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In Vitro Models of Chronic Obstructive Pulmonary Disease (COPD)

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1. Introduction

1.1 The lung

The lungs are situated at the air-blood interface and are a crucial boundary between the organism and the environment, protecting the host from a battery of potential insults such as inhaled particles, pollutants, carcinogens and infectious agents that deposit on airway surfaces during normal tidal breathing. The upper or conducting airways (tracheo-bronchial region) are covered with a columnar epithelium composed of ciliated cells and mucus producing goblet cells (Figure 1A). The apical surface of the epithelium is covered by a surface liquid which is comprised of two distinct layers. The outer mucus layer provides a physical barrier that traps inhaled particles. The underlying periciliary fluid is a low viscosity liquid which allows cilia to beat and continually move the mucus layer towards the pharynx. Thus inhaled particles trapped in the mucus are cleared from the airways. Under normal conditions mucus protects the lung airway epithelium; however abnormalities in mucus hypersecretion or clearance can lead to respiratory disease (Rogers 2007). In the lower bronchioles, the epithelium is simple columnar, containing secretary Clara cells and has progressively fewer ciliated cells. The alveolar epithelium is composed primarily (95%) of flattened alveolar type I (AT-I) cells that form a thin barrier for gas exchange. These cells are interspersed with rounded alveolar type II (AT-II) cells that secrete pulmonary surfactant to decrease the surface tension within the alveoli and prevent alveolar collapse during expiration (Figure 1B).

1.2 Chronic obstructive pulmonary disease (COPD)

COPD is an umbrella term that is used to describe chronic lung disease and includes the familiar terms of chronic bronchitis, small airways disease and emphysema. A more specific definition of COPD is; 'a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases' (Global Initiative for Chronic Obstructive Lung Disease, 2009).

Pathologically the small bronchi (structures 2-4mm in diameter), small airways (<2mm in diameter) and the lower airway lung parenchyma are the main sites in which chronic bronchitis, small airways disease and emphysema develop. To monitor airflow and the changes in lung function that occur as a consequence of COPD, the volume of air forcibly expelled from the lungs in one second (forced expiratory volume in one second [FEV_1]) is measured. Lung function alters due to changes in the distensibility (compliance) of the lung and in the obstruction of the small airways. Increased compliance (emphysema) and/or decreased airflow (small airways disease), results in prolonged emptying of the lung and thus reduced FEV_1. The classic description of the changes in FEV_1 in smokers and those smokers that develop COPD is summarized in the study conducted by Fletcher & Peto in 1977.
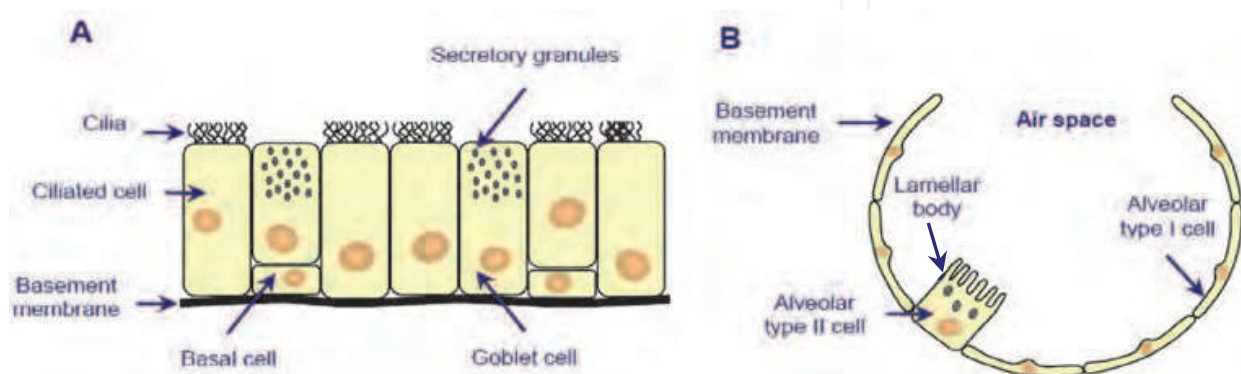

Fig. 1. A. Schematic representing the tracheal bronchial epithelium. The epithelium consists of ciliated, goblet and basal cells situated on top of the basement membrane. B. Schematic representing the alveolar epithelium. The epithelium consists of alveolar type I and type II cells situated on top of the basement membrane.

Inhalation of cigarette smoke, occupational and environmental pollutants are the main causes of COPD (Driscoll et al., 2005; Sethi et al., 2000) and affect all major compartments of the lung, including the central and peripheral airways, the parenchyma and the pulmonary vasculature. Smoking is known to be the most important risk factor for this disease (Oswald et al., 1955) but although some 80-90% of all COPD cases can be attributed to this activity (Sethi et al., 2000) considerable variation in the response to smoke is observed. It has been estimated that only 15% of the variation in lung function is explained by smoking (Beck et al., 1981) thus implicating a genetic predisposition to the disease.

Smoke exposure can directly injure the lung through the action of toxicants found within smoke but also through the attraction, activation and the release of pro-inflammatory mediators from cells of the immune system. These mediators, which can act locally to damage tissue, can also perpetuate the inflammatory response through the attraction of further inflammatory cells to the site of injury. In addition, smoke exposure also contributes to injury through an imbalance in the oxidant–antioxidant profile within the lungs of smokers. If the exogenous (cigarette smoke) and endogenous (inflammatory cells) oxidants outweigh the lung's antioxidant capacity, this can lead to injury and further inflammation. Thus oxidative stress and direct toxicant induced tissue injury drives inflammation and in susceptible individuals drives the disease process and the subsequent development of COPD. To fully understand the causes and elucidate the mechanisms associated with the pathogenesis of this disease and to develop appropriate therapeutic regimes, *in vivo* and *in vitro* studies are and have been vital.

2. *In vivo* models of COPD

Studying COPD using *in vivo* models is not ideal as there is no one model that encompasses all aspects of the clinical disease pathology. Instead there are a number of models that represent individual disease mechanisms or endpoints (MacNee 2005; Pauwels et al., 2001). Animal studies also possess the disadvantage of species variation including differences in respiratory anatomy, breathing patterns and lung protein expression profiles. The most common animal species used to date have been the mouse, rat and guinea pig; the mouse offering the unique opportunity for genetic manipulation, which have and will continue to help unravel key mechanisms underlying the development of cigarette smoke-induced COPD, including emphysema, small airway disease and chronic bronchitis.

2.1 Emphysema

Cigarette smoke-induced emphysema has been the one pathology of COPD that has attracted the most interest over the years and the use of animals to elucidate the mechanisms involved in this destructive process has been previously reviewed (Wright & Churg 2010; Yoshida et al., 2007). Studies conducted in mice, rats and guinea pigs demonstrate that prolonged cigarette smoke exposure results in the development of emphysematous like lesions. However, these lesions form primarily in the alveolar ducts, regions distinct from those seen in humans where the focus is mainly around the respiratory bronchioles. The degree of emphysema, even in the most chronically exposed animals is fairly mild compared to that seen in humans. This not only represents the intrinsic differences in the susceptibility of animals to cigarette smoke exposure but also to the fact that emphysema develops over many years of chronic smoke exposure, an exposure period that may not be fully captured *in vivo*.

