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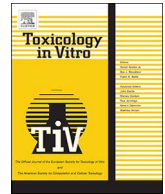
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Review

Human lung epithelial cell cultures for analysis of inhaled toxicants: Lessons learned and future directions

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ABSTRACT

The epithelium that covers the conducting airways and alveoli is a primary target for inhaled toxic substances, and therefore a focus in inhalation toxicology. The increasing concern about the use of animal models has stimulated the development of *in vitro* cell culture models for analysis of the biological effects of inhaled toxicants. However, the validity of the current *in vitro* models and their acceptance by regulatory authorities as an alternative to animal models is a reason for concern, and requires a critical review. In this review, focused on human lung epithelial cell cultures as a model for inhalation toxicology, we discuss the choice of cells for these models, the cell culture system used, the method of exposure as well as the various read-outs to assess the cellular response. We argue that rapid developments in the 3D culture of primary epithelial cells, the use of induced pluripotent stem cells for generation of lung epithelial cells and the development of organ-on-a-chip technology are among the important developments that will allow significant advances in this field. Furthermore, we discuss the various routes of application of inhaled toxicants by air-liquid interface models as well as the vast array of read-outs that may provide essential information. We conclude that close collaboration between researchers from various disciplines is essential for development of valid methods that are suitable for replacement of animal studies for inhalation toxicology.

1. Introduction

For many years, animal models were the central tools in scientific and regulatory research to assess the health effects of inhaled substances. However, the increasing concern that animal studies may not always be optimal predictors of human responses to inhaled substances, and the public concern about use of animals for research purposes in general, has prompted the search for alternatives. The aim of these efforts is to reduce, refine and/or replace these animal models (the 3Rs). Computer-modelling techniques in the analysis of the possible biological consequences of inhaled toxic compounds (so-called *in silico* models) might be a useful alternative in the future, but also *in vitro* culture systems have added value and can serve as an alternative for animal exposure studies.

Within the lung, epithelial cells that cover the surface of the airways and alveoli are the first targets for exposure of inhaled substances that may be derived from environmental, occupational or other external sources. Therefore, epithelial cells are the main focus in cell culture

models for inhalation toxicology. Furthermore, inhaled medications often target these epithelial cells, and appropriate models are needed to optimize delivery of such compounds. Four important elements determine the validity of an *in vitro* system for evaluation of inhaled toxicants: *i.* the choice of cells; *ii.* the cell culture system used; *iii.* the type of exposure; and *iv.* its possibilities for valid readouts to assess the effect of exposure on the cells. Rapid technological developments in both the culture of human airway and alveolar epithelial cells (and their co-culture with other cells), as well as the new exposure technologies developed for inhalation toxicology studies are the focus of this review. In addition, the hurdles for accepting such *in vitro* toxicology studies as an alternative to animal testing by regulatory authorities are discussed.

2. Structure and function of the airway and alveolar epithelium

The conducting airways transport oxygen-containing inhaled air to the alveoli for gas exchange, and remove carbon dioxide from the alveoli. However, inspired air also contains a variable amount of possibly

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toxic components and potential pathogenic micro-organisms. The body is protected from the penetration of such toxic, pathogenic and other unwanted substances by an epithelial layer that covers the internal and external surfaces of the body. These epithelial cells are positioned on a basal membrane and are tightly connected by intercellular junctional complexes that form a physical barrier. The human airways are lined with a pseudostratified epithelium that is composed of various cell types, including mucus producing goblet cells, ciliated cells, non-ciliated club cells, neuro-endocrine cells, and basal cells that act as progenitor cells for the various cell types of the airway epithelium (Crapo et al., 1982; Hiemstra et al., 2015). In contrast, the alveolar epithelium is composed of only two cell types: the cuboidal alveolar epithelial type 2 cells (AEC2) and type 1 cells (AEC1). AEC2 form the surfactant that is critical for the control of surface tension to prevent collapse of the alveoli during expansion and compression during ventilation, and act as progenitor cells for the flattened alveolar epithelial type 1 cells (AEC1) (Whitsett and Weaver, 2015). AEC1 cover 95% of the alveolar surface as a 0.2 µm thin layer to allow gas exchange between the alveoli and the blood (Crapo et al., 1982).

A very thin fluid layer, named the epithelial lining fluid or airway surface liquid, covers the epithelium of the lung. In the large airways, this airway surface liquid is composed of two layers: a periciliary liquid layer and a mucus layer. The periciliary layer allows movement of the cilia to propel the mucus that lies on top of this periciliary liquid. This promotes removal of inhaled particles that are trapped in the mucus layer in a process called mucociliary clearance. The alveolar surface is covered with an alveolar surface liquid that contains pulmonary surfactant. In addition, airway and alveolar epithelial cells produce other protective substances, including antimicrobial peptides, and a wide range of protein and lipid mediators that allow them to communicate with other cell types in the airway wall such as fibroblasts, endothelial cells, airway smooth muscles cells and a range of immune cells (Hiemstra et al., 2015; Whitsett and Alenghat, 2015). The composition of the airway epithelium differs based on the anatomical location, with goblet cells being more prominent in the large intra-thoracic airways, and club cells being more prominent in the distal airways. Furthermore, the composition of the airway epithelium is often changed in various disease states, as illustrated by an increase in goblet cells and decrease in ciliated cells observed in asthma (Hiemstra et al., 2015). Alveolar epithelial cells and the structure of the alveoli are markedly affected in lung diseases such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). Both are accompanied by loss of alveolar function: whereas breakdown of alveolar tissue in emphysematous lesions is a characteristic of COPD, IPF is marked by interstitial fibrosis (Chilosi et al., 2012).

The lung has a variety of mechanisms at its disposal for defence against inhaled toxicants (Fig. 1). Within the airways, mucociliary clearance is an important mechanism for removal of trapped inhaled toxic particles and aerosols, but also resident phagocytes may contribute by ingestion of particles. These systems are however not well suited to deal with inhaled gases. For this, a variety of additional mechanisms contribute to epithelial defence against such gases, as well as against particles that are not removed by mucociliary clearance or phagocytosis. First, epithelial cells express a range of metabolic enzymes, including those of the cytochrome P450 family, that are able to detoxify inhaled toxicants (Castell et al., 2005). Furthermore, defence against the adverse effects of inhaled oxidants is provided by an extensive epithelial anti-oxidant system that can be further activated by mechanisms including Nrf2-mediated transcription (Loboda et al., 2016).

