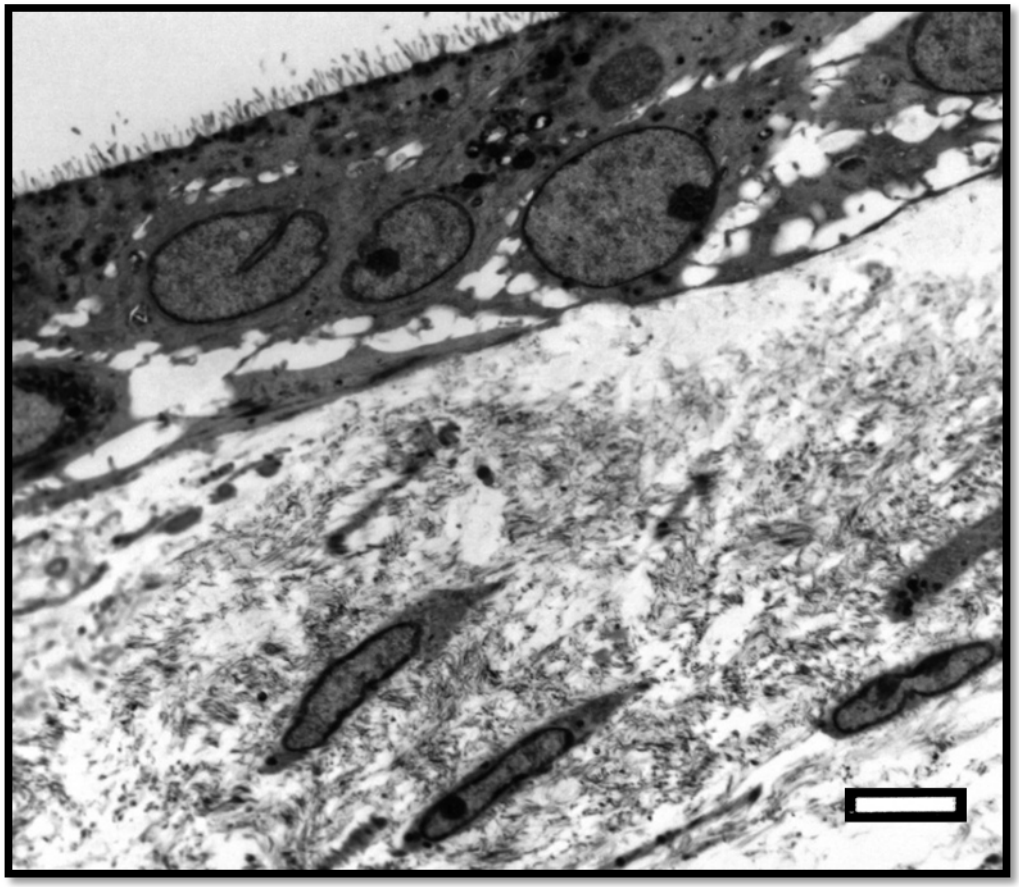


Figure 4.1 Phase-contrast observation of outgrowths in culture for 3, 7, 10 and 28 days.

4.1.2 TEM analysis

Figure 4.2 shows a panoramic view of a 30 days old outgrowth: it is possible to clearly identify two distinct strata, the top one formed of two layers of epithelial-like cells, a basal and an apical one, and the bottom one where fibroblast-like cells are dispersed in a highly organized ECM. The two layers are separated by a well-developed basement membrane.