Destruction of the connective tissue framework is the primary mechanism of alveolar destruction in emphysema which is brought about by an imbalance in the protease-antiprotease profile within the lung (Gross et al., 1964). In the Pallid mouse, a strain of mice with reduced alpha 1-proteinase inhibitor levels, cigarette smoke exposure leads to the development of emphysema (Takubo et al., 2002). Furthermore, neutrophil elastase null mice are protected against chronic cigarette smoke-induced emphysema and treatment with a neutrophil elastase inhibitor has been shown to decrease the airspace enlargement observed in control mice following cigarette smoke exposure (Wright et al., 2002). Hautamaki et al., 1997, demonstrated that matrix matelloproteinase 12 (MMP-12) null mice are also protected from the development of cigarette smoke-induced emphysema. The role for other MMPs in the development of emphysema is still under study, although early transgenic studies with MMP-1 indicated that this proteinase may also be involved in the development of emphysema (D'Armiento et al., 1992).

The important role that antioxidants play in protecting the lungs from smoke-induced emphysema has also been shown by the use of transgenic animal models. Nrf2, the key transcription factor involved in upregulating intracellular antioxidants is key in protecting the lungs against smoke-induced emphysema (Sussen et al, 2009). In rats, administration of antioxidants in smoke-exposed or elastase-treated animals decreases inflammation and ameliorates the emphysematous lesions that develop (Smith et al., 2002). These observations have been mirrored in chronically smoke-exposed transgenic CuZnSOD animals that are 100% protected (Foronjy et al., 2006). The use of antiproteases and anti-inflammatory compounds have been considered in the treatment regime of patients with emphysema and have been proved effective in animal models (Roh et al., 2010). However, few human trials

of compounds based on predictions from animal studies have been successful (Barnes, 2007). This discrepancy between human and animal data and the efficacy of anti-inflammatory and anti-oxidant therapy needs to be resolved.

2.2 Small airway disease

Small airway disease is an important cause of airflow limitation in smokers with COPD (Pare et al., 1991). Although little attention has been paid to the use of animal models of small airways disease many studies now show that small airways disease manifests in animals as an increase in airway wall collagen after smoke exposure. Studies conducted in mice (Churg et al., 2006) and guinea pigs (Churg et al., 2007) demonstrate that smoke exposure increases airway thickness and correlates with reduced expiratory flow and increased airway resistance (Wright et al., 2007).

Both B and T lymphocytes are increased in the small airway walls of humans with COPD (Cosio et al., 2002; Hogg et al., 2004). The interaction between inflammation and small airway remodeling has been addressed in rodent models (Lee et al., 2002). Interleukin (IL) 10 over expression causes mucus cell metaplasia, B and T cell inflammation and the sub-epithelial fibrosis of the airways. Fibrosis of the peribronchial region was also seen in mice over expressing IL-1β (Lappalainen et al., 2005) indicating that many of the mechanisms involved in the features of small airways disease are mediated by multiple mechanisms.

2.3 Chronic bronchitis

Chronic bronchitis is associated with an inflammatory response involving the small bronchi, leading to abnormal remodelling, chronic cough and the accumulation of excessive mucus in the airway lumen due to goblet cell metaplasia and/or hypersecretion. The use of animal models to explore the mechanisms associated with chronic bronchitis has not been extensive, but has been previously reviewed (Nikula et al., 2000). In the guinea pig cigarette smoke induces secretary cell metaplasia (Wright et al., 1992) which is analogous to that seen in humans. In contrast, cigarette smoke exposure has little effect in the mouse (Bartalesi et al., 2005) with only a few secretory cells appearing in the small airways. In the larger airways cigarette smoke-exposed rats (Rogers et al., 1986) and guinea pigs (Komori et al., 2001) exhibit significant goblet cell metaplasia which can be attenuated with treatment with anti-inflammatory agents and antioxidants.

2.4 Transgenic and gene targeted models and COPD

Gene depletion and over expression in mice is a way of identifying the function and role of distinct genes in disease. An early application of this approach was the over expression of collagenase 1 which resulted in airspace enlargement in the mouse (D'Armiento et al., 1992). This challenged the elastase-antielastase hypothesis and identified collagen as a potential player in the development of emphysema. However, mice do not express collagenase 1 but rather two other similar proteases, collagenase 2 and 3 and thus limited the interpretation of this study. Another transgenic mouse model that may prove useful in future COPD related research is the Marlboro mouse (Shapiro, 2000). These animals carry the null gene for macrophage elastase (MMP-12), which in man is expressed in the macrophages of cigarette smokers and in patients with emphysema. In chronically smoke-exposed MMP-12 null mice, macrophages are not recruited to the lung and nor do these animals develop emphysema. Over expression of IL-13 has been shown to lead to emphysema in adult mice (Zheng et al., 2000). The resultant inflammation and lung destruction is metallo- and cysteine proteinase

dependent. These mice exhibit airway remodeling with goblet cell hypertrophy, driven in part by the MMP-9 mediated activation of transforming growth factor-beta (TGF-β). A similar proteinase dependent pathway has also been established for emphysema through the effects of over expressed interferon- gamma (IFN-γ) (Wang et al., 2000). This also results in inflammation and suggests a potential Th1 pathway involvement in the development of emphysema.

Prior to the advent of gene targeting technology, several natural mutants were known to develop airspace enlargement and included the tight skin (Tsk) (Green et al., 1976), Pallid (de Santi et al., 1995), Blotchy (McCartney et al., 1988)) and Beige (Barbosa et al., 1996) mice. Tsk mice have a mutation in fibrillin-1, a matrix protein that is an important component of elastic fibres (Kielty et al., 1998), whilst the Blotchy mouse has a deficiency in copper metabolism that results in reduced lysyl oxidase activity, a key collagen and elastin cross linking enzyme. These naturally occurring mutations can help to uncover key pathways in lung development but also in the development of tissue injury and remodeling following cigarette smoke exposure. These gene targeting techniques are useful tools to examine potential molecular mechanisms underlying human COPD. In combination with cigarette smoke exposure new transgenic and gene-targeted models will help further elucidate the role of key inflammatory and immuno-regulatory molecules in the development of COPD.

Currently available animal models are restricted to investigating a limited number of the varied and extensive characteristic features of COPD. Although in future combined models of inhalation exposure, gene targeting techniques and naturally occurring mutations may provide more appropriate models of COPD, these may not necessarily be better. There is still concern with *in vivo* systems as to their utility in predicting the pathophysiology and pathogenesis of COPD. There are clear structural, biochemical and physiological differences between animal and human lungs that make translation of animal data to man difficult. However, in conjunction with other experimental approaches, such as the use of *in vitro* models utilising tissue derived from smokers, non smokers and individuals with COPD, a clearer understanding of the molecular and biochemical process involved in the development of COPD will be established.

