transatlantic think tank for toxicology

t4 Workshop Report*

Non-Animal Models of Epithelial Barriers (Skin, Intestine and Lung) in Research, Industrial Applications and Regulatory Toxicology

Sarah Gordon[1], Mardas Daneshian[2], Joke Bouwstra[3], Francesca Caloni[4], Samuel Constant[5], Donna E. Davies[6,7], Gudrun Dandekar[8], Carlos A. Guzman[9], Eric Fabian[10], Eleonore Haltner[11], Thomas Hartung[2,12], Nina Hasiwa[2], Patrick Hayden[13], Helena Kandarova[14], Sangeeta Khare[15], Harald F. Krug[16], Carsten Kneuer[17], Marcel Leist[2], Guoping Lian[18,19], Uwe Marx[20,21], Marco Metzger[8], Katharina Ott[10], Pilar Prieto[22], Michael S. Roberts[23], Erwin L. Roggen[24], Tewes Tralau[25], Claudia van den Braak[26], Heike Walles[8] and Claus-Michael Lehr[1]

[1]Department of Drug Delivery, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Helmholtz Center for Infection Research (HZI) and Department of Pharmacy, Saarland University, Saarbrücken, Germany; [2]Center for Alternatives to Animal Testing-Europe, University of Konstanz, Konstanz, Germany; [3]Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research, Leiden University, Leiden, The Netherlands; [4]Università degli Studi di Milano, Department of Health, Animal Science and Food Safety (VESPA), Milan, Italy; [5]Epithelix Sàrl, Geneva, Switzerland; [6]Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK; [7]NIHR Respiratory Biomedical Research Unit, University Hospital Southampton, Southampton, UK; [8]Translational Center Regenerative Therapies for Oncology and Musculoskeletal Diseases, Würzburg Branch of the Fraunhofer Institute Interfacial Engineering and Biotechnology (IGB) and Department of Tissue Engineering and Regenerative Medicine, University Hospital Würzburg, Würzburg, Germany; [9]Department of Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research (HZI), Braunschweig, Germany; [10]BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany; [11]Across Barriers GmbH, Saarbrücken, Germany; [12]Center for Alternatives to Animal Testing, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, USA; [13]MatTek Corporation, Ashland, MA, USA; [14]MatTek In Vitro Life Science Laboratories, Bratislava, Slovak Republic; [15]Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR, USA; [16]Empa - Swiss Federal Institute for Materials Science & Technology, St. Gallen, Switzerland; [17]German Federal Institute for Risk Assessment (BfR), Department of Pesticide Safety, Berlin, Germany; [18]Unilever Research Colworth, Sharnbrook, UK; [19]Department of Chemical and Process Engineering, University of Surrey, Guildford, UK; [20]Technical University Berlin, Germany; [21]TissUse Incorporated, Spreenhagen, Berlin, Germany; [22]EURL ECVAM, Systems Toxicology Unit, Institute for Health and Consumer Protection, European Commission, Joint Research Centre, Ispra, Italy; [23]Therapeutics Research Centre, School of Medicine, University of Queensland, Translational Research Institute, Brisbane, Queensland; University of South Australia, Adelaide, Australia; [24]3Rs Management and Consulting ApS, Lyngby, Denmark; [25]German Federal Institute for Risk Assessment (BfR), Department of Chemicals and Product Safety, Berlin, Germany; [26]Danone corporation, Nutricia Research, Utrecht, The Netherlands

Summary

Models of the outer epithelia of the human body – namely the skin, the intestine and the lung – have found valid applications in both research and industrial settings as attractive alternatives to animal testing. A variety of approaches to model these barriers are currently employed in such fields, ranging from the utilization of *ex vivo* tissue to reconstructed *in vitro* models, and further to chip-based technologies, synthetic membrane systems and, of increasing current interest, *in silico* modeling approaches. An international group of experts in the field of epithelial barriers was convened from academia, industry and regulatory bodies to present both the current state of the art of non-animal models of the skin, intestinal and pulmonary barriers in their various fields of application, and to discuss research-based, industry-driven and regulatory-relevant future directions for both the development of new models and the refinement of existing test methods. Issues of model relevance and preference, validation and standardization, acceptance, and the need for simplicity versus complexity were focal themes of the discussions. The outcomes of workshop presentations and discussions, in relation to both current status and future directions in the utilization and development of epithelial barrier models, are presented by the attending experts in the current report.

Keywords: *in vitro* models, epithelial cell culture, permeability, transport studies, cytotoxicity

Received October 5, 2015;
http://dx.doi.org/10.14573/altex.1510051

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*A report of t4 – the transatlantic think tank for toxicology, a collaboration of the toxicologically oriented chairs in Baltimore, Konstanz and Utrecht sponsored by the Doerenkamp Zbinden Foundation. The views expressed in this article are those of the contributing authors and do not necessarily reflect those of their institution of employment.

1 Introduction

Non-animal models of epithelial barriers are currently enjoying increasing interest from various groups, including scientists in academia, product developers in industry, regulatory authorities and, last but not least, society in general. They play a critical role for *in vitro* to *in vivo* extrapolation as they feed critical information beyond structure and modeling of kinetics (Basketter et al., 2012; Leist et al., 2014), which is a key element of translating *in vitro* results to a dose paradigm, thus enabling the implementation of a Toxicology for the 21st Century (Hartung, 2009). While ethical constraints, legislation, rising costs and concerns over the predictive value of animal experiments may be responsible for the altered level of awareness of *in vitro* models, the interest in epithelia as such has primarily to do with the duality and, by virtue of this, the importance of their function. Epithelial tissues represent the body's interface with the "outside world", and therefore must fulfill important biological barrier functions. These functions include protecting the body against potentially noxious compounds or microorganisms ("outside-in-barriers"), as well as preventing the loss of vital compounds such as water and solutes ("inside-out-barriers"). The latter function is perhaps most obvious with respect to the skin epithelium, but is also evident for other organs, and particularly notable in cases of malfunction as seen in various states of disease (e.g., diarrhea or pneumonia with reference to the intestinal and pulmonary epithelium, respectively). Besides their protective function against *unintended exposure* to chemicals or materials (e.g., from the environment or at the workplace), epithelia also provide the opportunity to apply compounds *on purpose* – for example, decorative and protective cosmetics, food and dietary supplements, and medical products such as drugs, diagnostic agents and vaccines. In the context of medical products, delivery via the transdermal, oral or pulmonary routes is non-invasive and therefore much preferred over any form of injection (intravenous, subcutaneous, intra-articular, etc.). While similar considerations of course also apply for some more specialized organs and epithelia such as the eye, the nose or reproductive organs, the predominant routes of either intended or unintended application/exposure are the skin, the gastrointestinal tract and the respiratory tract. As such, while the need for test methods and the considerable progress that has already been made regarding other epithelia are acknowledged, this report will focus only on the epithelia of skin, intestine and lung (Fig. 1A,B).

Despite the fact that interest in epithelial barriers is common to various applications and industries, each epithelial barrier model has both specific characteristics and caveats for application. For the safety of cosmetics, chemicals and new (nano!) materials, it is most important to show that no harm is done to the epithelia to which such compounds are applied intentionally or not. In addition, any transport across epithelia, which would lead to unintended local or systemic bioavailability, should be

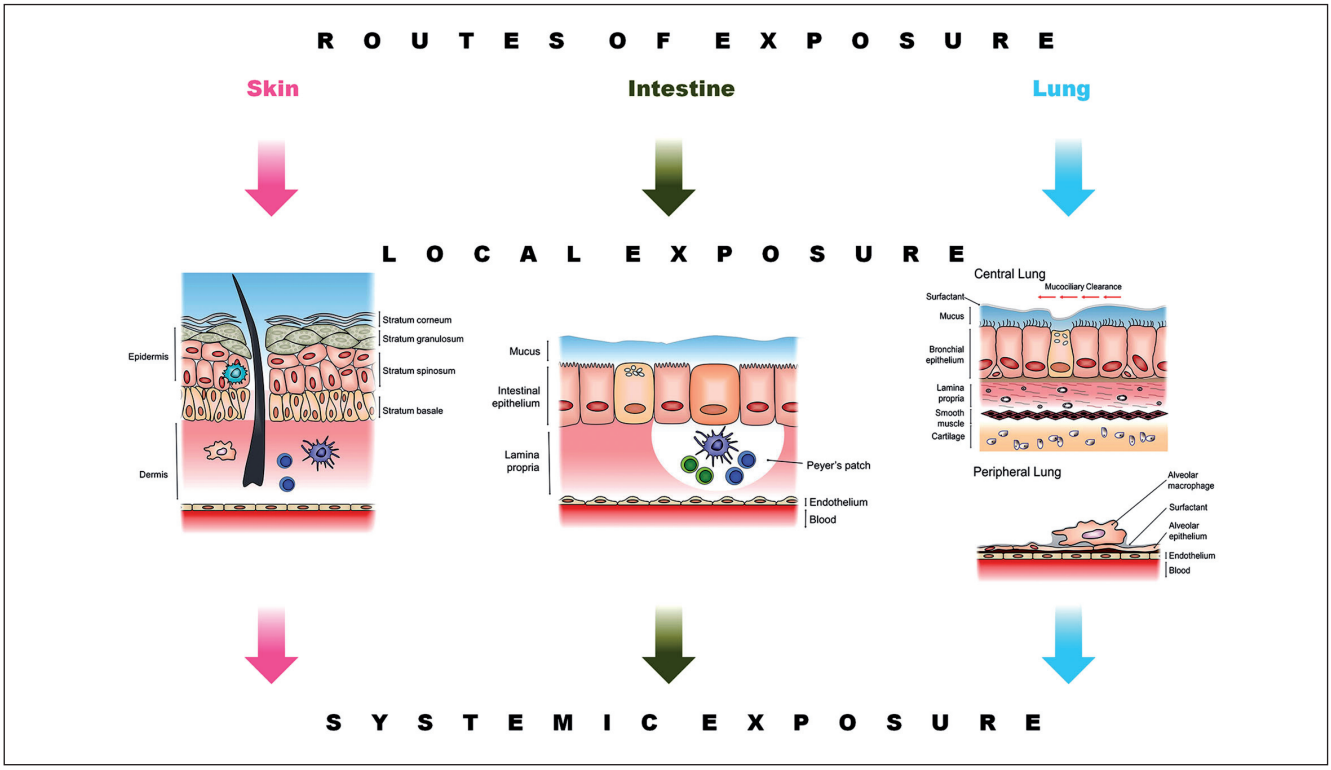

Fig. 1A: Depiction of the outer epithelial barriers of the human body – the skin, the intestine and the lung
Intended or unintended application of test chemicals to these epithelial barriers may result in either no effect, indicating a successful protective action of the barrier; a local effect, occurring at the barrier itself; or a systemic effect, resulting from penetration through the barrier structure. Figure modified in part from Ruge et al. (2013) and de Souza Carvalho et al. (2014), with permission.

as small as possible (ideally nil). Quantitative information on absorption across the external barriers is also needed to relate external exposures to internal (or systemic) threshold values in the context of regulatory risk assessment. This approach is followed to collectively assess the impact of simultaneous or sequential exposures through the different routes, e.g., for pesticides and biocides. It is also important to show that the natural barrier function of a given epithelium is not changed as a result of application or exposure – not even indirectly, for example via the induction of an immunological response – as this would not only cause unacceptable local irritation but as its consequence also inevitably lead to an increased absorption of potentially noxious compounds. Such considerations define the common interest of some industry sectors (i.e., cosmetics, industrial chemicals, biocides and plant protection products) in epithelial barriers in the context of their R&D efforts for new products. Marketing regulations in this case are therefore also different from those for food or pharmaceutical products.

In contrast, the pharmaceutical industry, interested in new drugs and related products, is obliged to demonstrate not only product safety, but also efficacy (Rovida et al., 2015). This includes providing evidence that an active pharmaceutical ingredient (API) becomes biologically available at its intended site of action (e.g., inner organ, tissue, cell or receptor), meaning that it is able to cross relevant absorption barriers which otherwise would limit its bioavailability and therapeutic efficacy. The marketing approval of drugs is regulated by very strict and complex laws worldwide. Besides their quality, the safety and efficacy of new products must be extremely well documented and demonstrated in several phases of clinical testing. Before even entering clinical trials, preclinical tests must however be passed successfully. The entire process from discovery to market typically takes 10 years or longer, and costs more than 1 billion €/$ for a single new drug molecule. The discovery to market process is also associated with extremely high drop-out rates (Hartung, 2013), an occurrence which is particularly notable in the case of new vaccine candidates. Preclinical, highly predictive models for use in vaccine development therefore constitute a significant and as yet unmet need within the pharmaceutical industry.

The needs and activities of the food industry are different again, in some way "in between" those of pharma and the cosmetic/chemical industry (Hartung and Koeter, 2008). While food products, including dietary supplements or so-called "probiotics," must of course be safe, there is on the other hand at least some expectation regarding their efficacy. The latter – like for pharmaceuticals, but unlike for cosmetics and chemicals – thus includes assessment of absorption and bioavailability. Compared to drugs and medical products however, the regulatory standards for foods are much less stringent. Yet, with the rise of new technologies (including but not restricted to "nano"), the need to demonstrate safety and efficacy, if only to convince potential customers, is certainly also present in the food industry.

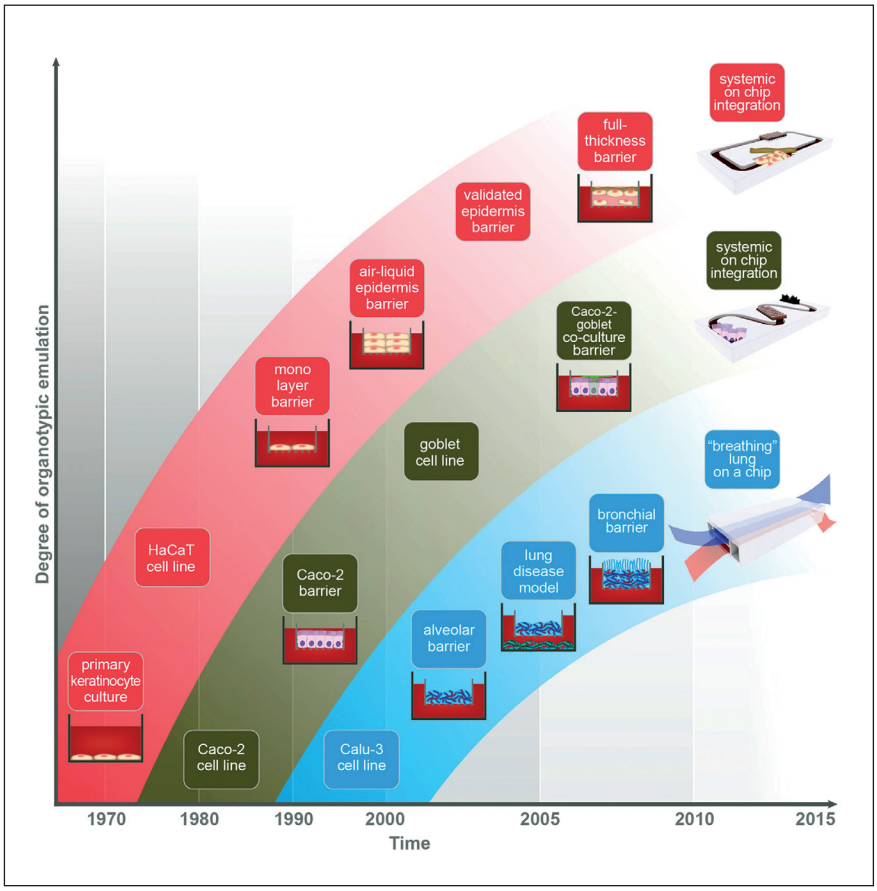

Fig. 1B: Development of *in vitro* models of human outer epithelia at a glance
Cell culture devices have improved from petri dishes, through membrane-based Transwell® tissue culture plates, to microfluidic tissue culture chips over the few last decades. This significantly influenced the development of human skin (pink boxes), intestine (green boxes) and lung (blue boxes) barrier models. All three evolved from the monolayer culture of respective primary epithelial cells or cell lines to subsequent levels of organotypic emulation. Skin models progressed from keratinocyte monolayers to full-thickness air-exposed skin cultures on-a-chip. Intestinal models progressed from monolayer Caco-2 cell line culture to systemic chip-based co-culture with liver and tumor cells. Lung models progressed from alveolar and bronchial epithelial barrier cultures to mechanically coupled alveolar models on-a-chip. Only a single epidermis skin model has been validated at an OECD level so far, fully replacing respective animal models. The detailed characteristics and impact of each barrier model are described in the corresponding sections.

The outcome goal of this workshop and report was to analyze the current status of barrier models (skin, intestine and lung) in the various areas of application introduced above (environmental chemicals/cosmetics, pharmaceuticals and food). Based on such analysis, it became possible to identify specific needs for new models, as well as to define further research needed to validate existing models. The long term goal in this respect must be to create valid, robust alternatives to animal testing.

2 Non-animal models of the skin in research, industrial applications and regulatory toxicology

2.1 General introduction

The skin, a complex living membrane, is the largest unfolded organ of the human body, accounting for approximately 15-17% of the body weight and with a surface area of approximately 1.5-1.7 m^2. Skin is a continuously self-renewing (via repair and desquamation), metabolically active organ providing the means for detoxification of chemical insults; it is immunologically relevant with regard to sensitization and skin allergies, provides a surface that hosts a dense population of microbial commensals (up to 10^7 CFU/cm^2) (Peiser et al., 2012; SanMiguel and Grice, 2015), while at the same time protecting the human body against adverse microbial colonization. Furthermore, the skin protects the body from heat (temperature regulation), water (-loss) and electromagnetic radiation. The relationship between the structure of the human skin and its mechanical and biological barrier properties has been the subject of extensive research in the last fifty years (Elias, 1981; Jensen and Proksch, 2009; Kirschner and Brandner, 2012; Loewenthal, 1963; Menon et al., 2012; Norlen, 2001; Wiechers, 1989).

The skin can be depicted as a bilayer organ, consisting of the epidermis (outer region) and the dermis (inner region). The avascular epidermis itself is composed of various cell layers, whereas the stratum corneum – the outermost layer – is identified as the main penetration barrier of the skin (Hadgraft and Lane, 2005). This structure consists mainly of dead corneocytes (derived from keratinocytes) embedded in lipids. The lower epidermis, the viable layer, poses the second main biological barrier, being both metabolically and immunologically active. The dermis is vascularized and innervated (including sensory cells); it also contains lymphatics, hair follicles, sweat glands and sebaceous glands. Substances penetrating the skin barrier have to pass through the lipid matrix of the stratum corneum, which forms a continuous structure. Protein-containing corneocytes within this lipid matrix form a hydrophilic second compartment within the stratum corneum, and can act as a substance reservoir (Hansen et al., 2009). Being significantly different from other biobarriers, the lipid matrix of the stratum corneum contains ceramides, free fatty acids and cholesterol in an approximately equimolar ratio (van Smeden et al., 2014; Weerheim and Ponec, 2001).

Information on skin toxicity and (trans)dermal penetration of compounds via healthy and diseased skin is of the highest importance for applied and safety sciences of consumer products, e.g., chemicals and their mixtures, plant protection products, drugs, vaccines and cosmetics. A number of EU regulations therefore require information on skin penetration, irritation, corrosion and sensitization. Within Europe, such regulations for example pertain to chemicals (EU, 2006, 2008), biocides (EU, 2012), cosmetics (EU, 2009a) and plant protection products (EU, 2009b). From a pharmaceutical point of view, topical delivery poses an important alternative to oral drug administration, which in many cases is associated with insufficient intestinal absorption. Delivery via the skin is also associated with a reduced pre-systemic metabolism (first pass), the possibility to achieve sustained drug penetration and the potential to avoid drug toxicity as seen with oral administration (Bouwstra et al., 2003). Currently there over 35 transdermal delivery systems are described and approved in the EU, USA and Japan (Murthy, 2012; Uchida et al., 2015). The use of *in vitro* diffusion systems to assess skin permeability therefore constitutes a useful tool for the development of further novel formulations, and for the purposes of toxicity testing and quality control.

The best model to study effects in human skin avoiding any species extrapolation would be native human skin. For dermal absorption studies excised human skin is the preferred model and accepted by many authorities, since the barrier properties are well preserved after excision. However, storage in a freezer can influence other properties of the skin tissue such as CYP450 isoenzyme activity (Henkler et al., 2012; Kao et al., 1985); this in turn may change the absorption or sensitization characteristics of a compound. Furthermore, restricted access to human material, limited availability and high costs are obstacles to the use of native human skin. Such restrictions also provide an explanation for the continuing use of *in vivo* and *ex vivo* models of animal skin, as well as the growing importance of *in vitro* reconstructed human skin models. Artificial skin surrogates (membrane systems with lipids applied on top of these membranes) and *in silico* models have also proven to be helpful when predicting the *in vitro* release of the compounds and modelling of compounds penetration and toxicity.

