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Human epithelial cells in vitro – Are they an advantageous tool to help understand the nanomaterial- biological barrier interaction?

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ABSTRACT

The human body can be exposed to nanomaterials through a variety of different routes. As nanomaterials get in contact with the skin, the gastrointestinal tract, and the respiratory tract, these biological compartments are acting as barriers to the passage of nano-sized materials into the organism. These structural and functional barriers are provided by the epithelia serving as an interface between biological compartments. In order to initiate the reduction, refinement and replacement of time consuming, expensive and stressful (to the animals) in vivo experimental approaches, many in vitro epithelial cell culture models have been developed during the last decades. This review therefore, focuses on the functional as well as structural aspects of epithelial cells as well as the most commonly used in vitro epithelial models of the primary biological barriers with which nanomaterials might come in contact with either occupationally, or during their manufacturing and application. The advantages and disadvantages of the different in vitro models are discussed in order to provide a clear overview as to whether or not epithelial cell cultures are an advantageous model to be used for basic mechanism and nanotoxicology research.

1. INTRODUCTION

Nanotechnology is a rapidly growing field, with the application of engineered nanomaterials in daily life is constantly increasing. Nanomaterials are defined by the European Commission as materials whose main constitutes have a dimension between 1 and 100 billionth of a metre (http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/what-is-a-nanomaterial-european-commission-breaks-new-ground-with-a-common-definition). In the past decade there has been a substantial

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increase in the debate regarding the potential harmful effects of nanomaterials [1-4], with particular concern expressed as to their possible adverse effects to both human health and the environment [5]. Thus, it is essential that the potential toxicity and the mechanism(s) of the interaction of nanomaterials with target cells that may lead to local and systemic (human) health effects [6] are understood. Possible cellular reactions such as the induction of oxidative stress, (pro-)inflammatory reactions [7] and genotoxicity [8] must be considered for such studies.

The specific routes by which nanomaterials may enter the human body, and potentially elicit adverse effects are the lung via inhalation, the gastrointestinal tract via digestion, the skin, and blood vessels via intravenous injection [4]. As nanomaterials gain contact with the skin, the gastrointestinal tract, and the respiratory tract, these biological compartments are "innately designed" to act as barriers to the passage of nano-sized materials into the organism [9]. Since the epithelium is "the" primary structural barrier, the aim of the present review is to provide an overview of this important anatomical structure as well as functional aspects of epithelial cells and furthermore to discuss, if *in vitro* epithelial cell cultures of human origins are advantageous models for gaining a basic understanding of the potential human health effects of nanomaterials.

1.1. The epithelium

The epithelium is besides the connective tissue, muscle tissue and nervous tissue one of the four types of mammalian tissue. Since the epithelium lines all body surfaces, cavities and tubes its main function is to provide an interface between biological compartments. Epithelial cells are closely bound to each other and supported by the basement membrane acting as scaffolding which separates the epithelia from the underlying connective tissue. Since the epithelium is innervated but never penetrated by blood vessels the epithelial cells are dependent on the diffusion of oxygen and metabolites from the underlying tissue. Epithelia are responsible for adsorption, secretion as well as protection and they are also often arranged into structures called glands [10].

Epithelial cells form continuous sheets within the epithelium which are attached to one another at many locations by tight junctions, adherens junctions and desmosomes [10] (Figure 1A).

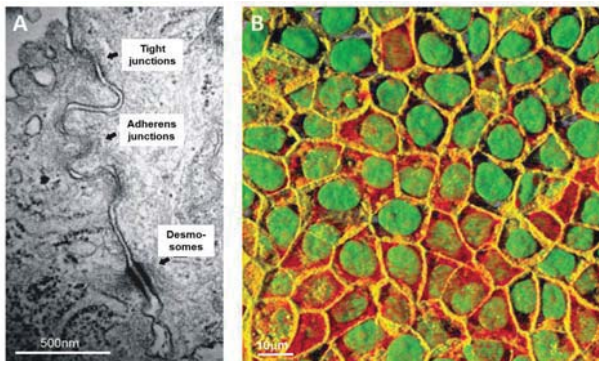

Figure 1: Cell-cell junctions between 16HBE140- bronchial epithelial cells. A) Transmission electron microscopy image showing cell-cell contacts in epithelial cells, i.e. tight junctions, adherens junctions, as well as the desmosomes (Adapted from [27]). B) Laser scanning micrograph of 16HBE14o- cells stained for the tight junction protein occludin (yellow), F-actin cytoskeleton (red) and cell nuclei (green).