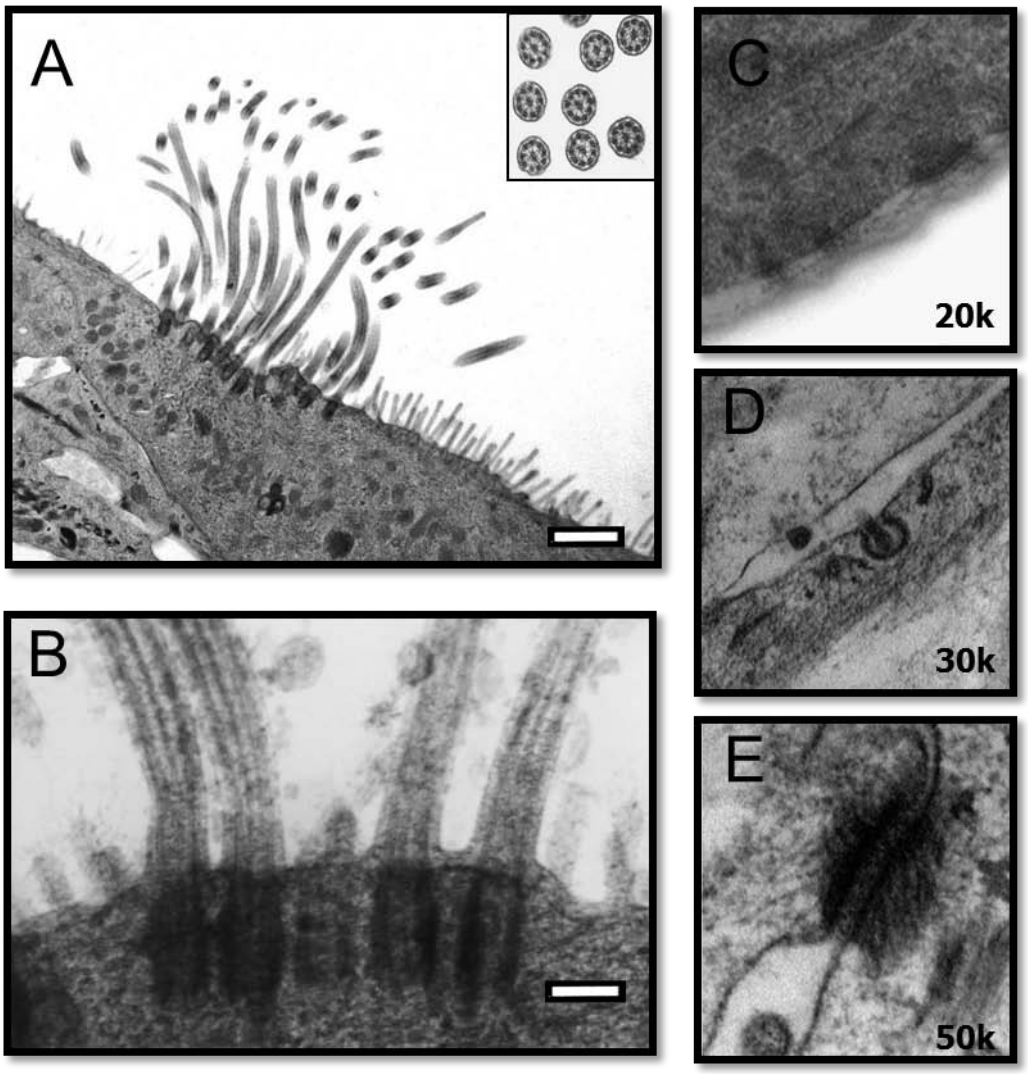
Figure 4.2



Overview by TEM of a 3D outgrowth after 30 days of culture. Clearly visible in the apical part of this image is the stratified epithelial layer, separated, by a basement membrane, from the layer of connective tissue with fibroblast cells surrounded by ECM.
Bar = 2um

In Figure 4.3 it is possible to analyze the epithelial component more in details. In particular, the apical epithelial elements present microvilli and cilia that are structurally well formed. The basal epithelial cells are separated from the mesenchymal layer by a well-developed basement membrane to which basal epithelial cells are attached via hemidesmosomes (Fig.4.3 C). The latero-lateral surfaces present adhesive junctions (desmosomes, clearly visible in fig.4.3 E) that keep the surfaces of adjacent cells well

attached, and enlargements where desmosomes are missing and where it is possible to discern exo- and endocytosis ccurrences (Fig.4.3 D).

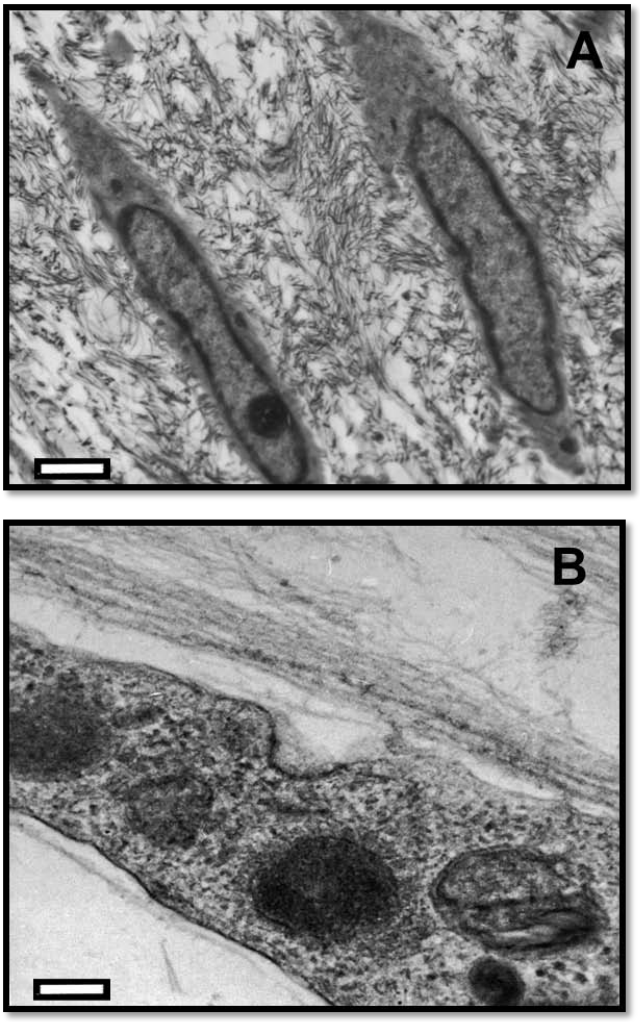


Epithelial elements with different apical structures. Both microvilli and cilia are visible (A), the inset shows a particular of the cilia, displaying their internal structure; in figure B, it is possible to observe the presence of basal bodies of the ciliated structures. C shows a well developed basement membrane to which basal epithelial cells are attached via emi-desmosomes. In D is possible to observe an enlargement of the later-lateral surface inside which is possible to detect exo/endocytosis of cytoplasmic materials. E shows a complete desmosome just above the abovementioned enlargement, that keeps the latero-lateral surfaces of two adjacent cells well attached. Bars = 1um in A and 200nm in B.

The lower layer (mesenchymal layer) has a completely different structure with fibroblasts dispersed in a well-defined ECM (Figure 4.4 A). In fig.4.4 B, it is possible to observe high magnification details of a fibroblastic cytoplasmic process: it is quite clear that the proteic material that will eventually form the ECM is extruded into the extracellular space from caveolae-like structures present on the cytoplasmic membrane of the fibroblasts.