3. *In vitro* models of COPD

In vitro tests are often used prior to or in place of *in vivo* and subsequent clinical studies. *In vitro* tests are designed to generate rapid, initial data that will give general insight into disease mechanisms and the biological effect of test compounds and materials. There is a general shift in employing human cells and tissues to ensure as many of the physiological parameters are maintained in the *in vitro* test systems. Typical *in vitro* systems modelling an organ such as the lung can include the use of established continuous cell lines, primary cells and tissues (such as organ slices). Primary cell cultures are explanted directly from either a healthy or diseased donor organism and can keep their functional differentiated state for a short period (days to weeks). These cells have a limited life span. However maintenance of the differentiated properties has been improved slightly with the addition of additives to the culture medium, the use of biological scaffoldings using components of the extracellular matrix or by different forms of co-culture. Permanent, continuous cell cultures have acquired the ability to proliferate indefinitely either through a natural or introduced mutation. Most continuous cell lines have originated from embryos, tumors or transformed cells. There are countless well established cell lines representative of different cell types, for example NCI-H292 that are specific for human lung epithelial cells. The disadvantages of

continuous cell lines are that they do not retain many features of the original tissue; they may not accurately represent the *in vivo* situation as the phenotype of immortalised cells often differs from that of the normal tissue. A major advantage of using immortalised cell lines is that they are readily available, stable, easy to handle and convenient. Cell lines are homogenous populations therefore reducing donor to donor variability.

In comparison to *in vivo, in vitro* studies offer a number of advantages including:

- more flexibility
- generation of reproducible data as *in vitro* studies can be better controlled
- avoidance of animal species variation and animal/human extrapolation due to the availability of human tissue
- direct access and investigation to cellular components and biomolecules
- easier and quicker to perform
- more economical
- reduction in the number of animals used in research.

Each *in vitro* system has advantages and disadvantages, but it is generally accepted that the closer the system is to the whole organ (as it functions naturally and with endogenous cell types), the more accurate the predictive results will be.

COPD is a multifaceted disease and one *in vitro* model would not be able to replicate the entire disease pathogenesis. Consequently informative *in vitro* models of COPD must utilise the different cell types involved in disease pathogenesis and model endpoints with clinical relevance such as pro-inflammatory mediator release, goblet cell hyperplasia, cilia dysfunction, squamous cell metaplasia and emphysema. The development of pulmonary *in vitro* models began in the 1990's (Sporty et al., 2008) and despite the large number of pulmonary *in vitro* models described in the literature, currently there are no validated *in vitro* models of COPD. To demonstrate their utility, we will describe a number of *in vitro* models of the respiratory tract commonly used by researchers to investigate the mechanisms of COPD and some developed in our laboratories investigating the effect of cigarette smoke. We will focus predominately on *in vitro* models utilising human tissue.

3.1 Modelling the tracheo-bronchial airways
3.1.1 Primary cell cultures

Publications documenting the differentiation of primary lung cells into a mucociliary epithelium found in the tracheo-bronchial airways have been reported as early as 1984 (Lee et al., 1984). Initial studies described primary epithelial cells cultured on plastic and submerged in medium, however, more recent protocols describe the culture of cells at an air-liquid interface (ALI) (Gray et al., 1996). Primary human bronchial epithelial cells (HBECs) obtained directly from surgical tissue or low passage primary cells are available from several commercial sources. Cells are seeded on collagen coated surfaces in hormone and growth factor supplemented medium. Initially cells proliferate quickly to form a monolayer of undifferentiated cells. Proliferation then decreases and, after placing cells at an ALI, cells undergo differentiation in 2-3 weeks, producing a columnar epithelium containing goblet, ciliated and basal cells (Figure 2). Well-differentiated tracheo–bronchial cultures have been described for a variety of species including mouse (Davidson et al., 2004), rat (Ostrowski et al., 1995) horse (Schwab et al., 2010) hamster (Lee et al., 1984), ferret, pig (Liu et al., 2007) and human (Gray et al., 1996; Haswell et al., 2010).

Commercial 'ready-to-use' fully differentiated HBEC cultures are now readily available and include EpiAirway® by MatTek (http://www.mattek.com/pages/products/epiairway) and

MucilAir™ by Epithelix (http://www.epithelix.com/content/view/4/4/lang,en/).The MucilAir™ product has a unique advantage over both in-house derived HBEC cultures and the commercially available EpiAirway® model: the cultures are able to remain fully functionally differentiated for more than one year. This provides the potential for long-term or repeat exposure studies which could help model clinically relevant COPD pathologies that require chronic exposure to agents such as cigarette smoke.
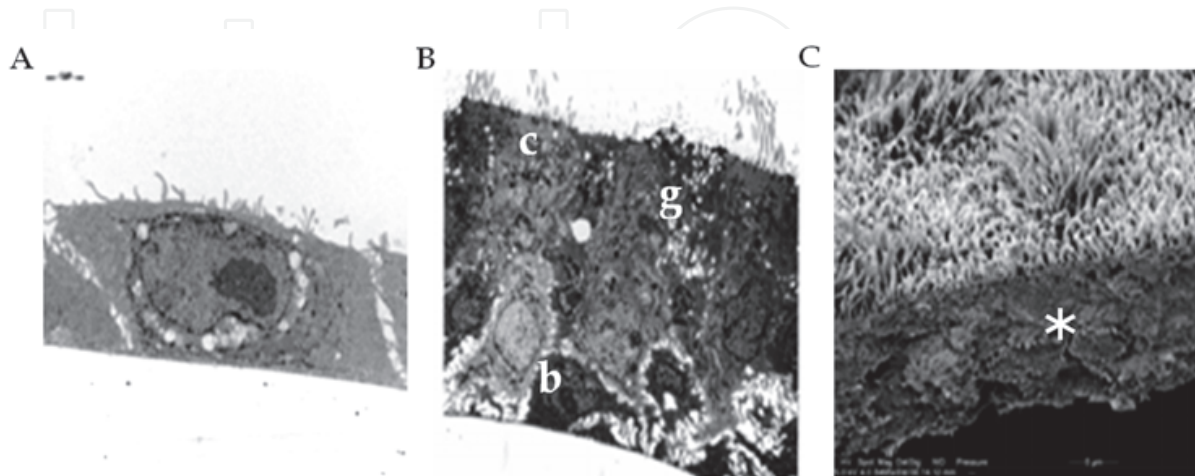

Fig. 2. Transmission (A and B) and scanning (C) electron micrographs of human bronchial epithelial cell air-liquid interface cultures at day 1 (A) and day 28 (B and C). At day 28 cultures had developed into a columnar epithelium containing basal cells (b), mucus containing goblet cells (g) and ciliated cells (c). The fractured culture edge of a culture is indicated by ✳ in the scanning electron micrograph. (Haswell et al., 2010).

The use of highly differentiated models of the conducting airways allows for the investigation of inhaled agents to specific cell types and the simultaneous interaction between different cell types in response to exposure. Moreover, culturing cells at an ALI supports the direct exposure of cultures to aerosols and gases thus better modelling an *in vivo* exposure. Despite these clear physiological advantages for using primary differentiated cells, HBEC cultures have several limitations including, tissue availability, limited number of cells harvested from each isolation, a limited replicative lifespan, donor to donor variation and relatively high cost. Regardless of these constraints, primary differentiated cell cultures have been used in a large number of studies to investigate the effects of cigarette smoke on the conducting airways.