Already in the early 1990's a number of scientists and institutes worldwide focused on the development of alternative methods to address topical toxicity, due to several revisions of the Cosmetics Directive 76/768/EEC. In 2003 a new revision resulted in the provision of specific testing and marketing bans related to using animals in the safety evaluation of cosmetics. In 2009, the new EU Cosmetic Products Regulation (EC) No 1223/2009 was adopted and has been fully applicable since 2013 (EU, 2009a). As a consequence, most of the *in vitro* assays available today have been developed as replacements dealing with hazard identification, and to a much lesser extent, with hazard characterization.

This chapter reviews the available models for studying the skin barrier, skin penetration and topical toxicity in pharmacology and toxicology. It gives examples of model employment in the various areas and discusses the current problems associated with their use.

2.2 *Ex vivo* skin models

Ex vivo human skin is usually obtained as a product of plastic surgeries either directly from hospitals or distributed by associated tissue banks. It is mainly used for the assessment of

percutaneous penetration, as required for the safety evaluation of chemicals, plant protection products, pharmaceuticals and cosmetics (Danso et al., 2015). *Ex vivo* skin may be prepared in various thicknesses using a dermatome. It is thus available as epidermis (split-thickness; approximately 100-400 μm) or as full skin thickness samples containing the dermis (depending on body location up to 1-2 mm). Deviations in the sample thickness however impact on the penetration of substances into the receptor fluid (Wilkinson et al., 2006), and therefore samples containing only part of the dermis with a defined thickness are preferred for testing by several authorities (EFSA, 2011, 2012; OECD, 2011).

Along with the pig skin model, employed due to its barrier properties comparable to human skin as well as a better availability (Herkenne et al., 2006), rat skin is also used for dermal penetration studies (Takeuchi et al., 2011). Rat skin is mainly employed in the plant protection area, for systemic toxicology studies and *in vivo* skin absorption studies. It has, however, to be noted that rat skin is more permeable than human skin (van Ravenzwaay and Leibold, 2004), and that skin metabolism differs between human and rat species. Thus, results obtained with rat models should be interpreted with care (Bartek et al., 1972; Jung and Maibach, 2015; Oesch et al., 2014). However, a triple pack using human *in vitro* data corrected by rat *in vivo* and *in vitro* data is an accepted refinement option for human risk assessment in the EU. If the ratio of rat *in vivo*:rat *in vitro* is close to 1, *in vitro* results with human skin and the triple pack are also accepted in NAFTA countries (NAFTA, 2009).

OECD test guidelines 427 (*in vivo*) and 428 (*in vitro*) and their associated guidance document No. 28 provide a basic framework for the practical methods used for dermal absorption studies, but they are not specific to a particular regulatory field or industry. Thus, different regulatory bodies may require different types of study structure, while some of the national agencies are reluctant to accept results from *in vitro* skin penetration tests in general (OECD TG 428). Some authorities have published additional guidance for the performance and use of *in vitro* tests for specific product groups, for example EFSA for plant protection products, or alternatively have published evaluation criteria, such as for cosmetic ingredients by the scientific committee on consumer safety of the European Commission (EFSA, 2012; SCCS, 2010). For some authorities a high donor-to-donor variability in the study is an exclusion criterion. On the one hand, this donor dependency is disadvantageous as it complicates the comparison of different studies; however, on the other hand, the inherent variability reflects the individual differences as in real life. In this respect, research focusing on the identification and definition of the range of an "average healthy human skin" (as referred to in the conclusion of this chapter) would help to standardize the method further and allow for comparison between different studies. A much simpler step in this direction is the performance of an accompanying integrity test that ensures the exclusive use of intact skin samples as well as skin samples with comparable properties (Wiegand et al., 2014; Guth et al., 2015).

Other limitations of *ex vivo* absorption studies are as follows: i) The receptor fluid may influence the transfer of the substance via the *ex vivo* model. Therefore, when using very lipophilic substances, for example, the acceptor phase should be carefully chosen so as not to affect the model. In such a case, the penetration may be very difficult to assess, and the absorption rate determining the drug effect concentration should be handled with care as a result. For toxicological evaluation the use of an organic receptor fluid may ensure solubility, but the resulting change in diffusion pressure may theoretically lead to an overestimation of the absorption for some substances. ii) The absence of blood vessels and the variable thickness of the dermis in dermatomed skin samples may pose additional shortcomings, especially in the case of lipophilic compounds. To minimize this effect and improve consistency and comparability, EFSA recommends the use of split-thickness skin of 200-400/500 μm (EFSA, 2012). iii) The penetration via hair channels and sweat ducts may be different *ex vivo* and *in vivo*. However, although fast absorption via hair follicles can be observed for selected molecules, e.g., caffeine (Otberg et al., 2007), there are indications that the fast absorption via shunts dominates the transport through skin only until the lag time for intra- or transcellular transport is reached (Scheuplein, 1972). Therefore, the overall contribution of absorption via skin appendages may be limited. iv) The period for which *ex vivo* skin will remain viable is limited.

In contrast, there are several advantages offered by employment of *ex vivo* models of human or animal in place of *in vivo* methods. These include: i) Conditions can be more precisely controlled and experiments are easier to perform. ii) Radiolabeled or presumed-toxic chemicals (e.g., as indicated by cheminformatics data) can be used. iii) A high number of experiments can be run simultaneously. iv) Species-relevant (i.e., human) skin can be used, providing the possibility to obtain quantitative data on intra-species variability. v) The impact of particular conditions (e.g., release from different vehicles) can be studied much more precisely. vi) Kinetics can be directly measured without dilution in tissue fluids or organs. vii) Removal of ethical costs by avoidance of animal experiments (as applies to the Cosmetics Regulation in the EU) (De Wever et al., 2015).

An overview of advantages, limitations and needs for excised human skin (in addition to other skin models as discussed below) is given in Table 1.

In conclusion, *ex vivo* dermal absorption testing is a well-investigated methodology that provides useful results. However, to reduce further the number of animals used in safety sciences, the standardization of *ex vivo* and *in vitro* experimental parameters is imperative (EFSA, 2012; Guth, 2013; SCCS, 2010; WHO, 2006). Furthermore, due to the increasing demand for skin absorption studies (EFSA, 2011, 2012) and the stated limitations of the use of excised human skin, robust, sensitive, cost-effective and validated alternatives to *ex vivo* testing are of considerable interest. In this sense, validated and regulatory body-accepted *in vitro* assays predicting dermal penetration would not only significantly reduce the number of animals used in experiments, but also dramatically save costs related to the pre-clinical testing of potential drug candidates.

Notably, different regulatory fields have differing requirements regarding acceptance of approach criteria, i.e., while

Tab. 1: Models for dermal absorption

	Examples	Advantages	Limitations	Issues and needs	Memo
Models for dermal absorption					
Excised skin	– human – rat – pig	– regulatory accepted for plant protection products and defined product groups (EU) – screening – native tissue – relevant species addressable – spectrum of donor and body region-specific sensitivities – metabolically competent	– limited acceptance in US – limited acceptance for defined product groups (also EU) – species extrapolation necessary in case of animal skin – overpredictive versus *in vivo* (based on, e.g., artificial receptor fluid to ensure solubility) – limited availability, high costs and donor variability in case of human skin	– definition of acceptance criteria (e.g., reference datasets, integrity tests,...?) – higher standardization of the method including standardized integrity tests (SOP?) – reduce overpredictivity (optimize IVIVC) – "what is normal" – define reference compounds and acceptable inter- and intra-laboratory variability (ring-trial) – better understanding of barrier formation and function (different species, role of tight junctions etc.)	– *in vitro* skin absorption for plant protection products in US, accepted if IVIVC is given for the rat – regulatory use of excised skin: exposure estimates, estimates on skin absorption and penetration (cosmetics and REACH)
Reconstructed human skin models	– EpiDerm™ (MatTek) – EpiSkin™ (L'Oréal) – SkinEthic™ (SkinEthic Laboratories) – StrataTest® (Stratatech) – Phenion®FT (Henkel) – Graftskin LSE™ (Organogenesis)	– easily available, standardized, reproducible – metabolically competent – human derived – applicable for screenings? – reflects mixture effects (?)	– overpredicts absorption (not applicable for risk assessments) – technical limitations (low stability, transition in diffusion cells, adsorptive underlying membrane, dependence on shipment) – limited applicability for screening purposes ?	– definition of acceptance criteria (e.g., reference datasets, integrity tests,...?) – higher standardization of the method including standardized integrity tests (SOP?) – reduce overpredictivity (optimize IVIVC) – "what is normal" – enhance barrier function and stability – solve technical problems – enable application for screening and risk assessments – "validation" against highly standardized results for reference compounds with excised human skin	
Artificial skin surrogates	– skin-PAMPA (Pion) – Strat-M (Merck Millipore)	– easily accessible, standardized, reproducible – screening for aqueous dilutions	– technical limitations (only aqueous solutions, sufficient water-solubility, UV-activity needed) – no metabolism – applicability for mixtures not yet shown	– definition of acceptance criteria (e.g., reference datasets, integrity tests,...?) – higher standardization of the method including standardized integrity tests (SOP?) – optimize IVIVC – show stability against formulation ingredients – prediction of mixture effects needed	

Tab. 1: Models for dermal absorption

	Examples	Advantages	Limitations	Issues and needs	Memo
Models for dermal absorption					
***In silico* models**	– Potts and Guy, 1992 – Abraham and Martins, 2004 – Riviere and Brooks, 2007 – Guth et al., 2014 – Dancik et al., 2013	– no experiments needed – time and cost effective – screening for aqueous dilutions	– limited applicability domain – no general mixture model available	– mechanistic insights in dermal absorption and mixture effects – prediction of dermal absorption from different mixtures	

for the chemical and crop industry overestimation of an effect may not represent a problematic issue (due to the aim of protection of the workers and consumers), for the pharmaceutical industry precise prediction of the penetration via healthy and compromised skin has consequences for the development of the new drugs.

2.3 Reconstructed *in vitro* human skin models

Reconstructed human "skin" models are usually fabricated from non-transformed human epidermal keratinocytes, grown either on artificial membranes in plastic inserts or on a dermal component (artificial or biological matrix). These *in vitro* cultures are in general (whether full or partial thickness models) referred to as human skin equivalents (HSEs). HSEs which specifically model the epidermis and, as such, consist only of differentiated keratinocytes on an artificial membrane, are known as reconstructed human epidermis models (RhEs). RhEs closely mimic the morphological, biochemical and physiological properties of the human epidermis, and can be relatively easily created in laboratories by following precedents in published literature (open source models); they have also been commercially available for more than 20 years (Tab. 2a). Characteristics of the commercially available tissue models are also generally well described in the literature (Eckl et al., 2014; Netzlaff et al., 2007, 2005; Ponec et al., 2002, 2001; Schaefer-Korting and Schreiber, 2008; Schmook et al., 2001; Shevchenko et al., 2010; Welss et al., 2004; Cannon et al., 1994; Rosdy and Clauss, 1990; Tinois et al., 1994) although not all details are disclosed in published work.

More complex tissues, composed of combinations of two or more cell types and/or at least two "skin" layers (epidermis and dermis), forming as a result either healthy or diseased models of human skin, are also available (Bannasch et al., 2005; Semlin et al., 2011; Zhang and Michniak-Kohn, 2012; El Ghalbzouri et al., 2009; Nischt et al., 2006; Ponec et al., 2003; van den Bogaard et al., 2014) (Tab. 2b,c). The most commonly used combinations for formation of complex tissue models are those of fibroblasts with a dermis model, or keratinocytes with an epidermis model – so-called full thickness models.

Gene knock-down tissues, reporter tissues, wound models and models populated with various pathogens have also been described in the literature (Geer et al., 2004; Jansson et al., 1996; Kuchler et al., 2011; Popov et al., 2014; Poumay and Coquette, 2007; Zhai et al., 2007; van Drongelen et al., 2013). Reconstructed human skin models for therapeutic purposes (grafting) have additionally been described since the 1970s, several of which have been commercialized (Tab. 2d).

The use of HSE is not necessarily cheaper than animal experiments or the use of *ex vivo* skin, however human-derived reconstructed tissues used under defined test conditions offer several advantages: i) since most models are composed of primary human cells, inter-species extrapolation is avoided; ii) in contrast to *ex vivo* human skin, repeated application of formulations can be performed for at least several weeks; iii) work with the commercially available epidermal HSE does not require advanced knowledge of cell culture techniques (models are delivered "ready to use"); iv) (three-dimensional-)RhE models manufactured for regulatory testing purposes are highly standardized, and quality controls are established as requested by the OECD TG 431 and 439; v) HSE are readily commercially available in most of the EU, USA and Asia, or can be constructed according to available literature as "open source" tissues; vi) several RhE models are accepted in a regulatory sense for skin irritation and corrosion testing of chemicals, as well as for assessment of phototoxicity as one component of a drug testing strategy; vii) the employment of HSE leads to the reduction of laboratory animal use in regulatory toxicology as well as in preclinical studies.

The main shortcomings related to the currently available reconstructed tissue models are: i) insufficient barrier properties, reflected in a modulated lipid composition and organization (Leroy et al., 2014, 2013; Thakoersing et al., 2012, 2013) and increased flux or absorption rate in skin penetration studies (Davies et al., 2015; EFSA, 2012; Hui et al., 2012; Schafer-Korting et al., 2008); ii) lack of vascularization, sweat glands and hair; iii) lack of representation of the physiologically-relevant desquamation process; iv) lack of reproducibility of immunocompetent models.

In spite of these shortcomings, reconstructed human tissue models are commonly employed for the purposes of regulatory toxicological testing. The reconstructed epidermis models EpiDerm™ , EpiSkin™ , SkinEthic™ and epiCS™ (EST-1000) have gained acceptability as suitable alternative models for skin corrosion testing as a result of extensive validation studies (OECD TG 431); these models have also recently gained approval for sub-categorization of corrosive classes (OECD, 2014). Similarly, EpiDerm™ , EpiSkin™ , SkinEthic™ and LabCyte™ models are considered as acceptable setups for skin irritation testing (OECD TG 439 (OECD, 2013)). A validation study addressing the skin irritation and sensitization potency of extracts from medical devices is furthermore underway (Casas et al., 2013; ISO, 2010; Coleman et al., 2015). Another example for the successful use of RhE is the phototoxicity assessment of topically applied substances and formulations, as demonstrated in the pre-validation study of the EpiDerm™ model (Liebsch et al., 1999). The use of RhE was furthermore recently implemented into the updated ICH S10 guideline for the assessment of the phototoxic potency of topically applied drugs (EMA, 2012).

An important point which remains to be addressed, however, is the matter of the barrier properties of reconstructed human skin models. The barrier function of HSEs is still not thoroughly described, making it difficult to credibly extrapolate determined penetration rates from *in vitro* data to the *in vivo* situation. Although all lipid classes present in native human skin are also present in HSEs, there is a deviation in free lipid composition and organization compared to native human skin. The most important of such deviations are a high level of unsaturated fatty acids, shorter lipid chain lengths (Mojumdar et al., 2014; Thakoersing et al., 2013, 2015) and an imbalance in the level of ceramide subclasses (Thakoersing et al., 2012). These changes in lipid composition are expected to contribute significantly to the impaired skin barrier of HSEs (Mojumdar et al., 2014). With respect to the bound lipid profile, this is similar to that of native human skin (Ponec et al., 2003) – however, it must be stated that measurements of this profile have been conducted with a lower level of precision. Furthermore, the presence of a leaky cornified envelope would also contribute to a reduced skin barrier property; while almost no data have been reported to date on the protein composition of the cornified envelope in HSEs, it has been shown that some cornified envelope proteins are expressed differently (involucrin) in the models as compared to native human skin (Malaisse et al., 2014).

HSEs (as well as the previously discussed rat skin) seem to overestimate compound penetration by a factor unrelated to compound molecular weight, lipophilicity and/or aqueous solubility. Notably however, using a strictly controlled protocol conforming to OECD test guideline no. 428, a validation study with three different RhE models – EpiDerm™ , EpiSkin™ and SkinEthic™ – indicated their general suitability for *in vitro* absorption studies, both in infinite and finite dose experiments (Schafer-Korting et al., 2008). Though the permeability rate determined using RhEs seems to exceed that found using *ex vivo* human skin, the absorption ranking of substances does reflect results obtained with human skin (Schafer-Korting et al., 2008). Furthermore, the results obtained with skin constructs were less variable than results with excised human skin; the transferability of the protocol between the skin models and the influence of the vehicle was also reflected (Schafer-Korting et al., 2008).

The improvement of HSE barrier properties is certainly the subject of current and future research and may in fact be enhanced by modulation of culture conditions; however, it must be emphasized that until the barrier properties of HSEs can approximate those of human skin, minimizing the current overpredictability, a formal validation and regulatory acceptance of HSEs for dermal absorption studies is not feasible. Therefore, further research is needed to understand the processes and mechanisms underlying the human skin barrier, which may then inform respective options for tissue engineering. Improvement of the barrier function of HSEs could increase the overall stability of the tissue, allowing for processes such as washing, swabbing and tape-stripping, which are at the current stage not feasible, and rendering such models more suitable for permeation studies. Further practical aspects that need to be addressed for a routine application of HSEs are tissue size, the possibility to transfer or adjust models to automated systems, storage possibilities and the assessment of the influence of the underlying synthetic membrane as an additional, adsorptive barrier (Guth, 2013).

A parameter of additional importance to barrier function is the metabolic competence of reconstructed human skin models. In this respect, HSEs show advantages when compared to, e.g., frozen pig skin, frozen human skin or membrane systems (described later in this chapter). It has been shown that cutaneous biotransformation can inactivate toxic agents; however, at the same time, biotransformation may also contribute to sensitization and genotoxicity. Comprehensive investigations into the expression and functionality of enzymes involved in cutaneous biotransformation of xenobiotics in human skin *ex vivo* as well as in reconstructed tissue models are ongoing, and knowledge continues to improve (Gotz et al., 2012; Huh et al., 2010; Oesch et al., 2014). Pre-validation studies have in fact been conducted with HSEs to address genotoxicity (Brinkmann et al., 2013; Fautz et al., 2013) and skin metabolism (Schafer-Korting et al., 2006). Two *in vitro* genotoxicity approaches using three-dimensional skin models have been proposed: i) the human reconstructed skin micronucleus (RSMN) assay using the EpiDerm™ model, and ii) the Comet assay, which detects complementary DNA damage, including damage indicative of the occurrence of gene mutations (EpiDerm™ , Phenion™). Both models are currently evaluated in a validation study coordinated by Cosmetics Europe (Aardema et al., 2010; Reus et al., 2013). Once validated, the RSMN and the three-dimensional skin Comet assay may be used as follow-up tests for positive results from the current *in vitro* genotoxicity test battery in a weight-of-evidence approach.

In terms of skin sensitization, a lack of reproducible HSEs has led to the development of the less integrated but hopeful-

Tab. 2: Commercially available reconstructed epidermis models

Name	Manufacturer	Comment
a) Commercially available reconstructed epidermis models with regulatory acceptance* – models of healthy human skin		
EpiDerm™	MatTek Corp. and MatTek IVLSL	OECD TG 431, EU B.41, OECD TG 439, EU.B46, ICH S10
EpiSkin™ and SkinEthic™	SkinEthic Laboratories	OECD TG 431, EU B.41, OECD TG 439, EU.B46, ICH S10
EpiCS	Cell Systems	OECD TG 431
LabCyte	J-TECH	OECD TG 431
b) Commercially available reconstructed skin models (FT-models and/or models combining at least 2 cell types) – models of healthy human skin		
EpiDerm - FT	MatTek Corp.	Keratinocytes, fibroblasts (full thickness)
Phenion - FT	Henkel	Keratinocytes, fibroblasts (full thickness)
FTM/FDM	Biomimiq	Keratinocytes, fibroblasts
Melanoderm	MatTek Corp.	Keratinocytes, melanocytes
RHPE	SkinEthic Laboratories	Keratinocytes, melanocytes
EpiCS	Cell Systems	Keratinocytes, melanocytes
c) Diseased tissue models		
Psoriasis	MatTek Corp.	Model of psoriatic skin
Melanoma	MatTek Corp.	Model of skin cancer dermatitis
AD-model	FU-Berlin, Biomimiq	Model of atopic dermatitis
d) Reconstructed tissue models for grafting (therapeutic purposes)		
Graftskin™LSE™	Organogenesis Inc.	For grafting
EpiDex and EuroSkin	EuroDerm	For grafting
StrataTech™	Stratatest	Keratinocytes, fibroblasts (full thickness) for grafting

*due to the number of available RhE tissue models, only those with regulatory acceptance are depicted. The regulatory acceptance is limited to certain endpoints/OECD guidelines stated in the "Comment" column.

ly more predictive co-culture systems, and also to a focus on the specific role of keratinocytes in skin sensitization events. Despite a lack of immune cells in the currently commercially available tissue models, there are indications that assessment of cell viability markers in combination with gene expression and direct reactivity (e.g., via the SenCeeTox method (McKim et al., 2010, 2012) and Sens-IS method (Cottrez et al., 2015)) could be predictive of both the risks as well as potency of skin sensitizers (Reisinger et al., 2015). Another approach focuses on the investigation of IL-18 production by keratinocytes in order to assess the *in vitro* sensitization potential of low molecular weight chemicals (Corsini et al., 2009, 2013; Guyard-Nicodeme et al., 2015).