It was not possible to identify any other cytotypes (such as immune or inflammatory cells) apart from the ones already described. As better detailed in the Discussion session of this chapter, this is another strong point of this model because it enables study of cellular processes devoid of the presence of inflammatory cells.

Figure 4.4



Particular of two fibroblasts from the mesenchymal layer. They appear to be surrounded by ECM proteins (A) that, as illlustrated in figure B, are produced by the fibroblasts themselves. The cytoplasms of the fibroblasts contain vacuoles filled with proteic material and their cytoplasmic membranes present caveolae-like structures, from where the assembled proteins are released into the extracellular space. Bars = 1um in A and 200nm in B.

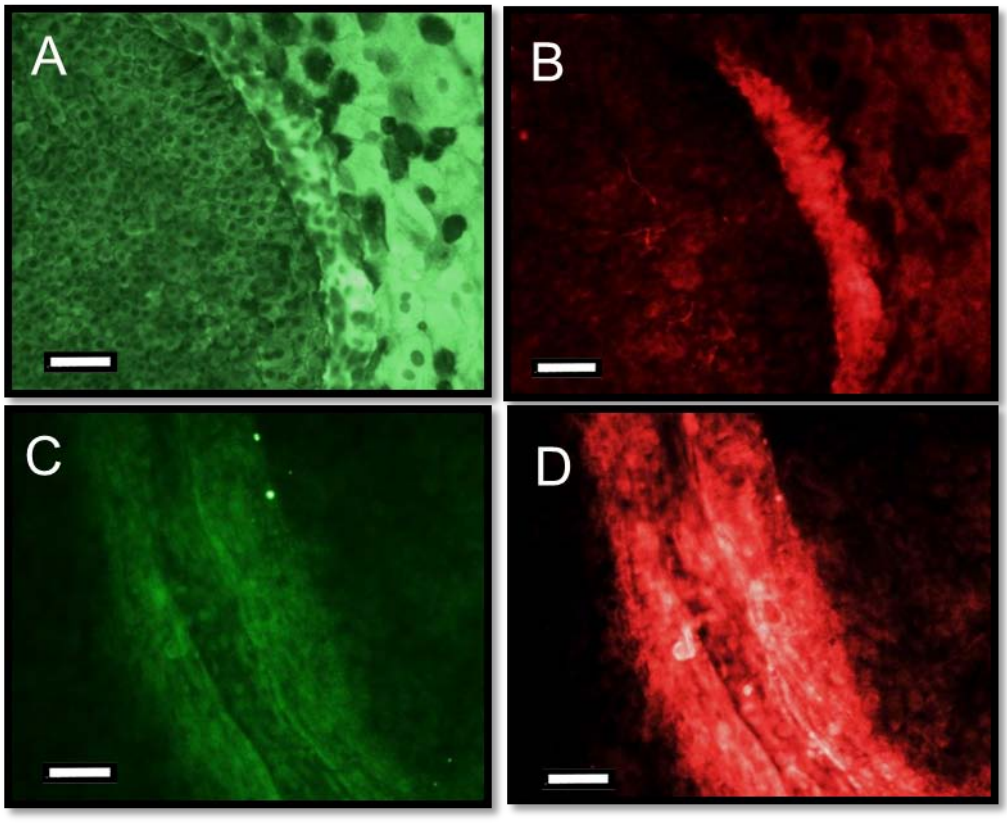
4.2 Immunocharacterisation

In order to properly characterize our 3D outgrowths, these were stained with a panel of antibodies directed towards some of the most common markers of the human bronchial mucosa, and visualized by immunofluorescence and immunogold in order to achieve precise localization at the different cell structures.

4.2.1 Immunofluorescence

Figure 4.5 shows that the epithelial layers were positive for CK13 (A) and CK18 (B), whereas the underlying fibroblast layer was positive for Collagen I (C) and Laminin (D). The outgrowths did not express CD3, CD4, CD8, CD18, CD28, CD 45, CD64, CD68 and MPO (markers of different kind of leukocytes, lymphocytes, macrophages and neutrophils; data not shown) and this was in accordance with the morphological observations.