If these defensive mechanisms fail to deal with the threat of the inhaled toxicant, a variety of cellular reactions may occur. These include cell death, loss of barrier function, production of cytokines and other mediators to recruit and activate inflammatory and immune cells, reduction of epithelial antimicrobial defence, as well as changes in epithelial differentiation such as loss of cilia and altered mucus

production (affecting mucociliary clearance) and squamous cell formation. Exposure to inhaled toxicants may also result in activation of the integrated stress response (van't Wout et al., 2014) and the unfolded protein response to endoplasmic reticulum stress (Walter and Ron, 2011). These responses may contribute to cell protection, but upon chronic stimulation may also contribute to injury.

3. Requirements for cell culture models for *in vitro* inhalation toxicology

In order to be adequate for *in vitro* inhalation toxicology studies, an *in vitro* epithelial cell culture system should mimic the functions of the lung epithelium in the best possible way. First of all, in order to determine the epithelial cell type (large or small airway epithelial cells, alveolar epithelial cells) for analysis of *in vitro* toxicology, the pulmonary deposition for especially aerosols and particles is highly relevant. Computational modelling of such deposition can be useful in deciding the proper test system (Rostami, 2009). In the previous section, we discussed the various defence mechanisms as well as possible responses of epithelial cells to exposure. Therefore, an ideal cell culture model should be able to recapitulate those defence systems and responses. However, the requirements may differ depending on the question that is of interest. For instance, if a compound is known to be metabolized (or if it is unknown whether metabolism contributes to its toxicity), an *in vitro* cell culture system must be metabolic competent. This is important, since epithelial metabolism contributes to detoxification of inhaled toxicants, whereas biotransformation of selected inhaled compounds may even generate toxicity, as illustrated by activation of pro-carcinogens (Castell et al., 2005). These processes are regulated by a series of enzymes that are involved in metabolizing xenobiotics, including members of the family of cytochrome P450 enzymes. Expression of CYP isoenzymes may be modulated in disease and is affected by e.g. epithelial differentiation (Boei et al., 2016), which may help to explain the differential susceptibility of patients with lung diseases and healthy subjects to inhaled irritants and toxicants. Therefore, cellular differentiation and disease state should be taken into account when developing a model. Importantly, in a 2004 workshop from the European Centre for the Validation of Alternative Methods (ECVAM) it was recognized that most *in vitro* test systems lack appropriate metabolic activity (Coecke et al., 2006), which still applies to many systems used to date. Likewise, if loss of barrier activity contributes to the (systemic) effects of an inhaled toxicant, the model system should display normal barrier activity. As discussed in the next section, many currently used continuous cell lines do not display such activity. These examples illustrate the complex requirements for designing appropriate *in vitro* models.

In view of the requirements for an optimal model to replace or reduce animal models, it is evident that many tumour or immortalized cell lines may not be optimally suited for the purposes for which they are currently used, since they lack *in vivo* characteristics such as mucociliary differentiation, barrier formation and metabolism of toxicants. In addition, it is increasingly realized that culturing of cells as thin (mono)layers on plastic surfaces under submerged conditions (the traditional two-dimensional (2D) method in cell culture) may not adequately allow studies of cell behaviour relevant to what is observed within tissues *in vivo*. This awakening has led to an increase in development of more complex three-dimensional (3D) models in which cells can grow in multiple directions in a way that better reflects cell interactions in their natural *in vivo* environment. In addition, in these models multiple epithelial and other cell lineages may be present, and the presence of an extracellular matrix contributes to cell behaviour. Culture of cells in organoids or spheroids as well as various air-liquid interface (ALI) culture models are examples of such 3D models that are discussed in the next section. An important advantage of the ALI models is that it allows delivery of toxic compounds using the air exposure route being representative for the inhalation route. Finally, the

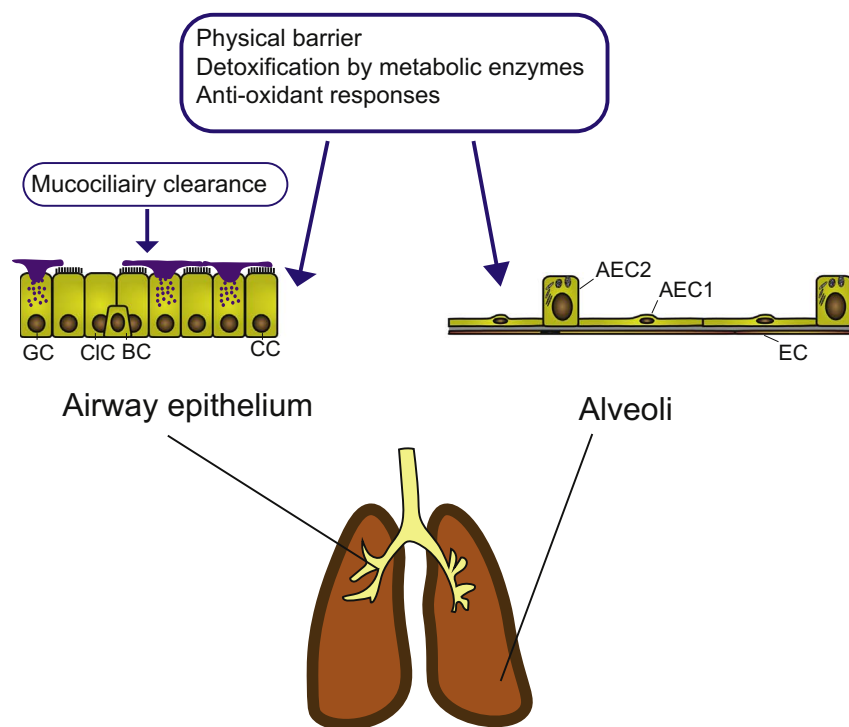


Fig. 1. Protective mechanisms of airway and alveolar epithelial cells against inhaled toxicants. The different cell types in the airway epithelium (CC: ciliated cell; GC: goblet cell; ClC: club cell; BC: basal cell), the mucus layer produced by goblet cells (purple), and in the alveolus (AEC1: alveolar epithelial type 1 cell; AEC2: alveolar epithelial type 2 cell; EC: endothelial cell) are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

traditionally used analysis of cell death as the main parameter of a result of toxicant exposure, may not fully capture the wide range of unwanted cellular reactions that may occur and may be relevant for human health. However, an extensive discussion of read-outs is beyond the scope of this review.