The use of reconstructed human skin models in pharmacology is associated with evaluation of the activity of drugs, including estimation of their adverse effects, in healthy as well as diseased skin. Studies describing the effects of long-term use of glucocorticoids in HSEs, which were found to be as suitable models, have been described (Gysler et al., 1999; Lange et al., 2000; Lombardi Borgia et al., 2008). As another example, specific HSEs have been developed to simulate the disease conditions of psoriasis and skin cancer (Tab. 2c), and utilized to further estimate effects and suggest possible treatments in such cases (Berking and Herlyn, 2001; Chamcheu et al., 2015; Datta Mitra et al., 2013; Eves et al., 2003; Li et al., 2011; Tjabringa et al., 2008).

As can be seen, reconstructed tissue models already provide valuable contributions to basic research as well as regulatory toxicology, and in light of the aforementioned progress, they continue to possess great potential for further applications.

2.4 Chip technology

The need for a high throughput testing approach in the areas of toxicology and pharmacology is motivating a number of research groups to develop organs-on-a-chip platforms as well as bioreactors (e.g., DARPA, USA; Human-on-a-Chip project, EU) (Hartung and Zurlo, 2012). Such a technology could allow for future high-throughput screening of novel drug candidates or for testing interactions of multiple organs in one experimental apparatus. Excised tissue, reconstructed tissue and cells lines are applicable for this purpose.

HSE can be produced in various sizes from very large samples (4 cm^2) for skin penetration studies in classic Franz cells to sizes of less than 0.1 cm^2 (96-well plates). Adjustment of the HSE and their implementation into the miniaturized conditions employed in chip technologies or into the environment of a perfused bioreactor is therefore feasible. In a recent study, scientists from TU Berlin demonstrated that perfused systems using reconstructed

human tissue provide better platforms for increased longevity of *in vitro* skin equivalents, and improve the tissue architecture if combined with underlying subcutaneous tissue. In addition, it has been shown that skin models can be co-cultured with other organ equivalents, such as liver, over periods of at least 28 days (Atac et al., 2013; Wagner et al., 2013).

2.5 Synthetic membrane systems

Other approaches aimed at gaining preliminary permeability data *in vitro* include the artificial membrane-based PAMPA (Parallel Artificial Membrane Permeability Assay), discussed in more detail in the following chapter in the context of non-animal intestinal models (Avdeef et al., 2007, 2008; Flaten et al., 2006b, 2007; Kansy et al., 1998, 2004), as well as Strat-M™ , available from Merck. The first commercially available PAMPA for performing penetration studies is the skin-PAMPA, as supplied by Pion Inc. Skin-PAMPA consists of a complete test system, including UV reader, as well as required technical support and is already in use for determination of temperature and protein-binding effects, for evaluation of new compounds and for predictive approaches studying quantitative structure-permeability relationships (Akamatsu et al., 2009; Bujard et al., 2014; Dobricic et al., 2014; Markovic et al., 2012; Vizseralek et al., 2014; Vucicevic et al., 2015). In contrast, the skin-mimicking artificial membrane setup Strat-M™ (Merck Millipore, USA) is composed of multiple layers of polyether sulfone, which is compatible with Franz chamber setups. A study addressing the applicability of the Strat-M™ approach, involving comparison of the permeability coefficients (Kp) of 14 chemical compounds in a Strat-M™ model with those found using excised human and rat skin, could show that the Strat-M™ approach appeared to be useful for prediction of permeability of compounds with a molecular weight between 151 and 288 g/mol and an octanol/water coefficient (log Ko/w) between -0.90 and 3.53 (Uchida et al., 2015).

While both PAMPA and Strat-M™ approaches exhibit considerable advantages for permeability assessment, they also naturally possess some limitations. For example, the composition and fluidity of PAMPA artificial membranes does not accurately mimic the physiological situation (Avdeef et al., 2001; Seo et al., 2006; Tanaka and Sackmann, 2005), as it does not contain all the barrier lipid subclasses present in human skin. Furthermore in those cases in which formulations are tested and the interaction between the formulation and the membrane has an effect on permeability, the predictive power of such approaches is expected to be limited. Additionally, PAMPA filters may become blocked, leading to inaccurate permeability data (Avdeef et al., 2001; Hamalainen and Frostell-Karlsson, 2004; Seo et al., 2006). These factors and possibilities lead inevitably to significant differences in permeability study outcomes (Chilcott et al., 2005; Frum et al., 2007; Khan et al., 2005) when compared to data present in the Flynn permeability database (Parnas et al., 1997, 1998). Furthermore, only Kp values presenting the permeation rate can be determined – the absorbed dose or the remaining compound in the skin layers, which are both relevant parameters for toxicological evaluation, cannot be predicted. Skin-PAMPA results can be used to prioritize and rank different compounds, but not yet for regulatory purposes; to date, a good correlation of Kp values derived from skin-PAMPA and excised human skin has only been shown for pure or aqueous solutions (Guth, 2013; Sinko et al., 2012) and, as mentioned above, such a level of correlation is not expected to extend to the testing of formulations.

2.6 *In silico* modeling

In general, *in silico* modeling is defined as predictions achieved by computer simulation or regression (Hartung and Hoffmann, 2009). Broadly speaking, all computer models are based on mathematical algorithms, with epithelial transport as described originally by mathematical principles (such as those espoused by Fick (passive diffusion), Michaelis and Menten (active transport), Hodgkin (ion transport), Huxley and Katz (solute structure), all based on membrane principles originally detailed by Meyer and Overton) now precisely defined in terms of a number of physicochemical parameters, including solute size, shape, polarity and flexibility. The rapid evolution of digital technology, high performance computing and big data has allowed epithelial transport to now be described accurately and rapidly in three-dimensional space over time and for precise constants, characterizing the transport processes to be derived by regression and applied to other solutes and epithelia. As a consequence *in silico* modelling has evolved to become an important component of non-animal testing methods, with *in silico* pharmacokinetic-pharmacodynamic model analysis being increasingly used in the regulatory evaluation, approval and personalized use of drugs.

Topical products are known to have existed since the dawn of time and have evolved through empirical dosage forms, sophisticated manufacturing and appropriate *in silico*, *in vitro* as well as *in vivo* studies to provide the range of sophisticated products on the market today (Pastore et al., 2015). Various mathematical and quantitative structure penetration models have been used to describe percutaneous absorption over the ages, directed particularly by the work of Takeru and William Higuchi, Alan Michaels and Robert Scheuplein in the 1960s and 1970s (Roberts, 2013; Scheuplein, 2013; Scheuplein and Blank, 1971). The use of (quantitative) structure-activity relationship (or (Q)SAR) models to estimate aspects of skin irritation, corrosion and sensitization has also been in practice for many years, with corrosion being related to the relative uptake of compounds within a given class of potentially corrosive compounds, such as the phenols and acids (Roberts et al., 1977), and irritation related to the reactivity of solutes with macromolecules in the viable epidermis and dermis (Golla et al., 2009; Hayashi et al., 1999). Development and use of (Q)SARs is however always empirical, due to a lack of understanding of the underlying biophysical and biochemical mechanisms of exposure, disposition and metabolic pathways. (Q)SAR models are limited by the defined applicability domain for which such a system can be used, and as such (Q)SARs are always seen as a component of the testing strategy in the particular field. (Q)SAR models have generally been developed for small datasets of specific groups of compounds, although in some cases more diverse and larger datasets have also been examined. The usual parameters that are used in the

development of (Q)SAR algorithms are compound molecular weight, melting point, aqueous solubility, vapor pressure, log Ko/w, pH, surface tension, and lipid solubility. With respect to skin sensitization, protein/electrophilic reactivity and the presence of alert groups are also of particular importance.

From the scientific literature, it appears that irritation and corrosion endpoints have largely been modeled on a separate basis. This is likely due to the fact that corrosion is considered to be more a physical effect (which can be captured by a simple description of acidity or basicity) than a biologically mediated effect such as skin irritation. One of the earliest (Q)SAR approaches to predict skin irritation is based on the TOPKAT methodology, however other methods such as BfR-DSS, DEREK and MultiCASE are well described, and approximately 20 additional models have been reported in the literature (ECHA, 2014). An overview of the systems developed for skin irritation and corrosion is provided by Saliner et al. (2006).

In the area of skin sensitization, the available (Q)SAR models are either chemical class-based/mechanism-based (local models), or are derived empirically using statistical approaches (global models). Some of these available (Q)SARs have been encoded into expert, knowledge-based systems that are available for the prediction of skin sensitization (e.g., Derek for Windows (DfW)), statistical systems (e.g., TOPKAT, MCASE) and hybrid systems (e.g., Tissue Metabolism Simulator (TIMES)). Comprehensive reviews and evaluation of expert systems, SARs and QSARs are available from ECETOC and in the form of publications from Patlewicz et al. and Teubner et al. (ECETOC, 2003; Patlewicz et al., 2008; Teubner et al., 2013).

Various overviews of *in silico* models for dermal absorption of chemicals can also be found in the literature (Fitzpatrick et al., 2004; Geinoz et al., 2004; Moss et al., 2002; Dumont et al., 2015). However, to date none of the available models are deemed acceptable from a regulatory viewpoint (EFSA, 2012; OECD, 2011). This is basically due to four reasons: i) Many reported models are focused on the prediction of permeability coefficients and not the absorbed dose (the relevant parameter for regulatory use), and often employ data devoid of appropriate cross-validation with various validated and standardized *in vivo*, *ex vivo* and *in vitro* experimental data sets. ii) Most of the reported models are derived for a tightly constrained applicability domain of pure and aqueous solutions, whereas such studies are in fact mainly needed for formulated products such as cosmetics, pharmaceutical creams or plant protection productsm. iii) Reported models are not integrated with the systems biology of biophysical and biological events leading to the epithelia endpoints. iv) Most of the models, including the evolving molecular dynamic models, appear to be limited in their generalizability and applicability. Several studies have been reported recently to address the above issues. A number of limitations appear to be intrinsic to the approach however – for example, it is generally questionable if a simple (Q)SAR model would be able to reflect the multilayered process of dermal absorption of a compound applied in a complex formulation (Guth et al., 2014). Recent approaches focus on mechanistic modeling of the transport and disposition kinetics of chemical compounds in multilayered skin at the cellular level (Dancik et al., 2013; Kasting et al., 2013; Chen et al., 2008a). With the mechanistic modeling approach, the transport and disposition properties of epithelia can be determined separately, e.g., by molecular dynamics simulation of chemical binding to keratin at the sub-cellular level (Marzinek et al., 2014). This represents significant progress of simple permeability models. It has been demonstrated that such mechanistic models are fully capable of predicting absorbed dose and subcellular distribution of chemicals from formulated products under *in vivo* exposure conditions (Lian et al., 2010).

2.7 Conclusion

To answer the question of what is needed to improve the currently available skin models, assays and test systems we first have to better understand the characteristics of a typical, "average" healthy human skin. Large scale studies focusing on the barrier properties of normal skin, skin metabolism, and potential for development of skin inflammation and irritation, sensitization and particularly skin diseases are required to define a normal, standard range that accounts for inherent donor variability. Such multidisciplinary research also needs to investigate detail that includes, but is not limited to, the average number of epidermis layers, tight junctions, cornified envelope composition, lipid composition and lipid organization in the stratum corneum in both healthy and diseased skin (taking into account genetic profiles and medication histories) and, finally, should also establish absorption profiles of representative marker molecules (i.e., for exposure and effects). A better understanding of the structures and mechanisms that determine dermal uptake and lead to toxicity events in human skin or even to disease conditions is of crucial importance when developing assays for testing drug candidates, active ingredients, chemicals and their mixtures or formulations. Moreover, such large epidemiological studies focused on characterization of human skin would ultimately bring unique knowledge backed by statistical power, and consequently also reference data needed for development of better *in silico* models and reliable assays, while accelerating the process of their formal validation – an achievement that is often hindered by a lack of reliable *in vivo* reference data.

It should also be noted that the currently available assays are inherently limited to substances applied topically, and as such do not allow for assessment of substances deposited intradermally. While the latter is a relatively rare circumstance, it is relevant, for example, in the case of tattoo inks. Long neglected by toxicologists and regulators, tattoos have increasingly lost their maverick image and are about to become a mainstream accessory, with up to 36% of adults under the age of 40 having at least one tattoo. Although this clearly makes them toxicologically relevant, we know very little about the metabolism and toxicology of the colorants and additives used in tattooing (Laux et al., 2015).

Also, the commonly applied eukaryo-centric perspective of toxicology makes it easy to forget that skin-associated metabolism does not necessarily stop at the epidermal surface, but potentially also includes the metabolic and signaling activity of vast numbers of skin commensals. An increasing number of studies show that the microbiota can have a profound impact on the human immune system, as well as on general wellbeing

and behavior (Arpaia et al., 2013; Foster and McVey Neufeld, 2013; Hsiao et al., 2013; Possemiers et al., 2009; Tralau et al., 2014). Yet, the microbial side of xenobiotic skin metabolism has so far not been addressed, and none of the aforementioned systems is fit to include the metabolic competence of the microbiome. A recent proof of principle study showed the formation of cytotoxic and carcinogenic metabolites from benzo[a]pyrene (B[a]P) by common skin commensals (Sowada et al., 2014; T. Tralau, personal communication). While it remains to be seen if and how this metabolism affects the human host, this example shows that it is necessary to further assess the influence of the microbiota and, concomitantly, the potential need for its inclusion into toxicity testing.

Having an understanding of both the possibilities and limitations of the currently available skin models is very important. Where available and feasible, the use of *ex vivo* human skin or *in vitro* skin models should not be limited by lack of experience or distrust, especially if evidence exists that such models may provide similar or even better predictions than their animal-based counterparts. Well-designed validation studies with clearly defined goals, target levels for sensitivity and specificity and sets of reference compounds with reliable *in vivo* animal or human data for a particular area are therefore the most effective way to facilitate and implement the use of such novel methods.

It has already been demonstrated by validation studies that for relatively simple toxicological endpoints, such as those employed for example for estimating the risk of skin corrosion or irritation for classification and labeling purposes, the use of relatively simple methods and models may suffice. However, for more complex endpoints such as skin sensitization or in investigation of a skin disease therapy, more complex models are required, and other approaches will be needed, combining suitable methods and tools into respective testing strategies. Novel biomarkers will also have to be investigated and incorporated into the researcher's toolbox.

3 Non-animal models of the intestinal mucosa in research, industrial applications and regulatory toxicology

3.1 General introduction

The gastrointestinal tract has a number of physiological functions including digestion, absorption, hormone and enzyme release, peristalsis, antigen presentation, control of microbial growth, and excretion. These vital functions are maintained by a diverse set of cell types and a unique tissue architecture. The intestinal mucosa is characterized by the presence of villi, which constitute the anatomical and functional unit for nutrient and drug absorption, and can be further subdivided into the epithelial layer (which constitutes the intestinal barrier), the lamina propria (collagen matrix containing blood and lymphatic vessels) and the muscularis mucosae (Carr et al., 1981; Hosoyamada and Sakai, 2005; Nelson, 2004). The intestinal epithelium is mostly made up of absorptive enterocytes that transport macromolecules, ions and water. Secretory cell types within the epithelium include goblet cells (which are responsible for the secretion of mucins that form an additional protective barrier), Paneth cells (secretion of antimicrobial peptides) and entero-endocrine cells (secretion of hormones regulating motility and release of digestive enzymes). Other important cell types present are involved in antigen processing and mucosal immunity, for example intraepithelial lymphocytes and microfold (M) cells, which transport organisms and particles from the gut lumen across the epithelial barrier to immune cells (Kucharzik et al., 2000; Wittkopf et al., 2014).

The epithelial cell layer not only provides a means for transport, but also constitutes the first layer of protection against foreign material at the intestinal mucosal surface. The commensal microbiome also plays an important role in the structural, barrier, immunological and metabolic functions of the intestine; however, a description of the microbiome at various mucosal surfaces is beyond the scope of this article (Brown et al., 2013; Rosenstiel, 2013). The junctions between the epithelial cells, tight and adherens junctions, which join cells to each other at the apical end of the lateral membrane (Schneeberger and Lynch, 2004), play very important roles in the homeostasis of the intestine and the maintenance of mucosal immunity. Tight junctions serve both as "gates" that seal the paracellular space and as diffusion "fences," to maintain the apical/basolateral polarity essential to perform asymmetrical exchanges (Sambuy, 2009) and maintain epithelial cell polarity by preventing intermixing of plasma membrane proteins and restricting diffusion of lipids in the exoplasmic membrane leaflet (Cereijido et al., 2008). These junction proteins also regulate epithelial cell proliferation and apoptosis. Furthermore, cell adhesion molecules are required in cell-cell and cell-matrix interactions, cell migration, cell cycle, and signaling. The epithelium is highly regenerative in nature, with reparative processes being driven by intestinal stem cells residing in the crypts of Lieberkuehn at the base of the villi (Clevers, 2013).

In drug discovery, knowledge of the absorption and metabolism at the intestinal barrier is of particular importance, since the oral bioavailability of a compound is defined as the fraction of an oral dose that reaches the systemic circulation. Since the oral route remains the most popular route of administration of drugs worldwide, the prediction of the *in vivo* performance of a drug candidate after oral administration to humans is one of the major goals and challenges in the drug discovery industry today. Furthermore, oral absorption has come into focus in pharmaceutical development in recent years, as new compounds tend to be larger and bulkier; their molecular weight and lipophilicity therefore increases, leading to both permeation and solubility issues. In general, intestinal absorption can occur via passive diffusion through the paracellular spaces and/or membranes of absorptive cells, vesicular uptake (endocytosis/pinocytosis), and release at the basolateral space (transcytosis). This transport can be receptor- or transporter-mediated across the apical domain, with subsequent passive diffusion into the basolateral space. Each transport mechanism depends on the physicochemical properties of the absorbed compound. In carrier-mediated events the drug molecule represents a substrate for (a) specific transporter(s) which localize(s) within the biological membrane (Kapitza et al., 2007). Carrier-driven translocation of trans-

porter substrates may be a passive occurrence, dependent on the existence of a concentration gradient, and results in either cellular uptake or efflux out of the cell. As this process is saturable, in the case of drugs showing low passive permeability, carrier saturation will give rise to a marked alteration in the absorbed fraction (Buckley et al., 2012). In addition, several carrier-mediated transport processes are coupled to a primary or secondary energy source (e.g., multidrug efflux pumps as primary active transport proteins), able to transport their substrates against concentration gradients (Buckley et al., 2012).

Besides knowledge about individual drug transport across the intestinal barrier, provision of toxicokinetic data is mandatory in pre-clinical drug development. To this end, animal experiments have a long tradition of use for pre-clinical risk assessment of new drugs. Similarly, *in vivo* toxicokinetic studies according to OECD test guideline 417 are part of data requirements for some sectorial chemicals regulations, such as those for plant protection products (Regulation (EC) No. 1107/2009) and biocides (Regulation (EU) 528/2012). An overview of regulatory provisions on ADME (absorption, distribution, metabolism and excretion) and toxicokinetics is available in the recently published EURL ECVAM (European Reference Laboratory for Alternatives to Animal Testing) strategy for achievement of 3Rs impact in the assessment of toxicokinetics and systemic toxicity (Bessems et al., 2015). However, it becomes more and more evident that animal-derived pharmaco-/toxicokinetic data are not always reliable for extrapolation to human safety assessment due to interspecies differences in physiology as well as in biochemical and metabolic pathways. Low-throughput, high costs and ethical considerations are further limitations associated with the use of animals (see also requirements in EU Directive 2010/63/EU on the protection of animals used for scientific purposes (Martinez, 2011)). To reduce animal experimentation and also the risk of failure of many drug candidates in the later phases of clinical trials, attempts have been made to provide inexpensive and convenient intestinal functional *ex vivo* and *in vitro* models to study toxicity and bioavailability of new substances and to study interactions between the host, pathogens and intestinal microflora. Advances have also been made in artificial membrane technology and *in silico* modeling systems, which may complement the cell/tissue models. An essential part of developing non-animal models is their validation, together with demonstration that their use provides equally viable (or even better) results in comparison to animal tests. Therefore, several national and international institutions like EURL ECVAM in the EU, the US validation body, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the Japanese Center for the Validation of Alternative Methods (JaCVAM) or the Organization for Economic Co-operation and Development (OECD) validate and keep track of new models (Hartung et al., 2004; Leist et al., 2012).