Goblet cell hyperplasia is a characteristic feature of the lung epithelium in patients with COPD contributing to the overproduction of airway mucus, including the mucin MUC5AC (Rogers, 2007). Chronic inhalation of mainstream cigarette smoke has been shown to increase the number of goblet cells, up-regulate MUC5AC at the gene level in the airways of smokers (Cosio et al., 1980; Innes et al., 2006; Saetta et al., 2000) and at the protein level in patients with COPD (Ma et al., 2005). We recently reported a study using primary HBECs as an *in vitro* model of differentiated lung epithelium, investigating the morphological and cellular changes in response to non-cytotoxic doses of cigarette smoke particulate matter (PM) and three mainstream cigarette smoke constituents: acrolein, formaldehyde and acetaldehyde (Haswell et al., 2010). HBECs from three different donors were exposed basally to cigarette smoke PM and the constituents for 28 days during the differentiation period. Using both flow cytometry and immunocytochemical techniques for identification

of MUC5AC positive cells, cigarette smoke PM treatment induced an increase in MUC5AC positive cells when compared to untreated control cultures. Treatment with acrolein also increased the percentage of MUC5AC positive cells in the HBEC cultures. However, formaldehyde and acetaldehyde (maximum dose 1µM) had little effect. This study demonstrated for the first time that cigarette smoke and acrolein, known lung toxicants, induce an increase in the percentage of goblet cells in an *in vitro* model of human lung epithelium. This response reflects, to an extent, the goblet cell hyperplasia observed in animal inhalation models, smokers and patients with COPD.

Another frequent observation in the tracheo-bronchial mucosa of cigarette smokers who develop COPD is squamous cell metaplasia (SCM) (Jeffery, 2000). SCM is the replacement of the normal mucociliary epithelium with a stratified squamous epithelium. SCM is considered to be an adaptive response, protecting the lumen from the effects of inhaled agents. However, the assessment of cigarette smoke on SCM induction often relies on human epidemiological data or *in vivo* animal inhalation studies. HBECs cultured using medium without retinoic acid became squamous, mucin secretion decreases and expression of the squamous cell markers transglutaminase-1 (Gray et al., 2007) and involucrin (BAT unpublished data) are elevated. Although culturing HBECs without retinoic acid has not been fully developed and characterised as an *in vitro* model of SCM it could potentially provide a method to allow the assessment of cigarette smoke and its constituents on SCM induction.

Mucociliary dysfunction is caused by mucus hypersecretion coupled with a decrease in mucus transport, and represents an important pathophysiological component of COPD. Effective mucociliary clearance requires both the appropriate amount of mucus and the co-ordinated cilia beating to clear mucus and remove inhaled agents from the lung. Smoking has been reported to adversely affect the function of cilia (Elliott et al., 2006; Simet et al., 2010; Sisson et al., 1991;Verra et al., 1995). Differentiated HBEC cultures are highly ciliated (Figure 2C) therefore the investigation of cilia beat frequency (CBF) following exposure to cigarette smoke could provide important information on mucociliary dysfunction. CBF is a measurable and tightly regulated function of the ciliated epithelium. CBF can be determined by high speed video microscopy; this requires specialised equipment, trained personnel and is highly time consuming. However, the development of the SAVA system, a high-speed all-digital video imaging system to measure CBF could shorten the analysis time and negate the need for expensive microscopy equipment (Sisson et al., 2003).

Primary HBEC cultures have also been used to investigate the effects of cigarette smoke on cell signalling and function. In a study by Maunders et al., 2007, HBECs from three different donors were exposed to air or non-cytotoxic doses of whole mainstream cigarette smoke for 1 hour and gene expression profiles were then determined post-exposure using whole genome Affymetrix microarrays. Many direct effects of cigarette smoke found in this study were consistent with previous reports of *in vivo* and *in vitro* cigarette smoke toxicity studies, such as increased epithelial permeability, activation of antioxidant responses, and cell signaling pathways (Boucher et al., 1980; Hackett et al., 2003; Mossman et al., 2006), thus demonstrating HBEC cultures are able to model key features of cigarette smoke-exposed conducting airways.

Many cigarette smoke toxicants are biologically inactive until transformed by metabolic enzymes into reactive intermediates. For example, the cigarette smoke constituent benzo(a)pyrene, when activated generates reactive forms capable of binding to DNA (Castell et al., 2005). Therefore the metabolic capacity of *in vitro* models of COPD is important. In a recent study Newland et al., 2001, characterised the expression and activity

of relevant cytochrome P450 (CYP) metabolizing enzymes, CYP1A1/1B1, and CYP2A6/2A13, in primary ALI HBEC cultures. HBEC CYP activity and inducibility was conserved over the 28 day culture period (Newland et al., 2011).

3.1.2 Cell lines

Immortal or continuous cell lines are commonly used to model COPD *in vitro*. NCI-H292 is a bronchial epithelial cell line derived from a mucoepidermoid carcinoma (Carney, 1985). Several studies have reported the responses of NCI-H292 cells to cigarette smoke are similar to that of primary HBECs and the airway epithelium *in vivo* (Baginski et al., 2006; Newland & Richter 2008; Phillips et al., 2005; Shao et al., 2004). In a study by Phillips et al., 2005, NCI-H292 cells were cultured on inserts and exposed at the ALI to whole smoke for 30 minutes. Low doses of smoke were shown to induce COPD associated markers including the up-regulation of MUC5AC mRNA and the production of the inflammatory mediators IL-6, IL-8 and MMP-1. NCI-H292 cells have also been shown to respond to a variety of agents associated with inhalation toxicity including cigarette smoke PM via various endpoints linked to inflammation (IL-6 and IL-8), airway remodelling (MMP-1, GM-CSF), and mucin overproduction (MUC5AC) (Newland & Richter 2008). Although NCI-H292 cells have been extensively used as a lung model for toxicological assessment, they lack critical metabolic activation capability, in particular they did not show CYP2A6/2A13 activity (Newland et al., 2011).

There are several other cells lines that have been used to model the tracheo-bronchial airways. 16HBE14σ cells originate from a normal human bronchial epithelial cell line that has been transformed by the SV40 large T-antigen. These cells retain differentiated epithelial morphology and functions, forming polarised monolayers with functional tight junctions (Cozens et al., 1994). The cell line BEAS-2B is another normal human bronchial epithelial cell line and was transformed using the adenovirus 12-simian virus 40 hybrid virus. Although continuous cell lines have been around for a while there is no general agreement as to which is the most appropriate.

3.2 Modelling the alveolar region
3.2.1 Primary cells

Currently there are no available or reported cell lines that possess significant functional properties of alveolar epithelial cells (Forbes & Ehrhardt, 2005). The primary culture of alveolar epithelial cells is therefore used for most *in vitro* studies of the alveolar epithelium. Human alveolar epithelial cells are isolated from human patients undergoing lung resection. These cells, when plated on permeable supports or plastic exhibit AT-II cell characteristics that include lamellar bodies, apical microvilli, tight junctions, and expressed surfactant (Witherden, 2004). After approximately eight days of culture AT-II cells can acquire the AT-I cell-like morphology (Elbert et al., 1999; Fuchs et al., 2003). As with primary HBECs, primary alveolar cells have the same limitations including a limited replicative lifespan, the limited number cells obtained from each isolation, tissue availability and donor variation.

3.2.2 Cell lines

The adenocarcinoma cell line A549 is the most widely used alveolar cell line, however, due the lack of tight junction formation, a key feature of the alveolar epithelium, the cell line potentially has limited value (Forbes & Ehrhardt 2005). In addition A549s have low levels of

P450 activities, a limited number of phase I enzymes (Castell et al., 2005) and they do not retain significant metabolic activity, having reduced CYP1A1/1B1 or CYP2A6/2A13 activity (Newland et al., 2011). Moreover studies have shown A549s are not as sensitive to cigarette smoke exposure as primary cultures (Kode et al., 2006; Newland & Richter 2008).

