Handbook of Experimental Pharmacology 265

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Organotypic Models in Drug Development

Springer

Handbook of Experimental Pharmacology

Volume 265

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Organotypic Models in Drug Development

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ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-030-70062-1 ISBN 978-3-030-70063-8 (eBook)
https://doi.org/10.1007/978-3-030-70063-8

Preface

Drugs have to be both efficient and safe. In the drug development process, comprehensive testing in animals is currently preceding first-in-human studies. The European Union reported the use of around 9.39 Mio animals in 2017 (latest report, released on the 5th of February 2020, covering the period 2015–2017), with basic biomedical research and drug development as the dominating purposes. Only agents found to be active and safe in preclinical (animal) testing proceed to clinical trials. Since rational drug development is based on detailed insights into the pathophysiology, basic biomedical research and drug development are inseparably connected. Yet, currently 66% (vaccines for infectious diseases) to 96.6% (antitumor drugs) of the drug candidates introduced into clinical testing fail, most often because of a lack of efficacy or due to inacceptable adverse drug reactions. The extreme attrition rate of drug candidates and the high cost of, e.g., improvements in cancer treatment must be rated against the particularly high number of animals used for this purpose. Drug candidates for other indications perform better to some extent. In light of this, there is a major need for translatable preclinical studies and disease models in order to save time and costs – as well as to reduce human exposure to failing agents and to avoid preventable animal suffering.

This book summarizes the current state of knowledge on "alternative testing" which means in particular preclinical or nonclinical studies on cell- and tissue-cultures. Well-defined cell-lines that reduce variability are an option in the preselection process. The use of primary cells of human origin also avoids the species gap and allows to introduce human heterogeneity into the test system by using cells of several donors. Human-induced pluripotent stem cells (hIPS cells) are expected to provide a major step forward. Generated from donors with hereditary diseases, hIPS appear to generate very precise results – yet being juvenile, hIPS cells may be less suitable for diseases of the elderly. In addition to human cells, the use of scaffold biomaterials, the exposure of the cultures to nutrients and signaling molecules, and the removal of waste products are of major relevance for disease model building in order to adequately reflect the target tissue in man. The mode of drug exposure to the skin model requires equally careful consideration.

This handbook outlines the benefits and limitations of current approaches and describes the frontier of knowledge with a strong focus on the testing of effects of drugs on the human skin. Historically, alternative testing for skin effects has been

established early – due to the harsh criticism of testing cosmetics in animals. Subsequently, additional organ-specific models were introduced and are presented in chapters of this book, including engineered heart, lung, and gut – all of them both healthy and diseased – which are highly needed in preclinical drug research. Because of the major relevance of the blood–brain barrier for the access of substances to the brain, a separate chapter is devoted to this topic. Models and methods for testing drug uptake following topical exposure to the lung and skin are described. Because of the very high failure rate of anticancer drugs in clinical studies, two chapters focus on cancer models: One is focusing on approaches to overcome resistance development. Another chapter is presenting a rather complex, though non-human, model for testing of anticancer drugs – the chicken chorio-allantoic membrane (CAM). In fact, the CAM has initially been used in one of the first non-animal testing methods to assess damages on conjunctival tissues.

A major challenge that remains is the inclusion of immune cells which is essential for modeling the large number of immune-driven diseases. Immune-competent models are a topic of the chapter on models of diseases of the gut and the skin. Specific aspects of co-cultures, gene knockdown, bioprinting, and microfluidic models are also addressed. Another chapter describes recent advances in tissue engineering to study tissue regeneration. The use of those human-based models in drug research is quickly becoming more feasible.

The introductory section of the handbook covers important topics of general relevance. Testing of cosmetics and their ingredients on animals is forbidden in the EU since 2013. Because of the resulting major need of the cosmetics industry, several companies started to offer reconstructed human epidermis, in part through automated production. Today, other test matrices are offered, too. The introductory section, therefore, also covers the specific aspects of standardized production and quality assurance of such commercial models. There are a still increasing number of OECD test guidelines for the hazard identification of chemicals using these tissue models. This illustrates the potential of human-based approaches for drug development. Currently, a discussion is initiated, whether patient-derived models may allow a step into precision medicine in order to improve success rates in tumor therapy.

To be included into OECD test guidelines, models and methods need to be validated. In validation ring-trials they have to demonstrate robustness and reproducibility and to prove that the results are in line with previous data generated in animals or humans. Yet, to the best of authors' understanding, the extensive validation effort required by the OECD is not feasible for preclinical drug development. We, therefore, propose an alternative approach to quality assurance which is applicable to the increasing complexity of the models in the early and advanced stages of preclinical research.

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Part I

General Topics – Quality Assurance and Predictability

Human-Derived In Vitro Models Used for Skin Toxicity Testing Under REACh

Susanne N. Kolle and Robert Landsiedel

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Abstract

In regulatory toxicology, in vivo studies are still prevailing, and human-derived in vitro models are mostly used in testing for local toxicity to the skin and the eyes. A single in vitro model may be limited to address one or few molecular or cellular events leading to adverse outcomes. Hence, in many instances their regulatory use involves the combination of several in vitro models to assess the hazard potential of test substance. A so-called defined approach combines different testing methods and a 'data interpretation procedure' to obtain a comprehensive overall assessment which is used for the regulatory hazard classification of the test substance.

Validation is a prerequisite of regulatory acceptance of new testing methods: This chapter provides an overview of the method development from an experi-

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_368

mental method to a test guideline via application of GIVIMP (good in vitro method practice), standardization, validation to the regulatory adoption as an OECD test guidelines. Quandaries associated with the validation towards reference data from in vivo animal studies with limited accuracy and limited human relevance are discussed, as well as uncertainty and limitations arising from restricted applicability and technical and biological variance of the in vitro methods.

This chapter provides an overview of human-derived in vitro models currently adopted as OECD test guidelines: From the first skin corrosion tests utilizing reconstructed human epidermis models (RhE), to models to test for skin irritation, phototoxicity, eye irritation, and skin sensitization. The latter is using a battery of different methods and defined approaches which are still under discussion for their regulatory adoption. They will be a vanguard of future applications of human-derived models in regulatory toxicology. RhEs for testing of genotoxicity and of dermal penetration and absorption, have been developed, underwent validation studies and may soon be adopted for regulatory use; these are included in this chapter.

Keywords

Dermal penetration and absorption · Eye irritation · Genotoxicity · Human-derived model · OECD test guideline · Phototoxicity · Skin corrosion and irritation · Skin sensitization · Uncertainty · Validation

Abbreviations

AOP	Adverse outcome pathway
cat.	Category
DA	Defined approach
DIP	Data interpretation procedure
DPRA	Direct peptide reactivity assay
e.g.	exempli gratia
EC	European Council
ECHA	European Chemicals Agency
EIT	Eye irritation test
et al.	et alii, et aliae
EU	European Union
F/RAND	Fair, reasonable, and nondiscriminatory
FI	Fluorescence intensity
FP	False positives
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GIVIMP	Good in vitro method practice
GLP	Good laboratory practice
h-CLAT	Human cell line activation test
IL	Interleukin

ITS	Integrated testing strategy
KE	Key event
LLNA	Murine local lymph node assay
MIE	Molecular initiating event (KE1)
MPD	Mean peptide depletion
NPV	Negative prediction value
OECD	Organisation for Economic Co-operation and Development
PPV	Positive prediction value
RhCE	Reconstructed human cornea-like epithelium model
RhE	Reconstructed human epidermis model
SCT	Skin corrosion test
SI	Stimulation index
SIT	Skin irritation test
STS	Sequential testing strategy
TG	Test guideline no.
TN	True negatives
TP	True positives
UV	Ultraviolet light

1 Introduction

Traditionally, in toxicology, animal models are used, which require the extrapolation from observations in animals to humans. On the other hand, animal models provide the complexity of a whole organism which is not offered by cell or tissue cultures. Effects on the skin and eye are, however, local and may not involve complex interactions of different organs in the body. Therefore, human-derived in vitro models using cell or tissue cultures could avoid interspecies difference and yet be sufficient to address local effects. Even for local effects, a single in vitro model may be limited to one or few potential effects or biological events leading to adverse effects on human skin; this can be addressed by usage of testing batteries of several in vitro models: Regulatory use of in vitro models usually involves the combination of several models to assess the hazard potential of test substance. This requires a battery of testing methods and a data interpretation procedure (DIP) to combine the outcomes of these tests. A pre-defined set of testing methods and the fitting DIP is called defined approach.

The present chapter provides an overview of regulatorily accepted test methods based on human-derived in vitro models and defined approaches. The validation of testing methods is a prerequisite of their regulatory acceptance. Adopted test methods are available at the OECD's webpage.[1] OECD-adopted test methods are

[1] At https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788.

usually taken up in the Annex to the EU Test Methods Regulation (Regulation (EC) No 440/2008 n.d.). Formal adoption of new methods or changes in existing methods by the OECD test guidelines and subsequently in the Annex to the EU Test Methods Regulation may take some time. However, "the latest version of an adopted test guideline should always be used when generating new data, independently of whether it is published by EU or OECD" (ECHA Endpoint Specific Guidance document. ECHA 2017a). In this chapter, we are referring to OECD test guideline methods.

2 Validation and Regulatory Acceptance

Since the early 2000s, several regulations include non-animal test methods (e.g., the European Chemicals Regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals); (Regulation (EC) No 1907/2006 n.d.)) or even completely rely on in vitro (and other non-animal) testing methods, such as the EU Cosmetics Regulation (Regulation (EC) No 1223/2009 n.d.).

The validation of an in vitro method and its adoption in an OECD test guideline do not inevitably lead to its global regulatory acceptance and use, much less the complete replacement of the respective in vivo study. The regulatory acceptance of (in vitro) test data depends on regional authorities' regulations and may also be sector specific (e.g., different for pharmaceuticals, chemicals, cosmetics, and agrochemical formulations). The differences in regional regulatory needs to address skin sensitization have been exemplified in Daniel et al. (2018), and hurdles in regulatory acceptance of in vitro skin irritation and sensitization methods and use have been described by Sauer et al. (2016) and Eskes (2019).

The so-called mutual acceptance of data (MAD) avoids unnecessary repetition of tests for individual countries. Instead, all OECD member countries accept a study, which was performed according to an OECD test guideline and under good laboratory practice (GLP). Until writing of this chapter (January 2020), the MAD applies to individual test methods only, as there are no adopted guidelines for DA yet (see Sect. 3.4.4 Defined Approaches: Combination of in vitro methods to assess skin sensitization). The lack of mutual acceptance for defined approaches hampers the full regulatory acceptance of data obtained with human-derived in vitro models and hence the replacement of in vivo studies (Sauer et al. 2016); the OECD is currently working on validating and implementing DAs into its test guidelines.

The principles of the modular approach of validation have been described and evolved in several publications (Hartung et al. 2004, OECD guidance document no. 34 (OECD 2005), Zuang et al. 2015). A central part of method validation is the assessment of the method's reliability (i.e., to determine the test's intra- and interlaboratory variability and transferability) and its relevance (i.e., analyzing the test's predictive capacity as well as understanding its applicability domain) (Fig. 1).

The intra- (i.e., within a certain lab) and interlaboratory (i.e., between different labs) reproducibility is typically determined biostatistically using the data generated in ring trial studies with at least three participating labs which are blinded for test substances' identities. The assessment of the predictive capacity of a testing method

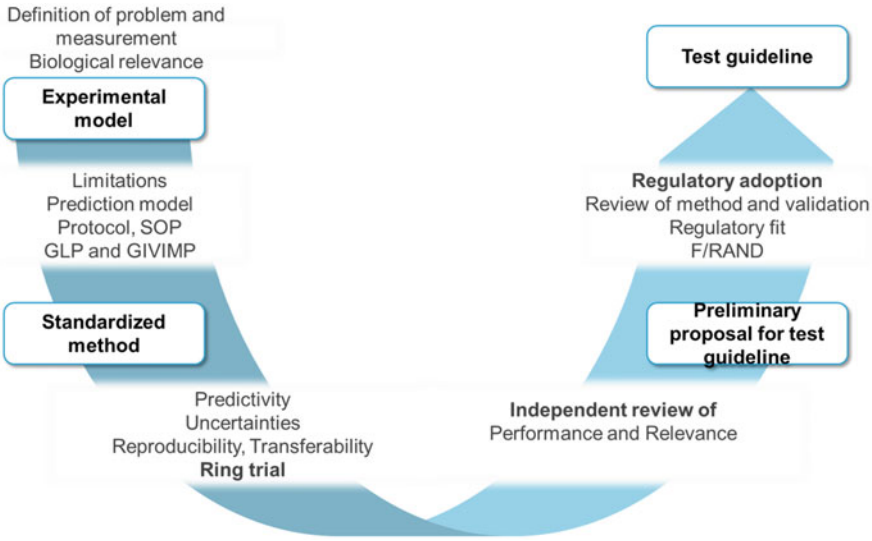

Fig. 1 Method development: From an experimental method to a test guideline via standardization, validation, and regulatory adoption. *F/RAND* fair, reasonable, and nondiscriminatory (ICH 2010; FDA 2011), *GIVIMP* good in vitro method practice (OECD 2018d), *SOP* standard operating procedure

or defined approach requires the testing of substances with well-known reference data (see also subchapter "Reference Data and Validation Sets"). The predictivity of the novel methods is assessed by comparing the results obtained with this method to the reference data. The predicitivity is described by the true positive rate (sensitivity), true negative rate (specificity), and the overall accuracy which are calculated according to Table 1 (Cooper et al. 1979).