4. Culture models of lung epithelial cells: Choice of cells

In this paragraph, various types of epithelial cells used in culture studies are discussed. Primary human lung epithelial cells are theoretically ideal, since they are directly obtained from tissue thus better maintain their physiological function than *e.g.* continuous cell lines, but unlike these continuous cell lines they have a limited life span. Furthermore, primary cells are difficult to obtain since access to human lung tissue is required, and once collected these cells are also more difficult to culture than cell lines requiring more complex, specialized and expensive cell culture media. Therefore, continuous cell lines (immortalized or tumour cell lines) are widely used for *in vitro* toxicology studies. However, as discussed below, use of these cell lines has various limitations, including a reduced or absent ability to differentiate *in vitro*. Novel, non-viral immortalization methods for transformation have been introduced resulting in cell lines that show more “natural” behaviour, including some degree of differentiation. Finally, as an alternative to classic culture of primary airway epithelial cells, novel pharmacological-based methods for extension of the life span of especially the basal cell population have been introduced that do not require gene-editing. Such developments may increase the availability of primary cells including those of specific patient populations. These developments are rapidly taking place for airway epithelial cells, but the situation is more complex when alveolar epithelial cells are of interest. The next paragraphs will discuss the use and potential applications of (induced) stem cells and co-culture models.

4.1. Cell lines

Continuous (immortalized or tumour) epithelial cell lines are widely used for *in vitro* toxicology studies. They offer the advantage that they *i.* are easier to handle than primary cells; *ii.* do not display inter-donor

variability since they are derived from a single donor (which however also makes it difficult to generalize the observed results); *iii.* have an extended life span and thus increased availability; *iv.* can be cultured in relatively simple, inexpensive culture media; and *v.* are usually suitable for high-throughput screening. Traditionally the tumour cell line Calu-3 and the immortalized bronchial epithelial cell lines BEAS-2B and 16HBE14^o are among the most widely used immortalized cell lines for *in vitro* inhalation toxicology. BEAS-2B is a cell line generated by immortalization of human bronchial epithelial cells using AD12-SV40 virus (Reddel et al., 1988). It resembles airway basal epithelial cells, but does not differentiate and does not form a strong barrier (Stewart et al., 2012). 16HBE14^o is a SV40^o transformed cell line generated from human bronchial epithelial cells (Cozens et al., 1994). It displays a cobblestone morphology, does form polarized cell layers and can be cultured at the air-liquid interface, however only build up limited barrier activity (Forbes et al., 2003). Comparison of BEAS-2B and 16HBE14^o cells indicates that 16HBE14^o showed slightly more differentiation than BEAS-2B with occasional periodic acid-Schiff (PAS) or alcian blue (AB) positivity (Zhu et al., 1999). The Calu-3 cell line was derived from a lung adenocarcinoma from submucosal gland serous cells. When cultured at the air-liquid interface for several weeks, the resulting airway epithelial layer expresses mucins and some cilia, demonstrating many characteristics a fully differentiated airway epithelium, and a reasonable barrier function (Kreft et al., 2015; BeruBe et al., 2007). However, none of these cell lines show normal differentiation with respect to ciliated, goblet and club cells. The A549 tumour cell line is widely used as a model for alveolar epithelial cells, and was developed 40 years ago when it was found to display some characteristics of AEC2 (Leiber et al., 1976). However, this cell line does not form tight junctions and using ALI exposures of such cells has therefore remained problematic (Cooney and Hickey, 2011).

Major drawbacks of the use of cell lines is that they only represent one donor, and that many cellular processes including cell cycle regulation and differentiation are deregulated in tumour cells and cells immortalized using viral oncoproteins such as BEAS-2B and 16HBE14^o. In the past decade, new methods of transformation of epithelial cells have been introduced that employ transduction of the catalytic subunit of telomerase (human telomerase reverse transcriptase; hTERT) to

bypass telomerase-dependent senescence. It has been demonstrated that premature growth arrest of other epithelial cells (keratinocytes and mammary epithelial cells) is associated with increases in the cyclin-dependent kinase (Cdk) inhibitor p16^{INK4a} (Ramirez et al., 2001). To counteract this increase, overexpression of Cdk4 (Ramirez et al., 2004) is combined with transduction of hTERT to obtain immortal bronchial epithelial cell lines. Such immortalized cell lines have been shown to differentiate into mucin-producing and ciliated cells, and thus better reflect the lung epithelium *in situ* (Vaughan et al., 2006). Use of these next-generation immortalized airway epithelial cell lines for *in vitro* toxicology is illustrated by a study by Bersaas et al. on the effects of tobacco smoke carcinogens (Bersaas et al., 2016). Studies on immortalization of primary alveolar epithelial cells are more limited. Kemp et al. developed an immortalized human AEC1-like (transformed type-1; TT1) cell line by transduction with the catalytic subunit of telomerase (hTERT) and simian virus 40 large-tumour antigen (Kemp et al., 2008), and this line was used in studies on nanoparticles toxicity (Sweeney et al., 2016).

4.2. Primary lung epithelial cells

Primary cells are directly isolated from human lung tissue and put into culture (Fig. 2), and thus better maintain their physiological function than continuous cell lines, but unlike these continuous cell lines they have a limited life span. Primary airway epithelial cells may be isolated from bronchial biopsies or bronchial brushes obtained during bronchoscopy, from resected lung tissue or from commercial sources such as Lonza, Mattek and Epithelix. Various protocols have been published to isolate and culture these cells, which can be sub-cultured by passaging a few times and differentiated at the ALI (Fulcher and Randell, 2013). Culture of primary airway epithelial cells at the ALI on permeable membrane inserts results in a polarized mucociliary differentiated airway epithelial cell layer that morphologically resembles the airway epithelium *in situ* in its organization and stratification. Indeed not only morphological studies, but also gene expression studies have shown that ALI cultures recapitulate the gene expression pattern of airway epithelial cells *in situ* (Pezzulo et al., 2011; Dvorak et al., 2011). Furthermore, such ALI-differentiated cultures do maintain some of the key metabolic enzymes (Baxter et al., 2015). We recently showed that the ALI-differentiated cultures used in our laboratory for exposures to e.g. diesel exhaust (Zarcone et al., 2016; Zarcone et al., 2017) or

cigarette smoke (Amatngalim et al., 2015; Amatngalim et al., 2017) show higher expression of various genes involved in biotransformation compared to undifferentiated cultures (Boei et al., 2016). Furthermore, we showed that undifferentiated cultures, in contrast to differentiated cultures, are incapable of metabolic conversion of a variety of different p450 substrates. These results clearly show that appropriate biotransformation requires the use of differentiated airway epithelial cell cultures, and also demonstrate the presence of inter-donor variation.