Such cell junctions are especially abundant in epithelial tissues. Epithelial cells form cellular barriers separating compartments of different composition. In forming such barriers the epithelial cells polarize and form intercellular junctions [11-13]. Tight junctions separate the epithelium in an apical and a basal site and are the most apical intercellular junctions [14]. They control paracellular transport, as for example, preventing macromolecules from easily passing through the epithelial layer. Besides the proteins forming the tight junctions there are also other cell-cell associating proteins, such as transmembrane proteins (cadherins) of adherens junctions, which are linked via intracellular proteins (e.g. catenins) to the actin cytoskeleton and cell adhesion molecules, desmosomes linking intracellular keratin cytoskeletal filaments to the cell membrane, and gap junction proteins (connexins) which are communicating junctions [10,15-17].

1.2. Epithelial cell culture models

Understanding the functional and pathological disorders induced by nanomaterials in different barrier systems requires the investigation of the direct effects of these "xenobiotics" on the state (i.e. homeostasis) and activity of cells present within such a biological barrier. So far, three approaches have been used: *in vivo* experiments, *ex vivo* studies of cells or biopsies isolated from animals, and *in vitro* cell culture systems to study the effect of pollutants under controlled conditions [18].

The exposure of animals, isolated tissues or *in vitro* cell cultures to nanomaterials requires the knowledge and understanding of several methodological challenges. The advantages and disadvantages of each model type need to be considered, as it is not possible to solve all problems and to answer all questions by conducting studies using animal models only [19,20]. Despite such methodological issues however, to study the interaction of nanomaterials with cells using *in vitro* models can be extremely powerful [21]. During the last years there have been sustained efforts to replace animal experiments by cell culture approaches in many fields of research. One of the major advantages of *in vitro* research is that, compared to animal models, cellular and sub-cellular functions (i.e. cell growth, cell interactions or metabolism, as well as the underlying molecular pathways) can be studied with ease in a simplified, biological model system, provided that the *in vitro* model is established appropriately to focus exactly on the mechanism of interest. Cultured human and animal cell can be better controlled and high standardization maximizes reproducibility. One has to bear in mind though that cell cultures are systems isolated from the normal environment and may hence behave in a way which is different from the *in vivo* situation. Guidelines for good cell culture practice are required, should be applied and documented, including the control of the starting material (e.g. the cultured cells, the culture medium, and the culture substratum) [21-23].

In vitro cell cultures may be established from freshly isolated tissue (primary cultures) or may stem from a continuous cell line (secondary cultures). Both systems have advantages and disadvantages. Primary cultures isolated from animal tissue represent a heterogeneous population of different cell types, although each isolation is unique and impossible to exactly reproduce. Primary cultures face the limitations such as the lack of availability of normal human airway tissue, the limited number of cells which can be received during each isolation, and an uncertainty due to donor variation [23].

Cell lines are genetically homogenous and more stable than primary cells and, hence, welcome to obtain minimal biological variation within the experimental setup. The disadvantage of cell lines, however, is that they retain little phenotypic differentiation. Nevertheless, if cell cultures are used properly they represent a sophisticated and reproducible system with which basic and mechanistic questions can be answered and which may help to understand what occurs within an *in vivo* environment.

1.3. Evaluating the barrier characteristics of epithelial cells

Cultures of mammalian cells can be used as the basis for simplified biological systems in basic science in order to obtain a more controllable and reproducible system compared to *in vivo* models [24]. However, if such a biological system is simplified to its absolute fundamental level, then it is paramount that the essential components of such a reduced system are clearly defined, understood and reproducible [23].

Certain biochemical markers can be used to make a positive identification of epithelial cells. The intermediate filament proteins in the cytokeratin group are almost exclusively found in epithelial cells, and thus, are often used for this purpose [24]. In addition, the expression and typical localisation of E-cadherin, a tissue specific protein expressed at sites of cell-

cell contact, and which is important for the formation of polarized epithelia [25] can also be verified to confirm the presence of epithelial cells in culture. Epithelial cultures can also be screened for tight junction proteins or adherens junction proteins using a variety of methods, such as real-time polymerase chain reaction, western blot, immune histochemistry and immunofluorescence [26,27]. By analyzing the fluorescently labelled proteins with laser scanning microscopy, combined with deconvolution of the dataset, valuable information regarding the spatial distribution of these proteins can be gained [28]. The cell-cell junctions can also be nicely demonstrated by transmission electron microscopy as shown in 16HBE14o- cells. In Figure 1A the junction complex consisting of tight and adherens junctions as well as desmosomes are well defined as determined with transmission electron microscopy. Such cells were examined in addition by laser scanning micrososcopy and the 3D reconstruction of the tight junction label showed that occludin was expressed at the cell-cell contacts and formed the typical belt-like structures (Figure 1B).