Overall, functional non-animal intestinal models play an increasing role in predicting and evaluating pharmacokinetic properties (in particular, oral bioavailability). The incentive for initial investigation as well as current application of intestinal absorption models continues to lie in the development and regulation of drug products. However, currently available models also appear interesting for the "animal-free" evaluation of many non-pharmaceutical products, such as dietary supplements, plant protection products, biocides or other chemicals, as well as nanomaterials. Physiology-based pharmacokinetic (PBPK) modeling is gaining importance as an alternative to animal studies for risk assessment and toxicology in general, setting the stage for in-depth evaluation of underlying mechanisms as well as factors of pathogenicity on a molecular and cellular level. Experimental model systems which are able to accurately provide toxicity assessment as well as a correlation between altered permeability and absorption and immunotoxicity are however a continuing and urgent need.

3.2 *Ex vivo* intestine models

Ex vivo intestinal cultures are important models for analyzing and assessing drug transport, cell-cell communication, safety aspects, and other interactions of orally consumed exogenous substances or pathogens in a three-dimensional tissue context of the gastrointestinal tract. Most substances first come into contact not with the epithelial cells themselves, but rather with mucus on the epithelial mucosa (Macierzanka et al., 2014). The mode of interaction with the biological barrier depends on the nature of the substance (i.e., particulate or soluble) – generally particulate substances cross the epithelial barrier via M cells or dendritic cells leading to accumulation in Peyer's patches (Brun et al., 2014; Schimpel et al., 2014). The gastrointestinal microbiota must also be considered in this respect as it plays an important role in the overall interaction and metabolism of orally consumed substances. As the gut is a highly organized and complex organ, it is challenging to maintain gut properties in an *ex vivo* situation for prolonged periods of time – changes may occur rapidly due to a lack of intact vascularization and nutrient supply. Lack of vascularization in particular quickly results in hypoxia, necrosis, loss of viability and subsequently functionality. Previously, freshly excised and perfused whole intestinal segments were used, obtained in the majority of cases from mice, rats or piglets. Whole organ segments or organotypic cultures can be maintained either as a free-floating culture or on a culture substratum *in vitro* (Jacobs-Cohen et al., 1987; Metzger et al., 2007; Quinlan et al., 2006; Rothman and Gershon, 1982). These segments have the advantage that physiological cell-cell contact and normal extracellular matrices are preserved, at least for a limited time. Most studies to date have however been restricted to fetal gut tissues and relatively short culture duration. When using the slice culture technique, maintenance of the three-dimensional environment of the fetal gut could be extended up to several weeks *in vitro* (Metzger et al., 2007). This offers the possibility for experimental manipulation and monitoring in long-term studies. Only a few groups have worked with adult intestinal tissue (Autrup et al., 1978a,b; Defries and Franks, 1977; Finney et al., 1986; Metzger et al., 2009; Moorghen et al., 1996; Shamsuddin et al., 1978). In this case, several biopsy punches can be obtained from the same intestinal tissue, placed in a Transwell® setup with nutritive media, and incubated with the test material. Besides transport activity, the

end points measurable in such a test system include changes in the histopathological assessment, as well as changes in the expression of genes at transcription or translation level.

More recently, whole jejunal gut segments from adult rats and 6 week old pigs with intact arterial and venous vessels could be maintained on a histological level for up to 2 weeks in specially constructed computer-assisted bioreactors and adapted medium conditions (H. Walles, unpublished data). If drugs were administered into the lumen, epithelial transport could be measured directly in the vessel circulation, which would provide a physiologically-accurate approach. Therefore, these advanced models could be useful in later phases of pre-clinical development and provide additional insights into drug absorption and metabolism processes.

For short duration drug absorption studies so far the most common applications of *ex vivo* gut cultures are the Ussing chamber and the (non-)everted gut sac model. Ussing chambers were introduced by Ussing and Zehran in 1951 for studying the active transport of sodium as a source of electric current in short-circuited, isolated frog skin. The usefulness of Ussing chambers for intestinal transport and barrier studies has also long been recognized. The chamber consists of an apical (mucosal) and basolateral (serosal) compartment separated by the mucosal/submucosal tissue, with the possibility for drug exposure at either side. The chambers are filled with physiological electrolyte solution, which is gas- and temperature-controlled. The simplicity of Ussing chambers makes them an attractive *ex vivo* model system for studying drug transport (Brun et al., 2014; Schimpel et al., 2014; Shah and Khan, 2004). When properly equipped with electrodes, effects of compounds on electro-physiological parameters of the intestinal barrier can be determined, which may provide additional information on the pharmacological behavior of the test compound (Shah and Khan, 2004).

The everted gut sac (mostly of rat) small intestine can be used to study drug transport across the intestine and into epithelial cells, and to determine kinetic parameters with high reliability and reproducibility. Integrity of the model can be monitored via measurement of transepithelial electrical resistance (TEER) and reference substances (Brun et al., 2014; Schimpel et al., 2014). A defined length of gut lumen is filled with test substances and closed at both ends. Oxygenated tissue culture media ensures tissue viability for up to 2 hours. Usually, sensitive detection methods are needed and therefore radiolabeled compounds are most appropriate and used in this approach. The gut sac model is suitable for measuring absorption at different sites of the intestine and is useful for estimating the first-pass metabolism of drugs in intestinal epithelial cells. In the past, mostly macromolecules and liposomes were studied; more recently the paracellular transport of hydrophilic molecules and the effect of potent enhancers has also been investigated (Feng et al., 2013; Neupane et al., 2013). A limitation of this model is that it does not reflect the actual intestinal barrier, as compounds pass from the lumen into the lamina propria and across the muscularis mucosa, which is usually not removed from everted sac preparations (Freag et al., 2013). Thus, transport of compounds might be underestimated.

In general, species-specific differences must be considered if data obtained from such models are translated to humans. The properties and, in particular, the dissolution properties of biopharmaceutical compounds themselves must also be considered as these also have a strong influence on the ability to establish an *in vivo-in vitro* correlation (IVIVC). Drugs belonging to class I of the Biopharmaceutics Classification System (BCS) can generally be expected to exhibit a good IVIVC, as they are both highly soluble and permeable; an IVIVC could also be expected for BCS class II drugs as dissolution is the rate limiting step in the absorption of such poorly soluble but highly permeable drugs. In contrast, for BCS class III (high solubility, poor permeability) and class IV (poor solubility, poor permeability) drugs, an IVIVC is generally unlikely (Lu et al., 2011). It must also be kept in mind that establishing a regular supply of freshly excised animal tissue still imposes logistical challenges to the implementation of such models for medium- to high-throughput applications in an industrial context.

3.3 Reconstructed human intestinal models *in vitro*

3.3.1 Cell lines (standardization and validation)

Immortalized human adenocarcinoma cell lines such as Caco-2 or T84 (Khare et al., 2009; Raffatellu et al., 2005; Tran et al., 2010; Khare et al., 2012) have been extensively used to study absorption mechanisms, as such cell lines have been shown to act as acceptable models for the investigation of enterocyte differentiation and function (Cencič and Langerholc, 2010). Cells are commonly grown on semi-permeable Transwell® inserts, where they form a polarized monolayer and exhibit villi formation. Earlier studies have shown that Caco-2 and T84 cells spontaneously express differentiation characteristics of mature enterocytes by forming a polarized monolayer with an apical brush border, tight junctions and the presence of brush border-associated hydrolases (Bolte et al., 1998). However, the Caco-2 cell line in particular is heterogeneous and highly dependent on culture conditions, leading to variable transport properties and permeability (Delie and Rubas, 1997; Hosoya et al., 1996; Vachon and Beaulieu, 1992). Caco-2 cells are cultured on porous filter supports until fully differentiated and polarized, as denoted by a TEER in excess of 300 $\Omega\,cm^2$ (van Breemen and Li, 2005), although lower values have also been reported (Buckley et al., 2012). Caco-2 TC7, a clone isolated from a late passage of the parental Caco-2 line, is as reliable a model for passive diffusion as the parental cell line; however it seems not to be predictable for intestinal absorption of highly lipophilic compounds and poorly absorbed compounds, or when transporter-mediated routes and/or first pass metabolism are involved (Turco et al., 2011). As the absorption of xenobiotics is not only restricted to passive diffusion, the expression of active transport and efflux systems (such as P-glycoprotein) in employed cell lines is certainly important (Yang, 2013).

When compared, it is evident that there are some significant differences between polarized Caco-2 and T84 cells. For instance, Caco-2 cell monolayers exhibit significantly lower TEER on confluence as compared to T84 cells. Decreases in TEER are

accompanied by an increase in the permeability of intestinal epithelial cells, and as such, variations in TEER have been used as an indicator of early sub-lethal epithelial toxicity (Ferruzza et al., 1999; McCall et al., 2009). There are also marked differences in Na^+-dependent nucleoside transport systems in T84 cells as compared to Caco-2 cells (Ward and Tse, 1999). Furthermore, T84 cells produce mucin in culture and thus more closely mimic the intestinal surface (McCool et al., 1990); they also respond to external stimuli to exert innate immune responses similar to those initiated at the intestinal luminal surface (Ou et al., 2009).

Several endpoints are utilized to evaluate the performance of intestinal system bio-barrier function. Some of these include the aforementioned TEER, increases in the permeability of epithelial cells, changes in gene expression of cell junction proteins (gap junctions, adherens junctions and tight junctions), immunotoxicology, and cell proliferation. Typically, specific components of seeded cell lines used to evaluate model function are apical and basal supernatants as well as cell lysates, which are used to monitor signaling molecules, activation factors, compound concentrations and numerous other parameters. While immortalized cells offer many advantages, extrapolation of data generated with these cell lines to *in vivo* conditions is often difficult, as such cells originate from tumors and may therefore not be representative of the true physiological environment (Le Ferrec et al., 2001).

3.3.2 Primary intestinal cells

Due to limitations of immortalized intestinal cell lines, many studies have focused on the use of primary intestinal epithelial cells as a more physiologically relevant cell-based approach (Aldhous et al., 2001; Booth et al., 1999; Pageot et al., 2000; Perreault and Beaulieu, 1998; Simon-Assmann et al., 2007). However, while isolated human intestinal epithelial cells in particular retain important *in vivo* anatomical and biochemical features, they are difficult to culture and have limited viability. As a result, until recently, no well-defined, robust culture system had been identified and long-term propagation of native, non-transformed primary cells from the intestine was not feasible. In most culture systems intestinal cells transiently divided, yet ceased proliferation after several days and finally underwent apoptosis. In the early stages of culture, these primary cells had the capacity to form monolayers with tight junctions and expressed general epithelial markers, such as cytokeratins 8 and 18 (Bosch et al., 1988); however, little characterization had been performed regarding their expression of intestinal cell type-specific markers or transport function. It was generally assumed that it would not be possible to establish long-term cultures of primary adult tissues without the introduction of genetic transformations promoting cell proliferation and survival. In 2009 however, Ootani et al. published details of an *in vitro* culture system that facilitated the long-term propagation (for several months) of intestinal fragments containing epithelial and mesenchymal cells from neonatal mice in a collagen gel with air interface supplemented with serum. Such cultured intestinal cells grew out into cystic structures, forming a simple epithelium comprising all major intestinal cell types associated with an underlying myofibroblast layer. However, this culture system failed to represent the characteristic crypt-villus morphology of the adult epithelium (Ootani et al., 2009).

More recently, several independent groups published exciting novel culture protocols allowing the long-term culture of primary intestinal epithelial cells *in vitro* as intestinal organoid structures (Jabaji et al., 2013; Sato et al., 2009). In this respect, mouse and human intestinal crypts are embedded in a three dimensional matrix such as Matrigel or Collagen I covered with medium basically mimicking the intestinal stem cell niche by providing factors of the Wnt-, EGF-, BMP-, and Notch-signaling pathways (Cao et al., 2013; Farin et al., 2012). Over several days crypt cells form spherical structures with a crypt-like lumen, referred to as human enterospheres. Small intestine and colon spheres can expand into multilobulated enteroids that mimic the ordered structure of the epithelium including crypts containing multipotent columnar base stem cells and Paneth cells. The remaining cell types such as enterocytes, goblet cells and enteroendocrine cells can be observed in larger cysts away from the crypt compartment. In these cysts, proliferation signals are down-regulated while differentiation is induced (Reynolds et al., 2014). For further promotion of differentiation into secretory lineages, Notch signaling can be reduced by γ-secretase inhibitors (such as DAPT) and omission of Wnt3A (Milano et al., 2004). Success and yield of the human culture protocol strongly depends on the donor tissue including age, gut region and pathology. While three-dimensional organoids derived from primary intestinal cells appear to possess physiologically relevant phenotypes, they cannot be used to assess classical functionality typically determined within 2-dimensional Transwell® cultures, such as the formation of monolayers with tight junctions and intestinal permeability and transport. However, through development of a real-time imaging system and mathematical modeling, the organoid system has been utilized as an efficient tool to evaluate the dynamics of P-gp efflux transporter-mediated drug transport (Mizutani et al., 2012).

3.3.3 Stem cell-derived intestinal cells

Stem cells have the capacity to self-renew and differentiate into the various cell lineages that build specific tissue types. In particular, multipotent intestinal stem cells have an enormous regeneration potential and are responsible for continuous self-renewal of the gut epithelium *in vivo*. Recently, based on the three-dimensional organoid protocols described above, several groups have independently published descriptions of methods by which leucine-rich repeat-containing G protein-coupled receptor 5 (LGR-5)- or Ephrin type-B receptor 2 (EphB2)-positive intestinal stem cells can be isolated from primary murine or human intestinal tissue and efficiently grown as organoids *in vitro* (Jung et al., 2011; Sato et al., 2009). Concerning the crypt culture protocol, flow cytometry-purified intestinal stem cells can be embedded in Matrigel™ and maintained in growth factor-enriched medium, the composition of which varies slightly depending on the gut region and species. Currently, the employment of Matrigel™ seems absolutely essential as it provides a three-dimensional laminin- and collagen-rich matrix mimicking the basal lamina. After organoid formation, new cells are continuously generated at the bottom of the crypt-like compartments,

where they proliferate, differentiate and are shed into the central cyst lumen some days later. Such organoids can be propagated for several months without significant changes of phenotype and karyotype. The culture protocol has also be demonstrated to be successful when employed with single stem cells in cell cloning experiments, although with less efficiency as important factors from crypt niche cells such as the Paneth cells are missing. Overall, the homeostasis of the generated organoids faithfully represents the *in vivo* situation and can be used for toxicity studies or, to some extent, also to study dynamics of drug transport in a three-dimensional tissue environment. However, the ability to grow enteroids as polarized monolayers instead of spheroids and without Matrigel™ would be advantageous, as this would permit direct and undisturbed apical exposure of compounds in transport studies. It is noteworthy in this case that enteroids contain only epithelial cell types, and do not represent additional cell and tissue types such as smooth muscle, supporting fibroblasts, endothelial cells, or enteric nerves.

Generation of more biologically-complex intestinal tissue has been accomplished through directed differentiation of (embryonic or induced) pluripotent stem cells to intestinal tissue (Kauffman et al., 2013; Ogaki et al., 2013). In general, pluripotent stem cells can be differentiated into all cells of the three embryonic germ layers. In particular, embryonic intestinal development can be mimicked by application of a temporal series of growth and differentiation factors. The process is initiated using the Nodal-related protein Activin-A, which directs differentiation of pluripotent stem cells into definitive endoderm up to 90% purity according to Sox17 and FoxA2 expression (D'Amour et al., 2005). Further, synergistic activation of the fibroblast growth factor and Wnt signaling pathways has then been used to promote formation of a posterior gut tube and promote growth of the intestinal mesenchyme. Intestinal cultures were then propagated under similar conditions as described for the organoid cultures under Matrigel™ conditions (Spence et al., 2011). The epithelium contained crypt and villus-like structures as well as all of the cell types normally found in the gut. Gene expression included caudal type homeobox2 (CDX2, a hindgut marker), E-cadherin (cell-to-cell junction marker), and Villin (epithelial brush border marker). The enclosed mesenchyme expressed markers for smooth muscle cells, myofibroblasts and fibroblasts. The organoids showed basic intestinal function including absorption of amino acids and secretion of mucus, and were able to be passaged for several months (reviewed by Finkbeiner and Spence, 2013), although cells were rather immature compared to fully differentiated epithelial cells. As for the adult stem cell-derived organoids, functional transport studies of drugs across epithelial monolayers using established Transwell® systems has not been demonstrated to date.

Nevertheless, pluripotent stem cells provide a promising defined and unlimited cell source for intestinal model generation and regenerative medicine. Furthermore, the recent progress of technologies utilizing human induced pluripotent stem cells (hiPSCs) may provide a valuable tool for oral drug discovery, as patient-specific somatic cells can be reprogrammed and directly differentiated to intestinal tissue for more physiologically relevant disease modeling. Given that protocols exist for generating other important cell types such as vascular endothelial cells and neural cells from hiPSCs (Lee et al., 2007; Levenberg et al., 2002), it should also be possible to incorporate vascular and neural networks.

3.3.4 Co-culture and pathophysiological models

The intestine exists in a fine balance of endogenous and exogenous factors provided by multiple cells in the mucosal niche. If this tightly regulated system is disturbed, inflammation, infection or cancer may result. Therefore, single cell based assays to mimic the intestinal system are overly simplified and in most cases do not adequately represent the complex interplay between the different cell types under pathological conditions.

Adding further important cell types to generate advanced three-dimensional co-culture models may help to improve the features of intestinal model systems or to better understand pathological symptoms. Within the intestinal stem cell niche, signals to maintain the undifferentiated nature of these cells, and thereby the epithelial integrity, are provided by surrounding mesenchymal cells. The alpha-smooth muscle actin expressing intestinal subepithelial myofibroblasts (ISEMFs) are known to support long-term epithelial growth *in vitro* and wound healing, for instance (Lahar et al., 2011; Seltana et al., 2010). This effect may be caused by factors such as R-Spondin-2, a Wnt-agonist, but it is still unclear if cell-cell contacts are necessary (Lei et al., 2014). More research is needed to understand the complex interplay between the different cell types to maintain the intestinal stem cell niche and gut homeostasis.

A number of investigations have been carried out previously to explore the use of co-culture systems in permeability studies (Walter et al., 1996; Wikman-Larhed and Artursson, 1995). From a commercial viewpoint, the small intestinal (SMI) tissue model available from MatTek exhibits more physiologically relevant TEER values as compared to conventional Caco-2 monocultures (Ayehunie et al., 2014; Maschmeyer et al., 2015a) and allows for conduction of relatively long-term (up to 4 weeks) permeability studies. The SMI model is also suitable for investigation of compound metabolism and toxicity in healthy situations, as well as in simulated acute and chronic conditions of inflammation and potentially also infection. Indeed, there is an increasing need for pathological models in addition to "healthy" gut modelling. Chronic inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease have an increasing incidence within industrial countries. The treatment options, however are still rather symptomatic than curative. Therefore, recently developed co-culture models involving cells of the immune system like monocytes, macrophages or dendritic cells could aid in understanding the role of certain cell types in these diseases, and in the development of novel treatment strategies (Leonard et al., 2010, 2012; Spottl et al., 2006). Similarly, autologous, humanized primary co-culture models (H. Walles lab, unpublished data) constitute a further improvement for the development and testing of new vaccines for more efficient and individualized treatment of gastrointestinal cancers, which show an extremely high prevalence worldwide. Finally, severe infections can disturb the tight balance of the microbiota leading to symptoms such as inflammation, dysmotility or even can-

cer. Currently, a clear limitation in studying human pathogens is the availability of relevant humanized infection models. This is particularly true for obligate human pathogens (e.g., *Salmonella*, *Helicobacter pylori* and *Campylobacter jejuni*) for which no animal reservoir is known (Dostal et al., 2014; Höner zu Bentrup et al., 2006; Naughton et al., 2013). Since simple cell culture systems and also animals are highly artificial models for obligate human pathogens, novel human infection models based on engineered human tissue are needed and are in fact currently under development (unpublished data).

3.4 Chip technology

Several new biological models mimicking the organ-organ interactions between the intestine and the liver have recently been developed, using coupled polycarbonate cell culture inserts and microfluidic biochips in integrated fluidic platforms which allows for the performance of dynamic co-cultures. In one such system, the Integrated Insert in a Dynamic Microfluidic Platform (IIDMP), the intestinal compartment is simulated using Caco-2 TC7 cells, while the hepatic component is represented by HepG2C3A cells (Bricks et al., 2014). Another novel multi-organ chip model (MOC, TissUse) utilizes primary small intestinal epithelial cells (SMI-100, MatTek) and HepaRG liver spheroids to reproduce intestine liver interactions (Maschmeyer et al., 2015a,b; Materne et al., 2015). Additionally, a "Gut-on-a-Chip" has been recently developed using Caco-2 cells (Kim and Ingber, 2013). In such a setup, cells are cultured within micro-fluidic channels of a clear, flexible, polydimethylsiloxane (PDMS) device created with microchip manufacturing that permits application of fluid flow as well as cyclic mechanical strain at levels similar to those experienced by epithelial cells in living intestine (Kim et al., 2012).