Some companies also provide cell cultures as differentiated ALI cultures to their customers, such as the EpiAir from MatTek and MucilAir from Epithelix. Interestingly, comparative studies between epithelial cell lines (BEAS-2B and A549) and such differentiated ALI cultures show that these primary cultures may be more resistant to the toxic effects than cell lines (Kooter et al., 2016). In addition to various studies using investigator-established ALI airway epithelial cell cultures (Zarcone et al., 2016; Aug et al., 2015), also these commercial ALI systems have been applied in *in vitro* toxicology studies (Reus et al., 2014; Kuper et al., 2015; Neilson et al., 2015). As an alternative to ALI culture, rotating bioreactors have been developed in which epithelial monolayers are alternately submerged in culture media or exposed to air on the apical side (Raredon et al., 2015). Analysis of the resulting cultures using immunohistochemistry and RT-PCR revealed evidence of differentiation in the cultured primary bronchial epithelial cells.

Finally, use of primary cells offers the advantage of studying cells from selected patient populations that may be at increased risk for a selected inhaled toxicant. The advantage of this option is underlined by the observation that ALI-cultured airway epithelial cells derived from patients with mild or severe asthma retain characteristics of the distorted airway epithelium present in the patients lungs, and that the phenotypic differences relate to the severity of asthma (Gras et al., 2012). Similar observations have been reported in COPD (Amatngalim et al., 2017; Staudt et al., 2014), suggesting that the lung micro-environment in these inflammatory lung diseases causes (epigenetic) changes in epithelial cells resulting in partial persistence of the phenotype in culture.

The possibilities to isolate and culture human alveolar epithelial cells are more limited. Isolation and culture of primary alveolar cells from human lung is hampered by the availability of human lung tissue, the isolation procedure needed to isolate the AEC2 that includes enzymatic digestion of tissue and a variable combination of differential attachment, density gradient centrifugation, magnetic cell sorting and/

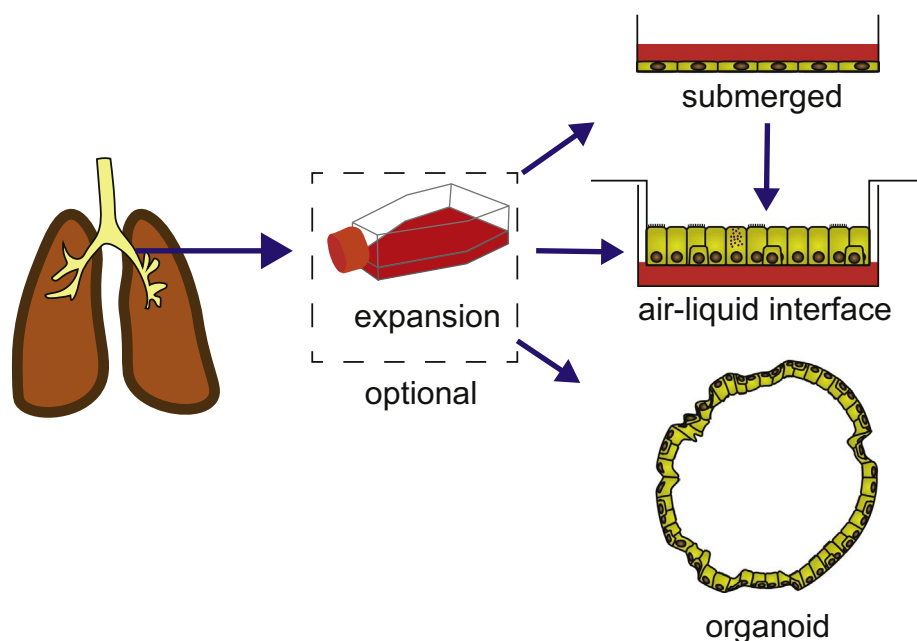


Fig. 2. Isolation and culture of human airway epithelial cells; submerged, air-liquid interface (ALI) and organoid models of airway epithelial cell culture. These cultures can be established directly from lung tissue, or there can be an optional expansion step.

or FACS. Fujino et al. developed a FACS-based method for isolation and culture of AEC2 cells based on the combination of high expression of epithelial cell adhesion molecule (EpCAM) and absence of T1 α (Fujino et al., 2012). Despite these advances in their isolation, isolated AEC2 have a very limited life span in culture and spontaneously differentiate into AEC1, and such terminally differentiated AEC1 cannot be passaged.

4.3. Non-genetic methods to expand the life span of primary epithelial cells

Primary epithelial cells have a limited life span, and once airway epithelial cells are put into culture, self-renewal becomes abnormal or is lost after only a few passages. This is even more evident when culturing AEC2. The life span of epithelial cells can be expanded using the immortalization techniques described above, but also non-genetic methods have recently been developed. Traditionally, feeder cells have been used to enhance performance of epithelial cells and various stem cells in culture, as also shown for human bronchial epithelial cells (De Jong et al., 1993). The life span of cultured airway epithelial cell can be markedly enhanced by inhibition of Rho-associated protein kinase (ROCK), a method that allows further expansion of basal cells (Horani et al., 2013). Furthermore, the combination of ROCK inhibition and use of a feeder layer of mitotically inactivated mouse fibroblasts (Butler et al., 2016) showed promising results. However, such fibroblast co-culture systems may complicate the interpretation of results obtained with such systems. Importantly, a recent study demonstrated the feasibility of prolonged culture of epithelial basal cells without the need for a feeder layer, using a combination of ROCK inhibition and dual TGF β /BMP inhibition to prevent spontaneous differentiation (Mou et al., 2016). These developments indicate that the life span of (airway) epithelial cells can be markedly expanded without the need for gene editing, and this will have important implications for future *in vitro* inhalation toxicology research as it will allow upscaling of these investigations. Further studies are needed to evaluate whether such methods are also applicable to alveolar cells.