Regardless of the *in vitro* model types utilized in transport or translocation studies the first priority is to ascertain the integrity of the model [29]. As a first step towards fulfilling assurance of the epithelial cell culture being used, one may begin with the measurement of the trans-epithelial electrical resistance of a given model. This method, which uses a pair of electrodes, enables the researcher to assess the difference in the electrical resistance across the epithelium, thus providing essential information regarding the tightness of the cell-cell contact within the epithelium culture. This process is mostly applied in a two chamber system, where the two chambers are separated by the epithelial layer grown on a filter insert. Finally the electrical resistance of the filter insert without cells is subtracted from the value measured with cells and the result is multiplied in regards to the surface area (as a factor of cm^2) of the insert (Figure 2A). The resulting unit measured is Ωcm^2 for trans-epithelial electrical resistance. For instance primary alveolar epithelial cultures display values of > 1000 Ωcm^2 [30] and, for bronchial cell lines, values ranging between 300 and 400 Ωcm^2 have been reported [31]. Another method to test the epithelial integrity is to measure the permeation of the epithelial barrier by a paracellular marker (e.g. ^{14}C-mannitol or different dextrans) which can be characterized by the apparent permeation co-efficient. Similar to the trans-epithelial electrical resistance evaluation, the apparent permeation co-efficient is usually determined in a two chamber system in which two compartments are separated by the model barrier. The compound to be studied is added to the donor compartment and the apparent permeation co-efficient is experimentally derived from the time-dependent linear increase of the amount of compound in the receiver compartment (Figure 2B).

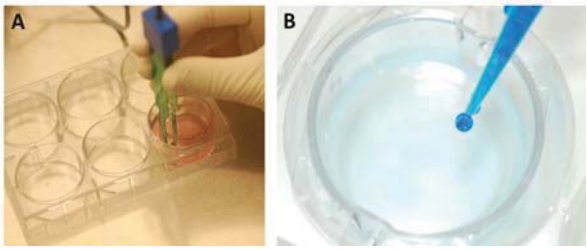

Figure 2: Measurement of trans-epithelial electrical resistance in epithelial cell cultures grown in two-chamber systems on a permeable membrane. A) Use of a pair of electrodes to measure the difference in the electrical resistance across the epithelium. B) Determination of the apparent permeation with Dextran-Blue. The compound is added to the donor compartment and the apparent permeation co-efficient is experimentally derived from the time-dependent linear increase of the amount of compound in the receiver compartment.

Mathematically, the apparent permeation co-efficient equals the fictive thickness of a buffer layer above the cells which is cleared from the compound within the indicated time interval; thus, the apparent permeation co-efficient represents the clearance of the compound divided by the area of the insert membrane cultured with cells. Numerous set-ups and several methods to determine apparent permeation co-efficient values are available [32]. The apparent permeation co-efficient in Madine-Darby canine kidney

cell cultures, which reflects a tight epithelial barrier, is 1.41 ± 0.13 x10^{-6}cm/sec [33], and for A549 cells 2.25 ± 0.4 x10^{-6}cm/sec [34].

2. Cell culture models

The use of cell cultures of the primary barrier systems, i.e. skin, gastro-intestinal tract, and skin, are reliable tools to study possible effects after exposure of those barriers to nanomaterials. After adequate characterisation and validation, such systems may be valuable alternatives to animal experiments. A lot of work to assess all the different scientific aims has been performed by establishing primary cell cultures or immortal cell lines and the focus of this review is to summarize the most used cell lines within the nanotoxicity field. Some ex-vivo systems which have been successfully applied for nanomaterial research are also mentioned.

2.1. Epithelial skin cell culture models

The external surface of the skin consists of a keratinised squamous epithelium, known as the epidermis, which is supported and nourished by a thick underlying layer of connective tissue referred to as the dermis, which is highly vascular and contains many sensory receptors [10]. A major function of the skin, especially the stratum corneum, which is the most outer layer, is to provide a protective barrier against the hazardous external environment. The skin is relatively impenetrable to lipophilic particles larger than 600 Daltons in size, whereas lipophilic particles any smaller than this may passively penetrate the skin [35].