3.5 Synthetic membrane systems

Artificial membranes represent attractive alternatives to the use of models employing native tissue. Such models may be highly predictive of *in vivo* conditions, are capable of expressing and allowing for the investigation of drug transport systems, and may also be relatively cost-efficient (Buckley et al., 2012). Artificial membrane technology is generally used to measure passive permeation through membranes composed of mixtures of phospholipids in dodecane, supported by an inert polycarbonate filter membrane, such as in the case of the Parallel Artificial Membrane Permeability Assay (PAMPA) (Avdeef et al., 2004; Kansy et al., 1998), or through lipid layers consisting of sedimented liposomes supported by filter membranes, as in the Phospholipid Vesicle-Based Permeation Assay (PVPA) (Fischer et al., 2011; Flaten et al., 2006a,b, 2007; Kanzer et al., 2010). PAMPA assays estimate the passive transcellular permeability (Buckley et al., 2012) of compounds from one compartment (donor microtitre plate) to another, overlying compartment (membrane acceptor), through an artificial porous membrane infused with biobarrier constituents and lipid solutions. The amount of a compound which diffuses into the acceptor compartment provides an indication of its passive penetration ability. As only approximately 5% of all drugs are estimated to be taken up via active transport (Artursson, 1998), PAMPA approaches are credible and useful for early-stage ADME screening, posing a low-cost and high-throughput non-cellular methodology. PAMPA models with different lipid compositions have been established; for instance models may consist of a single lipid such as lecithin (Kansy et al., 1998) or mixtures of phosphatidyl-choline, -ethanolamine, -serine, -inositol and cholesterol (biomimetic PAMPA, Sugano et al., 2003). A 20% dodecane solution of a phospholipid mixture (Avdeef et al., 2005), silicone oil and isopropyl myristate (Ottaviani et al., 2006) – which does not occur *in vivo* – systems of lipid bilayers (Nirasay et al., 2011), and also lipid triple-layers (Chen et al., 2008b) as well as synthetically generated amphiphilic phospholipids (Yu et al., 2015) also exist. An interesting example of a comparison between cell-based and artificial membrane assays was published by Kerns et al. (2004), where permeability data from Caco-2 cell monolayer and PAMPA methods was compared. This work showed a correlation between passive permeation as determined by the two models. Compounds which deviated from this correlation fell into two clear categories – compounds which were known to be subjected to absorptive transport mechanisms (associated with active transport, paracellular permeation, and gradient passive diffusion) and compounds known to be affected by secretory transport (related to efflux and gradient passive diffusion). Such a combination of assays as employed in the study in question presents an interesting approach to enable differentiation between permeation mechanisms.

3.6 *In silico* modeling

One of the earliest (if not the earliest) computer models used to describe intestinal absorption was an analog computer circuit for differential equations detailing a variable rate of glucose absorption from the intestine, taking into account the feedback effects of blood glucose levels and insulin production (Janes and Osburn, 1965). Not long after, many pharmaceutical scientists began using computer modeling to model biopharmaceutical and pharmacokinetic processes (Beckett and Tucker, 1968), including the kinetics of buccal absorption (Beckett and Pickup, 1975), as well as to solve intractable or difficult pharmacokinetic analysis of advanced models for oral absorption (Wagner, 1975). Later analyses included computer modeling of transport processes across human gastrointestinal epithelia, including the presence of an aqueous diffusion layer and saturable transport in the human jejunum (Read et al., 1977) and numerical simulation of differential equations describing absorption processes along the length of the intestine (Atkins, 1980). The principles applied in these studies then evolved to include digital computing and deconvolution methodology to assess gastric and intestinal absorption (Veng-Pedersen, 1987). A key step forward in this respect was the numerical analysis of transport in and from the intestine represented as a nonlinear, multi-compartment mixing tank model based on human physiological parameters (Luner and Amidon, 1993). This model is the forerunner to the now widely used computer program GastroPlus® (see below). At approximately the same time, simulations on the non-linear behavior of controlled products and the absorption of their contained actives along the length of the intestine were developed (Grass et al., 1994).

Modeling of solute structural determinants of intestinal penetration also represents a distinct phase in the development of *in silico* modeling. Studies carried out in this area utilized techniques such as dynamics simulations of the polar surface area of prodrug and drug conformations to correlate to Caco-2 cell apparent permeability coefficients (Krarup et al., 1998), and quantitative structure-property relationships as an alternative to experiments in estimating the percent human intestinal absorption (Wessel et al., 1998). These developments have led to *in silico* modeling that uses a combination of solute physicochemical properties (such as lipophilicity, polarity, polarizability and hydrogen bonding) and statistical analysis to predict intestinal penetration (Norinder et al., 1999).

In the current environment we see a continuation of concepts developed in early *in silico* modeling of intestinal absorption, however using more sophisticated computation and advanced modeling. Such *in silico* models can decipher complex, highly variable absorption and food interaction pharmacokinetics for a modified release drug product using an object- and agent-oriented discrete event system, which maps various physiological features and processes (Kim et al., 2014). The current-day pharmaceutical industry has a huge financial interest in the inclusion of computational (*in silico*) analysis in early stages of drug screening. Such *in silico* approaches support the development of tailored drugs that are optimized to overcome the intestinal barrier; for this reason *in silico* models concentrate on the prediction of adsorption, distribution, metabolism, excretion and toxicity (ADMET), properties of drug candidates which may be used to filter out substances that are unsuitable for further development and so minimize failure rates during drug candidate development and testing in further screening studies and clinical trials (Wang and Hou, 2015). Furthermore, the creation of "drug-like"-compound libraries was initiated over ten years ago in order to improve efficiency in drug development. Besides ADMET criteria, these libraries should incorporate medicinal chemistry knowledge as well as information about structure and metabolic stability (Matter et al., 2001). For classification neural networks are often used that are trained on a set of substances with known characteristics. After the training step the neural networks are used to predict the properties of new compounds (Guerra et al., 2010). With a well-trained network and enough training data, "Lipinski's rule of five" for optimal drug properties (which states that for oral activity, a drug should have ≤ 5 hydrogen bond donors, ≤ 10 hydrogen bond acceptors, a molecular weight of ≤ 500 daltons, and maximum logP value of 5) can thus be extended to any desired set of properties and substances (van de Waterbeemd and Gifford, 2003). Proteins are in several aspects the drug candidates of choice as they exhibit desired characteristics such as high selectivity and good response rates upon treatment, while causing fewer side effects (Antunes et al., 2013). In order to select intestinal barrier-permeable peptides from large peptide libraries, artificial neural networks have been applied to develop first models for prediction of intestinal permeability on the basis of peptide sequences (Jung et al., 2007). However, problems associated with proteins include their instability, high molecular weight and high hydrophilic character, which result in low permeability through biological membranes (Antunes et al., 2013). For passive permeation several *in silico* models have been established based on statistical models including multiple linear regression, partial least squares, linear discriminant analysis, genetic algorithms and support vector machines (Hou et al., 2006). Many computational models deal with the prediction of intestinal drug absorption and aim to identify important physicochemical properties in order to improve absorption of the investigated compounds (Hamalainen and Frostell-Karlsson, 2004).

Data analysis is often based on the assumption of a single permeation barrier separating donor and receiver compartment and simple diffusion according to the spatial gradient (Fick's first law). The application of such single barrier models can be an over-simplification of the cell monolayer however. Refinements include different active and passive absorption routes, and variation of pH as well as enzyme and transporter expression levels in order to improve the model of the absorption along the intestine (Heikkinen et al., 2010). Single barrier models are helpful for the basic analysis of concentration-independent mechanisms. More complex compartmental models have been developed for the simulation of intestinal transit, which describe the transfer of a compound between several kinetic compartments. Also, the cell monolayer can be subdivided by this approach into several compartments (Zhang et al., 2006). These mathematical models define the rates of compound movement by differential or other algebraic equations. If these equations are not analytically solvable, numerical methods have to be applied which require a high amount of computing power (Heikkinen et al., 2010). An example of a commercially available software package used for such modeling is GastroPlus®, which is a whole body model integrating compound distribution, metabolism and pharmacodynamics (http://www.simulations-plus.com/) utilized by a number of top pharmaceutical companies as well as various independent researchers (Heikkinen et al., 2012; Saxena et al., 2015). In contrast, the software Simcyp® simulates pharmacokinetic profiles and focuses on metabolic pathways of the cytochrome P450 enzymes (http://www.simcyp.com/). Combination of the Simcyp® population-based PBPK modeling platform with an intestinal pharmacokinetic model has also been utilized in order to take into account population variability, an area which is being increasingly emphasized (Hens et al., 2014).

Perhaps the most definitive outcome from the *in silico* modeling of intestinal transport has been the evolution of the aforementioned BCS, now being used to classify which generic drugs need to undergo *in vivo* bioequivalence studies and to provide accurate IVIVC predictions (Tsume et al., 2014). However, in spite of this achievement, as well as the various developments detailed above, it also needs to be noted that we are a long way from precise prediction of human bioavailability. Several examples are available of instances where *in silico* and *in vitro* predictive models based on Caco-2 permeability measurements do not correlate with human intestinal permeability (Larregieu and Benet, 2013).

3.7 Conclusion

Ex vivo, *in vitro* and *in silico* methods for oral drug toxicity and transport studies are available, as presented above and also summarized in Table 3. They differ with regard to expense, complexity, explanatory power, accuracy, etc., and are as such not

Tab. 3: Models for intestinal absorption

	Examples	Advantages	Limitations	Issues and needs	Memo
Models for intestinal absorption					
Intestinal tissue	Intestinal explant (*ex vivo*)	– mimics *in vivo* intestinal tissue – full architecture of the intestinal mucosa and submucosa is present – multiple biopsy punches can be taken from one tissue samples	– sample variability – technical limitations	– definition of acceptance criteria (e.g., applicability domains, reference datasets, integrity tests,...?) – higher standardization of the method including standardized integrity tests (SOP?) – think about new approaches (new *in vitro* models? *In silico* models? Combination of both?) that cover all pathways – simple system, applicable for screening purposes – applicable for mixtures	– no routine use in the regulatory context of chemicals and food, data from intestinal *in vitro* models can be treated as mechanistic add-on information – clarify transporter activity and extent of activity in Caco-2 model
Cell-based systems	Caco-2 monolayer (e.g., CacoReady, Advancell)	– easily accessible – regulatory acceptance for BCS classification – reproducible – metabolically competent – information on active transport – Papp useful for PBPK or bioavailability screening (e.g., BCS class)	– poor correlation to *in vivo* rat absorption data (since, e.g., no available human absorption data for pesticides) – technical limitations (sufficient water solubility needed, dependence on shipment)		
	T84 polarized cells	– depicts apical and basal surface – reflects villi like structures – well defined cellular junctions – easy to assess TEER for permeability measurements	– 7-10 days for polarization – technical limitations		
Artificial surrogates	GIT-PAMPA (Pion)	– easily accessible – standardized – reproducible – P_{app} useful for PBPK	– only passive diffusion – no metabolism – poor correlation to *in vivo* rat absorption data – technical limitations (sufficient water-solubility, UV-activity needed)		
***In silico* models**	– Matter et al., 2001 – Guerra et al., 2010 – Jung et al., 2007 – Hou et al., 2006 – Hamalainen and Frostell-Karlsson, 2004 – Heikkinen et al., 2010	– improved drug development by compound libraries – predictions of compound properties – prediction of intestinal permeability and improvement	– algorithms often based on simplifications (e.g., single barrier model, neglect of active and passive absorption) – more complex compartmental models require high computing power	– Standardized integration of *in silico* models into the preclinical process – collection of all available compound information before testing	

universally employable but rather tailored to suit specific applications. Despite their varying complexity, non-animal models of the intestine described so far still remain overly simplified compared to the *in vivo* situation, as they do not include feedback mechanisms or aspects of the gut microbiota, immune system, innervation or specific hormonal controls. Further efforts and technological innovations are therefore needed to further improve and exploit intestinal models in order to meet the growing requirements of industry and researchers. Several new methods and technologies are currently being developed (Alépée et al., 2014; Hartung, 2014) including (i) advanced human intestinal three-dimensional co-culture models with representation of intact vascularization, (ii) integration of non-invasive biochip and sensor imaging technologies, (iii) implementation of computer-controlled bioreactor and microfluidic systems and (iv) improved biomaterial and biomimetic approaches (e.g., 3D printing).

4 Non-animal models of the lung in research, industrial applications and regulatory toxicology

4.1 General introduction

The essential function of the lungs is gas exchange, involving transfer of oxygen from the air into the blood and carbon dioxide from the blood into the air. This is achieved via transport of air through the conducting airways of the upper and lower respiratory tract to the respiratory portion of the lung where gas exchange takes place. The pulmonary epithelial surface is the first site of contact for inhaled environmental materials (noxious gases, anthropogenic and natural particulates, including pathogens) and so its barrier properties are centrally crucial for tissue homeostasis; equally, the epithelium represents a barrier for inhaled drug delivery, and transport across it is required for drug efficacy. As in the intestinal tract, the junctional complexes (tight junctions and adherent junctions) between the epithelial cells play important roles in the homeostasis of the respiratory tract and the maintenance of mucosal immunity (Davies, 2014). However, it is important to recognize that the functions of the epithelium change along the respiratory tract, which is evident in the change in epithelial structure from proximal to distal regions of the lung.

The epithelium of the trachea and large bronchi is typified by pseudostratified epithelium with basal cells supporting columnar ciliated epithelium and mucous secreting goblet cells (McDowell et al., 1978). Secretions from the goblet cells and submucosal mucous glands form a viscous fluid layer overlying the respiratory epithelium to protect the epithelial surface. The fluid contains water, mucins, ions, cytoprotective molecules such as antioxidants, and antimicrobial substances including immunoglobulin A (IgA) and lysozyme (Swindle et al., 2009). This viscous layer traps inhaled particles, microorganisms and sloughed off epithelium, which are then moved via ciliary action of the respiratory epithelium towards the pharynx.

The bronchioles (< 1 mm diameter) have ciliated columnar respiratory epithelium with some Club cells (formerly known as Clara cells) and a few goblet cells. Club cells are non-ciliated cuboidal secretory cells. They are a source of secretoglobin and release surfactant apoproteins A, B, and D, proteases, antimicrobial peptides, several cytokines and chemokines, and mucins, to the extracellular fluid lining the airspaces. Their functions include xenobiotic metabolism, immune system regulation, and progenitor cell activity (Reynolds and Malkinson, 2010). Further branching of the respiratory tree gives rise to respiratory bronchioles, lined mainly by cuboidal ciliated epithelium and Club cells, followed by the alveolar ducts, which terminate in two or three alveolar sacs, the walls of which are comprised completely of alveoli.

The alveolus is the structural unit of gas exchange. It is a spherical or polygonal air space approximately 250 μm in diameter and its thin walls are surrounded by a rich network of pulmonary capillaries. The alveolar compartment is lined with membranous pneumocytes, known as type I alveolar epithelial cells (ATI), and granular pneumocytes, called type II alveolar epithelial cells (ATII) (Fehrenbach, 2001). ATI cells are large, thin squamous cells that cover 90-95% of the alveolar surface. These cells are involved in gas exchange and form an immunological barrier able to sense microbial products and generate inflammatory responses (Féréol et al., 2008; Williams, 2003). ATII cells are cuboidal, constitute around 15% of total lung cells and cover approximately 7% of the total alveolar surface. ATII cells synthesize, secrete and recycle all components of the surfactant that regulates alveolar surface tension (Fehrenbach, 2001). They are responsible for lung defense, immune regulation, epithelial repair and ion transport. In addition to surfactant phospholipids, they secrete antimicrobial products such as complement, lysozyme, and surfactant proteins (SP). Also present in the alveolus are the pulmonary macrophages, which play a key role in defense of this compartment.

4.1.1 Pulmonary epithelium for drug development

According to the European Lung Foundation and European Respiratory Society White Book (http://www.erswhitebook.org), the burden of lung disease in Europe remains as high today as it was at the turn of the millennium, and is furthermore likely to remain so for several decades. Each year in 28 EU countries, lung diseases cause two-thirds of every million deaths, and at least 6 million hospital admissions, accounting for over 43 million in-patient bed days. Therefore, there is an urgent unmet need for new lung disease treatments. For ethical reasons, studies in humans are restricted to some baseline investigations and a limited range of *in vivo* challenges (e.g., allergen, ozone, exercise, diesel exhaust) used in conjunction with morphological assessment of tissue biopsies or cells obtained by bronchoalveolar lavage (BAL) (Krishna et al., 1998; Salvi et al., 1999; Teran et al., 1995). Although providing valuable insight into inflammatory cell and mediator changes in the disease context, they only provide a "snapshot" and are not amenable to detailed mechanistic studies. Consequently, there has been a high dependency on *in vivo* animal models to study mechanisms of human respiratory diseases, where transgenic and knock out approaches permit more functional assessment of a given molecule (Shapiro, 2006), allowing for investigation of its relationship with the dynamics of the inflammatory process, or to study alterations in airway wall behavior. However, the utility of animal models

to understand human diseases such as asthma and chronic obstructive pulmonary disease (COPD) and for the development of therapeutics is a topic of considerable debate (Holmes et al., 2011). Of major concern is the failure of animal models to reproduce the complex interplay between genetic and environmental stimuli that underlies many chronic human diseases. Recognition of the importance of these gene-environment interactions in disease pathogenesis has led to a major shift in focus to the function of the pulmonary epithelium, since it is the interface with the external environment. As will be detailed below, access to human airway tissue has allowed development of *ex vivo* and *in vitro* models of the human airway epithelium that allow mechanistic studies that could not be performed ethically *in vivo* (Swindle and Davies, 2011). These studies range in their level of complexity from simple monocultures with little structural organization to complex three-dimensional cultures containing many cell types that are organized into a structure that retains (*ex vivo* tissue explants) or mimics (tissue engineered *in vitro* models) some *in vivo* elements (Blume and Davies, 2013).

4.1.2 Pulmonary epithelium for drug delivery

The inhaled route offers an efficient mechanism for delivery of drugs either for treatment of local disease or as a route of administration for systemic therapies. For local delivery, current technology offers efficient and reproducible pulmonary deposition of aerosol medicines allowing delivery of high local concentrations of active drug with a lower drug burden for the rest of the body (Ruge et al., 2013). For systemic delivery, the enormous surface area of the gas exchange region of the lung and relatively low enzymatic activity offers advantages for systemic absorption and avoids liver first pass metabolism, reducing potential side effects and achieving a quick onset of action (Labiris and Dolovich, 2003; Patton and Byron, 2007). However, regardless of the progress that has been made in pulmonary deposition of inhaled drugs, no significant advances have been made for pulmonary drug delivery beyond the treatment of some respiratory diseases (Ruge et al., 2013). One important reason for this is the limited knowledge about the fate of inhaled drugs or carrier particles after deposition in the lungs (e.g., absorption across the alveolar epithelium and targeting of specific regions or cells within the lungs). Furthermore, prior to contact with the epithelial cells, drug particles delivered to the respiratory tract have to overcome the non-epithelial pulmonary barrier of surfactant, alveolar macrophages, mucus and the mucociliary escalator. Mucus is a viscoelastic and adhesive gel that protects the surface of the airways by limiting contact of the epithelial cell surface with potentially damaging inhaled substances. Most foreign particulates, including conventional particle-based drug delivery systems, are efficiently trapped in human mucus layers. Trapped particles are then removed within seconds to a few hours depending on anatomical location. The penetration of mucus can be affected by particle size and surface chemistry (Schuster et al., 2013), and new mucus-penetrating particles that may avoid rapid mucus clearance mechanisms are under development for use in drug delivery (Lai et al., 2009). For these studies, access to systems that enable reliable assessment of drug transport and metabolism are essential.

4.2 *Ex vivo* lung models

Ex vivo lung cultures are convenient models for analyzing interactions of environmental matter or pathogens in a three-dimensional tissue context of the airway or lung parenchyma, as well as for evaluation of drug responses and toxicity. Human precision-cut lung slices (PCLS), an *ex vivo* model consisting of all relevant cell types in their microanatomical environment (de Kanter et al., 2002), are well-established in pharmacological testing (Sturton et al., 2008), analysis of allergic immune responses (Wohlsen et al., 2003) and studies of xenobiotic metabolism (Lauenstein et al., 2014). There is also emerging literature concerning the use of *ex vivo* lung perfusion as a tool for experimental research (Nelson et al., 2014). Originally developed to allow lungs to be assessed for their physiological and functional parameters prior to transplant, the technology is a powerful tool that enables isolated organ modification and evaluation, although it is currently applied only to small and large animal models due to limited access to human lungs. For disease-related studies, bronchial biopsies can be obtained from individuals with respiratory diseases or healthy control subjects by fiberoptic bronchoscopy, and have been used *ex vivo* for short term experiments to analyze disease mechanisms. For example, explant models have been used to demonstrate the involvement of T cells in allergic inflammation and to analyze signaling pathways involved in these responses (Hidi et al., 2000; Jaffar et al., 1999; Lordan et al., 2001; Vijayanand et al., 2010). While these models benefit from retaining the normal lung tissue architecture, they are limited by their relatively short term viability, and the barrier properties of the epithelial layer are compromised by exposure of the whole excised tissue sample to challenge agents.