Figure 4.5

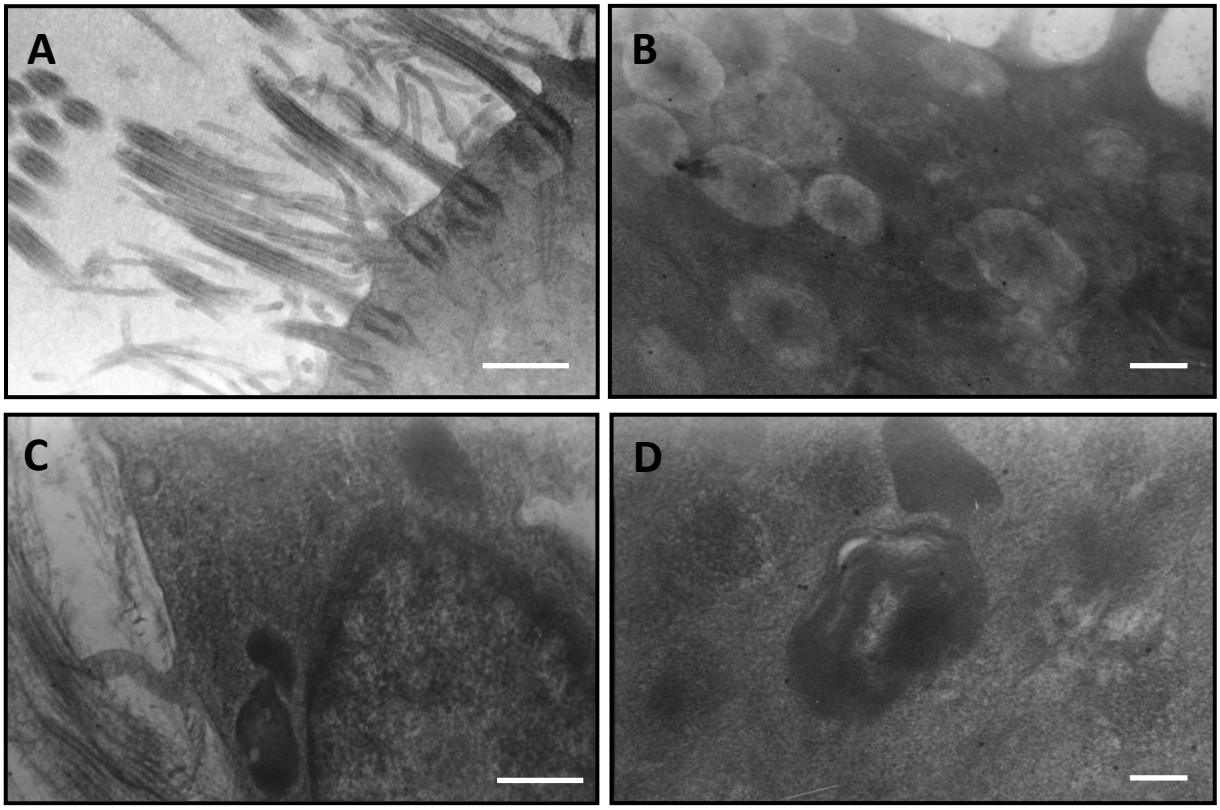


Immunofluorescence staining of the epithelial (A and B) and fibroblast (C and D) layers. The epithelial cells resulted positive for CK13 (A) and CK18 (B), whereas the fibroblasts expressed Collagen Type I (C) and Laminin (D). Bars = 40 um.

4.2.2 Immunogold

Immunological characterization of the bronchial outgrowths was also carried out by immunogold in order to obtain a better ultrastructural localization of certain markers. Figure 4.6 shows that the ciliated elements of the epithelial layer immunostained positively for cytokeratin 18 (a typical marker of columnar ciliated cells in the normal human bronchial mucosa, fig 4.6 A) whereas the goblet cells characteristically expressed MUC 5AC (fig. 4.6 B). The collagenous fibers released by the fibroblast in the extracellular space stained positively for collagen type I (the main collagen produced by fibroblasts in the lamina propria of the human bronchial mucosa, Fig. 4.6 C) while some of the fibroblasts (only 10%) expressed in their cytoplasm alpha-SMA (Fig. 4.6 D) suggesting a possible myo-fibroblastic differentiation of some of these cells.

Figure 4.6



Immunogold staining of human bronchial mucosa markers. A 30 days old bronchial outgrowth was immunostained for cytokeratin 18 (A), MUC 5AC (B), collagen type I (C) and alpha-SMA (D) using secondary antibodies labelled with colloidal gold particles 10nm in diameter and then analysed by TEM. Bars = 500nm in A and B, 200nm in B and C.

4.3 Effects of long term CSE exposure on 3D outgrowths survival and differentiation.

Having further confirmed that the bronchial outgrowths used in this study were effectively a good replica of the normal human bronchial mucosa in both its epithelial and fibroblastic components, we proceeded with a functional study aimed at the identification of the effects that long-term exposure to cigarette smoke could induce to these outgrowths.

4.3.1 Phase-Contrast analysis

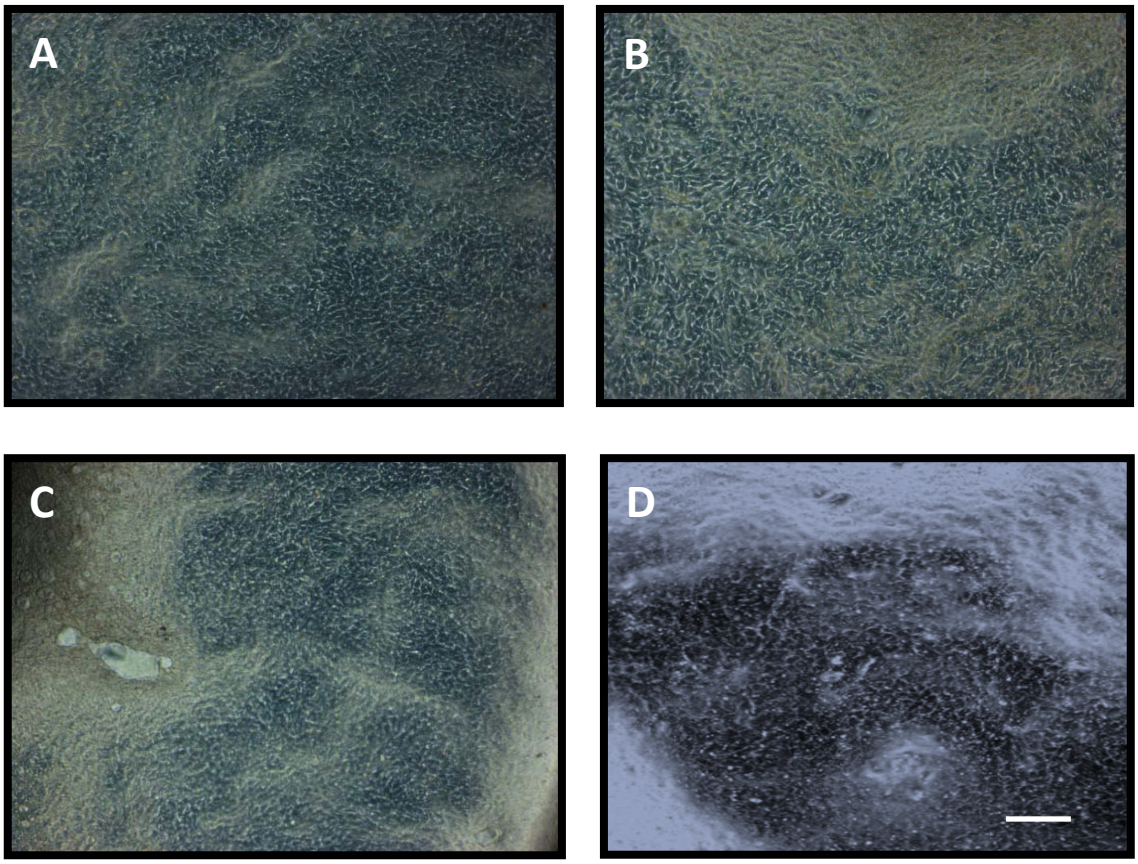
In order to find the correct doses and time points for the long-term exposure to CSE, 3D outgrowths were grown until complete differentiation (28 days) and then treated with or without (untreated control) different doses of CSE (10, 15 and 20%) for 1, 2 and 3 weeks.