The skin might be exposed to nanomaterials present in cosmetic products such as moisturisers and sunscreens. The skin is also a potential target for drug delivery via nano-carriers [36]. Nanoparticles (i.e. defined as a nano-object with all three external dimensions in the nanoscale [37]) have unique physical properties making them ideal for use in various skin care products currently on the market. Functionalized and/or surface modified metal oxide nanoparticles, specifically zinc oxide (ZnO) and titanium dioxide (TiO_2), are the primary nanomaterials used in sunscreen and skin care products as UV adsorber [38]. Most of the studies provide evidence that the skin is not the major target of nanoparticle delivery [39,40]. However, controversial discussions are ongoing concerning the benefits of nanoparticles in dermatological therapies and skin care products, as well as the potential disadvantages and possible mechanisms of toxicity [41].

Nano TiO_2 and ZnO formulated in topically applied sunscreen products exist as aggregates of primary particles ranging from 30 to 150 nm in size. These aggregates are bonded in such a way that the force of sunscreen product application onto the healthy skin would have no impact on their structure or result in the release of primary particles. Many studies using skin tissue (which is easily available from animal slaughterhouses) have also shown that under exaggerated test conditions neither nano-structured TiO_2 nor ZnO penetrate beyond the stratum corneum of skin using the “minipig” species [42]. Studies of the translocation of TiO_2 nanoparticles in histological skin sections suggest that these nanoparticles may only penetrate into the ‘horny’ upper layers of the stratum corneum [43]. However, other studies have shown that nano-sized particles can enter a small percentage of hair follicles and are stored in this location for a prolonged period compared to their location within the stratum corneum; a factor that may enhance drug delivery by this route, although it will also exacerbate any potential toxicity (i.e. dose kinetics) [44]. While such studies suggest little, if any epidermal or dermal penetration of these nanoparticles, recent work in live mice and pigs indicates that topically applied nano-sized TiO_2 particles (<10 nm) may indeed pass through the stratum corneum [45]. In addition, stretched porcine skin was far more susceptible to dermal translocation of a C_{60} fullerene substituted peptide, which could reach the intercellular spaces of the stratum granulosum in stretched skin [46].

Some studies have also been done using skin epithelial cell lines, such as the epidermal cell line A431. [47]. Another example is the immortalized human keratinocyte cell line HaCaT [48] which has been used to study the effects of silver [49], TiO_2 [50] or SiO_2 nanoparticles [51], just to mention some references.

Since the epidermis is composed of many different cell layers the optimal *in vitro* skin model is still lacking [52] and further research needs to be performed. However, since complete skin tissue can be easily obtained from the slaughterhouse this might be a better tool to study nanomaterial effects when applied on skin. In addition, commercially available models such as the EpiDerm™ skin model (www.mattek.com) as well as the EPISKIN™ (www.loreal.com) have been evaluated for corrosivity testing of chemicals and their potential in risk assessment for nanomaterials needs to be evaluated more thoroughly.

2.2. Epithelial (digestive) gastro-intestinal tract cell culture models

Throughout the digestive system the lumen is lined with the epithelium which changes structure and function according to the purpose of the corresponding part. The epithelium consists of a one-layer cell sheet which is however, altered in height and form in the different regions of the whole tract [10].

Humans continue to evolve with constant oral exposure to nanoparticles, such as to TiO_2 (designated E171 in Europe), which is used for whitening and brightening foods (especially for confectionary), white sauces and dressings, and certain powdered food products. Particulate silicates and aluminosilicates (E554, E556 and E559 in Europe) are also used in the food industry as anti-caking agents and to allow the flow of powders, and some are present in cheeses, sugars and powdered milks [53,54]. Overall, intake of dietary inorganic microparticles in the UK has been estimated to be about 40 mg/person/day (w35 mg for the silicates and w5 mg for titanium dioxide) which equates to a staggering daily exposure of $10^{12\text{-}14}$ particles/person [55]. Therefore, the gastrointestinal tract is regularly exposed to significant, milligram quantities of nanoparticles and microparticles (100–3000 nm) per day, including silicates and TiO_2 [54], contained in food, toothpaste and atmospheric sources. Following oral delivery, 'solid' particles (e.g. 50–100 nm polystyrene) can be actively transferred to the blood and lymphatic system and reach the liver and spleen [56].