4.3 Reconstructed human lung models *in vitro*

4.3.1 Cell lines (standardization and validation)

Immortalized human lung epithelial cell lines (exemplifying bronchial, Club and alveolar epithelial phenotypes) have been established from cancers or by viral transformation of cells from normal lung tissue (Tab. 4). Cell line-based models are widely used both for pathophysiological studies of responses of the barrier to endogenous and exogenous agents and their associated signaling pathways, and for transport studies across the epithelial barrier. They are convenient, provide high throughput capacity, and are more economical than *in vivo* experiments. However, they also have some limitations in that they are frequently used as a single cell type in monolayer culture, they may have an atypical phenotype (e.g., derived from adenocarcinomas) and their tight junctions may not be representative of the target tissue. Furthermore, their drug transporters may not be typical of normal lung epithelium, although these have been recently evaluated in five commercially available immortalized lung cell lines (Calu-3, BEAS2-B, NCI-H292, NCI-H441 and A549) by liquid chromatography-tandem mass spectrometry-based approaches (Sakamoto et al., 2015), allowing comparison with data from primary lung cells (Sakamoto et al., 2013). Although immortalized, ideally these cells should be used within a defined range of passages to ensure that their phenotypic characteristics remain consistent.

Tab. 4: Cell-based models for pulmonary research

	Examples	Advantages	Limitations	Issues and needs	Memo
Central Airway Models					
Cell Lines	Calu-3	– easily accessible – polarization – reproducible – information on active transport	– single cell type – no mucus layer – adenocarcinoma cell line may have untypical phenotypes	– should be standardized with respect to acceptable levels of TEER and P_{app} limits for specific reference compounds	– no routine use in the regulatory context of chemicals and food, data from *in vitro* models can be treated as mechanistic add-on information
	16HBE 140-	– reasonably accessible – polarization – information on active transport	– single cell type – no mucus layer – virally transformed cell line may have untypical phenotype	– variability dependent on culture conditions	
	H441	– easily accessible – polarized – reproducible – information on active transport	– single cell type – features of bronchiolar and alveolar epithelium	– express a number of key drug transporters and P- glycoprotein function comparable to human primary ATI-like pneumocytes	
	BEAS 2B	– allows for mechanistic studies, response to cytokines, pathogens, etc.	– single cell type – non-barrier forming: cannot be used for transport studies		
	H292	– Well suited for mucus/mucin gene expression studies	– single cell type – does not polarize: cannot be used for transport studies		
	1HAEo-	– Express tight junctions and mucin	– single cell type		
	VA10		– single cell type		
	9HTEo-		– single cell type – don't form tight barrier		
	HTE67tsa209o-		– single cell type – does not form a tight barrier		
	NCF3	– Defective DF transmembrane conductance regulator: allow for study of ion and fluid transport, pharmacological testing in diseased state	– single cell type		
	IB3-1				
	CFT43				
	CFT1				
	JME/CF15				
	CFBE41o-				
	CFTE29o-				
	CFDEo-/6REP-CFTR				
	CFT-1				
	CFT-3				

Tab. 4: Cell-based models for pulmonary research

	Examples	Advantages	Limitations	Issues and needs	Memo
Central Airway Models					
Primary Cells	Primary cultures, ALI models (e.g., MucilAir, EpiAirway)	– available commercially – mucociliary differentiation, – polarization – information on active transport	– inter-subject variability	– should be standardized with respect to acceptable levels of TEER and P_{app} limits for specific reference compounds	
Peripheral Airway Models					
Cell Lines	A549	– metabolic properties consistent with ATII cells *in vivo*	– do not form tight junctions – leakiness limits utility in examining the transport of low molecular drugs and xenobiotics	– differentiated function may be lost over time if not appropriately cultured	– clarify fields of (regulatory) acceptance
Primary Cells	ATI cells (via differentiation of ATII cells)	– polarized with good TEER – information on active transport	– not readily accessible – requires access to fresh lung tissue for preparation of ATII cells – inter-subject variability	– limited studies to evaluate this system	

A variety of human bronchial epithelial cell lines have been tested with respect to their transepithelial transport kinetics and their potential use in predicting *in vivo* lung absorption. These include cell lines such as the adenocarcinoma cell line Calu-3 (Shen et al., 1994) and the SV-40 transformed cell line 16HBE 14o- (Cozens et al., 1994), both of which are able to form tight junctions and polarize when grown on semi-permeable filter supports (e.g., Transwell® inserts). The barrier properties of these cell lines have been described (Ehrhardt et al., 2002; Forbes et al., 2003; Mathias et al., 2002) and detailed protocols for their culture, maintenance, growth and permeability assessment have been published (Meaney et al., 2002), but it should be noted that culture methods vary between laboratories, e.g., passage number, seeding density, submerged or air-liquid interface, use of extracellular matrices, media composition, duration of culture – see Forbes and Ehrhardt (2005).

The Calu-3 cell line is available from the American Type Culture Collection and is most frequently used for transport studies. Permeability data for small lipophilic molecules across Calu-3 monolayers also suggests that the cell line is a suitable model to examine the transport of low molecular weight substances and xenobiotics (Foster et al., 2000; Zhu et al., 2010). TEER values for Calu-3 monolayers grown on filter supports range from around 300 to 2500 Ω cm^2 (Foster et al., 2000; Loman et al., 1997). While there is an inverse relationship between TEER and Lucifer yellow flux, once the monolayers achieve an appreciable TEER (> 300 Ω cm^2), there is very little change in Lucifer yellow flux across monolayers with higher TEER values (Foster et al., 2000). Therefore, use of this cell line should be standardized (with respect to acceptable levels of TEER as well as P_{app} limits for specific reference compounds) to ensure reproducible results. The 16HBE 14o- cell line exhibits heterogeneous behavior which is highly dependent on culture conditions, leading to variable transport and permeability properties. However, by standardization of the culture conditions 16HBE 14o- cells can provide a discriminatory barrier to solute transport (Forbes et al., 2003). While 16HBE 14o- cells have been reported to exhibit morphological features including apical microvilli and cilia (Cozens et al., 1994), in our experience cilia are absent from polarized 16HBE 14o- cultures.

In addition to their use for toxicology (Forti et al., 2010, 2011) and drug transport studies, 16HBE 14o- and Calu-3 cell lines are frequently used for analysis of pathways involved in barrier function in response to environmental agents including allergens (Vinhas et al., 2011; Wan et al., 2001), particulates (Banga et al., 2012), cigarette smoke (Heijink et al., 2012) or viral infections (Harcourt et al., 2011; Rezaee et al., 2011). The other frequently used bronchial epithelial cell line, BEAS2B (Reddel et al., 1988), is not of value for transport studies as it fails to form an appreciable barrier (Forbes and Ehrhardt, 2005). However, this cell line is routinely used for mechanistic studies, responding to cytokines, danger signals and pathogens (Edwards et al., 2006; Hara et al., 2012). The NCI-H292 mucoepidermoid bronchial epithelial cell line (Carney et al., 1985) also fails to polarize, but is the cell line of choice for studies of mucus and mucin gene expression (Koff et al., 2008; Shao and Nadel, 2005). This cell line also can be used as a substitute for monkey kidney cells for isolation of a broad

spectrum of respiratory viruses, but not all strains of influenza viruses (Hierholzer et al., 1993), and for studies of rhinovirus infection (Heinecke et al., 2008).

A number of cancer cells have been identified which have features of peripheral lung cells (Gazdar et al., 1990). The most commonly used cell lines are A549 (Lieber et al., 1976) and H441 (Brower et al., 1986). A549 cells are commonly used as a model of ATII cells, as they were originally reported to contain lamellar bodies (Balis et al., 1984; Lieber et al., 1976). Over the passage of time this differentiated function may be lost, especially if specific culture conditions are not employed (Speirs et al., 1991). A549 cells do not appear to form functional tight junctions (Winton et al., 1998) but they exhibit some metabolic and transport properties consistent with ATII cells *in vivo*. However, the leakiness of the A549 cell line limits its utility in examining the transport of low molecular weight drugs and xenobiotics (Foster et al., 1998). H441 cells have also been evaluated as models of the peripheral lung. These cells, which were derived from a papillary adenocarcinoma of the lung, have features characteristic of Club cells (Gazdar et al., 1990) and have been used as a model of bronchiolar epithelium (Bruce et al., 2009). However, more recently, it has been found that culture of H441 cells on semi-permeable filter supports results in formation of electrically tight monolayers (peak TEER values of approximately 1000 $\Omega\,cm^2$ after 8-12 days in culture) which are able to differentiate paracellularly transported substrates according to their molecular weight (Salomon et al., 2014). In this model, the cells express a number of key drug transporters and P-glycoprotein function was found to be comparable to human primary ATI-like pneumocytes.

Immortalized cell lines derived from states of human disease are mainly from cystic fibrosis (CF) patients (Tab. 4) and several cell lines with defective CF transmembrane conductance regulator are available (Gruenert et al., 2004). These have utility for studies of ion and fluid transport and pharmacological testing.

4.3.2 Primary lung cells

Several protocols have been established for *in vitro* culture of primary human airway epithelial cells (PBECs) obtained from bronchial brushings or biopsies (Bucchieri et al., 2002; Devalia et al., 1999). These samples can be routinely obtained using fiberoptic bronchoscopy performed on volunteers after ethical approval and informed consent; however the requirement for bronchoscopy limits availability of this approach to those with access to appropriate clinical facilities. For cell collection by bronchial brushing, care must be taken not to be too vigorous, as this causes bleeding and will reduce cell viability. Alternatives to bronchoscopy include purchase of cells from commercial suppliers (e.g., Lonza, Epithelix, MatTek) or use of resected or transplant tissue. The cells are generally grown in monolayer culture on collagen I coated dishes using defined growth medium (e.g., BEGM from Clonetics) and can be expanded by 2-3 passages; early passage cells (P0) can be reliably cryopreserved without loss of viability for subsequent use. These cells express basal cell markers including cytokeratin 5 and the transcription factor p63 (Hackett et al., 2011a) and have been used as models of basal cells.

Many studies have employed primary human cells as monolayer cultures to evaluate responses of the cells to environmental agents. Most frequently, the cultures are plated directly onto collagen-coated culture plastic dishes and are not polarized. However, such approaches have allowed evaluation of cells derived from the airways of normal or diseased (asthma, COPD, CF) volunteers offering the potential to compare responses and identify dysregulated pathways that may contribute to disease. Several studies with PBECs from non-asthmatic and asthmatic individuals have highlighted differences in the cellular response after exposure to environmental agents with the potential to cause epithelial damage. For example, it has been shown that monolayer cultures of PBECs derived from moderate-to-severe asthmatic individuals have a deficient innate immune response to rhinovirus infection by producing less interferon (IFN)-β (Wark et al., 2005), and this is paralleled by higher levels of the Th2-promoting chemokine, thymic stromal lymphopoietin (TSLP) (Uller et al., 2010). Based on the observation that exogenous IFN-β restores the anti-viral response and suppresses viral replication (Cakebread et al., 2011), clinical trials using inhaled IFN-β as a new treatment for virus-induced exacerbations have reported a beneficial effect in a subgroup of patients with severe asthma (Djukanović et al., 2014). Modulation of the basal cell phenotype by cigarette smoke and growth factors such as EGF has also been extensively studied (Shaykhiev et al., 2013a,b).

Methods for the isolation and maintenance of ATII cells from human lung tissue are well established (Robinson et al., 1984; Witherden and Tetley, 2001). They all involve proteolytic (elastase, dispase and/or trypsin) digestion and many utilize density gradient separation and negative selection. This approach can provide at least 1×10^6 cells/g of lung tissue, yielding 95% pure ATII cells (Bingle et al., 1990; Witherden et al., 2004). Modifications of this method using different enzymes and positive selection by flow cytometry (Marmai et al., 2011), antibody-coated magnetic beads (Ehrhardt et al., 2004) or differential adherence (Witherden and Tetley, 2001) have been reported to yield higher ATII cell purity (up to 98%). The cells should be characterized with at least two epithelial markers, including Sp-C and E-cadherin, to determine purity (Jenkins et al., 2012). Cultured fetal lung epithelial cells differentiate into ATII cells following treatment with dexamethasone plus cAMP and isobutylmethylxanthine (DCI); similarly culturing adult ATII cells for 5 days on collagen-coated dishes with DCI for the final 3 days promotes lamellar body production and induction of a subset of hormone responsive genes in cultured adult cells, similar to those in the fetal cells (Ballard et al., 2010). In culture, ATII cells secrete the phospholipids characteristic of pulmonary surface active material and engage in immune signaling (Payne et al., 2004; Thorley et al., 2005, 2007). Primary alveolar epithelial cells have also been used to assess the pathogenesis of influenza infection, including H5N1 (Chan et al., 2009) and the interaction of alveolar epithelial cells with nanoparticles which can reach the periphery of the lung (Kemp et al., 2008). The major challenges of using primary ATII cells relate to viability and fragility (they rarely survive freezing), limited replicative capacity when cultured, and purity (mesenchymal cell "contam-

ination" in disease states) (Jenkins et al., 2012). ATII cells are the progenitors of ATI cells and acquire features of this cell type when cultured (Demling et al., 2006; Swain et al., 2008). Methods have been developed to promote this trans-differentiation: this involves purification of ATII cells by a combination of cell attachment, density gradient centrifugation and positive magnetic cell sorting followed by seeding the cells onto permeable supports. Under appropriate culture conditions, the ATII cells differentiate into an ATI-like phenotype within approximately one week, achieving TEER values of ~2,000-2,500 Ω cm^2 suitable for transport studies (Daum et al., 2012; Elbert et al., 1999) and showing an increase in caveolin-1 positive, Sp-C negative cells (Fuchs et al., 2003). ATII cells have also been obtained from fibrotic lung, however they are often contaminated, with only approximately 80% exhibiting classic ATII cell characteristics (Maher et al., 2010).

4.3.3 Fully differentiated 3D human airway epithelial models

As an extension of the PBEC monolayer model, use of *in vitro* differentiated PBECs offers an epithelial model that more closely recapitulates the airways *in vivo*. Different techniques have been developed to make three-dimensional cultures, by providing a microenvironment or architecture closer to the *in vivo* situation. Among these techniques are cellular matrix scaffolds, hanging drop cultures, perfusion culture chambers and air-liquid interface (ALI) cultures. However, in order to simulate *in vivo* lung conditions, ALI cultures seem to be most appropriate – the basolateral side of the epithelium is immersed in the culture medium and the apical side is exposed to a humidified air / 5% CO_2 environment. Placing PBECs at an ALI in the presence of retinoic acid causes the epithelial cells to form a pseudostratified structure with basal cells supporting ciliated cells and mucus-producing goblet cells (Pezzulo et al., 2010), as occurs *in vivo*. Transcriptomic studies have shown that ALI cultures provide a good representation of the *in vivo* airway epithelial transcriptome (Dvorak et al., 2011; Pierrou et al., 2007). Many in-house airway epithelial models based on primary human cells have been developed and used (Bérubé et al., 2010; Gray et al., 1996; Xiao et al., 2011). Two ALI systems named MucilAir (Epithelix) and EpiAirway (MatTek) are also commercially available and ready-to-use. MucilAir distinguishes itself by having a uniquely long shelf-life: it can be maintained in a homeostatic state for a year. Furthermore, cultures from distinct anatomical locations are also available (nasal, tracheal or bronchial) and cell cultures can be obtained from diseased donors (asthmatic, allergic, COPD or CF) in the case of both MucilAir and EpiAirway systems.

In-house, MucilAir and EpiAirway cultures start with de-differentiated cells, which then undergo a progressive differentiation with time. After 14-21 days (EpiAirway) or 20-45 days (MucilAir) of culture the epithelia are fully ciliated and electrically tight (TEER≈450 Ω cm^2). EpiAirway cultures are available in a number of useful insert formats, and have been successfully utilized for numerous applications including airway drug delivery (Agu et al., 2006; Leonard et al., 2005, 2007), airway disease modelling (Bai et al., 2015; Freishtat et al., 2011; Watson et al., 2010), mucociliary transport (Seagrave et al., 2012), airway toxicology (Mathis et al., 2013; Watson et al., 2010), electrophysiology (Hirsh et al., 2008), and bacterial and viral infection studies (Deng et al., 2014; Mitchell et al., 2011; Palmer et al., 2012; Ren and Daines, 2011; Ren et al., 2012). The MucilAir cultures have been characterized electrophysiologically and are fully functional: the activity of the main epithelial ionic channels, such as CFTR, EnaC, Na/K ATPase, etc. is preserved. Moreover, the epithelia respond in a regulated and vectorial manner to the pro-inflammatory stimulus TNF-α (Hardyman et al., 2013). A large panel of cytokines, chemokines and metalloproteinases has been detected (e.g., IL-8, IL-6, GM-CSF, MMP-9, GRO-α, etc.). Muco-ciliary clearance function is also present in MucilAir, with mucus velocity values comparable to the *in vivo* situation (around 2 mm/min – Huang et al., 2011). Furthermore, with clear PET Transwell® inserts, the model is very practical and convenient to use, and suitable for most applications such as imaging, immunocytochemistry, toxicity tests, electrophysiological studies (Ussing chamber measurements), assessment of drug permeation, drug formulations, etc.

The airway epithelium is a prime entry portal of xenobiotics into the body upon inhalation. Knowledge of toxicokinetics is needed to estimate the possible range of target doses at the cell or tissue level that can be expected from realistic external human exposure scenarios to inhaled compounds. This information is crucial for determining the dose range that should be used for *in vitro* testing. Kinetics in the *in vitro* system and dose-response information is also crucial to translate *in vitro* results to the human *in vivo* situation (Adler et al., 2011). Reconstructed human airway epithelium assays represent a promising *in vitro* tool to evaluate respiratory absorption, giving input parameters for PBTK modelling. By transposing the Caco-2 protocol (Hubatsch et al., 2007) to three-dimensional airway epithelial models, trans-epithelial permeability of drug candidates or xenobiotics can be used to assess the upper-airway permeability of potential drugs, formulations or xenobiotics. Measuring the rate of compound across EpiAirway (Agu et al., 2006; Leonard et al., 2005, 2007) and MucilAir (Reus et al., 2014) provides insight into the ability of different compounds to cross the respiratory epithelia. In permeability studies different compounds are utilized at known concentrations and in two directions (apical to basolateral and basolateral to apical). To evaluate the rate of absorption, the permeability coefficient (P_{app}) is calculated for each compound. To assess whether a compound undergoes active efflux, apical (A) to basolateral (B) transport is measured (A→B) and vice versa (B→A). The ratio of B→A/A→B measurements (asymmetry index) is then determined. If the value of the asymmetry index is greater than 1.5, the compound is subject to active efflux. Known P_{app} values range over two orders of magnitude: 4.94 x 10^{-5} (ibuprofen) to 2.06 x 10^{-7} cm/sec (nadolol) with a 240-fold difference between the highest and the lowest P_{app} value (Fig. 2).