These doses were chosen because they represent a good approximation of the amount of CSE present in the bronchial mucosa of an average smoker (subject that smokes 15-20 cigarettes per day).

Figures 4.7, 4.8 and 4.9 show the morphological features of the 3D outgrowths with the three doses analyzed after 1, 2 and 3 weeks of exposure, respectively.

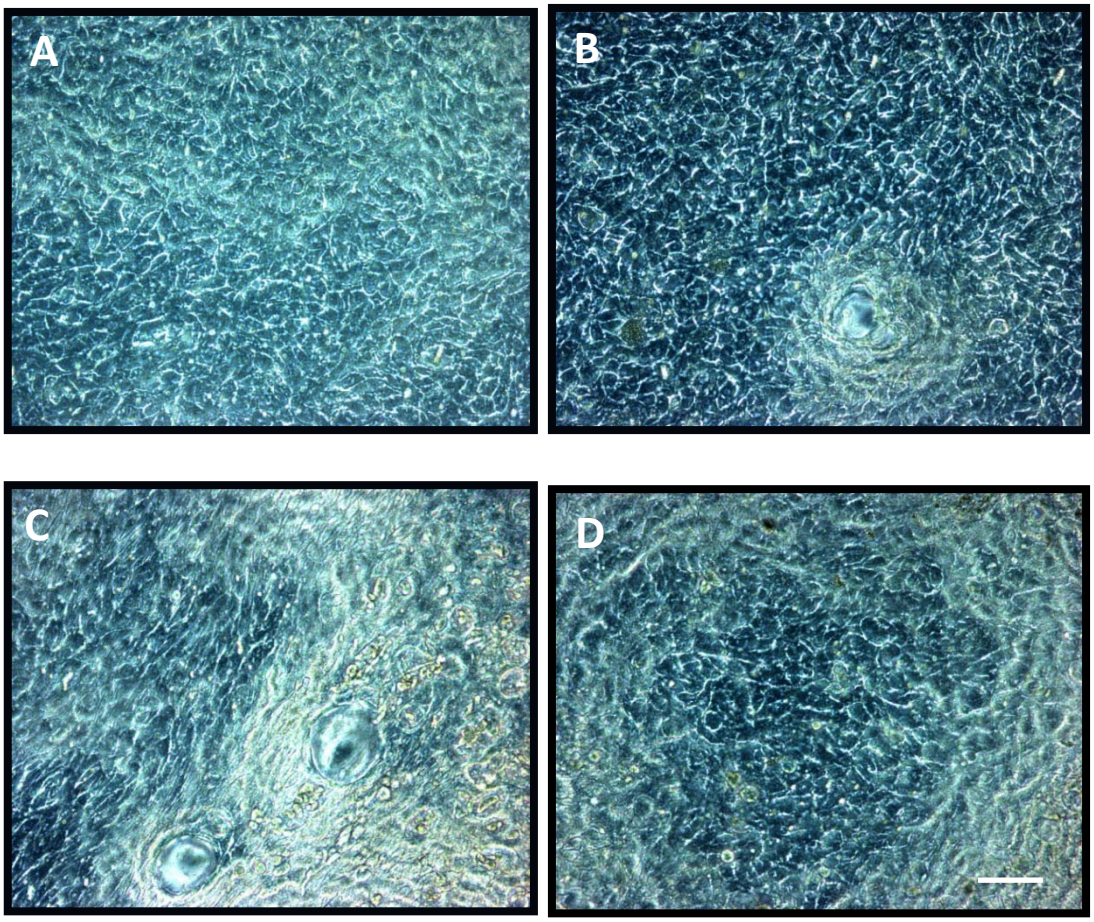
It is possible to observe that at all time points and doses studied CSE failed to induce significant amounts of cell death (whether necrotic or apoptotic) with only the highest dose, 20%, at the furthest time point, 3 weeks, presenting a few necrotic cells on the upper surface of the 3d outgrowth. For these reasons, the 15% dose of CSE and a three weeks exposure time were chosen for the following experiments.