It shall be mentioned that this comparison of new in vitro models with traditional in vivo models is questionable: The identification of hazard properties of a test substance and the classification and labelling criteria were defined according to the animal models, e.g., and it is grueling to try to accurately reproduce results defined by the parameters of an in vivo method by in vitro models. Moreover, the reproducibility of in vivo test results is limited, even though the test methods are highly standardized: The reproducibility of the refinement in vivo study, the murine local lymph node assay (OECD test guideline 429, OECD 2010), was found to be 89% (based on 296 test substances) and 73% for seriously eye-damaging findings in the Draize rabbit eye irritation (based on 46 test substances) (Luechtefeld et al. 2016a, b). As early as 1971, Weil and Scala reported on the intra- and interlaboratory variability of rabbit eye and skin irritation tests in 25 different laboratories and concluded that "The all-or-none, irritant or nonirritant, eye or skin rating of the reference samples was determined quite differently in different laboratories" (Weil and Scala 1971). In other words, it is a forlorn task to exactly reproduce the results of imperfect in vivo animal methods by in vitro models. Instead, we should strive for

Table 1 Calculation of a test method's predictive performance (confusion matrix)

		"True" condition		
		Reference data POSITIVE	Reference data NEGATIVE	
Predicted	Test data POSITIVE	TRUE POSITIVE	FALSE POSITIVE (Type I error)	$PPV = \frac{\sum TP}{\sum TP + \sum FP}$
	Test data NEGATIVE	FALSE NEGATIVE (Type II error)	TRUE NEGATIVE	$NPV = \frac{\sum TN}{\sum TN + \sum FN}$
		$Sensitivity = \frac{\sum TP}{\sum TP + \sum FN}$	$Specificity = \frac{\sum TN}{\sum TN + \sum FP}$	
		$Accuracy = \frac{\sum TP + \sum TN}{\sum TP + \sum TN + \sum FP + \sum FN} = \frac{\sum TP + \sum TN}{\sum Reference\ data}$		

With *FN* false negatives, *FP* false positives, *TN* true negatives, *TP* true positives, *PPV* positive prediction value, *NPV* negative prediction value

human relevance and address disturbance of relevant physiological processes in humans.

3 Regulatory-Accepted Human-Derived In Vitro Models

3.1 Skin Irritation and Corrosion

3.1.1 Testing Methods: Reconstructed Human Epidermis (RhE) Used in OECD Test Guidelines No. 431 and 439

The first regulatory-accepted non-animal method using a human-derived model is the in vitro skin corrosion test utilizing *reconstructed human epidermis model (RhE)*. Typically, the RhE are generated from non-transformed human epidermal keratinocytes forming a multilayered, highly differentiated model of the human epidermis. They consist of organized basal, spinous, and granular layers and a multilayered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to that found in vivo resulting in similar biochemical and physiological properties to human epidermis.

The skin corrosion test assay was first adopted by OECD in 2004 as OECD test guideline no. 431 (OECD 2019a). The corresponding skin irritation test was first adopted in 2010 (and revised several times since then) as OECD test guideline no. 439 (OECD 2019b). Skin irritation and corrosion tests using RhE are based on the experience that skin irritant and corrosive substances induce localized trauma as the underlying mechanism of skin irritation in vivo. The RhE-based tests are designed to predict a skin corrosion or irritation potential of a test substance after exposure on a RhE. Testing according to both OECD test guidelines can be conducted with several commercially available tissues (with similar but distinct exposure protocols and prediction models for each of the different models and irritation and corrosion endpoints).

After application of the test material to the *stratum corneum* surface of the reconstructed tissue, the induced cytotoxicity is measured by a colorimetric assay. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by the amount of reduced tetrazolium dye. After isopropanol extraction of the formazan from the tissues, the optical density of the extract is determined spectrophotometrically and compared to negative control values[2] to express relative tissue viability. Test substances reducing viability below certain cutoffs are then identified as skin corrosive or irritant according to the prediction models described in OECD test guidelines no. 431 and 439, respectively.

[2]Positive and negative controls are typically included in all in vitro assays (but not in all traditional in vivo assays) as well as lists of reference substances to demonstrate proficiency in a method ("proficiency chemicals") and a list of substances to demonstrate a novel assay performs similar ("performance standards").

The prediction models of the skin corrosion test according to *OECD test guideline no. 431* have been initially developed and adopted to identify substances not corrosive to the skin and those corrosive to the skin. In the EU, evidence of toxicological effects (at the time of writing this chapter, January 2020, mostly results of animal studies) trigger classification (and labelling) of substances (Regulation (EC) No 1272/2008 n.d.). The classification criteria were agreed at the UN level, the so-called Globally Harmonized System of Classification and Labelling of Chemicals, GHS (United Nations 2007). When toxicological data on a substance meet the classification criteria, the hazards of the substance are identified by assigning a certain hazard category; i.e., a substance is classified in skin corrosion category 1 if "Destruction of skin tissue, namely, visible necrosis through the epidermis and into the dermis, in at least one tested animal after exposure $\leq$ 4 h" is observed in rabbits tested according to OECD test guideline no. 405 (of note, the classification criteria are defined based on results of the animal studies). This can further be subclassified into subcategories 1A, 1B, and 1C.[3] This subcategorization of skin corrosion was initially not addressed by the OECD test guideline no. 431, but corrosives were distinguished from non-corrosives, only. Since the year 2015, the OECD test guideline no. 431 now supports the subcategorization into skin corrosives 1A and a combined 1B/C. Of note, an overprediction rate of approximately 30% for substances identified as UN GHS subcategory 1A actually belonging to subcategories 1B or C has been reported (OECD 2019a). In case subcategorization of the corrosive classes is needed and in particular in cases where UN GHS subcategories 1B and 1C have to be differentiated, the biomembrane-based *Corrositex* assay (OECD test guideline no. 435, OECD 2015a) can be conducted (as this assay is not using a human-derived model, it is not discussed further here).

OECD test guideline no. 439 provides a prediction model to identify substances nonirritant to the skin. In case the test is positive, additional testing is required to provide information whether a substance should be classified as skin irritant (UN GHS category 2) or skin corrosive (UN GHS category 1).

3.1.2 Combination of Methods to Assess Skin Irritation and Skin Corrosion

As can be concluded from the predicted UN GHS categories from OECD test guidelines nos. 431 and 439 and summarized in Fig. 2, in many cases a combination of assays (OECD guidance document no. 203, OECD 2014) is needed to cover the full irritation scale that was covered by the in vivo skin irritation test (OECD test guideline no. 404, OECD 2015b).

[3] Subcategory 1A "Corrosive responses in at least one animal following exposure $\leq$3 min during an observation period $\leq$1 h," Subcategory 1B "Corrosive responses in at least one animal following exposure >3 min and $\leq$ 1 h and observations $\leq$14 days," and Subcategory 1C "Corrosive responses in at least one animal after exposures >1 h and $\leq$ 4 h and observations $\leq$14 days" (ECHA 2017b).

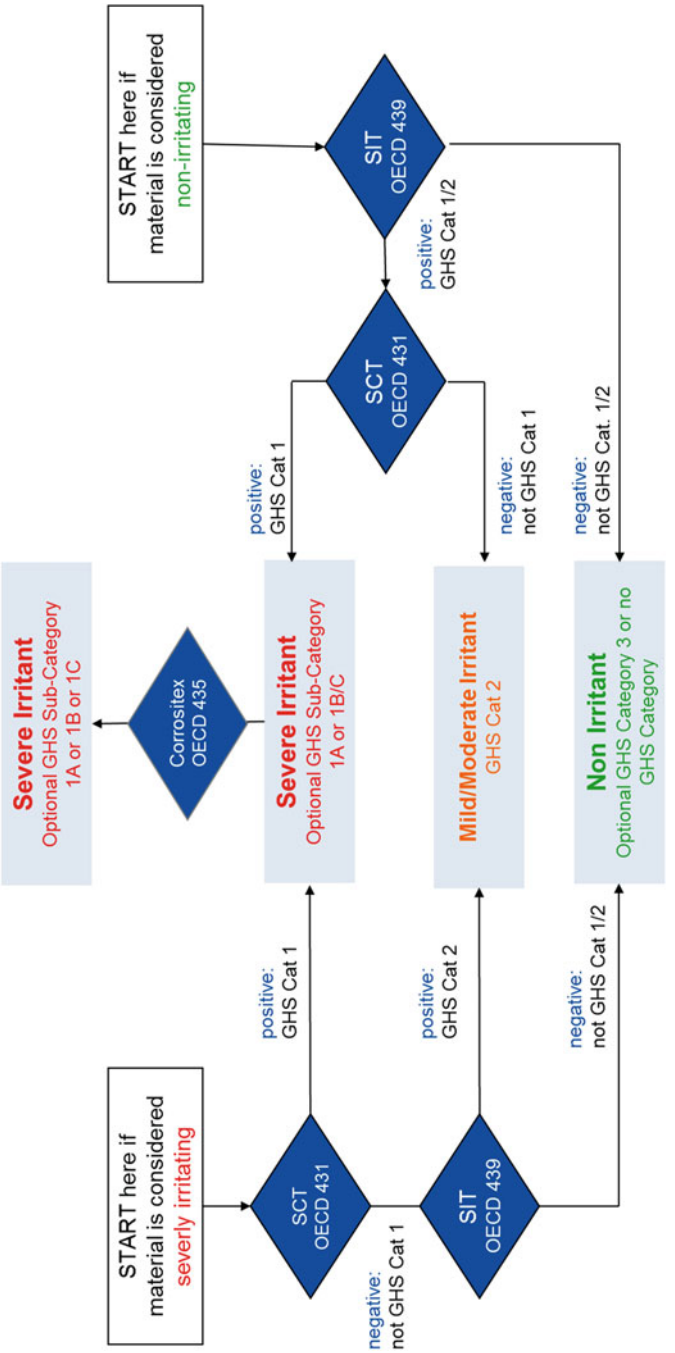

Fig. 2 Combination of in vitro methods to assess skin irritation/corrosion. Depending on the expected effects (severely irritating or nonirritating), the first test conducted is chosen. *Cat* category, *OECD* OECD test guideline, *SCT* skin corrosion test, *SIT* skin irritation test

3.2 Phototoxicity

Substances applied to the skin may form active substances by sunlight irradiation causing phototoxic (irritating) effects. Standardized and internationally harmonized in vitro methods and a tiered testing strategy are available, to test for these effects (Kolle et al. 2018). The 3T3 Neutral Red Uptake (NRU) phototoxicity test method (OECD test guideline no. 432, OECD 2019i) is using a mouse fibroblast line (BALB/3T3). Human-derived methods using RhE were developed and successfully pre-validated for phototoxicity assessment (Liebsch et al. 1999) and have been added to the OECD work plan in 2019. Both models were found to be overpredictive (Jirova et al. 2007), and today testing is usually performed according to a tiered testing strategy including light absorption, photoreactivity (formation of reactive oxygen species, ROS; *OECD test guideline no. 495*, OECD 2019j) of the test substance, as well as its distribution to the human skin (ICH 2013).

3.3 Eye Irritation

3.3.1 Testing Methods: Reconstructed Human Cornea-Like Epithelium Models (RhE) Used in OECD Test Guideline No. 492

The eye irritation test (EIT) based on *reconstructed human cornea-like epithelium models (RhCE)* was first adopted as *OECD test guideline no. 492* in 2015 (OECD 2019c). The RhCE tissue models are three-dimensional, non-keratinized tissue constructs composed of normal human-derived epidermal keratinocytes used to model the human corneal epithelium. RhCE have similar biochemical and physiological properties to human cornea epithelium.

After application of the test material to the surface of the RhCE, the induced cytotoxicity (= loss of viability, specifically of mitochondrial dehydrogenase activity) is measured by a colorimetric assay. Test substances that do not reduce viability below certain cutoffs are then identified as nonirritant to the eye according to the prediction model described in OECD test guideline no. 492. OECD test guideline no. 492 can be conducted with several commercially available tissues (with similar but distinct exposure protocols and prediction models for each of the different models and irritation and corrosion endpoints; OECD 2019c).

3.3.2 Testing Methods: Immortalized Corneal Epithelial Cells Used in OECD Test Guideline No. 494

In the *Vitrigel*-EIT, immortalized corneal epithelial cells are fabricated in a collagen *Vitrigel* membrane chamber. In this assay the time-dependent change in transepithelial electrical resistance is used to monitor the disruption of the barrier function. The *Vitrigel*-EIT assay has been adopted as *OECD test guideline no. 494* in 2019 for the identification of ocular nonirritants and seriously eye-damaging substances (UN GHS category 1) (OECD test guideline no. 494, OECD 2019d).

3.3.3 Defined Approaches: Combination of Methods to Assess Eye Irritation and Serious Eye Damage

In 2010 the concept of top-down and bottom-up approaches has been described for eye irritation (Scott et al. 2010) for the replacement of the in vivo eye irritation test (OECD test guideline no. 405, OECD 2017a). Like with skin irritation and corrosion testing, the first test to be conducted is selected based on the expected ocular irritant potential (Fig. 3, OECD guidance document no. 263; OECD 2019e). Both human-derived eye irritation test methods presented above could be employed to identify ocular nonirritants, while at least an additional method is needed to identify UN GHS Cat 1 seriously eye-damaging substances. Meanwhile several in vitro methods have been adopted to identify seriously eye-damaging substances (UN GHS category 1) by the OECD: the bovine corneal opacity and permeability test using bovine corneas (OECD test guideline no. 437 (OECD 2017b)), the isolated chicken eye test using chicken eyes (OECD test guideline no. 438 (OECD 2018c)), the fluorescein leakage test method using Madin-Darby canine kidney cells (OECD test guideline no. 460 (OECD 2017c)), the short-term exposure test method using *Statens Serum Institut* rabbit cornea cells (OECD test guideline no. 491 (OECD 2018e)), or the Ocular Irritection test method using a complex macromolecular matrix (OECD test guideline no. 496 (OECD 2019k)). As none of these assays is using a human-derived model, they are not discussed further here. Two defined approaches based on in vitro bottom-up approaches combined with physiochemical properties for ocular toxicity have been added to the OECD work plan in 2019.