4.4. Induced pluripotent stem cells

Use of stem cells could serve as an attractive alternative from primary lung epithelial cells isolated from lung tissue. Indeed, adult stem or progenitor cells can be used to generate lung epithelial cells, as demonstrated by the culture of AEC2 from peripheral-blood derived CD34 + cells (Srikanth et al., 2016). Pluripotent embryonic stem cells (ESC) can also be used to generate lung epithelial cells (Longmire et al., 2012), but ethical issues limit the availability of such cells from human sources. At present, especially induced pluripotent stem cells (iPSC) are considered as an attractive alternative and starting point for culture of human lung epithelial cells. These iPSC are somatic cells that have been reprogrammed to gain multi-potent-like features by introducing four pluripotency-associated genes that convert adult stromal cells into pluripotent stem cells: Oct4, Sox2, cMyc and Klf4 (Yamanaka factors; (Takahashi and Yamanaka, 2006)). Like ESC, also iPSC need to be guided through the different developmental stages of lung development using defined growth factors, inhibitors and other components. This directed differentiation of iPSC through the stages of definitive endoderm, anterior foregut endoderm, early lung progenitors to bronchial progenitors and alveolar progenitors forms the basis for generation of airway and alveolar epithelial cells from iPSC (Hawkins and Kotton, 2015; Ghaedi et al., 2015). Several protocols for development of human airway epithelial cells and alveolar epithelial cells have been reported, showing the feasibility of this approach (Ghaedi et al., 2015; Huang et al., 2015). The potential advantage of iPSC is that it offers a theoretically unlimited supply of subject-specific cells that can be generated from an easily accessible source, e.g. skin, blood or urine. The availability of a toolbox of gene-editing techniques now also vastly expands the potential use of such cells for both mechanistic studies as well as for

generation in reporter cell iPSC lines. Despite these advantages, currently generation of iPSC-derived lung epithelial cells is still very labour-intensive, requiring extensive optimization for each hiPSC cell line, and use of iPSC-derived lung epithelial cells is hampered by the remaining limitation of obtaining a mature, fully functioning epithelium (Hawkins and Kotton, 2015).

4.5. Co-culture models

Whereas isolated airway epithelial cells have been shown to differentiate into mucin producing and ciliary epithelial cells, signaling from the mesenchyme (a critical step in e.g. lung development and adult life) and from inflammatory and immune cells is absent. Therefore, a variety of co-culture models have been developed using epithelial cells, fibroblasts, endothelial cells, airway smooth muscle cells, as well as with e.g. macrophages and dendritic cells.

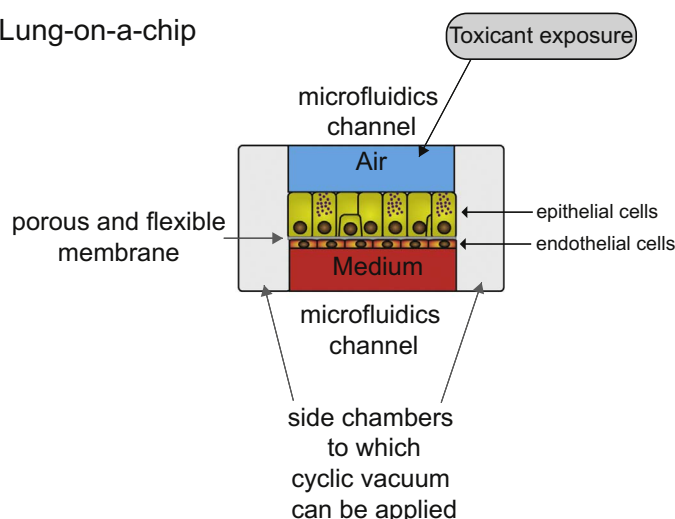
Choratra et al. demonstrated the usefulness of such a co-culture system, consisting of the A549 cell line with human monocyte-derived dendritic cells and macrophages, by demonstrating the feasibility of repeated exposure to carbon-nanotube-based aerosols (Chortarea et al., 2015). Klein et al. even extended this to a tetra-culture model based on the Vitrocell aerosol exposure system using four different cell lines: AEC2 line (A549), macrophage-like cells (THP-1), a mast cell line (HMC-1) and an endothelial cell line (EA.hy 926) (Klein et al., 2013). Many studies on co-cultures focused on the use of continuous cell lines, because these can be more easily controlled and usually do not require cell-type specific media. In contrast, it is more difficult to establish and control co-culture models using primary cells because of inter-donor differences in e.g. growth rates, and the requirement for cell-type specific media. Nevertheless, several studies have shown the feasibility of this approach using primary cells. In addition to the various co-culture models with primary epithelial cells developed in academic research laboratories, also commercial sources offer ALI co-cultures of e.g. airway epithelial cells with fibroblasts (e.g. Epithelix and Mattek).

5. Culture models of lung epithelial cells: Culture systems

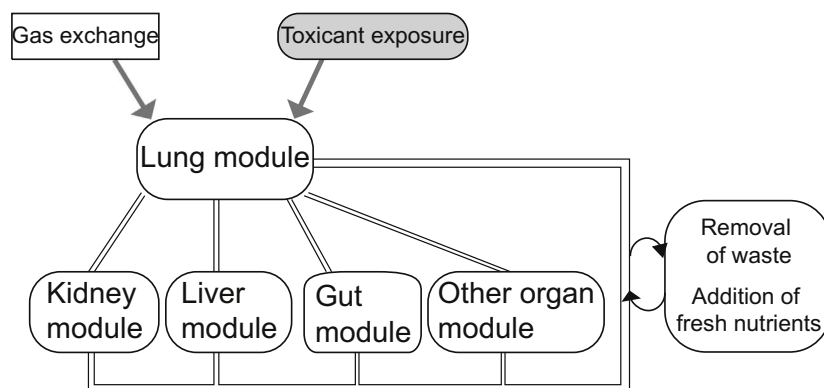
5.1. Organ-on-a-chip models and microfluidics