FIGURE 4.7



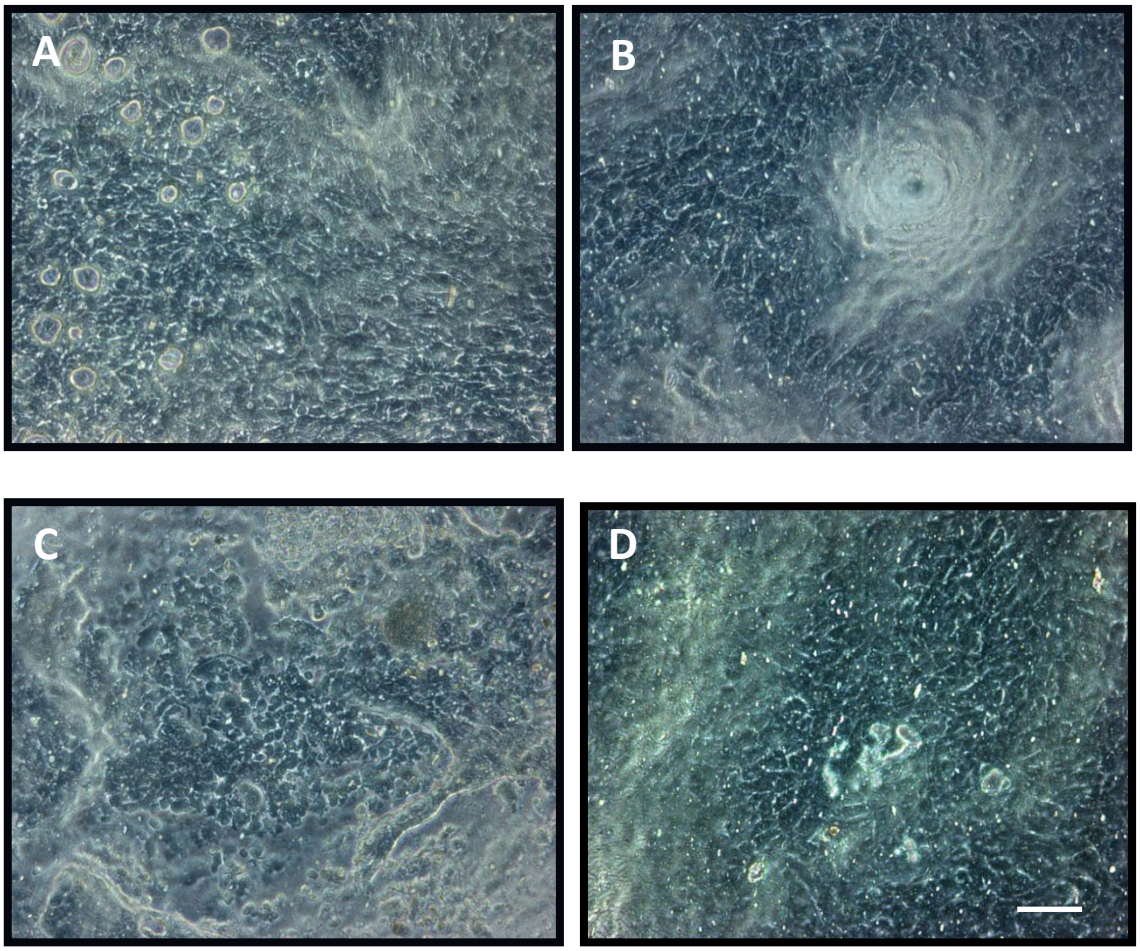
Phase-contrast observations of a representative 28 days old bronchial outgrowth exposed for 1 week to 10% (A), 15% (B) or 20%(C) CSE. D represents the untreated control. Bar=50µm

F**IGURE 4.8**



Phase-contrast observations of a representative 28 days old bronchial outgrowth exposed for 2 weeks to 10% (A), 15% (B) or 20%(C) CSE. D represents the untreated control. Bar=25µm.

Figure 4.9



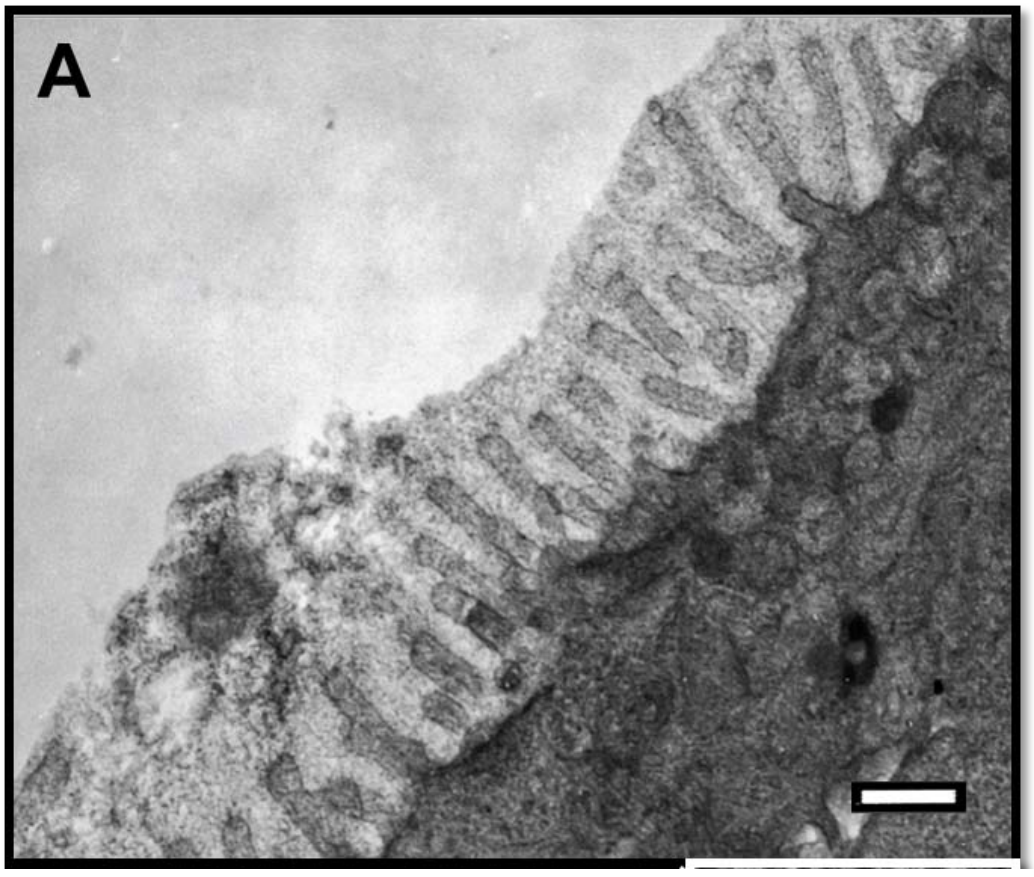
Phase-contrast observations of a representative 28 days old bronchial outgrowth exposed for 2 weeks to 10% (A), 15% (B) or 20%(C) CSE. D represents the untreated control. Bar=25µm.