3.4 Skin Sensitization

The underlying mechanism of skin sensitization is quite well understood and has been broken down into an adverse outcome pathway (OECD 2012a, b). Three of the key events can be assessed experimentally using non-animal methods (OECD 2018a, b, 2019f). Chemical reactivity has been shown to be well associated with allergenic potency and has been described as the molecular initiating event in the adverse outcome pathway. As a second key event of the skin sensitization adverse outcome pathway, keratinocytes must be activated to induce essential ("danger") signalling molecules. The third key event is the activation of the skin dendritic cells as antigen-presenting cells must upregulate cell surface markers to interact with T cells.

3.4.1 Testing Methods: Synthetic Peptides Used in OECD Test Guideline No. 442C

In the direct peptide reactivity assay (DPRA), the reactivity of a test substance towards synthetic cysteine- and lysine-containing peptides is addressed. For this purpose, a single test substance concentration is incubated with synthetic peptides for ca. 24 h at ca. 25 °C, and the remaining non-depleted peptide concentrations are determined by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm.

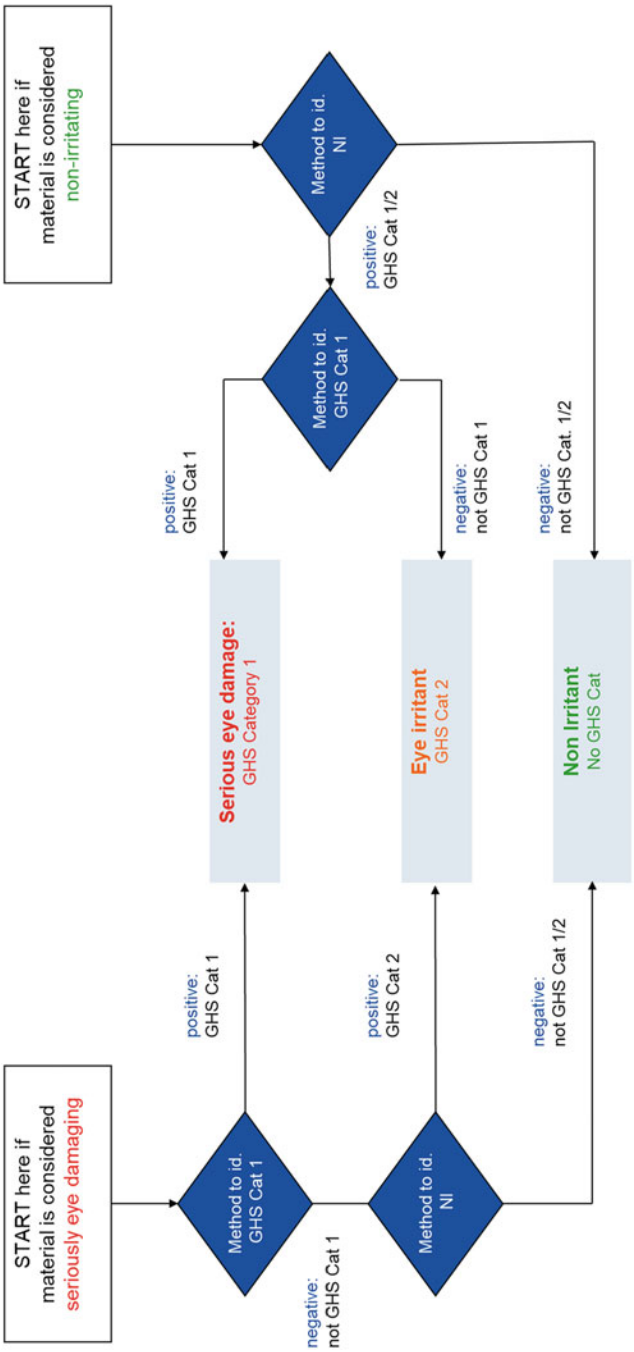

Fig. 3 Combination of in vitro methods to assess eye irritation/serious eye damage. Depending on the expected effects (seriously eye damaging or nonirritating), the first test conducted is chosen. Cat, category; NI, nonirritant; id., identify. Please note that a default GHS Cat 2 identification by exclusion of "nonirritant" and exclusion of "serious eye damage" may not be accepted by all regulatory bodies and additional information such as in vivo data may be required (OECD 2019e)

The peptide depletion of test substance incubated samples is compared to the peptide depletion of the negative control samples and expressed as relative peptide depletion. The DPRA has been first adopted as *OECD test guideline no. 442C* in 2015 (OECD 2019f).

The DPRA is not using a human-derived cell- or tissue model nor a biomacromolecule as test system, but rather a synthetic heptapeptide. The assay is, however, complementing human-derived models (described below, Sects. 3.4.2 and 3.4.3) in testing batteries to predict a skin sensitization potential in humans.

As the DPRA is not using biological systems, but rather a synthetic heptapeptide, it is often termed an *in chemico* rather than "in vitro" assay. Information on the reactivity of a test substance towards a peptide (as a proxy for skin proteins) can also be obtained by in silico methods. Several commercial and not-for-profit models have been evaluated (Teubner et al. 2013; Urbisch et al. 2016b; Fitzpatrick et al. 2018) and provided a lower overall predictivity, but good concordance with experimental results with specific models within their applicability domain. So far, peptide reactivity is used to predict a sensitization potential (presence or absence of hazard). Recently, the DPRA was extended to also predict potency classes (*kDPRA*). In the kDPRA several test substance concentrations are assessed after several incubation times to determine reaction rate constants which are then used to identify strong sensitizers (UN GHS category 1A) (Wareing et al. 2017).

3.4.2 Testing Methods: Human-Derived Keratinocytes Used in OECD Test Guideline No. 442D

As a second key event in the adverse outcome pathway for skin sensitization, keratinocyte activation can be assessed by the *KeratinoSens* and *LuSens* assays using the genetically modified human keratinocyte cell lines. Both assays employ the reporter gene for luciferase under the control of an antioxidant response element and hence monitor Nrf-2 transcription factor activity. The endpoint measurement is the upregulation of the luciferase activity after incubation with test substances. This upregulation is an indicator for the activation of the Keap1/Nrf2/ARE signalling pathway. The ARE-Nrf2 luciferase test methods have been first adopted in 2015 as *OECD test guideline no. 442D* (OECD 2018a).

3.4.3 Testing Methods: Human-Derived Dendritic-Like Cells Used in OECD Test Guideline No. 442E

Dendritic cell activation, the third key event in the adverse outcome pathway for skin sensitization, is addressed by the test methods described in *OECD test guideline no. 442E* first adopted in 2016 (OECD 2018b). The assays evaluate the potential to activate dendritic cells either by measuring changes in the cell surface marker expression (human cell line activation test (*h-CLAT*) and the U937 Cell Line Activation Test (*U-SENS*)) or by means of inducing the cytokine IL-8 in the interleukin-8 reporter gene assay (*IL8LUC*). The h-CLAT is performed using the human monocytic leukemia cell line THP-1 as surrogate for dendritic cells. As readout, the change in the expression of the cell membrane markers CD 54 and CD 86 is determined by flow cytometry after test substance exposure. Similarly, in

the U-SENS the change in the expression of the cell membrane marker CD 86 measured by flow cytometry after test substance exposure of U937 cells is determined. In the IL8LUC a THP-1 derived IL-8-reporter cell line, IL-8 dependent luciferase activity is determined after test substance exposure (OECD 2018b).

3.4.4 Defined Approaches: Combination of In Vitro Methods to Assess Skin Sensitization

Although non-animal methods addressing individual key events of the skin sensitization adverse outcome pathway are available as OECD-adopted test methods, none of the available methods should be considered as a stand-alone method to address the endpoint of skin sensitization, but rather the methods have to be combined in defined approaches. To conclude on the sensitizing potential of a test substance, the data from several methods are combined to a defined approach in which a fixed data interpretation procedure serves as prediction model for the combination of results. Several defined approaches have been described (Table 2, OECD 2016a, b), and in the following, we briefly describe one of the less complex defined approaches for the identification of the skin sensitization hazard (Fig. 4, Bauch et al. 2012; Urbisch et al. 2015).

In the *2 out of 3 approach* (Case study 1 in Table 2), assays addressing three of the key events of the skin sensitization adverse outcome pathway are conducted, and two concordant results determine the overall hazard prediction (i.e., if a test substance is positive in any two of the three assays, it is predicted to be a sensitizer).

This defined approach (and indeed most defined approaches) is combining the data of several test methods adopted by OECD. Yet, the adoption of defined approaches into OECD test guideline is still pending at the time of writing of this chapter (January 2020) and this is prohibiting defined approaches from providing the same regulatory recognition and mutual acceptance of data as the animal tests. Since

Table 2 Case studies of defined approaches described in OECD GD 256 (OECD 2016a, b)

1	An adverse outcome pathway-based "2 out of 3" integrated testing strategy approach to skin hazard identification
2	Sequential testing strategy (STS) for hazard identification of skin sensitizers
3	A non-testing pipeline approach for skin sensitization
4	Stacking meta-model for skin sensitization hazard identification
5	Integrated decision strategy for skin sensitization hazard
6	Consensus of classification trees for skin sensitization hazard prediction
7	Sensitizer potency prediction based on key event 1 + 2: Combination of kinetic peptide reactivity data and *KeratinoSens*® data
8	The artificial neural network model for predicting LLNA EC3
9	Bayesian network DIP (BN-ITS-3) for hazard and potency identification of skin sensitizers
10	Sequential testing strategy (STS) for sensitizing potency classification based on in chemico and in vitro data
11	Integrated testing strategy (ITS) for sensitizing potency classification based on in silico, in chemico, and in vitro data
12	DIP for skin allergy risk assessment (SARA)

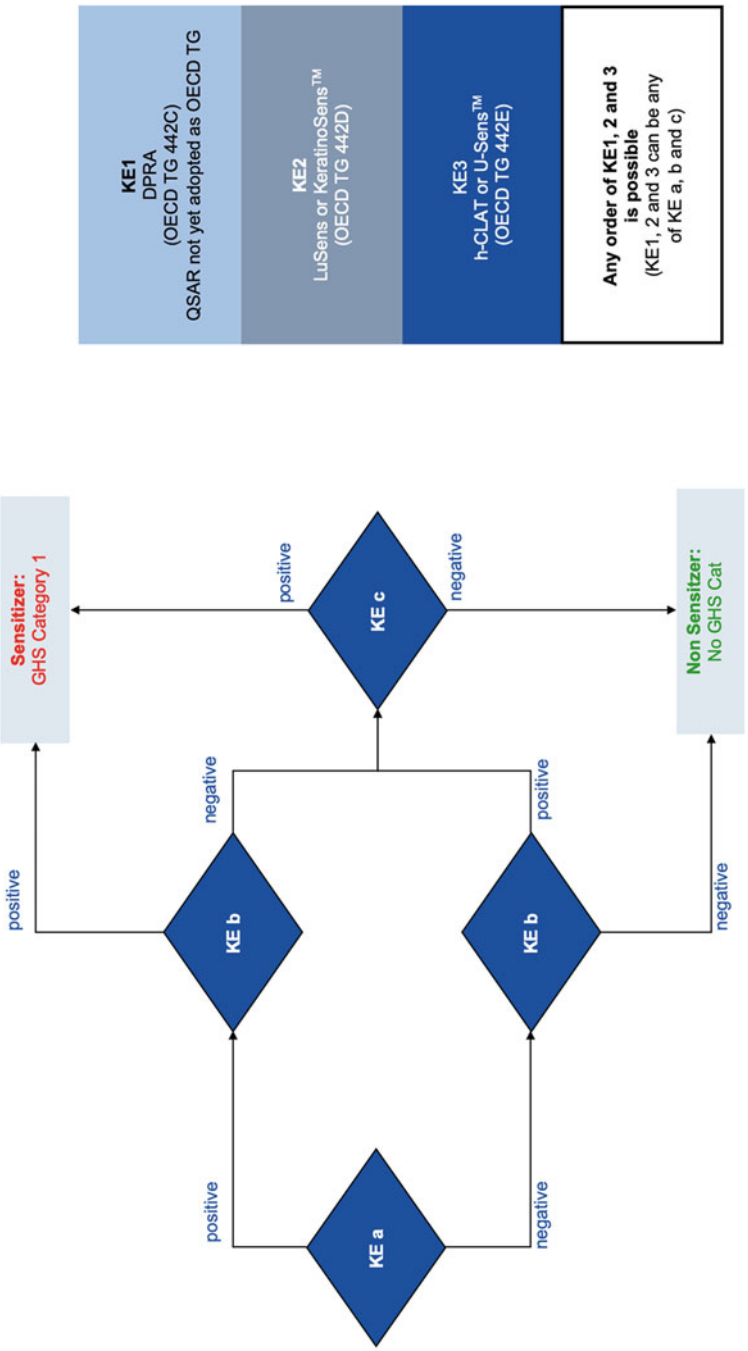

Fig. 4 The 2 out of 3 defined approach (according to Urbisch et al. 2015). KE, key event in the adverse outcome pathway for skin sensitization with key events (KE) A, KE B, and KE C being key events 1, 2, and 3 in an arbitrary sequence. Any two concordant results determine the overall prediction with at least 2 out the 3 KE addressed. KE1 is addressed by the DPRA (OECD test guideline no. 442C), KE2 by the LuSens/KeratinoSens™ assays (OECD test guideline no. 442D), and KE3 by the h-CLAT (OECD test guideline no. 442E)

the "2 out of 3" approach has been first submitted for regulatory acceptance to the European Centre for the Validation of Alternative Methods in 2011, a lot of progress has been made. A project to draft a guideline has been added to the OECD work plan in 2017, and a second draft guideline and supporting documents became available in September 2019 (OECD 2019g, h). The work undertaken to draft these documents included an extensive review of the human and mouse skin sensitization reference data (see also Reference Data and Validation Sets for a discussion of reference data in more general) as well as a discussion about the applicability domains (extending on the applicability domains of the individual assays).