Currently, there is only a small amount of literature that exists concerning the effects of nanoparticles on the gastrointestinal tract, most of which is contradictory. The upper aero-digestive tract, i.e., the nose and the oral cavity, on the one hand, acts as a complex barrier. Recently a buccal physicological *in vitro* system was developed using oral mucosa tissue with a Franz diffusion cell. In combination with human oral squamous epithelial cells (the H376 cell line) this system offers a valuable tool to evaluate the behaviour of nanomaterials in the buccal mucosa [57].

An evolving interest is ongoing however in studying nanomaterial interactions with the gastrointestinal tract *in vitro* such as the colonic carcinoma cell line, Caco-2 [58,59], which has differentiated features consistent with small intestinal enterocytes. During the last years many co-culture systems of the intestine have been developed and they will be described in more detail (see paragraph 3.1).

As an ex-vivo system the Ussing chamber has been described to provide a short-term organ culture method to study transport parameters of intact intestinal epithelium (for a review of the technique see Clarke, 2009 [60]) (. Only few studies have been found who have applied this method to assess nanoparticle apparent permeability across the epithelium [61,62] Since those studies aimed to develop drug carrier system the comparision with cell culture studies with the focus on basic nanoscience is difficult.

2.3. Epithelial lung cell culture models

Not only oxygen is inhaled but with every breath we take, millions of (nano)particles enter the respiratory system. The deposition of particles in the lung is size dependent [63,64]. Nanomaterials can be released into the environment from combustion-derived processes, or, in an occupational setting, by the use of nanomaterial-containing consumer products, such as aerosol sprays. Besides the geometry of the airways and the breathing pattern, the specific particle size is important for studying the deposition and clearance of particles in the respiratory tract. Following inhalation, airborne particles deposit in the different regions of the respiratory tract in a size-dependent manner [63,64]. Larger particles (1-10 μm) preferentially deposit in lager conducting airways (trachea, bronchi), whereas smaller particles (i.e. NPs) localize to more peripheral lung regions (alveoli) [4]. Once deposited, particles interact with the pulmonary surfactant and are displaced via wetting forces into the aqueous hypophase [65], where they can interact with pulmonary epithelial cells [66].

Because the lung is considered by far the most important portal of entry for nanomaterials into the human body, a lot of studies have been performed using epithelial lung cell culture models. The complex nature of the lung architecture means that the epithelium from the upper and the lower airways as well as from the alveoli is not readily accessible. Therefore, the use of *in vitro* cultures of airway and alveolar cells as a reliable tool for *in vitro* models to study the potential toxicity of nanomaterials has gained great interest during the last decade. Only if adequate characterisation and validation is performed, such systems may be valuable alternatives to inhalation experiments using animal testing strategies.

2.3.1. Airway epithelial cell cultures

The surface of the airways is lined by a pseudo-stratified epithelium. At the air-interface predominantly ciliated columnar cells lay interspersed with mucous goblet cells in the upper airways and cuboidal ciliated cells interspersed with the secretory active Clara cells in the lower airways [67,68].

Although primary cultures of tracheal and bronchial epithelial cells are technically feasible [69,70] a number of studies have been done using cell lines. Among them, the most popular human airway epithelial cell lines are the Calu-3 and the 16HBE14o-. The Calu-3 cell line is of human origin, commercially available from the American Type Culture Collection, and displays epithelial morphology as well as adherent growth. The presence of tight junctions and the secretory activity makes the Calu-3 cell line a promising tool for pulmonary drug absorption studies [71]. The immortalized 16HBE14o- cell line, a SV-40 large T-antigen transformed bronchial epithelial cell line, is a normal human airway epithelial cell line and only available as a gift from Dieter Gruenert (Cardiovascular Research Institute, University of California, San Francisco). The cells form polarized monolayers (Figure 3) with extensive tight junction belts [31,71] and when the cells are grown on collagen-supports at an air-liquid interface they retain important properties of differentiated airway epithelial cells [72].

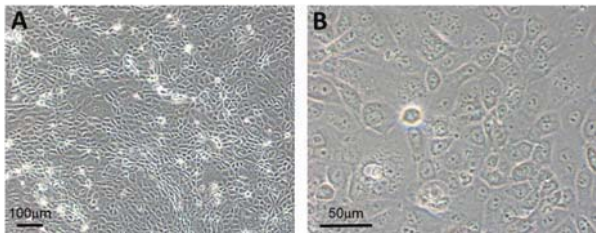

Figure 3: Phase-contrast micrographs of 16HBE14o- epithelial cell cultures after 4 days in culture. A) and B) represent images taken at different magnifications (10x and 40x, respectively). The cells formed a tight and confluent monolayer.