4.3.2 TEM analysis

3D outgrowths were cultured for 28 days, in order to let them properly differentiate, before exposing them to CSE 15% for 21 days. During the exposure time, fresh medium containing CSE 15% or not (untreated control) was added to the outgrowths every 48 hours.

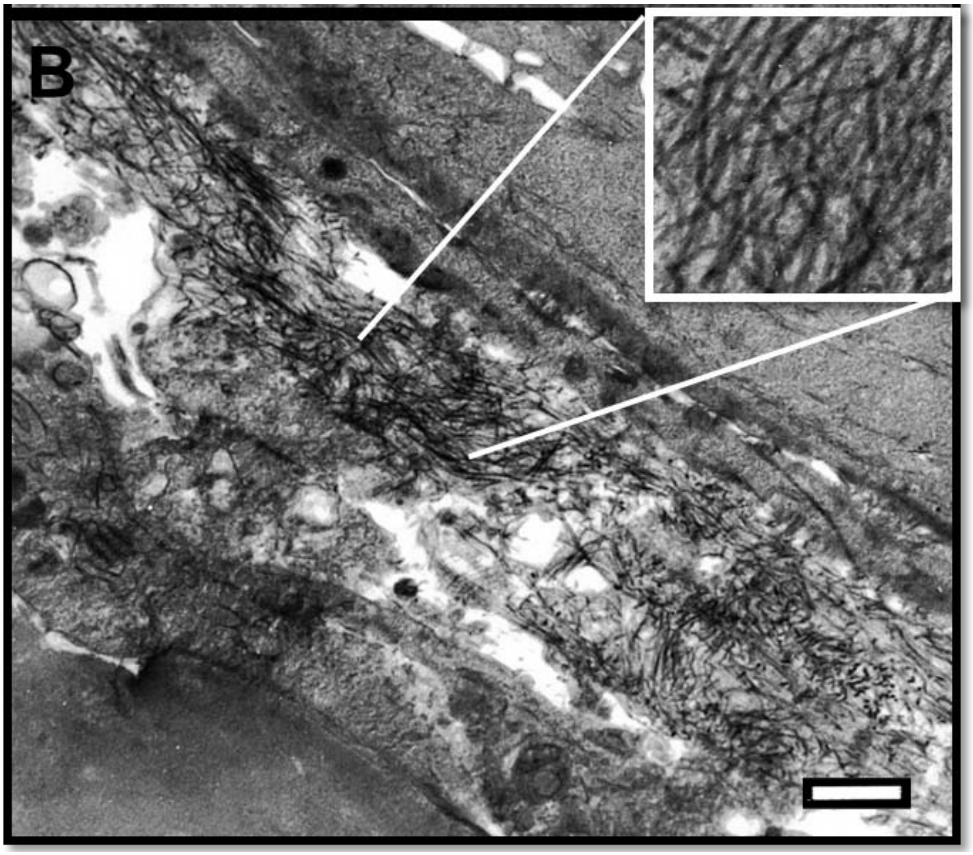
Long term exposure with CSE did indeed not cause any cell death amongst the two cell populations. However, the long term treatment caused a partial remodeling of the architecture of the 3D outgrowths. In particular, the apical epithelial cells completely lost their ciliated structures that were replaced by thick microvilli entirely covered by mucus (Fig.4.10 A).

Figure 4.10



Effects of CSE treatment on bronchial outgrowths' ultrastructure. Outgrowths were cultured for one month before being treated for 3 weeks with CSE 15%. It is possible to see modifications to the normal structures described earlier. In particular, the apical epithelial cells completely lose their ciliated structures, subsequently replaced by thick microvilli entirely covered by mucus. Bar=200nm.

Moreover, the fibroblast increased their production of collagens and this resulted in a clear thickening of the basement membrane and in a complete disarray of the fibroblast layer (Fig.4.10 B and inset). These structural modifications are compatible with those occurring after a chronic inflammatory insult. Interestingly though, our 3D outgrowth models are void of any immune or inflammatory cell components, as shown earlier.