3.5 Genotoxicity

There is a variety of in vitro genotoxicity and mutagenicity models available and used within testing batteries (Kirkland et al. 2006; ICH 2011; SCCS 2014). Interestingly none of the regulatorily accepted models is using human-derived models except for the so-called HuLy assay which is utilizing primary human lymphocytes (OECD test guideline no. 487, OECD 2016c). Recently, methods to detect genotoxic and mutagenic effects in human-derived reconstructed epidermal models were developed and validated in ring trials to detect genotoxicity (Reisinger et al. 2018) and chromosomal aberrations (Curren et al. 2006; Aardema et al. 2010). These methods await the finalization of their validation processes and inclusion in OECD test guidelines to be used within genotoxicity and mutagenicity in vitro testing batteries with the in vitro genotoxicity test for dermal exposure using 3D models added to the OECD work plan.

3.6 Dermal Penetration and Absorption

Dermal penetration and absorption methods are not testing for adverse effects on human skin but are assessing the penetration of dermally applied substance into the skin and through the skin to become systemically available in the human body. The OECD-adopted in vitro method (OECD test guideline no. 428; OECD 2004; Fabian et al. 2017) is utilizing human skin preparations. Non-viable skin can also be used provided that the integrity of the skin can be demonstrated. Either epidermal membranes or split-thickness skin (typically 200–400 μm thick) prepared with a dermatome are acceptable. The principal diffusion barrier of the skin is the non-viable stratum corneum; active transport of chemicals through the skin has not been identified, and dermal metabolism (Bätz et al. 2013; Oesch et al. 2018) is not rate limiting in terms of actual absorbed dose (OECD 2004). Methods to utilize human reconstructed epidermal or full-thickness skin models have been developed and pre-validated (Schäfer-Korting et al. 2008; Ackermann et al. 2010) but are not yet regulatorily accepted.

4 Limitations

4.1 Technical Limitations

Specific test substances may not be applicable to certain test systems. Some examples of these technical limitations are listed below (using the example of the in vitro skin sensitization methods).

In the DPRA (OECD test guideline no. 442C), the depletion of the synthetic heptapeptide is quantified by its UV light absorption after HPLC elution. Test substances co-eluting at the same retention times as the model peptides may hamper the peptide quantification.

The cell-based assay methods (OECD test guidelines no. 442D and 442E) use luciferase-generated bioluminescence or fluorescence of fluorochrome-labelled antibodies as detection methods. Test substances quenching the fluorescence or otherwise interfering with the optical detection may hamper the quantification of the luciferase induction or the identification of labelled cells.

Dendritic cell activation (OECD test guideline no. 442E) is analyzed by flow cytometric determination of the cell surface marker expression. Insoluble particles and polymers may however limit the technical applicability by clogging the flow cytometer.

4.2 Predictive Limitations

4.2.1 Mechanistic Limitations

In the following we present (and partially discuss) known mechanism limitation of in vitro assays. This is not to be understood as a comprehensive list; we'd like to present a few examples based on the in vitro skin sensitization assays.

OECD Test Guideline No. 442C The DPRA is based on reactivity of a test substance with cysteine and lysine residues. Metals do not form covalent bonds with those two amino acid residues and hence are out of the applicability domain of the assay. Also, test substance reacting with amino acid residues different from cysteine and lysine will not be detected in the DPRA. It has been described that some test substances favor the dimerization or oxidation of the peptide leading to an overestimation of a true peptide depletion (or non-covalent, specific binding, e.g., Roediger and Weninger 2011).

OECD Test Guideline No. 442D The underlying mechanism for the antioxidant response element pathway addressed in both the *KeratinoSens* and *LuSens* assays is closely linked to cysteine reactivity. Therefore, test substance primarily reacting with other amino acid residues (such as acylating agents reacting with lysine) would be expected to be underpredicted in the *KeratinoSens* and *LuSens* assays.

Metabolic Capacity

The three in vitro skin sensitization tests described above do not contain any external source of metabolic capacity. Nevertheless, the test systems can detect most pre- and pro-haptens. In vitro investigations (Urbisch et al. 2016a; Patlewicz et al. 2016) using test substances requiring molecular transformation to attain a sensitizing potential have shown that pre-haptens can readily be detected in the DPRA, many of which involve autoxidation processes. Moreover, many pro-haptens are also activated by nonenzymatic oxidation (and therefore are pre- *and* pro-haptens). The cellular models h-CLAT and *LuSens* have been shown to detect pro-haptens more efficiently; respective enzyme activities were detected in the cell lines (Fabian et al. 2013; Oesch et al. 2018). Thus, it can be concluded that potentially relevant molecular transformations are generally sufficiently considered using the in vitro skin sensitization tests DPRA, *LuSens* or *KeratinoSens*, and h-CLAT.

Water Solubility and Lipophilicity

OECD test guideline no. 442E describes that the h-CLAT result may be underpredictive (false negative) for test substances with log $K_{OW} > 3.5$. In this case a "negative" result is interpreted as "inconclusive." However, a "positive" result will be accepted. While with increasing log K_{ow}, less solubility is expected, it should however not be neglected that OECD test guideline no. 442E (OECD 2018b) and OECD test guideline no. 442D (OECD 2018a) allow the testing of homogenous but non-completely dissolved test substances.

4.2.2 Agrochemical Formulation in In Vitro Skin and Eye Irritation Tests

(Non-animal) tests are typically validated against well-characterized individual reference chemicals. Different chemistries, use classes, and, e.g., special types of mixtures can, however, not always (comprehensively) be included in validation exercises. Therefore, important post-validation experience is gained during routine testing (frequently after test guideline adoption and regulatory acceptance). We present here two examples of test methods both based on reconstructed human tissues. Agrochemical formulations are a special type of mixture (which as such fall under the GHS mixture definition and could generally be considered within the applicability domain of the OECD test guidelines) containing a variety of ingredients to alter the properties of the active ingredient. Oftentimes thereby toxicity is also affected, and rules of additivity do not simply apply. In 2015 Kolle et al., based on a comparative dataset of 97 agrochemical formulations, have reported excellent sensitivity of the *EpiOcular* eye irritation test assay to predict agrochemical formulations nonirritant to the eye (until the writing of this chapter (January 2020), there is still no non-animal method to reliably predict seriously eye-damaging agrochemical formulations) (Kolle et al. 2015, 2017a). This would lead to the notion that maybe reconstructed human tissues work well for the lower end of the irritation scale also for skin. This could unfortunately not be confirmed for a comparative dataset of 65 agrochemical formulations which showed that the in vitro skin irritation test was

neither sufficiently sensitive nor specific (Kolle et al. 2017b). Also proof of concept with five formulations assessed using a protocol modification of the SIT (using a 15 min exposure followed by a 24 or 42 h post-exposure instead of a 60 min exposure followed by a 42 h post-exposure in the EpiDerm SIT according to OECD test guideline no. 439) was not successful (unpublished data). Therefore, most unfortunately, the in vivo assay (OECD test guideline no. 404) is still needed to evaluate the skin irritation potential of agrochemical formulations.

At the time of writing of this chapter (January 2020), there is no regulatory-accepted method available to reliably predict the skin irritation potential of agrochemical formulations; the development and validation of such methods should be fostered.

4.3 Uncertainty

4.3.1 Reference Data and Validation Sets

When evaluating or implementing novel (non-animal) methods, it is of upmost importance to use substance with critically reviewed reference data. There have been reports on cases where method implementation based on so-called proficiency chemicals provided in OECD test guidelines has been hampered (Kolle et al. 2019a, b). Another example is the evaluation of the defined approach for skin sensitization which started with 128 substances for which human as well as local lymph node assay data was available. During the review of the guideline, the reference data was extensively reviewed and resulted in a reduced curated dataset of 105 substances with local lymph node assay data and 76 substances with human reference data (OECD 2019h).

4.3.2 Borderline Range: Uncertainty Arising from Technical and Biological Variance

The borderline range depicts the variance of the individual test methods, including technical and biological variability (Leontaridou et al. 2017). It addresses the uncertainty of the three assays around their respective classification thresholds and represents a range in which the likelihood to obtain a positive or negative result just below or above the classification threshold is equal (Fig. 5).

The borderline range can be determined statistically (e.g., using pooled standard deviations), using historical intra-laboratory data (Leontaridou et al. 2017). It is useful especially for assays for which no individual statistical analysis is possible due to low number of replicates per treatment (e.g., h-CLAT and DPRA). This evaluation is an amendment to the evaluation given in the respective OECD test guidelines, and it also influences a method's precision (Leontaridou et al. 2019) (Table 3).

The definition of a borderline range allows the possible prediction as "ambiguous" underlining the fact that a result close to a classification cutoff is rather random.

Table 3 summarizes the borderline range for the in vitro skin sensitization test methods. Borderline ranges rather than discrete cutoff values should be used in

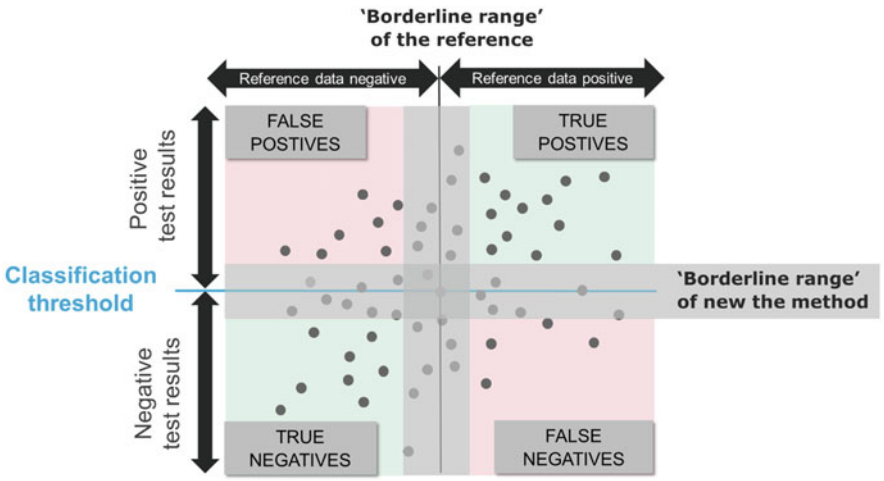

Fig. 5 The borderline range of new and reference methods

Table 3 Pooled standard deviations and borderline ranges of in vitro methods to predict skin sensitization in humans (Leontaridou et al. 2017)

Testing method	Borderline range
DPRA (OECD test guideline no. 442C)	MPD = {1.35%, 11.41%}
LuSens (OECD test guideline no. 442D)	FI = {1.26, 1.74}
h-CLAT (OECD test guideline no. 442E)	CD54 FI = {1.74, 2.26}, CD86 FI = {1.2, 1.81}
LLNA (OECD test guideline no. 429) for comparison	SI = {2.20, 3.71}

Test results in the range of 3.38% $\leq$ MPD $\leq$ 9.38% (DPRA), FI $\leq$ 3 (LuSens), and CD86 FI $\leq$ 3 as well as CD54 FI $\leq$ 3 (h-CLAT) were considered for quantifying the pooled standard deviation (Leontaridou et al. 2017)

DPRA direct peptide reactivity assay, *h-CLAT* human cell line activation test, *FI* fluorescence intensity, *MPD* mean peptide depletion, *SI* stimulation index

prediction models (or data interpretation procedures, DIP), and potential outcomes of studies which dichotomize continuous data into classes are really "positive," "negative," and "inconclusive."

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How Qualification of 3D Disease Models Cuts the Gordian Knot in Preclinical Drug Development

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
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Abstract

Preclinical research struggles with its predictive power for drug effects in patients. The clinical success of preclinically approved drug candidates ranges between 3% and 33%. Regardless of the approach, novel disease models and test methods need to prove their relevance and reliability for predicting drug effects in patients, which is usually achieved by method validation. Nevertheless, validating all models appears unrealistic due to the variety of diseases. Thus, novel concepts are needed to increase the quality of preclinical research.

Herein, we introduce qualification as a minimal standard to establish the relevance of preclinical models and test methods. Qualification starts with prioritizing and translating scientific requirements into technical parameters by quality function deployment. Qualified models use authenticated cells, which resemble the corresponding cells in humans in morphology and drug target expression. Moreover, disease models differ from normal models in the expression of relevant biomarkers. As a result, qualified test methods can discriminate effects of treatment standards and the effects of weakly effective or ineffective substances. Observer-blind readout, adequate data documentation, dropout inclusion, and a priori power studies are as crucial as realistic dosage regimens for qualified approaches. Here, we showcase the implementation of qualification. Adjusting the level of model complexity and qualification to three defined phases of preclinical research assures the optimal level of certainty at each step.

In conclusion, qualification strengthens the researchers' impact by defining basic requirements that novel approaches must fulfill while still allowing for scientific creativity. Qualification helps to improve the predictive power of preclinical research. Applied to human cell-based models, qualification reduces animal testing, since only effective drug candidates are subjected to final animal testing and subsequently to clinical trials.