3.3 Airway epithelium co-cultures

COPD involves the interplay of several systems including the respiratory, immune and cardiovascular systems. Therefore to model more complex endpoints and to investigate the underlying mechanisms by which cigarette smoke and other agents cause disease requires more complex culture systems that model the interactions between different cell types. Co-cultures contain either primary or continuous cell lines of epithelial origin in culture with either primary or continuous cell lines from a variety of different sources including the endothelial cells, fibroblasts and immune cells (Table 1). The co-culture of different cell types can be achieved in different ways. The simplest is the culture of two different cell types in the same medium e.g., a collagen gel, but more commonly inserts are used to separate the cell types. The cells can be seeded both on the insert or either side of the semi-permeable membrane, thus creating a bi-layer or co-culture system, as in Figure 3. The establishment of co-cultures is not easy as the differing culture requirements of each cell type creates a technical challenge. The development of co-cultures has allowed the cell-to-cell communication and interactions of differing cell types to be modelled *in vitro*, which is not possible using mono-cultured cells.

Airway cell	Other cell type	Interaction being modelled	Reference
16HBE14o	Human umbilical vein endothelial cells	Epithelial-endothelial	(Chowdhury et al. 2010)
A549 or NCI-H441	Human pulmonary microvascular endothelial cells	Epithelial-endothelial	(Hermanns et al. 2004)
NCI-H441	ISO-HAS-1	Epithelial-endothelial	(Papritz et al. 2010)
NCI-H441	Human pulmonary microvascular endothelial cells	Epithelial-endothelial	(Hermanns et al., 2009)
Calu-3 or A549	Peripheral blood mononuclear cells	Epithelial-immune	(Korpi-Steiner et al. 2010; Torvinen, Campwala, & Kilty 2007)
HBECs	Monocytes	Epithelial-immune	(Korpi-Steiner et al., 2010)
16HBE14o or A549 or primary human AT-I	Human monocyte-derived macrophages and dendritic cells	Epithelial-immune	(Blank et al. 2011; Lehmann et al. 2011; Rothen-Rutishauser, Kiama, & Gehr 2005)
16HBE14o or A549	Human monocyte-derived macrophages or dendritic cells	Epithelial-immune	(Blank, Rothen-Rutishauser, & Gehr 2007)
A549	Fibroblasts	Epithelial-mesenchymal	(Noguchi et al. 2007)
HBECs	Fibroblasts	Epithelial-mesenchymal	(Araya et al. 2007)
A549	Fibroblasts	Epithelial-mesenchymal	(Liu, Gao, & Zhang 2010)
HBECs	Fibroblast cell line Wi-38	Epithelial-mesenchymal	(Pohl et al. 2009)
Primary human AT-II	Human pulmonary microvascular endothelial cells	Epithelial-endothelial	(Hermanns et al. 2009)

Table 1. A summary of various *in vitro* co-culture COPD systems described in the literature.

To date only two studies have reported exposing airway co-cultures to cigarette smoke. Both studies were co-culture models of A549 cells with fibroblasts. Fibrosis of the small airways and respiratory bronchioles has been found to cause increased airway wall thickness in smokers compared with nonsmokers (Kim et al., 2008) and it is thought that epithelial cells and fibroblasts are involved in matrix deposition at the sites of lung injury. In one study human foetal lung fibroblasts and A549 cells were cultured in a collagen gel and exposed to cigarette smoke extract (Noguchi et al., 2007). The authors found these co-cultures prevented the inhibition of fibroblast-mediated collagen gel contraction induced by cigarette smoke and suggest that the epithelial cells protected the fibroblasts from cigarette smoke induced injury. The effect of cigarette smoke extract on the interaction of the alveolar epithelial cells and fibroblasts was also investigated by Liu et al., 2010. Human lung fibroblasts were cultured below an insert containing A549 cells and differing responses between both mono-cultured cells and co-cultured cells were observed. Low concentrations of cigarette smoke extract produced epithelial-mesenchymal transition in co-cultured A549 cells but not in mono-cultured A549 cells. This co-culture system may resemble the *in vivo* situation more closely than in mono-cultured cell systems by allowing cell-to-cell interactions important in disease pathogenesis to be modelled.
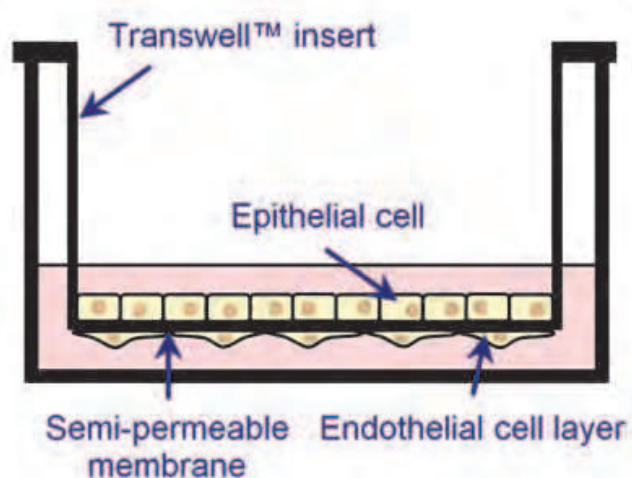

Fig. 3. Schematic of a bi-layer co-culture model. Lung epithelial cells and endothelial cells are grown either side of a semi permeable membrane.

Several other co-culture systems have been reported that model endpoints key in the development of COPD. The co-culture of HBECs with airway fibroblasts, to model human airway-mesenchymal interactions, has allowed the investigation of mechanisms by which SCM induces a fibrotic response in the adjacent airway fibroblasts (Araya et al., 2007). HBECs and human monocytic cell co-cultures have modelled interactions between the airway epithelium and the immune system during human rhinovirus infection, which is a major cause of exacerbations in patients with COPD (Korpi-Steiner et al., 2010). In addition co-culture of HBECs with fibroblasts have indicated that co-culturing these cell types extends the culture life of HBECs. HBECs grown in a bi-layer model with the fibroblast cell line Wi-38 were shown to differentiate and maintain a mucociliary phenotype for at least 3 months (Pohl et al., 2009). This type of culture system could permit the investigation of epithelial-mesenchymal interactions in a chronic or repeated exposure situation.

Recently a bi-layer system with primary human AT-II cells with human pulmonary microvascular endothelial cells has been described (Hermanns et al., 2009). In this system AT-II cells partly differentiated into AT-I like cells establishing a bi-layer model that reflects the cellular composition of the alveolar epithelium *in vivo*. This complex co-culture system could provide a suitable *in vitro* model to investigate the effects of cigarette smoke on the structural and functional behaviour of the alveolar epithelium using primary tissue. Studies using co-culture systems have not been limited to just two cell types. The cell lines 16HBE14σ and A549 and human primary AT-I cells have all been co-cultured with macrophages and dendritic cells (Blank et al., 2007; Lehmann et al., 2010; Rothen-Rutishauser et al., 2005). Co-cultures are becoming increasingly more complex and enable the study of the interactions between the immune system and cells of the human airway barrier.