Organ-on-a-chip models are micro-engineered organ models that usually contain multiple cell types and a microfluidics device for continuous delivery of nutrients and removal of waste products. Microfluidics can also be used for fully-differentiated ALI-cultured airway epithelial cells alone, and such cultures allowed continuous sampling of secreted products and were found to show a stronger response to pollen exposure than conventional static ALI cultures (Blume et al., 2015). The first lung-on-a-chip was reported by Ingber and colleagues (Huh et al., 2010), and included an air-exposed epithelial cell line co-cultured with endothelial cells and allowed the application of mechanical stress to mimic the effects of respiration (Fig. 3A). Cells were grown in separate microfluidics channels on opposing sides of a barrier composed of a thin and porous polydimethylsiloxane membrane (PDMS). The flexibility of the PDMS membrane enabled the application of mechanical stretch to mimic breathing movements, and that was found to markedly affect cellular responses to 12 nm silico particles that were used as a simulant of airborne pollutants. Indeed, transcytosis of these nanoparticles across the alveolar epithelial and endothelial layer was found to be sensitive to mechanical stress, and was not observed in static or conventional Transwell co-cultures. The relevance of these findings on the importance of cycling breathing was confirmed in an *ex vivo* mouse ventilation perfusion model. These findings on the importance of mechanical stretch for cellular behaviour in an alveolus-on-a-chip model was confirmed and extended to include metabolic activity and cytokine production by other groups (Stucki et al., 2015). Furthermore, the group of Ingber at the Wyss Institute reported on application of their model for studies on IL-2-induced pulmonary oedema,

A. Lung-on-a-chip



B. Body-on-a-chip



showing enhancement of modelled vascular leakage by mechanical stretch (Huh et al., 2012). While providing essential and new information on alveolar biology, a limitation of these alveolar organ-on-a-chip models is that an alveolar tumour cell line (A549) was used. Therefore development of a small airway-on-a-chip based on the use of primary differentiated mucociliary bronchiolar epithelial cells and primary microvascular endothelial cells was an important next step (Benam et al., 2016). Furthermore, the authors showed that epithelial cells from patients with chronic obstructive pulmonary disease (COPD) cultured in this model displayed disease-specific features such as increased inflammation in response to viral and bacterial exposures.

These organ-on-a-chip systems are ideally suited for toxicology research and the present studies support the importance of mechanical stretch, co-culture and microfluidics offered by these systems. Future studies are needed with more toxicants and scaling up to allow parallel screening of multiple compounds using cells from various donors. Furthermore, the coupling of connected organ-on-a-chip devices to create a body-on-a-chip (Ingber, 2016; Skardal et al., 2016) (Fig. 3B) as well as the combination of iPSC and organ-on-a-chip technology are technically demanding, but holds great promise for the future of inhalation toxicology. An example of the usefulness of such a body-on-a-chip approach is described by Esch and co-workers, who demonstrated that “ingested” nanoparticles that were absorbed through a gastrointestinal chip could cause injury to cells in a linked liver chip (Esch et al., 2014).

Fig. 3. Organ-on-chip models for inhalation toxicology. A. Airway-on-a-chip model of the human airway epithelium (Benam et al., 2016). B. Body-on-a-chip.

5.2. Organoids

Organoids are defined as cultured structures that consist of multiple organ-specific cell types, exhibit some of the functions of the organ it represents, and in which the cells are grouped and spatially organized similar to the organ (Lancaster and Knoblich, 2014). These organoids may be derived from stem cells (ECS or iPSC) or from organ specific progenitor cells that differentiate and self-organize through cell sorting and lineage commitment, similar to the process *in vivo*. It should be noted that this recent definition is not yet strictly used in the literature and the terms organoids and spheroids are widely used for a variety of structures. Organoids have been used especially for functional studies of tissue stem or progenitor cells, but also promising applications for disease modelling and host-microbe interactions have been reported (Fatehullah et al., 2016; Clevers, 2016). A specific advantage is that organoids can be kept in culture for months through propagation of stem/progenitor cell populations (Sato et al., 2009; Rock et al., 2009; Tadokoro et al., 2016). So far, only a few studies have reported on the development of lung organoids that include cells from multiple cell lineages (Longmire et al., 2012; Mou et al., 2012; Lee et al., 2014; Mondrinos et al., 2014; Dye et al., 2015). However, these systems are not readily applicable for studies aimed at resembling inhalation exposures such as ALI cultures, and provide additional technical challenges due to the 3D nature of the culture and its matrix components. Culture of cells in so-called hanging drop systems also leads to some organization of cells, that however lack the often complex architecture of organoids. Such a model has been described for A549 alveolar tumour cells in which the system was adapted to an air exposure set up,

allowing direct contact between benzene in the air and the cells (Liu et al., 2013).

5.3. Precision cut lung slices

Precision-cut lung slices (PCLS) derived from human lung tissue offer an intermediate between *ex vivo* and *in vitro* exposure systems. They form an attractive alternative to human epithelial cell cultures, since they contain all cell types in their *in situ* environment. Lauenstein et al. reported on the use of human PCLS for chemical-induced toxicity, but concluded that it was not suitable for simple screening (Lauenstein et al., 2014). Use of this method is limited by the complex 3D structure of the model, the variable life span of the different cell types cultured, and the requirement for regular availability of fresh (healthy) human lung tissue. Comparable limitations exist for *ex vivo* lung perfusion as a model.

6. Culture models of lung epithelial cells: Exposure systems

In standard toxicology research, exposure is simply achieved by adding substances to the medium of submerged cultures. This is however hardly relevant for inhalation toxicology studies, in which the exposure route is a far more critical issue. Nevertheless, many studies on respiratory toxicology do use either submerged 2D cell cultures to which test substances are added, or use 3D ALI cultures to which a small amount of fluid is added to the apical side. However, to optimally mimic the respiratory exposure route, an ALI culture of primary lung epithelial cells and ALI exposure to a gas or aerosol are required. A typical ALI set-up for evaluating the cellular effects of a test atmosphere is provided in Fig. 4. This ALI should be well-controlled and methods should be available to assess the composition of the applied mixture and its deposition on the cell surface. This deposition is determined by properties of the particles such as size and charge, and by dissolution of gases in the lining fluid. Furthermore, when performing such studies, it should be realized that interactions of particles and gases with components of the epithelial lining fluid, such as mucus, affect the ultimate exposure of the apical surface of the epithelial cells.