Keywords

Aging · Analytical methods · Data quality · Neoplasms · Nonalcoholic fatty liver disease · Pharmacology · Preclinical drug evaluation · Validation study

Abbreviations

3R	Replacement, reduction, and refinement of animal experiments
ADME	Absorption, distribution, metabolism, and excretion
AUC	Area under the curve
CRISPR	Clustered regularly interspaced short palindromic repeats
DXM	Dexamethasone
EU	European Union
FDA	U.S. Food and Drug Administration
HOQ	House of quality

ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
iPS	Induced pluripotent stem cells
LC	Liquid chromatography
MS/MS	Tandem mass spectrometry
N/TERT	Human keratinocyte cell line
NASH	Nonalcoholic steatohepatitis
OECD	Organisation for Economic Co-operation and Development
QFD	Quality function deployment
RhS	Reconstructed human skin
STXM	Scanning transmission X-ray microscopy

1 Current Efficiency in Preclinical Research

Despite major efforts in 3R from academia, industry, and legislation, the number of animals being sacrificed in research every year still amounts to almost 9.4 and 1.8 million in the EU and Germany, respectively. Fundamental and applied research account for 44% and 15%, respectively, of the animals in Germany (European Commission 2019; Federal Ministry of Food and Agriculture 2019). Concomitantly, preclinical testing often fails to collect data, being relevant for human patients. Success rates in clinical trials are as low as 3% in oncology or 15% in neurology and immunology and question the current methodology of assessing investigational new drugs in preclinical science. The major shortcoming of preclinical research is related to the complex architecture of organs like the brain or the immune system as well as the heterogeneous nature of diseases. The translation from bench to bedside is only sightly more successful for vaccines and drugs against cardiovascular and infectious diseases (Table 1, Wong et al. 2019). Poor efficacy and safety stand out as major reasons for the termination of drug development projects (Arrowsmith and

Table 1 Disease-group-related success rates (%) in clinical Phase 1 and overall (success in Phases 2 and 3) in clinical drug development; from Wong et al. (2019)

Disease groups	Clinical Phase 1 passed transfer to Phase 2 (%)	Overall success rate (%)
Oncology	57.6	3.4
Central nervous system	73.2	15.0
Autoimmune/inflammation	68.8	15.1
Metabolic/endocrinology	76.2	19.6
Genitourinary	68.7	21.6
Infectious disease	70.1	25.2
Cardiovascular	73.3	25.5
Vaccines	76.8	33.4
Ophthalmology	87.1	32.6

Miller 2013), besides economic considerations of the pharmaceutical company (Waring et al. 2015).

Despite the marked reduction of drug candidates in early and advanced preclinical testing, still too many substances pass the preclinical phase but fail in clinical studies. High numbers of volunteers and patients exposed to non-efficacious or unsafe drugs demand a stricter preclinical selection. Recent developments in tissue engineering should enable addressing these questions with human cell-based disease models and rejecting unqualified drug candidates. Rethinking preclinical drug development will avoid expendable applications to human and animal test subjects and should reduce costs, from now 2,800 $\times$ 10^6 US dollars per year for preclinical research that is not reproducible (DiMasi et al. 2016).

1.1 Phases of Preclinical Research

Preclinical drug research comprises all tests from drug discovery to the first-in-human studies. The current approaches encompass in silico methods and high-throughput screenings, tests in disease models and pharmacokinetic investigations in vitro and in vivo, as well as regulatory toxicology and safety pharmacology studies. Preclinical research starts with simple models to save time and costs, while sophisticated approaches are used in later phases. This stepwise approach could be grouped into three phases. We suggest summarizing in silico methods and high-throughput screening in *Phase I*; simple pharmacological tests, regulatory toxicology, and safety pharmacology in *Phase II*; and sophisticated pharmacokinetic and pharmacodynamic tests in *Phase III*. Currently, animal experiments are predominantly used in *Phases II* and *III* (according to our definition) and still remain the backbone for preclinical drug research.

1.2 Models and Test Methods

Here, we define a preclinical model (normal, disease) as a system recapitulating the hallmarks of the human tissue in animal or cell culture. A test method is an approach to identify drug effects in the preclinical model, respectively. An efficient selection of drugs, suitable for human use, requires a tiered procedure with models and test methods that are as simple as possible but as complex as necessary. This means an increasing complexity from *Phase I* to *III*.

2 Reasons for Poor Translational Success

Five reasons stand out from the causes which limit the translational success of investigative new drugs from bench to bedside:

- *Animal models* are confounded by a major gap between animal and human biology (Seok et al. 2013; Warren et al. 2015). The animal-based disease frequently aligns poorly with the human indication of interest. New technologies like CRISPR/Cas offer new opportunities for more human-like disease models, but transgenic mice and rats remain genetically engineered rodents, except for the drug target.
- *Heterogeneity* is excluded, since young male animals from single inbred strains are preferred (Hartung 2013). In contrast, diseases affect male and female, young, adult, and senior patients with different genetic backgrounds.
- Currently, *cell culture practice* faces limitations with cells subcultivated in high passages or not authenticated (Hartung 2007) and generally the lack of quality control (OECD 2018).
- The use of *cell lines* can be unrepresentative of complex diseases. Moreover, monolayer cultures lack the tissue-specific extracellular matrix (Nallanthighal et al. 2019).
- *Study design* in preclinical research frequently does not apply to the same standard as in clinical trials. In particular, randomization and blinding (van Luijk et al. 2014) as well as statistical tests for differences are rarely considered.
- *Quality assurance and validation* for both the model itself and the pharmacological test method appear to be expendable with respect to disease models, although preclinical studies lack reproducibility (Begley et al. 2015; Simeon-Dubach et al. 2016).

Now the time has come to transform preclinical drug development into relevant and reproducible research, while avoiding suffering animals wherever possible (German Research Foundation 2019). Nevertheless, even less stressful testing in the animal will not overcome the genetic differences between animals and humans.

Toxicologists have addressed this issue by the development and validation of alternatives to animal testing (Leontaridou et al. 2017). Validated toxicological methods use reconstructed human epidermis for the evaluation of skin corrosion (OECD 2019a), skin irritation (OECD 2020), and phototoxicity (tier-2; OECD 2019b). Yet, it took about 25 years from the development of reconstructed human skin (Green et al. 1979) to regulatory acceptance of the respective test methods, and there is still a gap in fully accepting these in regulatory toxicology (Sauer et al. 2016).

Currently the so-called investigative toxicology shifts pharmaceutical toxicology from a descriptive to an evidence-based, mechanistic discipline. Outside the boundaries of *regulatory toxicology*, investigative toxicology embraces new technologies to predict human responses. European leaders in the pharmaceutical industry propose humanized in vitro test systems to improve preclinical decisions (Beilmann et al. 2019). However, toxicity studies in animals remain essential for *regulatory toxicology* because of the limited number of organs which can be reconstructed and the approach to investigation of the whole organism is at its infancy. In 2018 the European Medicines Agency started a consultation on the regulatory requirements for drug development, and the pharmaceutical industry requested harmonization with the US Food and Drug Administration.

3 From Validation to Qualification

The ICH M3(R2) guideline clearly states that in vitro alternative methods can be used to replace current standard methods, if validated and accepted by all regulatory authorities.

3.1 Validation

A validation study provides the documented approval that a model or a test method reproducibly shows the desired effect. The extensive requirements make validation highly time-consuming and costly (Basketter et al. 2012) and might prevent innovative methods from their application in preclinical drug development. Moreover, the broad spectrum of drug effects and the heterogeneity of diseases increase the complexity and stand against a timeline in using in vitro disease models.

The overarching goal of validation, proof-of-concepts, performance standards, and best practice guidelines is to demonstrate the *quality* of a model or test method. According to the Latin origin of *qualitas,* quality is defined by the nature of an object. Modern perceptions of quality assume that *quality* must be produced rather than assured in retrospective validations (Kamiske and Brauer 1993). *Quality* should originate from a company- or research group-wide spirit with a clear vision of *quality*; it is an inherent responsibility which cannot be delegated or outsourced, as it is the basis of scientific and industrial success. To apply the vision of *quality* into real work, management tools such as "quality management systems" or "failure mode and effects analysis" have been developed for industrial applications and recently translated to scientific and academic use (Dirnagl et al. 2018). Even evidence-based medicine strives to improve model and test method development (Lefevre and Balice-Gordon 2019). Most often the high aims of such guidance are perceived to stand against scientific creativity, publication output, and fundraising. Consequently, the compliance to such guidelines varies among research institutions which might contribute to the overall low success of preclinical research.

Since the best strategy is useless if not applied, the two major questions to be answered are:

- How to deploy quality planning and management in the development of novel in vitro methods for preclinical research?
- Which level of certainty is required for the model and test method, respectively?

We suggest starting with compiling the scientific requirements, which a model or test method must fulfill. According to the industrial definition "*Quality* is conformance to customer requirements" (Crosby 1996), these scientific requirements should be in accordance to the user of the model or test method, from industry or academia.

3.2 Quality Function Deployment: Learning from Industry

A relevant model or test method depends on the *quality* of its planning. Researchers aiming for a high impact and short time to application should focus on structured method planning, since in industry 80% of product flaws, which are occurring during product assembly and product use, originate from insufficient product design. The car industry took major profit from introducing quality function deployment (QFD) into the product planning. Thereby, QFD reduced the costs for product development to 40% of the initial value and diminished the changes necessary to optimize the original product design (Fig. 1).

The major parts of QFD include the formation of a quality planning team and the correlation matrix "house of quality" (HOQ, Zoschke 2009). The quality planning team should consist of the leading and first-stage researchers as well as technical assistants to involve all concerned group members into the planning. The HOQ fosters a systematic assessment, categorization, and prioritization of scientific requirements and technical parameters and clearly documents the results of the quality planning team's discussion. To the best of our knowledge, QFD has not yet been introduced to the development of in vitro models. Here, we present the first example with the development of an immunocompetent model of head and neck cancer for the evaluation of local drug effects (Fig. 2). The selection of scientific requirements and technical parameters and their weighting are the author's choice to serve as an example to further develop a recently published tumor oral mucosa model (Gronbach et al. 2020). However, the definition and weighting of requirements and parameters must be adapted to the disease model or test method of interest.

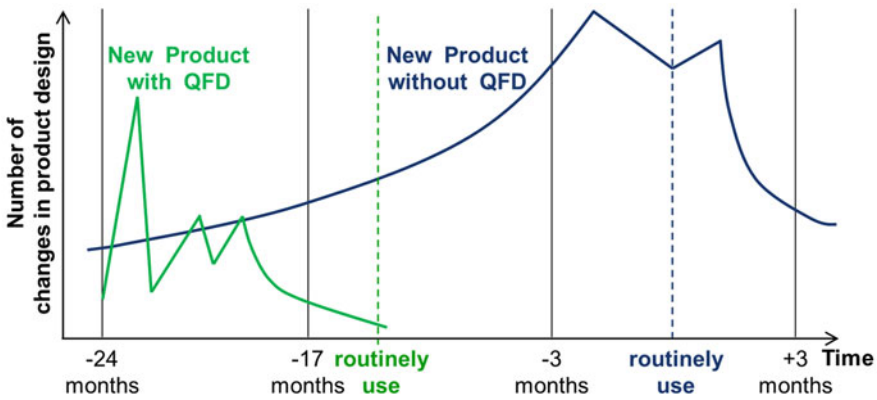
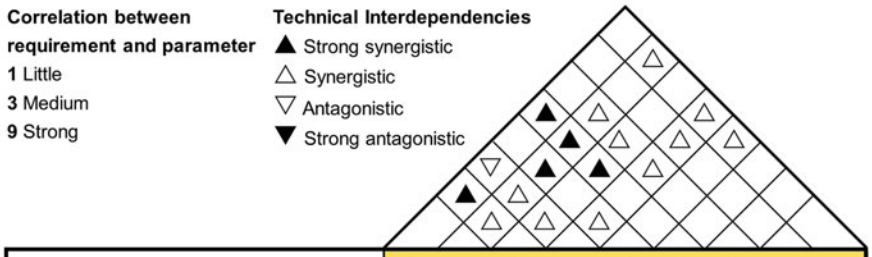


Fig. 1 Impact of quality function deployment in industry. The higher efforts and costs of structured product planning with quality function deployment (QFD) in the beginning of a car development are counterbalanced by shorter development times and less flaws of the product following start of production. Transfer to in vitro model and test method development should reduce the time-to-application similarly, from Zoschke (1993)



Optimization (↑ max ↓ min ⊙ aim)			↓	↑	↓	↑	↓	↓	↓	↑	⊙
Technical Parameters / Scientific Requirements		Weight	A Model size	B Number of cell types	C Number of cells	D Number of ECM components	E Number of media components	F Culture medium volume	G Preperation time	H Number of drug applications	I Drug target
M1	Immunocompetent model	4		9	3		3		1		9
M2	Extracellular matrix	2	3	3	1	9			9	3	3
M3	Tumor heterogneity	6	3	9	9	9			1		9
M4	Tumor metastasis	3	1	9	1	9			1		3
M5	Tumor progression	6	1	9	3	9			3	1	3
M6	Vascularization	2	3	9		9	3	1	9	9	3
R1	Drug effects - Verum	9		9	1	9	1			3	9
R2	Drug effects - Placebo	9		9	1	9	1			3	9
R3	Drug effects - Borderline	9		9	1	9	1			3	9
P1	Model availability	7	9	9	9	9	3	3	9		
P2	Model Reproducibility	6	1	9	3	3	1				9
Importance of technical parameters		total	108	555	197	495	72	23	130	111	426
		%	5	26	9	23	3	1	6	5	20
		class	3	1	3	1	3	4	3	3	2