The BEAS-2B cell line was derived from normal human epithelial cells immortalized using the adenovirus 12-simian virus 40 hybrid virus [73] and is available from the American Type Culture Collection. These cells have been often used to study airway epithelial structure and function, although they do not form

tight junctions (for a review see Forbes [74]).

All of these 3 cell lines are often used to assess particle cell interactions [26], and to study the potential toxicity of nanoparticles [75,76].

Pig trachea can be easily obtained from the slaughterhouse and used for several hours as an easy ex-vivo system to study effects of nanomaterial shape, size and charge on mucociliary clearance [77] and further studies with this system should be emphasized.

2.3.2. Alveolar epithelial cell cultures

Primary alveolar epithelial cell cultures provide a tight epithelial barrier resembling the pulmonary barrier *in vivo*. It has been described that human alveolar epithelial type II cells isolated from normal human lung tissue undergo morphological and histochemical changes, differentiating from type II to type I like cells [30]. In case alveolar epithelial cell cultures are used for *in vitro* drug transport studies a high trans-epithelial electrical resistance is a pre-requisite and it has been shown that in primary cultures of isolated alveolar type II cells, monolayers with high trans-epithelial electrical resistance (>1000Ωcm^2) can be generated [30,78].

In contrast to primary cells, cell lines are often preferred due to the ease of cell culture and the purity of cell types. The cell line A549, which originates from human lung carcinoma [79], belongs to the most well characterized and most widely used *in vitro* models [80]. It is also available from the American Type Culture Collection. It has been shown that the A549 cells have many important biological properties of alveolar epithelial type II cells (e.g. membrane-bound inclusions), which resemble lamellar bodies of type II cells [81]. Other ultra-structural characteristics that are common to type II epithelial cells have also been described, as for example distinct polarisation, tight junctions, and extensive cytoplasmic extensions [88]. Contradictory results concerning the capability of this cell line to express tight junctions and to generate trans-epithelial electrical resistance have been published. We have shown that trans-epithelial electrical values between 140 and 180 Ωcm^2 have been developed and remained stable from day 3 to day 12. Elbert and colleagues [30] found that A549 cells did not express Zonula occludens-1, an intracellular protein of the tight junction complex. We were also not able to detect Zonula occludens-1 when assessed via inmmunofluorescence. However, we did find that A549 cells are positive for Zonula occludens-3, occludin and also claudin-2 [89,90]. Comprising a comparison between the different published results is extremely difficult since in the Elbert study the A549 cells were used between passage 88-95 and after cells were grown to confluent monolayers. In our study the cells were used for 10-70 passages and kept in culture for a minimum of 7 days. Since it has been shown for other epithelial cell lines that various factors influence the growth and appearance [33] it is very important to evaluate the cells under strictly controlled conditions. Thus, it is the opinion of the authors that when using A549 cells under controlled conditions they are an appropriate epithelial alveolar model.

In addition, the A549 cells have already been used not only to assess acute effects but to study possible adaptive mechanisms during long-term consequences [82]. This is an important aspects since humans have to deal with chronic exposures to ambient nanoparticles as well as chronic occupational exposure to engineered nanomaterials at the workplace and needs to be studied in the future in more detail.

Just recently, the immortalization of human type II cells for the use in nanotoxicity studies has been reported by Kemp and co-workers. This new cell line exhibited a type I like phenotype, no longer expressed alkaline phosphatase and pro surfactant protein C, but showed enhanced levels of caveolin-1 and RAGE (receptor for advanced glycation endproducts). The uptake of latex particles was studied with these cells and the cell line was postulated to be important for particle translocation studies [83].