Various ALI exposure modules have been developed for *in vitro* air exposure, ranging from relatively simple systems developed in research laboratories composed of closed incubation chambers with a ventilator

to distribute the applied components (Amatngalim et al., 2015; Herr et al., 2009) to commercially available more complex CULTEX and VITROCELL exposure systems. Specifically for the analysis of effects of nanoparticles, the air-liquid interface cell exposure (ALICE) system was developed (Lenz et al., 2009). The system uses a nebulizer to create a cloud that deposits the particles by droplet deposition onto an air-liquid interface culture. Another modification of the ALI exposure system was introduced by the development of the Electrostatic Aerosol *in Vitro* Exposure (EAVES) system, which was used to increase deposition of particles present in diesel exhaust onto epithelial surfaces (de Bruijne et al., 2009). Based on an “all-in-one-plate” concept, Ritter and Knebel described the P.R.I.T. ExpoCube as a system in which all the phases of the experiment (cell culture, exposure and read-out) and various test substances and controls are included in one multi-well plate inserts in a 12-well plate that is temperature-controlled by a heating plate (Ritter and Knebel, 2014). However, duration of exposure beyond 6 h is a limitation in ALI systems, partly because of drying of the epithelial surface resulting in cellular stress and cell death. As an alternative, hanging drop (HD) culturing was used to produce spheroids from A549 cells, followed by exposure using sealed conditions to allow long-term (up to 20 days) exposures to even volatile compounds (Liu et al., 2013). The results of the exposure were more variable with A549 cultures pre-cultured before exposure for 7 days (designated 3D by the authors) than after 1 day (designated 2D). Whether such exposure systems can also be reliably used for primary lung cells, and whether the morphology and function still resembles that of lung epithelium *in situ* needs to be determined. Other alternatives are e.g. bioreactor-like systems using alternating exposure of the cells to culture medium or to air (Raredon et al., 2015).

7. Culture models of lung epithelial cells: Read-outs

Traditionally, cell death, DNA breaks and barrier function are used as primary read-out for *in vitro* inhalation toxicology studies, but also e.g. cytokine release and ciliary activity (for airway epithelial cells) are used. Whereas these may address some relevant endpoints, they do not fully cover all possible endpoints. When considering smoking-induced lung disease, important characteristics such as mucus hypersecretion, tissue remodelling and impairment of host defence against infection (Crotty Alexander et al., 2015) are not covered by these traditional

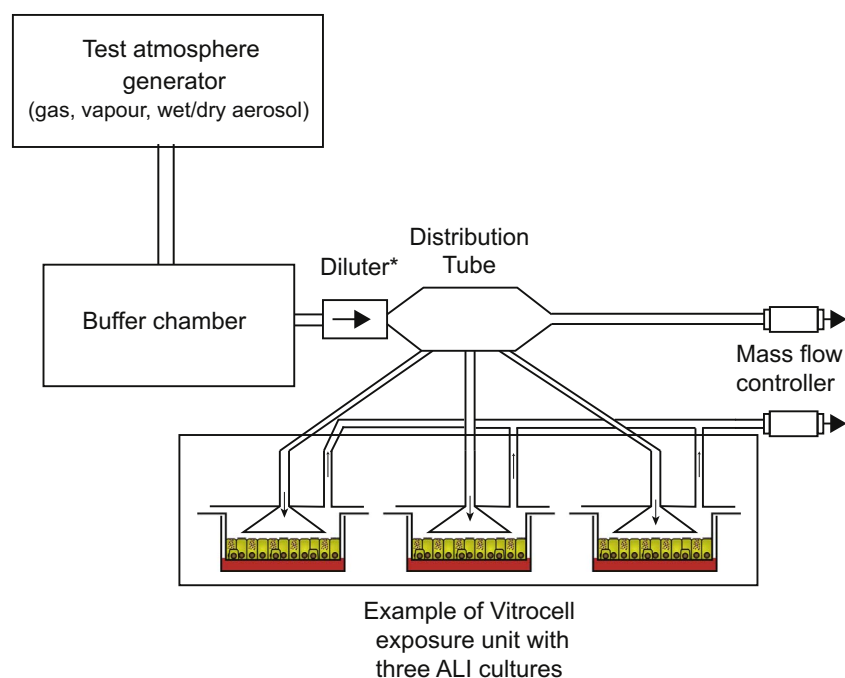


Fig. 4. Typical exposure setup for air-liquid interface (ALI) exposure of cells cultured at the ALI to freshly generated test atmosphere. A test atmosphere (gas, vapour, wet/dry aerosol) is generated, guided to a buffer chamber and diluted using Air-Vac eductors (the Diluter indicated by * in the figure is optional; only if the mixture is diluted). The diluted test atmosphere is guided to the exposure chamber using mass flow controllers. The exposure chamber may be composed of e.g. three Vitrocell exposure units that are each connected separately by tubing to the system. Test atmosphere is guided to the cell culture in this Vitrocell-based example through “trumpets” that are located at close proximity to the cell surface of the ALI culture. Modules are maintained at 37 °C during exposure.

Table 1
Overview of *in vivo*, *ex vivo* and *in vitro* experimental models for inhalation toxicology.