Fig. 2 House of quality (HOQ) for an immunocompetent 3D model of head and neck cancer. *red*: Scientific requirements with weighting. *yellow*: Technical parameters of the in vitro model. *blue*: Correlation between scientific requirements and technical parameters. *green*: Importance of technical parameters. *white*: Interdependencies between technical parameters, from Zoschke (1993)

First, the scientific requirements need to be listed in the left part (Fig. 2, red). Since not all requirements are equally important, the next step is to prioritize them. Therefore, a decision must be made for each requirement if it is less, equal, or more important than the other requirement. Having done this paired comparison for all requirements, a rank order of the requirements as well as a weighting factor will be obtained. Next the technical parameters need to be listed in the upper part of the HOQ (Fig. 2, yellow). Technical parameters can always be measured and quantified, which makes them very specific in contrast to scientific requirements. Next, the quality planning team determines the correlation between the technical requirements and the scientific requirements. Use an exponential scale with 1 for little correlation, 3 for medium correlation, and 9 for strong correlation; multiply the correlation by the weighting of the scientific requirements. The results are noted in the correlation matrix (Fig. 2, blue). Furthermore, the direction of optimization of each technical parameter is listed in the yellow part to better fulfill the scientific requirements. The roof of the HOQ serves to list the interdependencies between each technical parameter, since the optimization of one parameter can affect the optimization of another parameter synergistic or antagonistic (Fig. 2, white). If there is an antagonistic interference between two technical parameters, this will be a major target for innovation to overcome this antagonism. Moreover, in a synergistic interference, the deterioration of one parameter can also impair the other. The target values for each technical parameter are listed at the bottom of the correlation matrix (Fig. 2, green). The values are either minima, maxima, or a range which should be achieved for the in vitro model. The quality planning team can also assess the level of difficulty to achieve these target values. The final output of the HOQ is the importance of technical parameters. Therefore, the weighted correlations between the technical parameter and each scientific requirement are summed up. An overestimation of single parameters can be avoided by dividing the sum of one technical parameter by the sum of all technical parameters (relative relevance). Almost equally relevant technical parameters should be clustered together. The technical parameter with the highest value is most important, while the parameter with the lowest value needs to be addressed at last. After completing the HOQ, the quality planning team should check for plausibility by confirming that:

- Every scientific requirement correlates strongly to at least one technical parameter (no empty rows)
- Every technical parameter correlates strongly to at least one scientific requirement (no empty columns)
- The direction of optimization fits to the target value for each technical parameter
- Antagonistic technical interdependencies should be solved or prioritized
- The correlation matrix should be filled to at least one third to be able to prioritize technical parameters.

Additionally, the HOQ can be extended by comparisons of the approach to already existing models or test systems (Zoschke 2009). In conclusion, the HOQ cannot take the decision for the researcher, but the HOQ helps to systematically

translate vague scientific requirements into quantifiable and prioritized technical parameters. According to QFD, the outcome of the HOQ is the basis for the planning of the in vitro model or test method parts. Quality planning for the parts is the basis for planning the processes, which is finally leading to the final test method protocol. This deployment ensures the translation of the scientific requirements for the in vitro model or test method into feasible protocols for each step of the model or method development.

3.3 Qualification

We define *qualification* as minimal standard in model or test method development. *Qualification* comprises the sum of evidences that a model or test method is relevant for the disease at hand. *Qualification* is based on QFD and uses the state of the art in tissue engineering and testing in molecular medicine and pharmacology. *Qualification* does not replace validation but will provide a sufficient basis for decision-making in preclinical drug development.

3.4 Qualification of 3D In Vitro Models

Irrespective of the envisioned use in fundamental research or drug development, a qualified 3D in vitro model has to fulfill the following key features:

- Use of authenticated human cells
- Comparability between tissue morphology and function with the human disease
- Expression of drug targets in accordance to the human disease
- Concurring endpoints in models and patients, i.e., the change of biomarkers relevant for disease outcome

If relevant for the disease at hand, assessing graded drug effects should be preferred over simple yes or no assessments. The model features have to be reproducible over time and in different laboratories. *Qualification* is not limited to disease models but also applicable to models for normal tissue and the target structures used, e.g., in molecular modeling and high-throughput testing. Moreover, we suggest applying the same requirements for qualified normal models as described for qualified disease models. On the one hand, the normal models will serve as control to assess a potential *restitutio ad integrum* and on the other hand to provide insights into local adverse effects of drugs.

3.5 Qualification of Test Methods

Test procedures need to be qualified for preclinical drug research. Changes observed due to drug exposure are only signified if a suitable test protocol is used and the test

is run under quality assurance. Protocol adaptations during larger test series have to be avoided as they exclude comparisons over time. Hallmarks of a *qualified test method* in all phases include if not indicated otherwise:

- Relevant controls
  - Untreated
  - If available, an already approved standard treatment with maximum efficacy (*Phase III* only)
  - If available, an already approved treatment with minimal efficacy (*Phase III* only)
  - A treatment that showed no or insufficient efficacy in clinical trials (*Phase III* only)
- Observer-blind readout when using subjective endpoints
- Adequate data documentation, including dropouts
- Evaluation by explorative data analysis
- A priori definition of the relevant effect size and adapted sample size (power study, *Phase III*)
- Relevant dosage regimen and treatment period (*Phase III*)

Test methods can be qualified only by a range of performance standards, which are related to the respective drug targets. Thus, test methods designed for evaluating anticancer drugs are unlikely to be suitable for evaluating, e.g., antimicrobial endpoints. Yet, the transfer of a test method from one disease model to another disease model appears easier to achieve than a *qualification* from scratch. Testing of investigational new drugs with targets unrelated to the *qualification* process requires a requalification for the new performance standards.

3.6 Selection of Relevant Drug Doses

Currently, the extrapolation of drug doses from animal studies to first-in-human studies remains empirical. The most common approaches are dose-by-factor based on no observed adverse effect levels (NOAEL or benchmark dose), pharmacokinetically guided approaches, based on minimal anticipated biological effect levels, pharmacokinetic-pharmacodynamic models, similar drug approach, and data from human microdosing (Nair et al. 2018). Nevertheless, interspecies differences impede the calculation of human equivalent doses from animal data, despite of the introduction of correction factors.

A failed translation of preclinical dosage regimen into clinical treatments results in severe toxicity, prolonged dose escalation procedures or patients exposed to ineffective doses. Dose finding for anticancer drugs is in particular challenging, since they have steep dose-response curves and narrow therapeutic windows (Mathijssen et al. 2014). In vitro studies frequently use drug doses far higher than the maximum tolerated dose in cancer patients (Smith and Houghton 2013) and contribute to the highest attrition rate of anticancer drug candidates in clinical trials

(Wong et al. 2019). Moreover, ambiguous dosing in cell culture experiments due to different physical conditions, like volume of medium and number of cells used, hamper the reproducibility of in vitro experiments in different phases of preclinical research (Doskey et al. 2015).

Testing the efficacy of anticancer drugs in 3D in vitro models that recapitulate the tumor-specific extracellular matrix is crucial to emulate the drug uptake and metabolism in the tissue. The dense tumor stroma with extracellular matrix and cancer-associated fibroblasts (Mueller and Fusenig 2004; Minchinton and Tannock 2006) as well as the high interstitial fluid pressure may reduce drug uptake into the tumor despite their endothelial hyperpermeability (Saleem and Price 2008; Dewhirst and Secomb 2017). Thus, the failure of anticancer drugs in 3D models despite drug efficacy in monolayer cultures could be related to the absence of a tumor stroma (Cruz Rodriguez et al. 2019).

The determination of the drug concentration which is high enough to be active in 3D in vitro models, e.g., by automated UHPLC-MS/MS approaches (Joseph et al. 2020), might help to improve the translation of preclinical into clinical dosage regimen. Another approach uses time-dependent or maximum biomarker modulation as the matching metric, rather than a minimal threshold concentration (Spilker et al. 2017).

4 Current Strategies to Rethink Preclinical Drug Research

Our concept of qualification can be applied to various approaches in preclinical research. We highlight already existing strategies using human cell-based 3D in vitro models and novel test methods. These five strategies in preclinical research fulfill the criteria of qualification to different extents.

4.1 Strategy 1: Characterized Cell Lines

Studying the N/TERT keratinocyte cell line provides a deep insight into the MAPK/ERK pathway and revealed the impact of histone deacetylase modulation in skin diseases like psoriasis, atopic dermatitis, and cancer (Robertson et al. 2012). Inducing filaggrin knockdown in the N/TERT cell line and supplementing the Th-2 cytokine IL-31 result in a skin model with clinical signs of atopic dermatitis: fostered *Staphylococcus aureus* colonization, increased IL-8 levels, and reduced human β-defensin upregulation (van Drongelen et al. 2014a, b). Moreover, patient-derived material served to generate an iPS cell line for future use of, e.g., in vitro atopic dermatitis models in drug development (Devito et al. 2018).

In cancer research, human-based models revealed the impact of the dermal equivalent and the presence of a basement membrane on melanoma invasion (Commandeur et al. 2014). A 3D in vitro model of cutaneous squamous cell carcinoma was generated from primary human keratinocytes and fibroblasts as well as SCC-12 tumor cells, recapitulates the tumor histology, and predicts the activity of ingenol mebutate (Fig. 3). Ingenol mebutate induced abundant epidermal

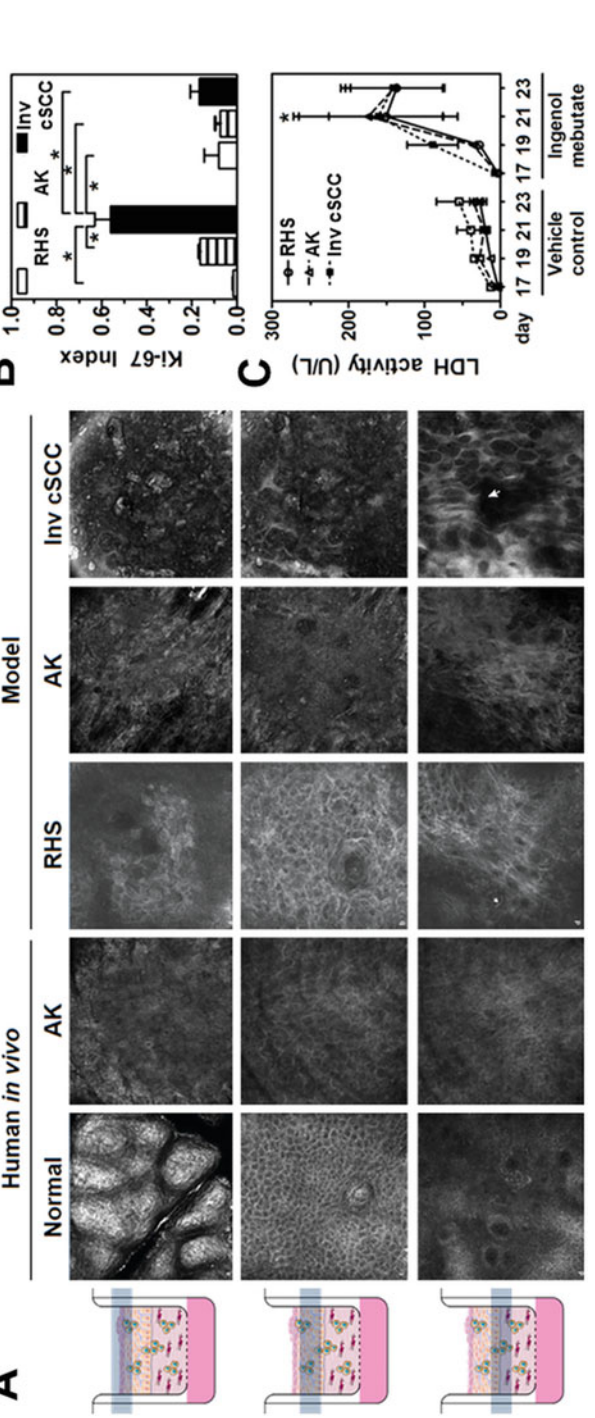


Fig. 3 3D skin cancer models and ingenol mebutate effects. (**a**) First row, stratum corneum: normal reconstructed human skin (RhS) with homogenous reflectance, actinic keratosis (AK) with beginning disruption, inhomogeneous appearance and detached cells. Second row, stratum granulosum: RhS with regular honeycomb pattern; AK with distinct epidermal foci of nuclear atypia; invasive cSCC model with dissipated honeycomb pattern. Third row, dermis: RhS with homogenous reflectance of collagen; AK model with few and invasive cSCC model with many irregular, bright collagen bundles. (**b**) RhS with slightly increased and skin cancer models with significantly decreased Ki-67 index ($p \leq 0.001$) upon ingenol mebutate treatment. (**c**) Lactate dehydrogenase (LDH) activity in the culture media of RhS and skin cancer models peaks after second ingenol mebutate treatment ($p \leq 0.05$). Graphs depict data of three batches and are presented as mean ± SEM, from Astner and Ulrich (2010) and Zoschke et al. (2016)

cell necrosis, acantholysis, and microvesicles in normal RhS (Zoschke et al. 2016). The epidermal growth factor receptor inhibitor erlotinib induced beneficial effects in another model of cutaneous squamous cell carcinoma and induced severe desquamation in the normal RhS (Commandeur et al. 2012).

This strategy is not limited to skin models but is also used for chronic kidney disease models. A human podocyte injury model of chronic kidney disease indicated that the renoprotection induced by sodium-glucose co-transporter 2 (SLGT-2) inhibitors is linked to normalized podocytes renewal and not to the lowering of blood glucose in type 2 diabetes. Correction of podocyte morphology and of associated cytoskeletal architecture renews the adhesion to the glomerulus membrane. Inhibitors of adenosine kinase reduce AMP formation and rescue cell adhesion and the actin cytoskeleton (Abraham et al. 2017).