Effects of CSE treatment on bronchial outgrowths' ultrastructure. Outgrowths were cultured for one month before being treated for 3 weeks with CSE 15%. It is possible to see modifications to the normal structures described earlier. There is a clear increase in the production of collagens by the firoblasts that results in the thickening of the basement membrane and a disarray of the fibroblast layer. Bar=1µm.

5. Discussion

Primary cultures or cell lines still represent the most commonly used *in vitro* human models to study responses of cell systems to specific stimuli. However, the main limitations of these models are the absence of the extracellular components and the loss of proper cell-cell communication that arises from the former. For this reason, our laboratory has been constantly driven by the need to develop better and more complex *in vitro* models of the human bronchial mucosa. In the last few years this necessity has been motivating other groups, as evidenced by the progressively increasing number of publications on this regard. Of particular interest, in the field of tissue engineering of the lung, is the research work by Huh and colleagues on the alveolar-endothelial membrane, or more anatomically relevant to the objective of this thesis, the research by Choe and colleagues on human bronchial airways. In the latter paper, the Authors developed a tissue-engineered human airway wall model, where differentiated bronchial epithelial cells were sitting on top of a collagen gel containing lung fibroblasts. Lateral compressive strain was then applied using a novel straining device and responses studied in terms of ECM remodelling. Their interesting results showed that dynamic strain induced increased deposition of collagen type III and IV as well as secretion of matrix metalloproteinase-2 and -9. Their conclusion was that "in a physiologically relevant three-dimensional model of the bronchial wall, dynamic compressive strain induced tissue remodeling that mimics many features of remodeling seen in asthma, in the absence of inflammation and dependent on epithelial–fibroblast signalling" (40).

In this experimental model however, bronchial epithelial cells and fibroblasts were first cultured separately and then co-cultured in an artificial collagen gel. This model, along with recently employed similar ones, is limited by the fact that the ECM is artificially laid out, the cells are already phenotypically modified before to even enter the three-dimensional system, and their life is quite short and therefore is not possible to undertake long-term experiments. In our model instead, both PBEC and fibroblasts outgrow autonomously from a bronchial biopsy into a 3D gel (Matrigel), whose composition is very similar to that of the normal ECM of the bronchial airways. Moreover, after the initial expansion phase, fibroblasts start to lay out a newly formed ECM that is architecturally and structurally compatible with that of the human bronchial airways. In the meantime, the PBEC start differentiating because of the air-liquid interface, and after around 30 days of culture the outgrowths present a properly differentiated bronchial epithelium, separated, by a functional basement membrane, from a newly constituted *lamina propria* where

fibroblasts lay the ECM. However, in our model there are two limitations. First of them is the complete absence of the immune cells, and the second being the lack of circulation. The latter could be easily overcome by the employment of microfluidics (41) while the former is may be considered an advantage, giving us the opportunity to selectively add, whether in the epithelial or in the mesenchymal layer, cytokines, chemokines or any other factor whose effects one might want to research. In our case, this lack of any immune or inflammatory elements has permitted us to verify that after a long-term exposure to CS, the newly formed bronchial mucosa undergoes a series of morphological changes (such as thickening of the basement membrane, loss of ciliated cells, increase of mucus production, disarray of the *lamina propria*) typical of the inflamed and remodelled mucosa seen in chronic inflammatory lung diseases, but without immune cell intervention.

Diseases such as asthma and COPD are difficult to be studied using animal models *in vivo* since there are too many important differences between animal and human cells at various levels. The epithelial response to cigarette smoke may represent an attempt by the airway epithelium to protect itself and repair the smoke-induced injury (42). These damages may lead to the development of squamous metaplasia, which is the reversible replacement of the columnar epithelium by squamous epithelium, an effect that has been correlated with airflow obstruction (43). Squamous cell metaplasia impairs mucociliary clearance and contributes to the increased risk of squamous cell carcinoma in COPD. A significant increase in airway smooth muscle in small airways of patients with COPD has been reported in several studies (44-46) The airway smooth muscle mass in the small airways seems to be the only differentiating feature when comparing nonobstructed patients with COPD with patients with asthma (47). Moreover, it has been shown that cytokines and chemokines are involved in many aspects of disease processes in COPD, including recruitment of neutrophils, macrophages, T-cells and B-cells, airway wall remodelling, goblet cell metaplasia, epithelial cell hyperplasia and the induction of emphysema. Other studies also showed an increase in alveolar epithelial and endothelial cellapoptosis in emphysematous lung tissue (48–50), associated with an increase in activated subunits of caspase-3 and loss of the anti-apoptotic protein Bcl-2 (48) reported that expression of vascular endothelial growth factor (VEGF) and VEGF receptor 2 protein and mRNA was significantly reduced in emphysema and, since these are maintenance factors for endothelial cells, this reduction may lead to endothelial alveolar septal death.

Unfortunately, due to the long time necessary to characterise the 3D outgrowth model, little time was left during the course of this PhD study for functional studies using this

model. Future work will therefore be focused on other typical bronchial mucosa stressors of interest in the COPD (RV infection, oxidative stress and eventual protective effect of antioxidants). The outgrowth model could also be applied to other tissues in order to study other diseases, where, similarly to COPD, little is known about the true interactions between resident and immune cells. The model would for example be of great interest for studies of cystic fibrosis. Furthermore, it would be of interest to test whether similar models of the enteric mucosa can be developed and used for the study of the pathogenesis of the celiac disease or other chronic inflammatory diseases of the bowel.

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