3.4 Lung slices

Organ slices have been used for a wide range of biochemical studies for several decades (Parrish et al., 1995). Organ-slice cultures can be particularly beneficial for modelling the pathological processes and the underlying mechanisms of a complex disease such as COPD for several reasons. Organ-slice cultures maintained *in vitro* have the potential to preserve all cell types present in the original tissue in the correct spatial configuration. Data derived from organ slices maintained *in vitro* could provide an important link between studies on isolated cells and *in vivo* models. Unlike many continuous cell lines, lung slices retain the composite metabolic activity of the lung parenchyma and can be used in studies requiring bioactivation (Freeman & O'Neil 1984). Relatively large numbers of slices, up to 30 from one resection, can be generated from each donor (Wohlsen et al., 2003). However, large variation exists between sequential slices and also from donor to donor. One major disadvantage of lung slices is their limited life span, approximately one week *in vitro* (Parrish et al., 1995). Therefore their utility is currently restricted to short term studies. Lung slices for *in vitro* culture have been produced from a variety of species including rats, hamsters, guinea pigs, rabbits, horses and humans.

In the literature there are many different methods available that have not been standardised with respect to slice preparation, thickness, stabilisation and culture media. Organ slices can be prepared by either tissue slicers or mechanical slicers, the latter are often referred to as precision cut lung slices. An important consideration when deciding upon slice thickness is that cut surfaces will contain damaged cells (Freeman & O'Neil 1984). As the slice thickness increases the percentage of damaged cells will decrease. However, as slice thickness increases diffusion pathways are extended, potentially leading to inadequate gas diffusion and substrate delivery. Optimal lung slice thickness has been described as between 500-700μm, this is relatively thick when compared to 200-350μm for slices of the liver, kidneys and heart (Parrish, et al 1995). Stabilisation of the organ slices prior to exposure can reduce the impact of slicing induced cell damage. A large number of different culture media have been used to maintain the lung slices *in vitro* but as yet there is no consensus on which is the best approach.

To date there have been very few studies that have utilised lung slices to model cigarette smoke exposure. In a recent study precision-cut lung slices from guinea pigs exposed to cigarette smoke were used to detect endothelial dysfunction in pulmonary arteries (Wright & Churg 2008). Several studies have also used lung slices to examine the effects of cigarette smoke constituents including acrolein (Fisher et al., 1994), benzo(a)pyrene (Harrigan et al., 2004) and cadmium (Lin et al., 2010).

4. Exposure systems

Appropriate exposure of *in vitro* models to cigarette smoke, diesel emissions and other aerosols and particles implicated in the development of COPD is crucial. How aerosols and particles are collected and presented to the *in vitro* model system needs careful consideration. In the field of cigarette smoke toxicity and biological testing many studies have attempted to develop relevant and appropriate cigarette smoke test substances and systems. The impotance and intricacies of these systems will be discussed.

4.1 What is cigarette smoke?

Cigarette smoke is a concentrated, complex and dynamic aerosol consisting of several thousands of chemicals (Rodgman & Perfetti, 2008). The smoke aerosol is divided into two phases: a particulate and a gas/vapour phase. The particulate phase is the minority fraction and constitutes 4-9% of the total smoke by weight; the gas phase is the majority fraction and comprises the remaining 91-96% by weight (Clunes, 2008). The combination of the particulate and gas phase is termed 'whole smoke', capturing any interactions or synergies between the two. The exact number of chemicals in cigarette smoke is unknown, and this is due to the technical challenges in identifying and quantifying the chemical constituents present in smoke. Some researches have speculated that as many as 100,000 chemicals are present (Wakeham, 1972, as cited in Liu et al., 2011), however a more conservative estimate would put the number at 5,300 identified compounds (Rodgman & Perfetti, 2008). Examples of chemicals in the gas phase include formaldehyde, acrolein, and hydrogen cyanide (associated with COPD); examples of chemicals in the particulate phase include polycyclic aromatic hydrocarbons and tobacco specific nitrosamines (TSNAs) (associated with cancer) (Hoffmann et al., 1997). The leading smoke toxicants identified as relating to disease are largely products of combustion and are found in the gas phase rather than in the particulate phase (Laugesen & Fowles, 2005), hence the importance of performing biological assessment using whole smoke rather than any individual phase alone.

4.2 Generating smoke for *in vitro* testing

For *in vitro* testing cigarette smoke can be generated on smoking machines, of which there are many commercially available and will be described later. There are several methods to trap either or both of the particulate and gas phases of the smoke for exposure to cell and tissue cultures. The three main types of 'smoke' generated for *in vitro* tests described here are PM, aqueous extracts of smoke also termed cigarette smoke extract (CSE), and direct whole smoke exposures in an exposure chamber.

PM is trapped on a Cambridge filter pad (CFP) when inserted directly in-line of the smoke generation. The pad efficiently traps 99.9% of all particles >0.1μm (Johnson et al., 2009) which can later be eluted using a solvent such as dimethyl sulphoxide (DMSO) and diluted in cell culture medium prior to exposure to submerged cell culture systems (Figure. 4A). This is a relatively simple, quick and robust method for the biological assessment of cigarette smoke exposure (Haswell et al., 2010; Newland & Richter 2008) but crucially it only captures <5% of whole smoke (Clunes, 2008) and will not contain volatile compounds (Johnson et al., 2009). Solvents used to extract PM from filters can also affect the way in which cells respond, for example DMSO is a known antioxidant. Furthermore, these cell cultures are submerged and this type of exposure method lacks in physiological relevance to the human lung where epithelial cells are exposed to air.
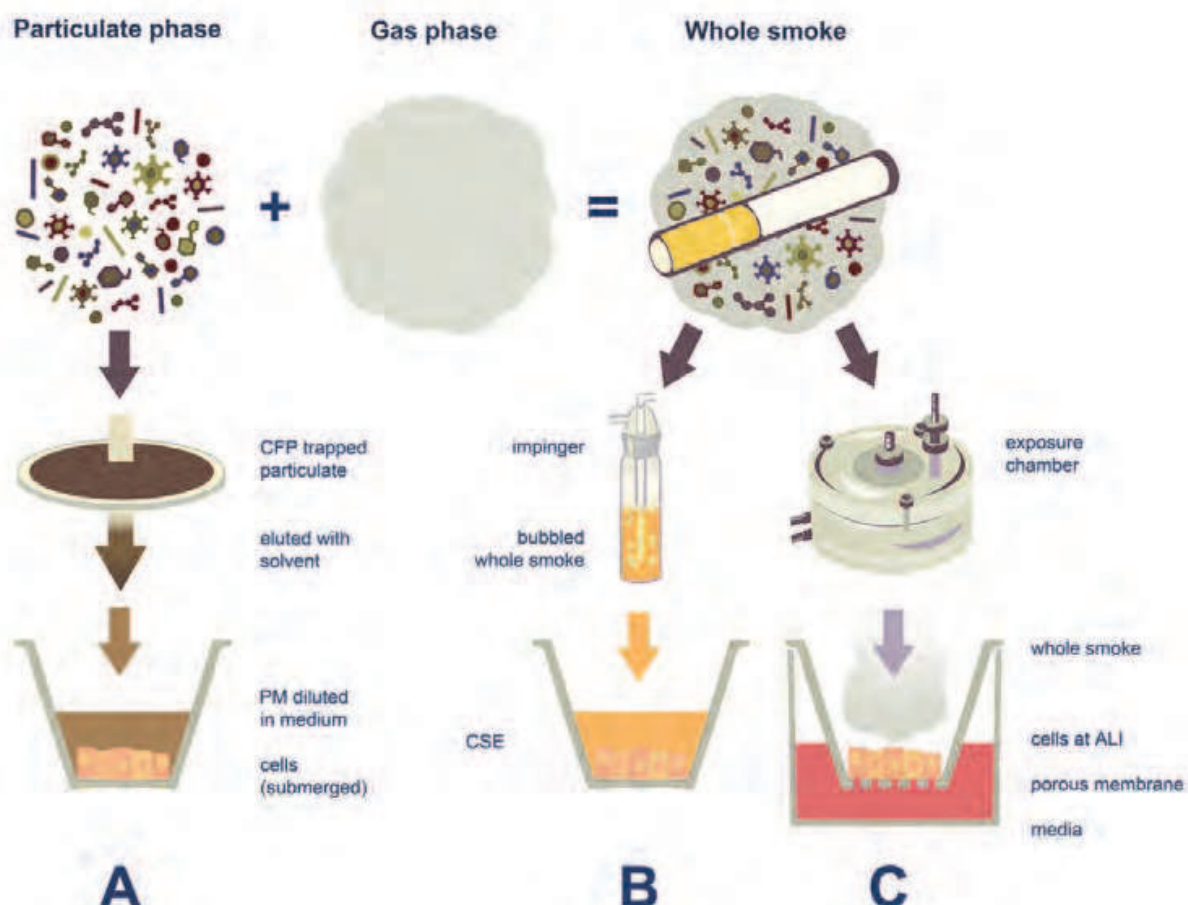
Fig. 4. Three general methods of generating smoke for *in vitro* testing: (A) particulate matter (PM), (B) Aqueous cigarette smoke extracts (CSE) and (C) whole smoke.