4.2 Strategy 2: Primary Cells to Recapitulate Human Heterogeneity

An advanced 3D in vitro model of nonalcoholic steatohepatitis (NASH) was designed by co-culturing primary human hepatocytes in collagen sandwich with macrophages and stellate cells, separated by a porous transwell membrane (Fig. 4, Feaver et al. 2016). Tissue exposure to glucose, insulin, and free fatty acids corresponding to plasma levels in NASH patients repeatedly induced the lipotoxic milieu by activating key pathways spanning liver dysfunction in the hepatocytes. Triacylglycerides, diacylglycerides, cholesterol esters, and glucose levels increased significantly, and markers of inflammation (alanine amino transferase and caspase-generated cytokeratin 18, IL-6 and IL-8) as well as of fibrosis triggering TGF-β and osteopontin did so, too. Moreover, the secretion of smooth muscle α-actin increased.

Next, the model was challenged by the exposure to steady-state serum levels of obeticholic acid that targets the farnesoid X receptor in hepatocytes. The responses were compared to the vehicle control and the outcome in a clinical *Phase II* study (Hirschfield et al. 2015). Lipid accumulation declined by 25% with the most significant decrease in triacylglycerides. IL-6 and IL-8 declined significantly by 48% and 25% and other parameters of NASH including TGF-β and osteopontin were also reduced, indicating beneficial effects. Yet, intracellular cholesterol and several apolipoproteins including ApoB and ApoE increased (Feaver et al. 2016).

The interim analysis of 931 patients after 18 months treatment in clinical *Phase III* study "REGENERATE" indicated a significant improvement of key NASH factors and fibrosis by obeticholic acid 25 mg/d compared to placebo (Younossi et al. 2019). According to the positive outcome of the interim analysis, rapid drug EMA approval of obeticholic acid for NASH is applied for. The improvement of the intermediate endpoint (histology of liver biopsies) regarded as risk factors for the long-term outcome might be acceptable despite the lack of formal validation of intermediate clinical endpoints (Angulo et al. 2015). Yet already today, the *Phase III* study demonstrates the predictive power of this NASH model.

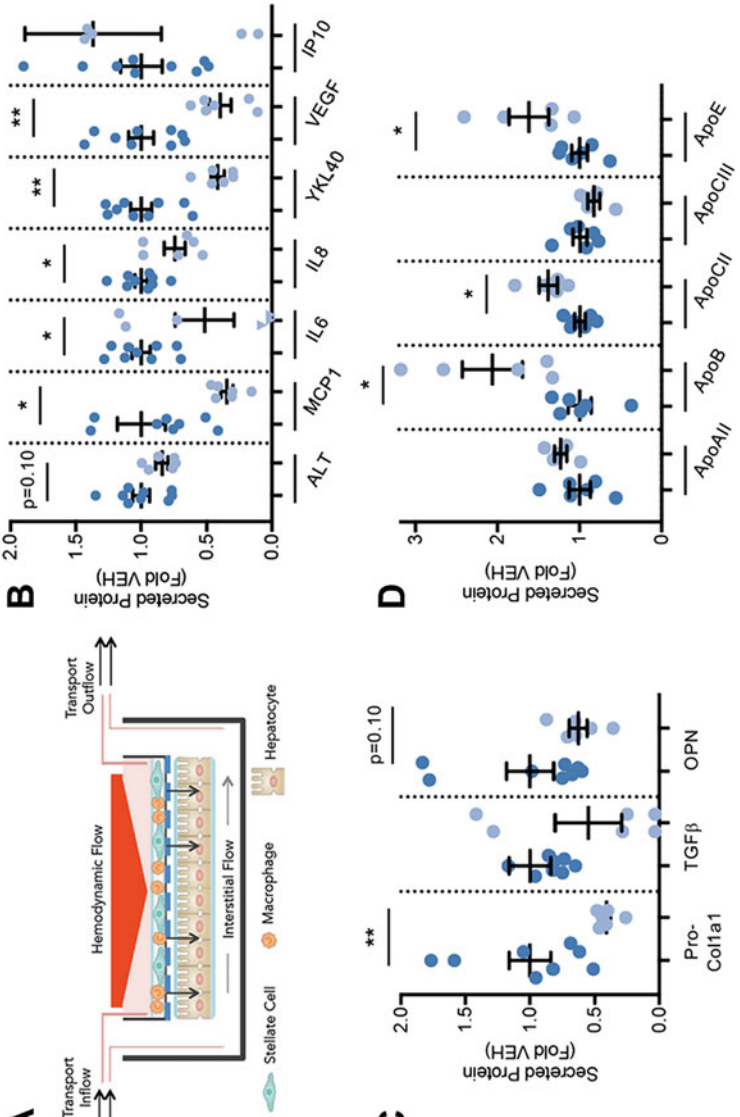


Fig. 4 NASH model used to evaluate obeticholic acid effects. (**a**) Liver sinusoidal hemodynamics were applied to the human liver system using a cone-and-plate viscometer incorporated into a transwell co-culture model of nonparenchymal cells (NPCs) (top of transwell) and hepatocytes (bottom of the transwell). Rotation of the cone (orange triangle) imparts shear stress onto the transwell. Medium is continually perfused to recapitulate interstitial flow, as indicated by the inflow and outflow ports. (**b**, **c**) Secreted analytes were measured in the media effluent from devices at day 10, $n \geq 5$ experiments, 3 donors. (**d**) Secreted apolipoproteins were measured in the media effluent from devices at day 10. $n = 4$ experiments, 2 donors. Triangles indicate samples that were below the lower limit of quantification. $^{*}p < 0.05$, $^{**}p < 0.01$, student's two-tailed t test, from Feaver et al. (2016)

Another NASH model used hepatic cells generated from human skin-derived precursors. Exposure of these cells to lipogenic (insulin, glucose, fatty acids) and pro-inflammatory factors (IL-1β, TNF-α, TGF-β) resulted in a characteristic NASH response. Elafibranor attenuated in vitro key features of NASH and significantly lowered the lipid load as well as the expression and secretion of inflammatory chemokines, being responsible for the recruitment of immune cells in vivo. This reduction in inflammatory response was mediated NFκB (Boeckmans et al. 2019). The clinical outcome, however, failed to meet the hepatic endpoint (Ratziu et al. 2016).

Treatments for end-stage liver disease, nonalcoholic liver disease in particular, are allograft liver or hepatocyte transplantation. Main obstacles are donor organ shortage and the need for efficient immunosuppression. While hepatocytes are more available than entire livers, the transplantation of hepatocytes tends to be less successful and requires more immunosuppression than the organ replacement. Allogeneic hepatocytes appear to be highly antigenic; alternatively, liver sinusoid endothelial cells or hepatic stellate cells may induce a loss of the antigenicity of hepatocytes in an allogeneic environment (Iansante et al. 2018). Immunosuppressive therapy following hepatocyte and liver transplantation include calcineurin inhibitors (cyclosporine, tacrolimus), everolimus, glucocorticoids, and basiliximab.

The suppression of immune responses was studied in a co-culture of primary human hepatocytes and allogeneic peripheral blood mononuclear cells (PBMC). Hepatocytes were isolated from six patients undergoing partial hepatectomy and grown as monolayers, while PBMC were isolated from blood of healthy donors and were added to the hepatocyte culture. Drug concentrations matched blood levels in patients receiving solid organ transplantation. Hepatocyte co-culture for 10 days strongly enhanced PBMC proliferation, and the secretion of Th-2 cell-associated cytokines strongly increased. Immunosuppressive drugs like everolimus efficiently suppressed the pro-inflammatory responses. A reduced metabolic activity of the hepatocytes, however, may indicate a potential toxicity of everolimus (Oldhafer et al. 2019). This interesting model demonstrates the immunosuppressive activity of the clinically used drugs. Given the correct identification of agents failing in the prophylaxis and therapy of allogenic rejections, the test may enable preclinical drug research on drug candidates most suitable for use in hepatocyte transplantation. The introduction of the missing innate immune system may improve predictive capacity. Moreover, these insights may allow for a pretest of the suitability of hepatocytes from donor livers for transplantation. Currently, hepatocytes are often isolated from livers unsuitable for transplantation, which appears to explain the lower success rate compared to liver transplantation (Iansante et al. 2018).

Primary cells are also essential to study the heterogeneity of aging processes and to evaluate differences in drug effects within the groups of aging. Monolayer cultures of fibroblasts from intrinsically aged human skin exhibited more signs of aging including DNA segments with chromatin alterations reinforcing senescence versus dermal fibroblasts from middle aged and young donors. Forty-three proteins confirmed the known hallmarks of aging and led to a consistent picture of eight biological categories involved in fibroblast aging, e.g., development and differentiation, cell death, and response to stress. Most of the age-associated alterations are

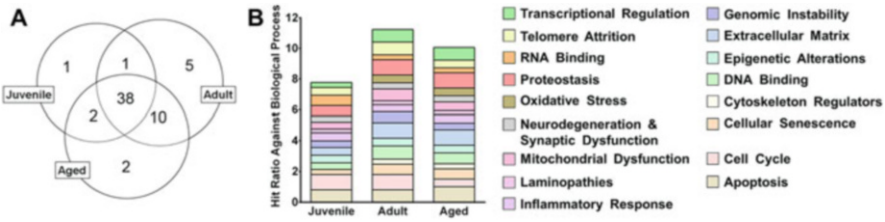


Fig. 5 Impact of normal human dermal fibroblast culture (fibroblast monolayers and reconstructed human skin) on gene expression. (**a**) Venn diagrams showing the number of genes altered due to culture conditions. (**b**) Hit ratios of the altered genes for different biological processes. The diagrams consider fold changes in gene expression $> |1.3|$ and Ct values ≤ 35 for the 19 groups of biological processes; the maximum proportion of altered gene expression per biological process (hit ratio) $= 1$; from Hausmann et al. (2019)

likely caused posttranscriptionally (Waldera-Lupa et al. 2014, 2015). Next, fibroblasts from the donors aged 20–30 or 60–70 years were used to investigate the impact of age and body region on skin homeostasis, epidermal differentiation, and drug uptake on cell monolayers and reconstructed human skin. Fibroblasts from juvenile foreskin (<10 years old) served as control. 3D in vitro models containing aged fibroblasts differed from its juvenile and adult counterparts, especially in terms of the dermal extracellular matrix composition, IL-6 levels, and wound healing (Fig. 5). The region of the body from which fibroblasts are derived appears to affect the epidermal differentiation of the construct. Emulating patient heterogeneity in preclinical studies might improve the treatment of age-related skin (Hausmann et al. 2019).

4.3 Strategy 3: Patient-Derived Cells

The access to patient-derived cells is limited, and only a few subcultivations are feasible without cellular dedifferentiation. Plucking hair follicles offers a noninvasive approach for the generation of skin disease models. Only minor differences in morphology, ultrastructure, expression of important structural proteins, or barrier function are observed between normal reconstructed human skin and the in vivo counterpart generated from hair follicle-derived or interfollicular keratinocytes and fibroblasts (Löwa et al. 2018). Next, fibroblasts were isolated from plucked scalp hair follicles of six healthy volunteers and six atopic dermatitis patients. Some of the RhS with fibroblasts from atopic dermatitis patients show epidermal thickening and parakeratosis independent from filaggrin mutations. Moreover, the thymic stromal lymphopoietin and protease-activated receptor 2 are significantly upregulated in hyperproliferative RhS (Löwa et al. 2020).

For cancer research, tumor cells are used to generate patient-derived organoids in vitro and patient-derived xenografts in vivo. One of the largest collections of patient-derived material is the OncoTrack preclinical platform for colorectal cancer.

The biobank consists of 116 resected tissue samples with matched blood samples, comprising 89 primary tumors (stage I to IV) and 27 metastases from 106 patients. Organoids and xenografts are treated with drugs representing the therapeutic gold standard and experimental substances that address major pathways relevant in colorectal cancer. The OncoTrack study provides an unprecedented repository of data and models, which can be exploited further for improved drug discovery and understanding of cancer biology (Schütte et al. 2017).

4.4 Strategy 4: New Technologies in Tissue Engineering

The ongoing change in drug development will significantly increase the need for standardized tissues in high numbers. *Bioprinting* of the tissues, in particular, has the potential to enhance the delivery of the essential test platforms. For example, functional cardiac constructs can be printed. The inclusion of conductive gold nanorods improved the electrical propagation between adjacent cardiomyocytes (Zhu et al. 2017). Inter alia, bioprinted cardiac tissue reflects the activity of β-adrenoceptor and m-receptor antagonist as well as the reversibility of the effects after removal as reviewed recently (Lind et al. 2017). Bioprinting allows for the generation of models closer to the human morphology and the control of culture environment. Injecting the cell suspension into a micromold can ensure cell cluster growth sufficient nutrient supply to avoid cell death and the formation of blood vessel (Huh et al. 2013; Prabhakarpandian et al. 2013; Hagiwara and Koh 2020).

Transforming the human-on-the-chip technology to the *patient-on-the-chip* by the use of miniaturized disease models is ahead of us. For example, a cancer chip has been developed for drug testing in a vascularized tumor model (Nashimoto et al. 2020). Tissue banks, providing vital tissues and replicable cells of defined quality over years (Palechor-Ceron et al. 2019), should allow the inclusion of human heterogeneity into *Phase III* of preclinical drug development.

The implementation of human-based testing may even open up the path to improve the therapeutic outcome of the most severe, non-acute diseases by *personalized therapy*.