In addition to consideration of culture models, methods for delivery of drugs, particles or gases to the pulmonary surface also require reliable, easy-to-handle and efficient technologies for direct aerosol-to-cell delivery. The well-characterized commercially available VITROCELL® exposure chamber sys-

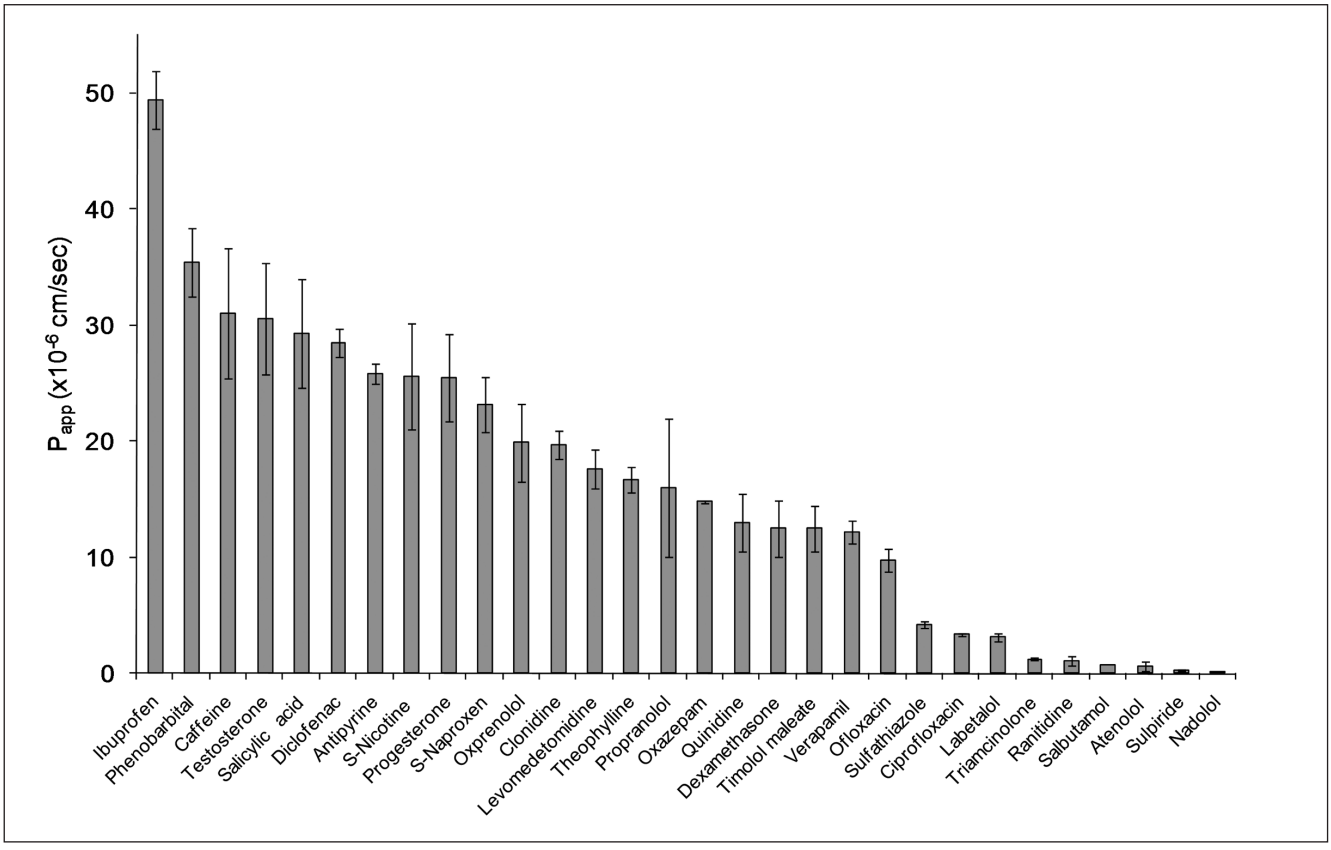

Fig. 2: Comparative apparent permeability coefficient (P_{app}) of 30 compounds obtained on MucilAir airway epithelium
The absorption of test items was assessed after apical exposure, and the P_{app} of the chemicals across airway epithelium was measured. A panel of 30 compounds was tested to evaluate the ability of the assay to rank relative permeability (P_{app} A→B from 2.06 x 10^{-7} to 4.94 x 10^{-5} cm/s).

tem is specially designed for direct contact between cells and components of the test atmosphere at the air/liquid interface. Recent studies suggest that application of an electrostatic field (referred to as ALI deposition apparatus, ALIDA) facilitates particle deposition efficiencies that are higher by factor > 20 than the unmodified system (Panas et al., 2014). Another approach, the ALICE-CLOUD technology, utilizes principles of cloud motion for fast and quantitative delivery of aerosolized liquid drugs to pulmonary cells cultured under ALI conditions (Lenz et al., 2009, 2014). Aerosol-to-cell delivery was shown to be highly efficient, reproducible and rapid using aerosolized fluorescein as a surrogate drug. This type of approach may pave the way for screening of inhalable drugs under more physiologically relevant and hence potentially more predictive conditions than the currently used submerged cell culture systems (Lenz et al., 2013). Primary human alveolar epithelial cell (hAEpC) models can also be used for toxicity and transport studies for newly developed compounds and delivery systems (Daum et al., 2012). The so-called Pharmaceutical Aerosol Deposition Device On Cell Cultures (PADDOCC) system (Hein et al., 2011) enables pharmaceutical formulations to be aerosolized and deposited as a dry powder aerosol onto ALI-grown alveolar epithelial cells.

ALI cultures have provided insights into pathogenetic mechanisms, for example the ability of Th2 cytokines, IL-4 and IL-13, to skew bronchial epithelial cell differentiation towards goblet cell hyperplasia, increased mucin gene expression and mucous hypersecretion (Kanoh et al., 2011; Thavagnanam et al., 2011; Zuyderduyn et al., 2011). Furthermore, use of ALI cultures has allowed for comparisons of barrier properties, mucus production and other responses of epithelial cells from asthmatic and healthy subjects, as well as the demonstration that tight junctions are reduced and mucus production and inflammatory cytokine induction are increased in cultures from asthmatic subjects (as observed *in vivo*). Such changes have been seen to correspond to an increase in ionic and macromolecular permeability (Xiao et al., 2011; Bai et al., 2015; Freishtat et al., 2011; Watson et al., 2010). It has also been shown that the permeability of the bronchial epithelial cell layer is only increased in asthmatic subjects after exposure to ozone or nitrogen dioxide (Bayram et al., 2002), and that mediator release is altered after exposure of ALI cultures from asthmatic donors to respiratory syncytial virus (RSV) or particulate matter (Hackett et al., 2011b). Using cells derived from patients with COPD, ALI cultures show higher baseline levels of cytokine expression and increased susceptibility to RSV infection, despite an increased IFN response

(Schneider et al., 2010). ALI cultures are also useful to analyze ciliary beat frequency in response to various stimuli (Bayram et al., 1998; Devalia et al., 1992).

While the focus of this section of the current review is on the surface epithelium, it is also worth noting that primary human bronchial epithelial cells can differentiate into three-dimensional spheroids with a lumen and cells that secrete MUC5B mucin (a marker for glandular mucous cells) together with lysozyme, lactoferrin and zinc-a2-glycoprotein (markers for glandular serous cells) when grown on Matrigel™ (Wu et al., 2011). This *in vitro* model system of respiratory tract glandular acini is relevant for investigations into mechanisms that lead to the submucosal glandular hyperplasia seen in COPD, CF and chronic rhinosinusitis. Spheroids can also be derived from bronchial brushing and are free floating with ciliated cells on the outer surface of the spheroid (Deslee et al., 2007). These models have been used for studying processes involved in morphogenesis and branching (Hayden, 2012; Hubatsch et al., 2007) and may also offer pharmaceutical companies potential for high throughput screening using approaches similar to those employed for evaluating anti-cancer drugs (Kunz-Schughart et al., 2004). ATII cells have also been cultured as spheroids and expressed mature morphological characteristics including lamellar bodies. SP-C mRNA, a specific protein for ATII cells was reported to be expressed only in cells forming spheroids (Takahashi et al., 2004).

4.3.4 Immortalization of primary cells without loss of differentiation capacity

While monolayer and ALI models contribute significantly to our understanding of disease mechanisms, they are limited due to the relatively low number of cells that can be collected and grown from each subject and there is the potential for cells to lose their characteristics after long term culture (Yoon et al., 2000). However, recent advances involving induced expression of human telomerase reverse transcriptase (hTERT) to prevent replicative senescence and expression of the cell cycle protein cdk4 (Ramirez et al., 2004) has enabled immortalization of epithelial cells in culture without loss of their ability to differentiate when taken to an ALI (Vaughan et al., 2006). More recently, a human airway epithelium-derived basal cell line with multipotent differentiation capacity has been established by immortalization using a retrovirus expressing hTERT (Walters et al., 2013). Such an approach might be useful to generate cell lines from asthma-derived cells, although any such lines would need careful phenotypic characterization.

An immortalized ATII cell line has been generated by transduction with the catalytic subunit of telomerase and simian virus 40 large-tumor antigen (Kemp et al., 2008). Immunochemical and morphologic characterization demonstrated an ATI-like cell phenotype with expression of caveolin-1 and receptor for advanced glycation end products (RAGE). This cell line has been used to study innate immune responses to bacterial ligands and the role of TLR signaling (Thorley et al., 2011), however, the TT1 cell line did not form tight junctions and was not suitable for drug transport studies (van den Bogaard et al., 2009).

4.3.5 Stem cell-derived lung cells

While primary cell culture models are more able to recapitulate differentiated functions of the pulmonary epithelium, they are usually limited by the relatively small amount of material that it obtained at bronchoscopy or from resections. More recently, traditional stem cell differentiation protocols have been applied to human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) to evaluate their use for lung specific differentiation. Thus, transfection and culture procedures have been developed which facilitate the differentiation of hESCs into a pure (> 99%) population of ATII cells with morphological characteristics including lamellar body formation, expression of surfactant proteins A, B, and C, alpha-1-antitrypsin, and the CF transmembrane conductance receptor, as well as the synthesis and secretion of complement proteins C3 and C5 (Wang et al., 2007). Protocols have also been developed whereby human embryonic stem cells can be differentiated into the major cell types of lung epithelial tissue (Van Haute et al., 2009). ATII cells have also been derived from iPSCs and have phenotypic properties similar to mature human alveolar type II cells (iPS-ATII) (Ghaedi et al., 2014). Of note, use of a rotating bioreactor culture system that provides an ALI is a potent inducer of ATI epithelial cell differentiation for both iPS-ATII cells and human ATII cells. iPS-ATII progenitor cells retain the ability to adhere to and proliferate within three-dimensional lung tissue scaffolds and display markers of differentiated pulmonary epithelium (Ghaedi et al., 2013). This approach may provide a method for large-scale production of alveolar epithelium for tissue engineering and drug discovery. However, much research is needed to establish optimal conditions for growth of specific cell types and, as with the immortalized cell lines, to characterize their phenotypic properties.

4.3.6 Co-culture and pathophysiological models

A significant limitation of many *in vitro* cell culture models is that they investigate responses only from one isolated cell type whereas *in vivo*, the interplay between many different cell types is crucial for cellular homeostasis. In order to address these complex interactions in experimental models, considerable effort is currently being invested into the development of co-culture models and three-dimensional tissue constructs integrating cells and extracellular matrix components to more closely mimic the *in vivo* state. A variety of co-culture models have been described using many different combinations of cell types. The simplest co-culture models use differentiated or undifferentiated airway epithelial cells in the apical compartment of semi-permeable filter support units and other cell types in the basal compartment. Adherent cells such as fibroblasts or endothelial cells can also be cultured in close contact with the epithelial cells on the under-surface of the filter insert (Pohl et al., 2010). A commercially available co-culture model that allows direct contact between the differentiated airway epithelium and a fibroblast-containing subepithelial matrix is also available (EpiAirwayFT, MatTek). These co-culture models represent a valuable tool to analyze epithelial-mesenchymal signaling, which is believed to play an important role in airway inflammation and remodeling in asthma. For example, fibroblast activation has been observed

in response to epithelial challenges including physical damage (Malavia et al., 2009; Thompson et al., 2006), viral infection (Tomei et al., 2008) or mechanical strain to simulate the effect of bronchoconstriction on the epithelium (Choe et al., 2003). Similarly, by co-culturing airway epithelial and endothelial cells, it has been shown that cell-cell communication involving soluble endothelial-derived factor(s) causes a significant reduction in paracellular permeability compared to that seen when epithelial or endothelial monolayers are studied in monoculture (Chowdhury et al., 2010).

The complexity of these models can be increased further by combining more than two different cell types, however one critical step in these approaches is optimization of media compatibility, as different cell types have differing requirements for supplements and co-factors and their behavior can be markedly modified by changing culture conditions. Using membrane filter supports, a triple cell culture model of the bronchial epithelium has been developed with airway epithelial cells cultured on the upper surface of the filter support until confluent, followed by introduction of macrophages onto the apical surface and dendritic cells basolaterally (Lehmann et al., 2011; Rothen-Rutishauser et al., 2005). In this model, macrophages and dendritic cells were able to make contact with each other without breaking down the epithelial barrier by expression of tight junction proteins; an exchange of particulates between macrophages and dendritic cells was also possible (Blank et al., 2011). Another triple cell culture model is described by Farcal et al. (2013). The authors established an *in vitro* alveolar barrier by co-culturing three human cell types (epithelial, endothelial and monocytes), and studied the mechanisms of toxicity induced by amorphous silicon dioxide nanoparticles. The induction of a pro-inflammatory state was shown, as evidenced by a high release of TNF-α and IL-8. To specifically study the interaction between epithelium and subepithelial cells, a co-culture model has been developed using bronchial fibroblasts embedded in extracellular matrix overlaid first with dendritic cells, and then with bronchial epithelial cells at the air-liquid interface; this model has been used to show that the structural cells regulate the capacity of dendritic cells to produce the chemokines CCL17, CCL18, and CCL22 (Hoang et al., 2012).

A primary co-culture system to simulate the human alveolar-capillary barrier has been developed using human pulmonary microvascular endothelial cells and primary human ATII cells on opposite sides of a permeable filter support. Within 7-11 days of co-culture, the ATII cells partly transdifferentiated to ATI-like cells, and tight junctions and adherent junctions were evident. The model demonstrated polarized cytokine release and may be useful for studying mechanisms of lung injury in both the epithelial (intra-alveolar) and the endothelial (intravascular) compartments (Hermanns et al., 2009). A triple cell co-culture model composed of primary ATI-like cells isolated from human lung biopsies, macrophages and dendritic cells has also been developed (Lehmann et al., 2011). In this model, epithelial integrity was high (1113 ±30 Ω cm2), demonstrating the potential of the model to offer a novel and more realistic cell co-culture system to study possible cell interactions of inhaled xenobiotics and their toxic potential on the human alveolar type I epithelial wall.

4.4 Chip technology

Currently, substantial effort is being invested into the development of new devices and multicellular 3D co-culture models (Andersen et al., 2014) to study airway epithelial barrier functions *in vitro* using tissue engineering approaches. Since airway epithelial cells are cultured at an ALI, alternative methods have been developed to monitor barrier functions instead of using chop-stick electrodes that require the cultures to be submerged, allowing electrical contact to be made across the epithelial layer. By using electrical impedance, the electrical barrier properties of epithelial cells can be monitored continuously without submerging the cultures (Sun et al., 2010). A more advanced model of the pulmonary epithelium is the so called "lung-on-a-chip" model, a device with alveolar epithelial cells on the apical and endothelial cells on the basolateral side of a porous membrane (Huh et al., 2010; Stucki et al., 2015). In this model, the cells are supplied with nutrition via microfluidic flow of medium which also allows introduction of immune cells into the model. Additionally, the device is built of flexible materials, which allow controlled stretching of the cells on the membrane to simulate the cyclical stretch of the lung tissue during inhalation and exhalation. Other models have used tissue engineered matrices to support airway cells in a three-dimensional tissue equivalent (Choe et al., 2006). Using biocompatible hydrogels as an ECM analogue offers the advantage of modulating the ECM stiffness, which is of interest in asthma remodeling processes. For example, it has been shown that increased ECM stiffness induces fibroblast differentiation and results in increased expression of α-smooth muscle actin (Huang et al., 2012).

4.5 *In silico* modeling

Computer modeling has also long been used to study kinetics of absorption in the lung. Underpinning this work is the computer modeling of gaseous exchange in the lung and in the body (Conway, 1986; Joyce et al., 1993), and an understanding of the detoxification of toxins by the lung (Evelo et al., 1993). Computer modeling and multimodality medical imaging have also been used to study the three-dimensional spatial distribution of a metered dose inhaler and two nebulizers within the lung of humans (Fleming et al., 1996). In more recent times, an emphasis on population pharmacokinetic modeling has been seen with respect to the assessment of the absorption of pulmonary-directed active compounds (Bartels et al., 2013); three-dimensional imaging of particle distribution in and clearance from the lung also constitutes an area of recent interest (Burgreen et al., 2010; Longest et al., 2012; Sturm, 2013). This work is complemented by somewhat simpler compartmental models (Weber and Hochhaus, 2013). There are also a limited number of *in silico* quantitative structure-activity relationships to predict lung retention of solutes after inhalation (Cooper et al., 2010), or chemical sensitization of the lung (Mekenyan et al., 2014).

While the presented material both within this chapter in general as well as in the current section has focused on epithelial barrier function in the lung, there is a need to understand how this cellular function fits into the integrated function of the pulmonary system. To date, relatively little attention has been paid to modeling at the cellular or subcellular level in the lung, or to

link information from the protein structure/interaction and cellular levels to the operation of the whole lung (Burrowes et al., 2008). Multi-scale modeling provides a powerful approach to investigate interdependent processes that span many orders of magnitude. For example, the interactions responsible for epithelial injury during airway reopening are fundamentally multiscale, since air-liquid interfacial dynamics affect global lung mechanics, while surface tension forces operate at the molecular and cellular scales. Consequently, a combination of computational and experimental techniques is being used to elucidate the mechanisms of surface-tension induced lung injury (Ghadiali and Gaver, 2008). Computational approaches have also been applied to toxicodynamic modeling of silver and carbon nanoparticle effects on mouse lung function (Mukherjee et al., 2013) and to uncover underlying mechanisms of disease, such as emphysema (Suki and Parameswaran, 2014).

4.6 Conclusion

In vitro cultures of pulmonary cells can be used for a wide range of scientific and regulatory studies. Even simple models with one cell type are of predictive value for many studies; however rapid advances in tissue engineering approaches will enable development of more complex models that better reflect the *in vivo* situation. Careful phenotyping of cells (passage number, calibration of barrier properties, molecular markers) and donors (age, clinical characterization, smoking history) is essential, as is standardization of methodologies to achieve reproducibility of results between laboratories. Access to tissue banks and/or stem cell technologies will significantly advance the field (Cozens et al., 1992; Franks et al., 2008; Gruenert et al., 1988; Halldorsson et al., 2007).

5 Regulatory considerations regarding non-animal models of biological barriers

In vitro biological barrier models have been used mainly in the pharmaceutical sector, and as yet have not been fully exploited and developed to generate information used for chemical risk assessment. When moving from the classical toxicological safety assessment based on animal models to approaches using alternative methods (*in vitro* and *in silico*), toxicokinetics becomes a key element to assess systemic effects (Bessems et al., 2014; Schroeder et al., 2011). This is particularly relevant for the cosmetics sector in which an animal-free human risk assessment is mandated in the EU (EU, 2009a); however, it is also important for other sectors (industrial chemicals, biocides, plant protection products) in which there is an increasing need for the use of non-animal based methods for compound safety assessment. Passage of chemicals through biological barriers such as intestine, skin and lung constitutes the first ADME (i.e., absorption) process and is the prerequisite for all direct systemic toxicities. The rate of absorption will vary depending on the type of chemical under evaluation, e.g., pharmaceuticals which are designed to be absorbed versus industrial chemicals which are not. In this regard, knowledge about absorption is very important for proper development of intelligent and integrated testing strategies for priority setting (Marquart et al., 2012). It can also serve for risk assessment, such as route-to-route extrapolation, interspecies extrapolation, and internal exposure-based waiving, as outlined in the EURL ECVAM strategy for achieving 3Rs impact in the assessment of toxicokinetics and systemic toxicity (Bessems et al., 2015).

5.1 Skin

5.1.1 Acceptance

Of all non-animal barrier models, the *ex vivo* test for dermal absorption using human skin has probably found the widest regulatory acceptance. Today, this method is considered by many authorities as stage 2 in a tiered testing strategy, triggered if the worst case default assumptions (stage 1) do not lead to a satisfactory assessment (Niemann et al., 2013). We could substantiate this observation by analyzing the type of information on dermal absorption that applicants submitted to the German Federal Institute for Risk Assessment (BfR) in order to satisfy data requirements of pesticide and biocide regulations (source: eASB, internal study data base; date accessed: 12/03/2014). Of all studies with a report date in 1993, i.e., for which the study report was finalized in 1993 and which were – accordingly – performed in the month before, 47% used an *ex vivo* method and 41% were performed *in vivo* in animals. A similar picture was obtained for the report date in 2003 with, again, 47% *ex vivo* and 46% *in vivo* in animals. This ratio changed dramatically in the years following the publication of the OECD test guideline 428 in 2004 (OECD, 2004a) and the accompanying guidance document (OECD, 2004b). For report dates in 2010, 2011, 2012 and 2013, remarkable percentages of 84, 71, 67 and 85% of *ex vivo* studies, respectively, were counted, compared to 7-15% of *in vivo* studies. Interestingly, there also seemed to be a tendency towards submission of more non-testing data (21% in 2011/2012).

Similarly, alternative tests are well established when testing for skin/eye irritation and corrosion under REACH. Meanwhile the combination of OECD TG 439 (*in vitro* skin irritation) and OECD TG 431 (*in vitro* skin corrosion) allow the classification of corrosive and irritant substances, as well as the identification of substances that do not require classification and labeling. With regards to activated carcinogens, the use of metabolically competent skin models in conjunction with the Comet assays is currently pre-validated for regulatory suitability in an ongoing collaboration between Cosmetics Europe and the partners of a project funded by the German Federal Ministry of Education.

5.1.2 Needs

In reality, exposure to a certain drug or chemical via the skin occurs over a wide range of conditions that differ with regard to factors known to influence the extent to which the substance becomes systemically available. Examples include the volume/mass per surface area, the concentration and the vehicle in which the substance is present, the anatomical site, the duration of exposure, etc. The resulting, large number of exposure scenarios contrasts with the standardized conditions under which *ex vivo* (as well as *in vivo*) skin penetration testing is performed.

Therefore, the development – and ultimately validation as a prerequisite for regulatory acceptance – of mathematical models would be desirable to improve extrapolation from testing conditions to the exposure scenario, thus enhancing the overall utility of the method.