2.3.3. Air-liquid cultures

The cultivation of epithelial cells on permeable supports enables the culture medium to be separated on either side of the cultured epithelium leading to an increased differentiation of the cultured cells [84]. Furthermore, the medium can be removed from the upper side to expose the cells to air on one side, allowing the cells to 'feed' from the medium in the chamber underneath [85,86]. The air-liquid culture technique has been described in different cell culture models [87-89]. Air-exposed cell cultures allow studying the interaction of inhaled nanomaterials with cells in an environment that more closely mimics the *in vivo* situation. Of particular importance is that the cells are covered by a very thin liquid lining layer with a molecular surfactant film at the air-liquid interface since surfactant plays an important role in particle displacement and retention [90]. In recent nanoparticle-cell interaction studies air-exposed A549 cells were used and exposed to air and the test substances simultaneously [91,92]. However, the cells need time to release the surfactant into the liquid layer [93]. Also the bronchial epithelial cell line 16HBE14o- as well as Calu-3 cells can be exposed to air [94]. In these studies the air-exposed cultures exhibited a clear epithelial morphology and integrity as in *in situ* conditions. Such *in vitro* cell systems combined with various air-liquid exposure systems that allow a dosimetrically accurate delivery of aerosolized nanomaterial offer a reliable method for the investigation of nanomaterial-cell interactions and possible cellular responses (for reviews see [20,95]).

3. Co- culture models

Studies have shown that when cells are removed from their host tissue and are grown as monolayers on impermeable surfaces, they undergo dedifferentiation and lose specialized functions, which is thought to be, in part, due to the disassociation of cells from their native three-dimensional (3D) tissue structure *in vivo* [24]. During the last years it has been recognized that not only the three dimensional structure seems to be important for the differentiation of certain cells [96] but also the culturing of different cells together is an important issue. Not only is 3D structure important, but also co-cultures of different cell types have been shown to have an influence on the outcome of the results, since cells continuously cross-talk *in vivo* through intercellular signalling to maintain homeostasis and to coordinate immune responses [97].

3.1. Intestine mucosa

To study the interaction of dendritic cells with gut epithelial cells during the process of particle sampling, a co-culture system was developed using the human enterocyte cell line caco-2 combined with bone marrow-derived dendritic cells [98,99]. Another co-culture system of the gut mucosa was recently developed by using Caco-2 cells that are cultured in close contact with Raij-B-cells (B lymphocytes). During co-culture 15 to 30 % of the Caco-2 cells are converted into M-cells which are differentiated epithelial cells, specialised in the transcytosis of macromolecules and particles across the gastro-intestinal tract [100,101].

Of interest is also if diseased tissue reacts more susceptible than healthy tissue to the exposure of nanomaterials. To reveal more insights into this, a complex *in vitro* model of the inflamed intestinal mucosa has been developed composed of Caco2 enterocytes, macrophages and dendritic cells. By applying fluorescently labelled particles to the co-cultures they found significantly more nanoparticles adhered to inflamed cells compared to the non-stimulated control [102]. Further studies need to be done to evaluate the differences between mono- and co-cultures.

3.2. Lung mucosa

So far, *in vitro* co-cultures to mimic the alveolar epithelial barrier with two cell

types have been described in the literature. Two cell co-culture models with epithelial (A549 cells) and endothelial cells were established to examine events in the pathogenesis of bacteria [103,104]. Recently, a primary co-culture system to simulate the human alveolar-capillary barrier by using primary cells was developed. Human pulmonary microvascular endothelial cells were co-cultivated with primary isolated human type II alveolar epithelial cells on opposite sides of a permeable filter support, to study the impact of nanocarriers [105,106].

Recently we developed a triple cell culture *in vitro* model of the human airway wall to study the cellular interplay and the cellular response of epithelial cells, human blood monocytes derived macrophages and dendritic cells to particles [107]. In this model, monolayers of two different epithelial cell lines, A549 [34] and 16HBE14o- epithelia [26] as well as primary epithelial type I cells [108] can be grown on a microporous membrane in a two chamber system. In addition, a quadruple-culture containing epithelial, endothelial, macrophages as well as mast cells has been established [109]. Studies using such co-culture cell systems have reported that they observe different reactions compared to monoculture analysis when the cells were exposed to nanomaterials [27,110]; however, such reactions observed from a culture containing two, three or four different types of cells merely cultured in the same dish, is not specific to that as it would occur in the human body. Thus, the architecture of the *in vitro* cell co-culture model in regard to the specific organ they represent is essential when nanomaterials effects are studied.

4. Nanomaterial- epithelial cell interactions: Biological mechanism and responses

Epithelial cell systems in vitro have become an important tool for the study of biological mechanism upon nanomaterial exposure as well as a pre-screening system for nanomaterial risk assessment. A complete overview about nanomaterial uptake mechanism and induction of cell responses is however beyond the remit of this review article and for a clear understanding in these fields it is suggested that other publications are considered.