Model	Advantages	Limitations
<i>In vivo</i> human (experimental exposures)	- Most relevant	- Ethical considerations - No detailed mechanistic studies feasible
<i>Ex vivo</i> human (e.g. PCLS)	- Normal tissue architecture maintained - Allows functional assessment of tissue function (e.g. airway constriction)	- Viability upon prolonged culture - Availability of tissue
<i>In vivo</i> animal	- Whole body response	- Ethical considerations - Predictive nature of animal models for human responses - Difficulty in modelling complex gene-environment interactions that determine human responses
<i>In vitro</i> human Cell lines	- Inexpensive - Ease of use - Amendable to high-throughput screening	- Inadequate differentiation - Genetic alterations - Mostly limited barrier activity - Not representative for populations (one donor only)
Primary lung cells	- Better resemble properties of cells in tissue than cell lines - Allow comparison of inter-donor differences - Allow analysis of cells from specific patient populations	- Availability of tissue - Limited life span - More expensive than cell lines
Stem cell-derived lung cells	- Potential to generate any kind of cell type or tissue - Generation of cells from specific patient populations	- Labour intensive - Costs - Ethical sensitive when using ESC - Difficulty in obtaining mature cells

Abbreviations: PCLS, precision cut lung slices; ESC, embryonic stem cells.

read-outs. Although an extensive discussion of the various read-outs is beyond the scope of this review, it needs to be stressed that a range of other parameters are currently assessed to determine the biological effect of an inhaled toxicant in *in vitro* cell culture systems. These include the various -omics techniques, as illustrated by the effect of electronic cigarette liquid on the metabolome of airway epithelial cells (Aug et al., 2015), markers of oxidative stress, induction of an oxidative stress response and inflammatory response (Zarcone et al., 2016; Zarcone et al., 2017; Kuper et al., 2015; Kooter et al., 2013), induction of the integrated stress response (Zarcone et al., 2016; Zarcone et al., 2017), impairment of autophagy (Shivalingappa et al., 2015), and impairment of epithelial antimicrobial defence (Amatgalim et al., 2017; Herr et al., 2009; Wu et al., 2014). Adsorption of test compounds can be specifically addressed by using radiolabeled substances that are applied via the apical surface, as demonstrated by Reus et al. using the Muc1Air™ model of differentiated human bronchial epithelial cells (Reus et al., 2014). The analysis by Schamberger et al. of the effect of repeated exposures of ALI-cultured airway epithelial cells to an aqueous extract of cigarette smoke also illustrate the importance of repeated exposures and epithelial differentiation as a read-out (Schamberger et al., 2015). In addition to these more or less traditional methods, also engineered reporter cell lines may be useful to assess readouts. This includes the use of iPSC single or multiple reporter lines (Pei et al., 2015) that can be differentiated to lung epithelial cells, which offers future novel opportunities for relevant models that are amendable to high-throughput screening.

8. Regulatory considerations

There is general consensus that *in vitro* methods for inhalation toxicology testing offer great advantages over current animal testing and have the capacity to ultimately replace the use of animals. However, at present *in vitro* culture models are used either for scientific purposes, for in house screening and/or to support *in vivo* animal data in regulatory dossiers. In order for *in vitro* cell-based modelling systems to be accepted by international regulatory authorities and industry as an alternative to animal testing, the validity of the model should be demonstrated. This means that the robustness, reproducibility and predictive value should be assessed. The combination of *in silico* methods to model pathways and *in vitro* cell culture models as an integrated system appears most promising based on available

information. Indeed, risk assessment of chemicals requires data on adsorption, distribution, metabolism and excretion (ADME) and such data can be generated by a combination of *in vitro* methods and *in silico* analysis.

In the validation process, the reliability and relevance of the novel method for a selected purpose is established. Therefore, agreement is needed on the endpoints that best predict the local and systemic consequences of exposure, since validation for all possible endpoints is not feasible. What should be predicted and what is the gold standard, to which the new *in vitro* method needs to be validated? This is preferably not the animal model. The early phases of validation (usually referred to as pre-validation) should focus on development of a standardized protocol and its optimization for maximal accuracy and reliability. The pre-validation phase is especially useful to assess and refine the validation process, as supported by the European Union Reference Laboratory for Alternatives to animal testing (EURL-ECVAM). Additionally, it is valuable input for the discussions to establish EU regulatory guidelines, and global test guidelines such as those from the Organization for Economic Co-operation and Development (OECD). Based on these discussions with regulatory authorities and stakeholders, validation criteria should be established (including selection of readouts, robustness, reproducibility, sensitivity and specificity, availability of the model), and a validation process involving multiple international laboratories needs to be designed. Only if these adequate validation studies have demonstrated that the proposed new method is useful, it will be considered for acceptance by regulatory authorities.

In view of this process, it is evident that the acceptance of cell culture models for inhalation toxicology is still in its early phase, compared to e.g. *in vitro* methods for skin toxicology that have made their way into OECD test guidelines. Therefore, harmonization and standardization resulting in validation of these models are all keys to progress in the field of inhalation toxicology.

9. Concluding remarks

Many important lessons have been learned from the application of the various *in vitro* and *in vivo* models used in inhalation toxicology (Table 1), and it has become clear that current *in vitro* test systems are not often optimally suited for their task. Advanced models, such as primary airway epithelial cells cultured and 3D differentiated at the ALI, are now more widely available, even from commercial sources, and

will largely replace continuous cell lines such as the widely used A549 line. However, for selected research questions, use of such cell lines that are easy to handle may still be of some relevance for screening purposes. In addition, technological advances in ALI exposure of cell culture systems now allow a better control of exposures of cultured epithelial cells. Application of such well-controlled exposure systems is not yet feasible in many research laboratories and is often difficult to combine with medium- to high-throughput screening. These issues need to be addressed, but it is evident that the use of primary human epithelial cells cultured and exposed at the ALI using well-controlled exposure systems offers many important advantages over conventional testing with cell lines in submerged conditions. Whereas these models using primary cells are state-of-the-art in *in vitro* toxicology research, they require validation as discussed in the previous section before being accepted by regulatory authorities.

Further improvements are on the horizon. Rapid developments in the application of iPSC-derived epithelial cells and novel cell culture systems such as the organ-on-a-chip microfluidics system, offer new possibilities for *in vitro* inhalation toxicology research. The feasibility of these new technologies for inhalation toxicology has been demonstrated and may have added value, but these tools are not yet widely available in the inhalation toxicology community. Furthermore, many technical issues in the use of iPSC-derived cells and organ-on-a-chip technology still limit their use in inhalation toxicology and need to be addressed.

It is evident that cell culture models offer a valid alternative or supplement to animal exposure studies. It is important to stress that the selection of the cell source, its culture conditions and exposure conditions require careful matching with the aim of the study. The future is in the validation of the current state-of-the-art models, and further development, optimization and implementation of novel integrated cell models. Well-organized case studies assessing the effect of a single compound in various models may be useful in this respect. To achieve these ambitious goals, the joint effort of multidisciplinary teams is needed because of the complex nature of the systems used.

Transparency document

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