Cigarette smoke aqueous extracts (CSE) are collected using impingers, a piece of glassware designed to hold a liquid medium and which can be attached to a smoking machine. As the machine puffs on the cigarette, whole smoke is drawn through the impinger, bubbles through and dissolves into the cell culture medium or buffer within it (Figure 4B). The CSE can be diluted and added to cells in submerged culture conditions (St-Laurent et al., 2009). The benefit of this method is that it captures both particulate and gas phases of smoke, although there is uncertainty as to exactly which chemicals are trapped effectively and at what concentrations. Currently we are performing analyses on the collected CSE to quantify and qualify its composition. As before, cell cultures exposed to CSE are submerged and again this type of exposure method lacks in physiological relevance to the human lung. However, this method is useful when exposing endothelial cell types or anchorage-independent cell types where a submerged exposure is preferred.

Lastly, cells can be exposed to whole smoke within a specially designed exposure chamber which holds cells at the ALI (Maunders et al., 2007; Phillips et al., 2005; Thorne et al., 2009). This exposure method was developed in response to the challenges of making *in vitro* exposures akin to the *in vivo* situation. ALI exposures are more physiologically relevant, where the cells or tissues are exposed apically to smoke and supported on an

insert (porous membrane) basally with cell culture medium (Figure 4C), resembling more closely the *in vivo* configuration. This method has many advantages over the previous two methods described, and is especially relevant to *in vitro* models of COPD using mono or bilayer models, 3D lung tissue constructs or whole lung slices, all of which can be supported at the ALI. Furthermore, assessments can be made on the contribution of the gas phase alone within this exposure set-up, simply by placing a CFP in-line of smoke generation to occlude the particulate phase from the exposure chamber. There are many types of exposure chambers designed to be used in conjunction with smoking machines for this purpose; they are available commercially, ranging in design and complexity, and will be described later.

4.3 Smoking machines

Laboratories within academia, specialist tobacco research groups, pharmaceutical and tobacco industries generally use smoke engines to reliably generate cigarette smoke for *in vitro* studies. Ranging in design, engineering, capability and price, all are intended to generate, dilute and deliver whole cigarette smoke to one or multiple exposure chambers housing cells at the ALI. Examples include the Borgwaldt RM20S smoking machine (Figure 5), (Adamson et al, in submission, Kaur et al., 2010; Maunders et al., 2008, Phillips et al., 2005; Thorne et al., 2009), the Borgwaldt RM 1/G and LM1 single port diluter (Clunes, 2008), the Burghart Mimic Smoker-01® (Scian et al., 2009) and the Vitrocell® VC 10® Smoking Robot (www.vitrocell.com). These machines vary in design and capability but in principle their purpose is shared. The specification on how the cigarette is smoked by the machine, or smoking regime, has been standardised. For example, the ISO regime states cigarette puff volume is 35ml, taken over 2 seconds every minute and that the vents on the filter paper are unblocked. In contrast, the Canadian Intense/Health Canada regime takes a 55ml puff over 2 seconds, every 30 seconds, and the vents are blocked (usually with a ring of tape). Furthermore, various smoking machines are freely programmable and are even capable of human smoking puffing profiles. As a result of many years of development, some of these devices are very sophisticated and capable of smoking cigarettes with a high level of repeatability and reproducibility.