5.1.3 *In silico* models – SAR/QSARs

Although a large number of QSARs have been developed for the prediction of dermal absorption, their use is rarely accepted in regulatory frameworks. According to the review of an OECD working group, the reason for this situation is not solely the lack of stringent validation, small training and testing data sets or the limited chemical space covered by the model, but, in part, also of a technical nature. For example, many QSARs do not take into account factors known to influence the extent of dermal absorption such as the concentration of a substance in its vehicle, co-formulants or solvents used, or the amount applied (OECD, 2011). Furthermore, the currently available QSARs only predict Kp values, which are not the relevant endpoint in areas such as risk assessment of plant protection products. In the context of the COSMOS (Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of Cosmetics to Optimize Safety) project, funded by the Seventh Framework Program of the European Commission, Gajewska et al. have reported a strategy for the oral-to-dermal extrapolation of toxicity data based on PBTK modeling using three cosmetic ingredients (coumarin, hydroquinone and caffeine) as case study compounds. The authors evaluated the role of quantitative structure-property relationships (QSPR) as predictors for skin penetration and concluded that QSPR provided a reliable alternative to *in vivo* dermal penetration experiments (Gajewska et al., 2014). With respect to exposure by potential skin penetration, mathematical modeling has already been successfully established in parallel to newly developed *in vitro* systems, with the most recent models also accounting for the three-dimensional microstructures of the skin (Tralau et al., 2015). Such models have been successfully established in the R&D environment; however, translation into regulatory practice is still needed.

5.2 Intestine

5.2.1 Acceptance

EURL-ECVAM has been involved in studies of intestinal barrier models (Le Ferrec et al., 2001; Zucco et al., 2005) and, in particular, an ECVAM pre-validation study on *in vitro* models for the prediction of gastro-intestinal absorption was finalized in 2008 (Prieto et al., 2010). With respect to the oral route, the non-animal models available (e.g., Caco-2, TC7 clone) have been used for screening purposes for many years, mainly by the pharmaceutical industry as mentioned above. However, the models have not been properly evaluated for compounds with relatively poor absorption (e.g., below 30% within a short time period). Within the framework of the EU ACuteTox project, which aimed to develop a non-animal testing strategy to predict acute oral toxicity of chemicals in humans, the suitability of the Caco-2 cell line and the TC7 clone for prediction of intestinal transport of selected drugs and chemicals was investigated (Prieto et al., 2013; Turco et al., 2011). A number of difficulties were encountered in such investigations: Caco-2 permeability values were not able to be obtained for 44% out of 32 tested chemicals due to poor or no detection, lack of stability, and/or lack of available analytical methods (Prieto et al., 2013). Several compounds (1,2-benzanthracene, phenantrene, hexa-, penta- and tetra-chlorobenzene) were excluded from the performed transport experiments due to their high lipophilicity, which created problems with respect to solubility and non-specific surface adsorption (Turco et al., 2011).

5.2.2 Needs

The problem highlighted above is particularly relevant in the case of industrial chemicals, as these are frequently lipophilic. Additional efforts are therefore needed to change the conditions of permeability assays to allow for analysis of lipophilic compounds in such application areas. A suitable strategy to measure compound-specific biokinetics (including chemical and metabolic stability, lipophilicity, adsorption to plastics, binding to protein) should also be considered as this information would prove helpful in data interpretation (Broeders et al., 2012). Moreover, there is an urgent need to optimize biobarrier models with respect to chemical applicability domains (not only drug-like chemicals) as well as with respect to the biological response domain in the sense that they should be able to quantify extremely low, low and medium membrane permeability and not only high permeability as holds for the Caco-2 cell line.

As available non-animal biobarrier models may have different barrier properties (e.g., transporter expression and level of metabolism) in comparison to their *in vivo* counterparts, it is necessary to thoroughly characterize such models in terms of metabolic competence and activity of specific transporters, in order to determine possible effects on absorption as well as correlation with the *in vivo* situation. By knowing the differences between *in vitro* and *in vivo*, the performance of the cellular model could be improved by treating cells with known inducers/inhibitors, or by using complementary *in silico* tools (Turco et al., 2011). The key parameters of a particular biobarrier model critical for each application setting also have to be established. In general, it would be important to identify a set of chemicals with reliable human data to calibrate the model under evaluation. It would also be important to have different sets of calibrators for different sets of reference chemicals with particular physicochemical properties (e.g., industrial chemicals, pharmaceuticals, nanoparticles and also for ranges of physicochemical properties in general: MW, log $K_{o/w}$, pKa, pKb, number of H-donors and H-acceptors, etc.). The performance of different barrier models with different levels of integration (e.g., PAMPA versus human cell models versus tissue) also needs to be compared.

The biobarrier models should also be optimized if necessary to enable the delivery of information that can be used ultimately in the risk management and risk assessment context. Approaches developed for this purpose should ideally have a clear implementation domain and be designed for straightforward application in order to keep regulators on board. There might be scientific support that the chemical under investigation will not be subject to any active transport as the chemical does not fit the structural requirements for being a transporter sub-

strate (e.g., based on SAR tools that also need to be developed). If so, then simple PAMPA models may by sufficient and the use of a Caco-2 model or similar superfluous. This also holds for the reciprocal situation. There is also ultimately a need for establishment of a model that mimics not only the cell components of the intestine, but also the presence and impact of the commensal microbial population. The diverse commensal microbiota harbors a genome a 100-fold the size of its host, providing genes for essential functions that the human host is incapable of performing, such as vitamin production and metabolism of indigestible dietary polysaccharides.

5.3 Lung

5.3.1 Acceptance

The use of *in vitro* lung models has been mainly driven by the pharmaceutical industry. As such, tobacco and chemical industries are now investing considerable effort into the evaluation and adoption of novel predictive *in vitro* models for lung toxicity assessment. Among the panel of tests used, *in vitro* evaluation of xenobiotic permeability is an important piece of information in an integrated testing strategy in order to give reliable information regarding potential (non)-toxicity upon breathing. However, as most of the available models to assess respiratory and pulmonary absorption are still under development, no model has yet found regulatory acceptance.

5.3.2 Needs

Pulmonary absorption is an area that needs further consideration since no methods that have reached an appreciable level of regulatory acceptance are readily available (Adler et al., 2011; Bessems et al., 2014). EURL-ECVAM has done some preliminary investigations on the MucilAir™ *in vitro* human 3D airway epithelium model to assess the inhalation absorption of chemicals; MatTek's EpiAirway model has also been successfully utilized in several drug delivery applications (Agu et al., 2006; Leonard et al., 2007, 2005). Other pulmonary models, such as the Calu-3 human bronchial epithelial cell line grown on permeable supports at the air interface, and the human alveolar barrier have been used to evaluate *in vitro* pulmonary toxicity (Farcal et al., 2013; Forti et al., 2011). In this area, the use of more appropriate culturing conditions such as air liquid interface culture and the way the cells are exposed should be carefully considered, in particular when the tested compound is volatile or insoluble, e.g., (nano)particles (Rach et al., 2014; Adamson et al., 2013). Ongoing efforts worldwide aim to investigate the application of biobarrier models to study the *in vitro* absorption of nanomaterials for safety testing (Ahlberg et al., 2014).

6 Overall Conclusions/Outlook

As the conducted workshop and this resulting report have hopefully revealed, non-animal models of epithelial barriers have reached impressive levels of technical and scientific sophistication. Also, their implementation by various industries has made considerable progress. While there obviously is considerable interest in such models in each of the cosmetic/chemical, drug and food industries, it would be intriguing to conduct an analysis into why the state of the art, the choice (skin, intestine or lung) and the level of model implementation are rather different across these three industries.

It is well known that the major drivers for changes in an industrial context are laws, money and time. In the case of the cosmetic industry, efforts to establish alternative testing models have been driven considerably by the introduction of the Cosmetics Regulation (EU, 2009a), which banned animal experiments and literally forced this sector to investigate (and to invest!) in alternative testing methods; likewise, the REACH legislation with its aim to reduce live animal testing has been proven to be a driver for the development and regulatory application of alternative testing approaches (EU, 2006; Hartung, 2010; Tralau et al., 2015). Together with the fact that there had already been quite some progress in reconstructing skin equivalents as an alternative to native human skin transplants for treating severe burns, this has led to the advanced status of non-animal models for safety testing of cosmetics and other topically applied chemicals. Similarly, for other toxicological endpoints (e.g., skin sensitization, phototoxicity), methods validated by, e.g., EURL ECVAM and accepted at an international level (e.g., OECD TGs) are in place. Besides the cosmetics sector, the concept of the 3Rs (replacement, reduction and refinement of animal use) is present in other relevant EU legislation.

In the case of the pharmaceutical industry, the situation is quite different (Rovida et al., 2015). Firstly, testing the safety and efficacy of drugs and related products before entering first clinical trials in man is not only ethically accepted, but even legally requested. For this reason, preclinical R&D – either in an industrial or academic context – is typically performed using animals. A substantial part of serious R&D efforts is even made to develop new animal models, especially in the context of certain diseases such as cancer, infection or neurodegenerative processes. The expectation in these models is to correctly predict both benign and adverse effects of novel therapeutic modalities when applied to humans. While it cannot be denied that animal models have made a major impact on the development of new drugs since the beginnings of the pharmaceutical industry and rational drug research, there is also increasing evidence that results observed in animal models cannot always be directly translated to humans – a fact that has already caused a number of major setbacks (Hartung, 2013).

The need to overcome the intrinsic limitations of any non-human animal model has perhaps been the major driver in pharma to look for human-based – and thus non-animal – *in vitro* testing alternatives. In addition, working with human cells and tissues avoids the "black-box effect" of whole animal experiments. Such an effect dictates that, while it appears very convincing and intuitive when a given treatment shows an improvement of characteristic symptoms, a prolongation of lifespan or even a curative effect in a particular animal disease model, in the case of a negative outcome of such experiments, it is often not easy (and at times, may even be impossible) to identify the reason for the failure or to suggest rational improvements to either the treatment itself or to the testing procedure. This is particularly

true for research on novel systems and technologies for improving delivery of APIs across biological barriers, especially when the barrier in question is not easily accessible (as in the case of the intestinal and respiratory mucosa). The pharmaceutical industry has of course noticed and taken advantage of existing skin models – the implementation of *in vitro* skin models for the development of transdermal or dermal drug products is in fact rather routine, and the obvious advantages of such models have initiated and continue to drive further model research and development considerably, as seen in this review.

As regards oral drug delivery, which is still by far the most preferred route of administering a drug to a patient, the introduction of the Caco-2 cell line as a model of the intestinal mucosa in the late 1980s (Hidalgo et al., 1989) sparked a virtual revolution within the pharmaceutical industry. Optimizing permeability in contrast to receptor affinity according to "Lipinski's rule of five" (Lipinski et al., 1997) has become a well-accepted new paradigm in drug discovery for medicinal chemists; such *in vitro* approaches are also widely used in pharma for the approval of generics, in conjunction with the BCS as pioneered by Amidon (Amidon et al., 1995), where so-called "biowaivers" (regulatory exemptions from expensive and also ethically sensitive bioavailability studies on healthy humans) can be obtained under certain conditions. Specialized companies have even been founded based on *in vitro* expertise and have established themselves as "*in vitro* CROs" (Goldberg and Hartung, 2008), while numerous research activities both in industry and academia are currently directed towards better and more complex (e.g., disease-relevant) *in vitro* models of the intestinal mucosa.

It is interesting to note (as alluded to above) that the major driving force for implementation of non-animal models in the pharmaceutical industry was the clear expectation that utilization of *in vitro* tools would help to accelerate the translation of new candidate drugs into clinical trials, and to reduce candidate attrition. The same is likely also true for the food industry, which has of course witnessed the ongoing development and level of implementation of *in vitro* models in cosmetics and pharma. Despite the lack of confinements conferred by a strictly regulated registration process (as for pharmaceutical products) and the absence of a ban on animal experiments (as for cosmetic products), the use of intestinal *in vitro* models in particular must remain of interest in the food industry, offering possibilities to investigate and demonstrate the advantages of new diets and food processing technologies.

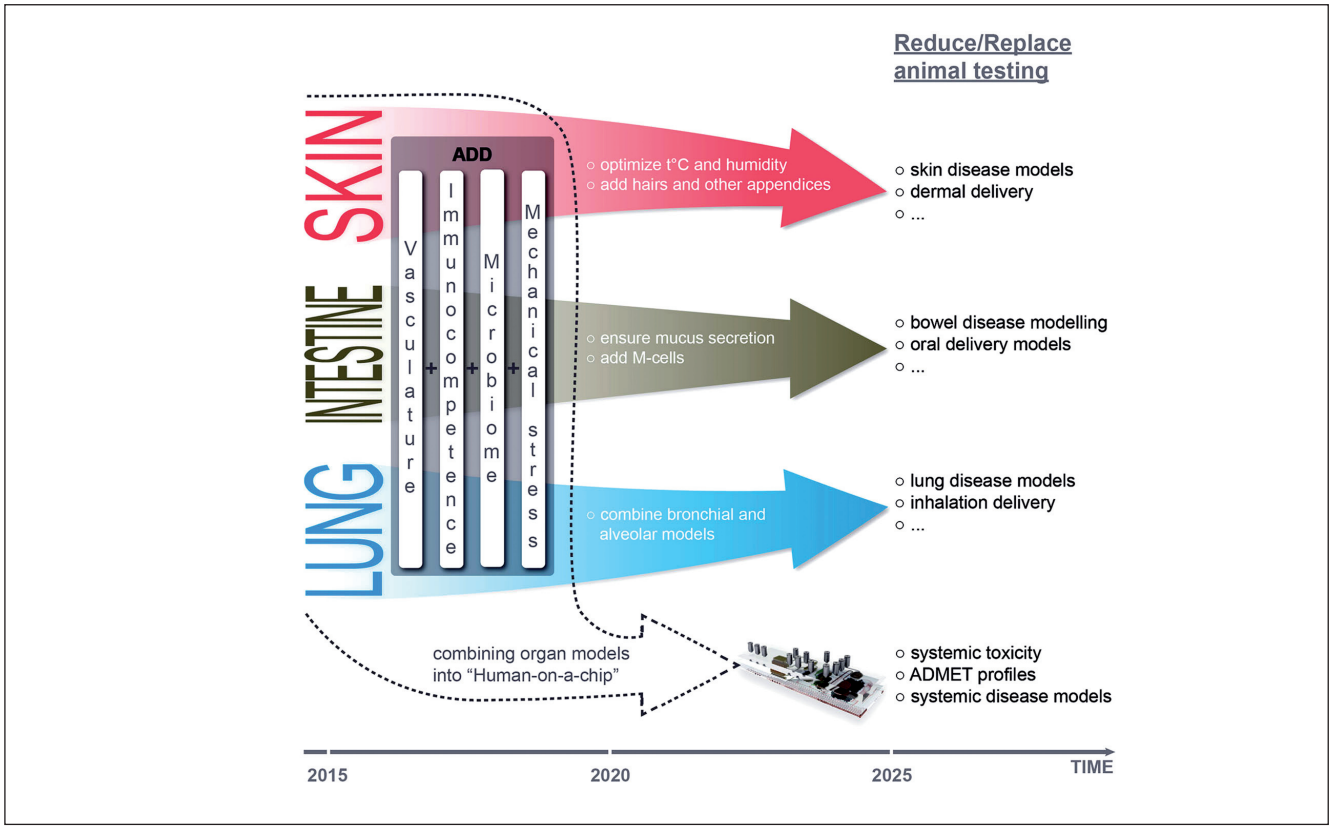

Fig. 3: Future outlook

The next hurdles to overcome in order to achieve significant reduction and replacement of animal testing are the integration of vasculature, immunocompetence, microbiome, and physiological mechanical forces into each barrier model. With specific respect to the skin, models with improved barrier function also are required. Combined with organ-specific optimization, this opens the floodgates for replacing human disease modeling and delivery testing methods in animals. In parallel, these developments will strongly support the multi-organ "human-on-a-chip" concepts aiming to replace systemic animal testing.

With respect to the lung, one can reasonably conclude that the state of the art for models of the so-called "air-blood barrier" is less advanced in comparison to intestinal or skin models. The reason for this is largely the anatomical complexity of the pulmonary tissue. The sponge-like nature of the mammalian lung structure conferred by the millions of alveoli makes working with native *ex vivo* preparations much more challenging than skin or intestinal tissue, which exhibit a comparatively simpler sheet- or tube-like structure. Useful *in vitro* models of the bronchial and alveolar epithelium are therefore generally only accessible by reconstituting them, which requires a rather high level of expertise in cell and tissue culture. Difficulties aside, there is certainly an interest in such models, for example from the chemical industry in the context of novel nanomaterials which can easily reach even the deep lung after unintended exposure. Some – still emerging – interest also comes from pharma, along with the realization that inhaled aerosol medicines cannot only be used for the treatment of pulmonary diseases like asthma or COPD ("air-lung delivery"), but also for the non-invasive delivery of macromolecular biopharmaceuticals for the treatment of systemic diseases. A case in point for the latter is the approval of aerosol formulations of insulin for diabetes therapy (Leone-Bay et al., 2010). Progress in the fields of microfluidics and lab-on-a-chip technologies continue to counteract the difficulties associated with lung *in vitro* model development, and the accompanying ever-improving approximations of complex lung anatomy as well as the dynamic processes of blood perfusion and ventilation promise some interesting new developments in the next few years.

At this stage, it must also be questioned what still needs to be done in the field of non-animal epithelial barrier models, and also where possible limits might be encountered (Fig. 3). Besides the work on models of single barriers, there is clearly also an incentive to combine barrier models in an integrative way with other organs and compartments of the human body (e.g., liver, heart, brain). This would allow the study of various routes of exposure/administration simultaneously and in combination with physiological PK/PD models that also include metabolism, elimination and pharmacological effects at the target site. While such developments appear at least in reach, building in a further level of complexity by integration of immune system components into such models appears rather far away. And yet, given the significant role of the immune system in facilitating uptake and disposition (not only of vaccine candidates) across biological barriers, together with its potential to impact on the intrinsic function of the barrier itself in states of disease or abnormality, development of models which allow for simultaneous assessment of barrier penetration and immune cell function would offer considerable advantages. Even further evolution of barrier models could involve incorporation of the vascular system or elements enabling a faithful reconstruction of a true neuro-immune-endocrine synapsis (Dustin, 2012), or representation of the host microbiome, which has been suggested to play a central role in the host's immunological processes, metabolism, cellular differentiation and endocrine function (Belkaid and Hand, 2014; Oh et al., 2014; Tralau et al., 2014). To what extent miniaturized and more complete models of the human body ("Human-on-a-chip") might indeed be practical and useful also remains to be seen once the first prototypes of such sophisticated systems become available. Indeed, the issues of practicality and usefulness must always be considered in any enterprises aimed at increasing model complexity. A cost-benefit analysis must always be conducted, involving careful consideration of the extent to which it is worth to invest considerable resources to obtain the "perfect model" – or whether implementation of rather more robust and simple models, which additionally may find greater acceptance with industry and regulators, would be of greater value.

Specific mention should also be made here with respect to the future development of *in silico* models of epithelial transport, which, to date, have not evolved sufficiently to be acceptable from a regulatory point of view (noting, however, that the FDA has in progress a number of studies that focus on models of various epithelia, with the aim of exploring how such models may inform the regulatory process). One of the main issues that continue to haunt existing *in silico* modeling is that many models, especially early models, are mainly empirical and/or statistical in their nature. Another drawback is that many models are limited to either a single or low number of biophysical and biochemical processes of epithelia, while the efficacy and safety of epithelia exposure often involves multiple biophysical and metabolic processes. The main future challenge of *in silico* modelling is therefore to develop mechanistic multi-scale and multi-process models capable of predicting the whole biophysical and biochemical events of epithelia from source exposure to endpoint outcome. Indeed, this is the recent trend in epithelia modelling, from molecular dynamics of sub-cellular disposition to systems biology of metabolic pathways.

Finally, the development of non-animal models is affected by a certain dilemma, which arises from the fact that – in contrast to the discovery and creation of novel models – it is not so attractive for academics to validate such models in detail (Hartung, 2007), as validation data alone are difficult to publish in high-ranking scientific journals. On the other hand, regulatory bodies cannot accept any new models until they are properly validated. Obviously, academia, regulators and industry must therefore collaborate in this respect, and the necessary funding to finance such work must also be made available.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

D. E. Davies is supported by grants from Medical Research Council (UK), Biotechnology and Biological Sciences Research Council, British Lung Foundation, Asthma, Allergy and Inflammation Research (AAIR) Charity, National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and National Institute for Health Research (UK).

Correspondence to

Claus-Michael Lehr, PhD
Head, Dept. of Drug Delivery (DDEL)
Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS)
Helmholtz Center for Infection Research (HZI)
Saarland University, Campus E 8
D-66123 Saarbrücken, Germany
e-mail: claus-michael.lehr@helmholtz-hzi.de