Briefly, different uptake mechanisms of nanomaterials into cells and intracellular trafficking described so far have been discussed in detail by various reviews [66,111-113]. Depending on size, shape, material and coating of the material and the cell type possible uptake can occur by phagocytosis, macropinocytosis, clathrin- and caveolae-mediated endocytosis, clathrin and caveolae independent endocytosis and nanomaterial diffusion / transport across the cell plasma membrane. In addition it has been shown that the kinetics of all known processes depends largely on nanomaterial surface as well as on in vivo surface modifications such as interactions wih endogenous proteins [4].

Once inside the cells, nanomaterials may cause several biological responses including the generation of reactive oxygen species [114], the enhanced expression of pro-inflammatory cytokines [115], and DNA strand breaks [116]. However, the precise mechanism of possible nanomaterial toxicology is still not fully understood [19]. Currently, the hypothesis that nanomaterials induce adverse cellular effects via oxidative means (oxidative stress paradigm) [117] is used as a basis for many nanomaterial-based investigations. Recently, additional paradigms have been suggested for nanomaterials, such as the fibre paradigm [118] and the theory of genotoxicity [119]. In addition, some important aspects about the use of reliable methods and realistic test conditions to study possible risks of nanomateriales have recently been reviewed in several publications. For instance the in-depth characterization of the nanomaterial, the use of suspension versus air-liquid exposures, the use of realistic doses, and the validity of the selected test methods have been highlighted [95,120].

5. Conclusions

There is an ascertainable trend towards the development of more predictive and reliable *in vitro* testing systems using smart biotechnology to reduce time and cost investments. The discussed epithelial cell culture models may help to elucidate the mechanism of how nanomaterials can interact with cells, the primary barrier system encountered following exposure of nanomaterials by ingestion, inhalation and application to the skin. Even though epithelial cell culture models exhibit a number of limitations, they can be used for high-throughput screening and the screening of large numbers of newly developed nanomaterials and to study basic nanomaterial-cell interactions, such as uptake and induction of cellular responses. An essential disadvantage is, however, that the complex environment cannot be satisfactorily been reproduced since cell culture models often do not exhibit all the differentiated and functional characteristics of the corresponding native epithelium or the entire organ. Finally, any model needed to study the epithelium for a certain question should be selected very carefully, considering its limitations and taking these limitations into account for the experimental design and the interpretation of the results.

For studies with skin-models it seems to be feasible to use skin-tissue from animals, i.e. pigs, which can directly be obtained in the slaughterhouse. The use of cell cultures to mimic the gastro-intestinal or lung tissue helps to evaluate basic mechanisms such as nanomaterial-cell interactions or induction of cellular responses. However, this might not be sufficiently covered by only performing monoculture analysis and for both tissues there are sophisticated co-culture models available mimicking more realistically the specific organs. Table 1 summarizes the epithelial cell culture models discussed in this review. Considering the attempts to initiate the reduction, refinement and replacement of *in vivo* experimental approaches, many *in vitro* epithelial cell culture models have been developed during the last decades. By using well defined *in vitro* approaches combined with a controlled nanomaterial exposure such systems are of great importance to help understanding how nanomaterials interact with living matter.

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Table

Table 1: Human epithelial cell lines mimicking primary exposure barriers. This table only represents a selection of available cell lines.

Skin epithelial cell lines	References
• Keratinocyte cell line HaCaT	[49-51]
• Epidermal cell line A431	[47]
Gastro- intestinal tract epithelial cell lines	
• Oral squamous cell line H376	[57]
• Intestine cell line Caco2	[58,59]
Lung epithelial cell lines	
Airway epithelial cells	
• Calu-3	[71,88,121-123]
• 16HBE14o-	[26,27,74,88]
• BEAS-2B	[75,76]
Alveolar epithelial cells	
• A549	[34,80,82,93]
• Immortalized human alveolar type 2 cells with alveolar type 1 phenotype	[83]
Co- Cultures	
Gastro-intestintal tract	
• Co-cultures of Caco2 with dendritic cells	[98,99]
• Co-cultures of Caco2 with Rauj-B-cells	[100,101]
Lung	
• Co-cultures of A549 with endothelial cells	[103,124]
• Triple cell co-culture model (epithelial cells, macrophages, dendritic cells)	[26,34]
• Quadruple co-culture model with epithelial, endothelial, mast cells and macrophages	[109]

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