4.4 Exposure chambers

As with smoking machines, there are many different types of exposure chamber to be used with *in vitro* cultures. There is a great diversity available and range significantly in design, sophistication, physiological resemblance, ease of use, flexibility in exposure design, fragility, sterility, price and compatibility with an individual smoking machine. An exposure chamber in its basic form is a container housing cells grown on a cell culture dish or commercially available insert, with an inlet for smoke to pass through and interact with the cultures. Simple examples include a small hermetic chamber big enough for a 12-wellplate containing two holes for ventilation and a small fan for smoke distribution (St-Laurent et al., 2009), or a rocking platform system which exposes half a submerged culture at a time to whole smoke as the liquid is rocked side to side (Bombick et al., 1997). Very sophisticated and engineered examples include Cultex® Laboratories exposure modules (Aufderheide & Mohr, 2000) and Vitrocell® linear modules (Walsh et al., 2008); both of which are commonly used with the Vitrocell® VC 10® Smoking Robot, and have special design features such as individual warmed media supply to each cell culture insert within the chamber, and specialised chamber docking to the dilution systems of the smoking robot.
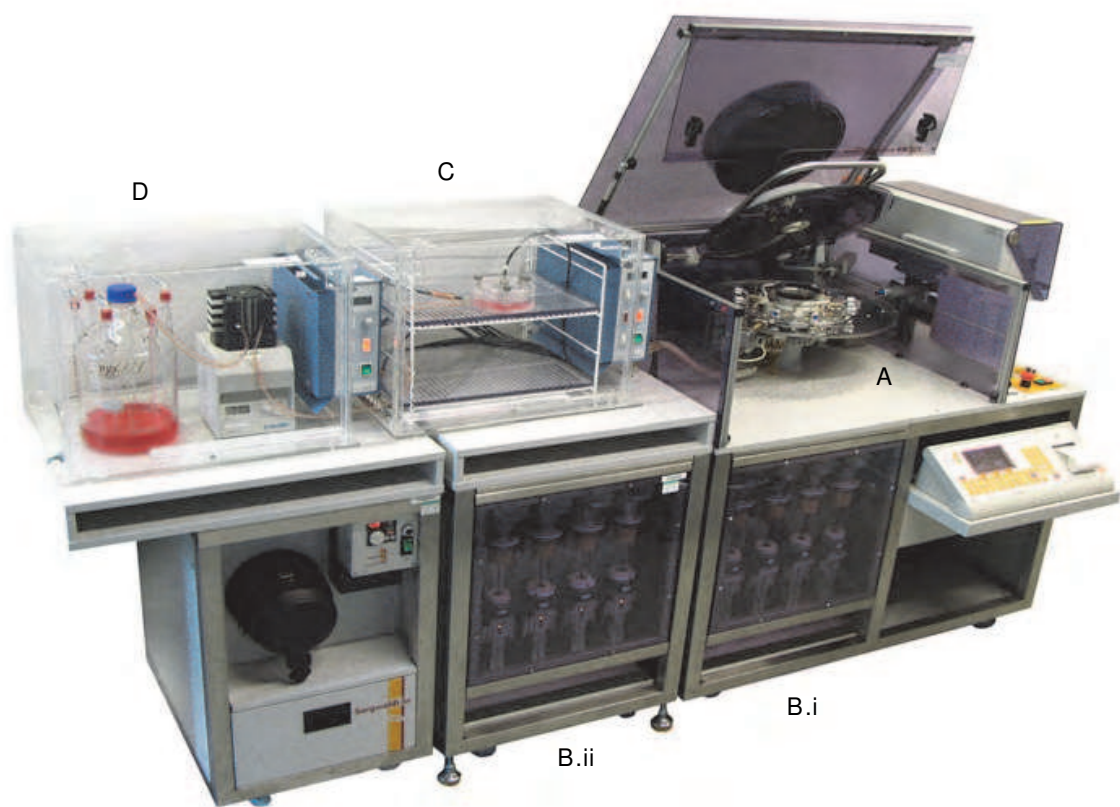
Fig. 5. The Borgwaldt RM20S eight syringe smoking machine. (A) cigarette smoke generator; (B.i) integral 4-syringe unit; (B.ii) additional 4-syringe unit to increase output; (C); incubator at 37°C to house exposure chambers containing cells/tissues at the ALI; (D) an incubator at 37°C holding the cell culture media which is supplied to the chambers using a pump (Adamson et al., in submission, http://www.bat-science.com/)

At BAT we have designed and developed an exposure chamber to enable ALI *in vitro* exposure to cigarette smoke and other aerosols (Phillips et al., 2005, Patent publication number WO 03/100417 A1). As shown in Figure 6 the exposure chamber is very simple in
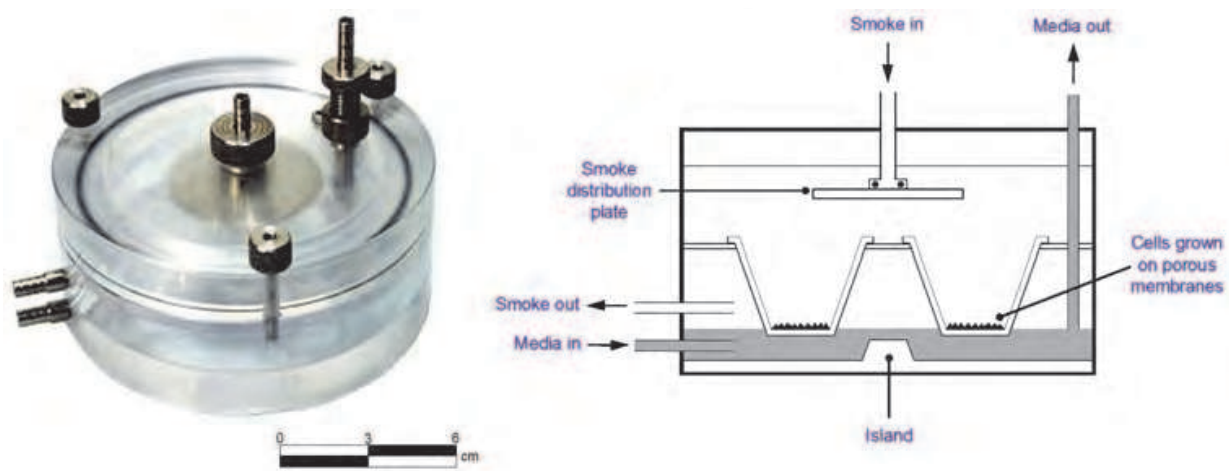
Fig. 6. BAT's exposure chamber, left, and a schematic cross-section, right (Adamson et al, in submission, http://www.bat-science.com)

design. It is therefore compact, robust, easy to clean and relatively economical. It can accommodate commercially available culture inserts, allowing flexibility in experimental design and replicate number: 3 large (24mm ø), 6 medium (12mm ø) or 8 small (6mm ø) fitting symmetrically in a single chamber simply by changing the insert support. Media is supplied into the chamber basally so it contacts with the porous membrane of the cell culture inserts, but does not flood the apical/air surface of the cultures. This chamber has been characterised and used extensively with Borgwaldt smoking machines (Adamson et al, in submission, Maunders et al., 2008, Phillips et al., 2005; Thorne et al., 2009) and most recently with the Vitrocell® VC10® smoking robot.

5. Conclusion and future research

As described in this chapter, there are many tools available to the biologist and toxicologist to evaluate and understand COPD disease processes and causative agents. There are advantages and disadvantages in the utility of these tools for risk and disease prediction. However it is important to note that each tool should be evaluated as a component part of an integrated or 'weight of evidence' approach and not solely in isolation. To add further reliability to the data and confirm the robustness of *in vitro* systems, there is a need to validate *in vitro* models of the whole respiratory system and appropriate exposure devices. This will only be achieved through closer working with the developers, users and producers of these systems along with better and more communication with regulators and opinion leaders in the field of *in vitro* testing and application.

At British American Tobacco, as part of our approach to tobacco harm reduction, we are developing a portfolio of appropriate pre-clinical assays for product assessment and investigating the components of cigarette smoke. We have described a number of these *in vitro* assays and presented an exposure system that is well characterised and demonstrates a physiologically relevant method of generating and exposing *in vitro* cultures to whole cigarette smoke at the ALI. This system allows all phases of cigarette smoke, particulate and gas, to be assessed separately or in combination with the possibility of assessing the effects of single aerosol or individual gas phase components. We propose to further use this system and our portfolio of *in vitro* models at BAT for the biological assessment and evaluation of future tobacco products designed to reduce the harmful effects of cigarette smoke. Furthermore, this exposure system could be a useful *in vitro* method in other industries for evaluating the effects of different aerosols and gaseous mixtures such as air pollutants, desiel particulates, inhaled pharmaceuticals, cosmetics and to examine occupational exposure scenarios.

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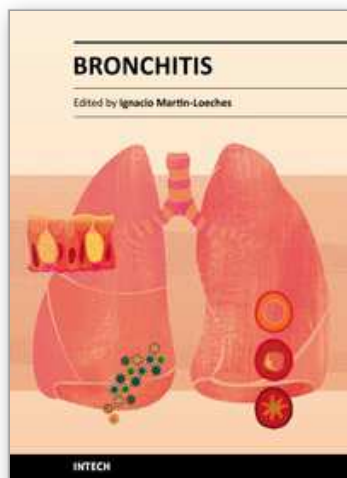
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