4.5 Strategy 5: Comparing New Test Methods to Current Standards

The ongoing introduction of high-end analytics will allow for a much more detailed insight into pharmacokinetics and pharmacodynamics. Recently the label-free quantification of drugs at the highest local resolution of 70 ± 5 nm became possible by scanning transmission X-ray microscopy (Fig. 6a, Yamamoto et al. 2015, 2017). STXM and LC-MS/MS quantified dexamethasone equally in reconstructed human skin (Fig. 6b). Moreover, this study compared the drug penetration into reconstructed human skin with human and SKH1-mouse skin ex vivo. SKH-1 is reported to be the most human-like (Radbruch et al. 2017). The inter-model

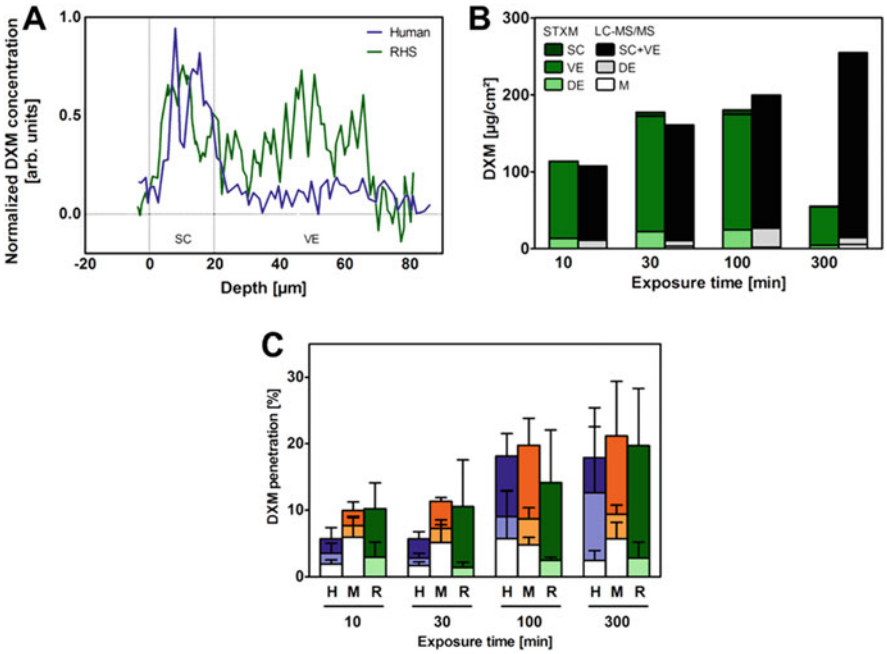


Fig. 6 Dexamethasone penetration of RhS, human, and murine skin determined by LC-MS/MS and STXM. Dexamethasone (DXM) in hydroxyethyl cellulose gel (600 µg/cm^2 DXM, 70% ethanol) was applied topically for up to 300 min. (**a**) Spatial analysis of DXM concentrations in human skin and RhS (STXM) following 10 min of exposure. Tissue surface: 0 µm. (**b**) STXM quantification shows the same skin penetration results as observed by LC-MS/MS for $t \leq 100$ min. ($n = 1$). (**c**) DXM slowly penetrates into human skin compared to murine skin and RhS. Grouped bars in order from left to right: human skin (H), murine skin (M), reconstructed human skin (R). Stacking order from top to bottom: epidermis (dark), dermis (light), heat separation water (white; human and mouse). LC-MS/MS measurements, mean ± SD, $n = 3$, from Wanjiku et al. (2019)

comparison revealed an overall similar dexamethasone uptake with minor differences in the penetration rate (Fig. 6c, Wanjiku et al. 2019).

5 Phases of Innovative Preclinical Drug Research

To keep the efforts for the qualification of novel models and test methods as low as possible, we suggest categorizing preclinical research in three phases and defined the requirements for qualified approaches accordingly (Fig. 7). Human cell-based models are preferred in all phases of preclinical testing. The combination of different in vitro models will provide higher levels of predictive power than relying on only one sophisticated model. Although human cell-based models are already revolutionizing fundamental and applied research, today, the entire organism cannot be recapitulated in vitro. Although animal tests have the clear advantage of getting an insight into systemic drug effects, risk will remain as seen in the almost fatal

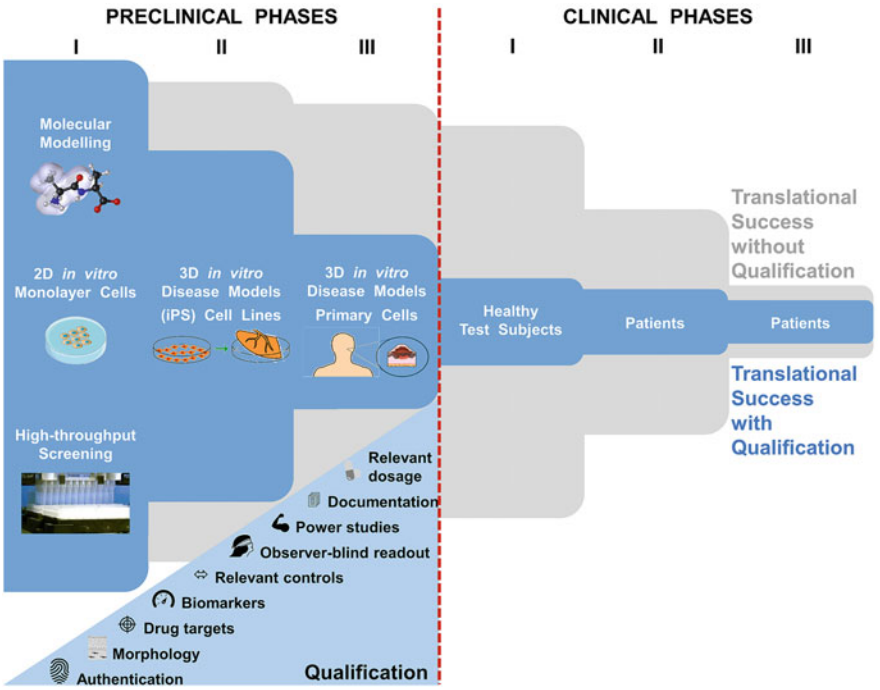


Fig. 7 Assumed impact of qualification on the predictive power of preclinical research. *iPS* induced pluripotent stem cells

cytokine release in the first-in-human study of TGN 1112 (Costello et al. 2018). To date, we consider final toxicology testing in animals prior to first-in-human studies indispensable for those drug candidates which passed preclinical efficacy tests.

5.1 Preclinical *Phase I*

In *Phase I*, physicochemical parameters of drug candidates like molecular size and hydro-/lipophilicity are the basis for in silico methods like molecular modeling, read-across, and quantitative structure-activity relationship screening. This is exemplified by the in silico identification of hit and lead structures for G-protein-coupled receptors (Wacker et al. 2017) as well as by recent breakthroughs in the treatment of HBV, HCV, and HIV as well as of cancer and severe eosinophilic asthma.

The vast knowledge of essential physicochemical features of drugs (Egner and Hillig 2008) helps to predict their bioavailability and the drugability of the pharmacological target (Zuang et al. 2018). Progress in machine learning allows calculating drug absorption, distribution, metabolism, and excretion very quickly (Tao et al. 2015).

Subsequently, high-throughput screening provides a first insight into the profile of drug effects. Moreover, biotransformation has to be assessed. The metabolism of substances into carcinogenic intermediates would complicate the therapeutic use of this drug candidate. Furthermore, the FDA has recently published a guidance document to plan and evaluate studies on drug-drug interactions (FDA 2020).

Focusing on anticancer drugs, screening can be done in authenticated well-characterized human tumor cell lines, but the genetic aberrations and epigenetic modifications will increase with the number of subcultivations. The effects of the tumor-specific extracellular matrix on tumor progression and drug efficacy cannot be captured by monolayer cultures at all. Tumor cell lines are known to be more sensitive to drug treatment than patients, as observed in the false-positive prediction of effects for more than 90% of the test agents in large surveys (Palechor-Ceron et al. 2019).

If substances appear active without severe adverse effects in *Phase I*, the drug candidate will pass to *Phase II* preclinical development.

5.2 Preclinical *Phase II*

The desired drug effects predicted by in silico approaches and high-throughput screening need to be verified in qualified models that reflect the human disease. Using the right targets, adequate biomarkers and endpoints will allow narrowing the panel of drug candidates generated in *Phase I*. Models in *Phase II* include different cell types, extracellular matrix, and tissue architecture to obtain a more precise effect profile of the drug candidate. However, the complexity of models should increase stepwise with models in *Phase II* still based on co-cultures of cell lines and/or iPS cells (Zoschke et al. 2016; Wolff et al. 2019). Another example is the investigation in the barrier function of skin models generated from the N-TERT keratinocyte cell line, which corresponds to skin models generated from primary human cells (van Drongelen et al. 2014a). Yet, the filaggrin knockdown did not alter *stratum corneum* lipids in the cell-line-based skin models (van Drongelen et al. 2013), but in primary cell-based, skin models (Vávrová et al. 2014). Slight deviations to the human patient as well as the loss of patient heterogeneity will be tolerated in *Phase II* studies to limit the numbers of repeats, necessary to observe effects with statistical significance. Nevertheless, disease models in *Phase II* need to be qualified as outlined in the qualification section.

5.3 Preclinical *Phase III*

Models in *Phase III* include different cell types, extracellular matrix, and tissue architecture to obtain a more precise effect profile of the drug candidate. Testing in *Phase III* needs to consider patient heterogeneity by using primary human cells as well as pharmacodynamics and pharmacokinetics. Models and test protocols must be qualified for predicting human responses.

The lack of sufficient amounts of patient-derived cells is increasingly addressed by the establishment of various biobanks (Simeon-Dubach et al. 2016; Palechor-Ceron et al. 2019), but cannot satisfy the needs yet. Together with potential ethical concerns in biopsy taking, e.g., in children, this limitation further supports testing only the most promising drug candidates in on primary cell-based models in *Phase III*. Current approaches to retransform iPS cells of various donors might facilitate the use of patient-derived material, but the potential dedifferentiation as well as the fact that all these cultures are juvenile (embryonic) tissues already showed some limitations in aging research (Christensen et al. 2018).

The use of flow-through chambers in organ-on-a-chip cultures continuously supplies fresh medium, removes waste, and induces sheer stress related to the blood flow (Prantil-Baun et al. 2018). These dynamic culture conditions increase the cultivation times to 28 days, potentially useful for evaluating the efficacy and toxicity of several treatment cycles. Moreover, microfluidic platforms are suitable to investigate cancer metastasis (Lin et al. 2020) as well as hematopoietic stem cells (Sieber et al. 2018). The human-on-the-chip technology can connect several tissue chambers to an in vivo organ system (Maschmeyer et al. 2015).

The major challenge in preclinical drug development will be the transition to the *patient-on-the-chip*. Beyond efficacy testing, the consideration of the higher vulnerability of patients can provide a more relevant toxicological risk analysis, currently lacking in preclinical toxicity testing (Menshykau 2017). Finally, the drug candidates need to pass the standard tests of regulatory toxicology and safety pharmacology.

In particular, *Phase III* studies must be conducted in accordance to clinical trial protocols: blinding, randomization, and proper controls. Dosage should consider both the effective concentrations as derived from *Phase I and II* studies as well as pharmacokinetic calculations of *Phase I*.

6 The Price of Quality

The implementation of qualification and quality function deployment into model or test method development involves higher costs in the beginning and might slow down the time to the first publication. Moreover, these concepts require the availability of clinical data. Taking the development of models for skin aging as an example, reliable clinical data are scarce (Hausmann et al. 2020). Preclinical models for evaluating drug effects can hardly be better than the clinical knowledge of the disease. Once human cell-based in vitro models have been qualified, their modular design offers the opportunity to manipulate single parameters to better understand the underlying mechanisms of the disease. Despite several strategies in nonclinical research that already use parts of qualification, the final proof-of-concept is still to be awaited. Therefore, we suggest developing and qualifying a disease model first and perform a full validation subsequently. The best proof of our concept will be an increased success rate of investigative new drugs in clinical trials that have been evaluated before by qualified models in preclinical drug development.

The time of wasting costs and time for poorly predictive models and test methods in preclinical research should come to an end. The increased efforts in model development will pay off, since publishing "just another disease model" will cost more time and money than developing a model and test method that fulfill and have a real impact on preclinical research.

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Standardised Reconstructed Skin Models in Toxicology and Pharmacology: State of the Art and Future Development

Helena Kandarova and Patrick J. Hayden

Contents

Abstract

Three-dimensional (3D) reconstructed human skin (RhS) models featuring fully-differentiated characteristics of in vivo human epidermis have been known for almost 40 years. In this chapter, the topic of commercial in vitro tissue models is described, taking RhS models as an example. The need for highly standardised models is evident for regulatory testing purposes, e.g. the classification and labelling of chemicals and formulations, as well as for pharmacology-oriented

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Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_417

research and drug development. Following the standardisation of RhS model production by commercial developers, international validation studies and regulatory acceptance, 3D RhS models are now used globally in both industrial and academic research laboratories. Industrial production of standardised 3D RhS models involves GMP-compliant processes together with ISO 9001 documentation in order to control and ensure reproducibility and quality. Key biological, functional, and performance features that are addressed in industrial production include barrier properties, histological and immunohistochemical characterisation, lipid profile characterisation, and tissue viability before and after transport. An up-to-date survey of commercial RhS tissue producers and the regulatory acceptance status of major safety, hazard, and efficacy assays currently available to chemical and pharmaceutical industries is presented in this chapter. Safety and ethical concerns related to the use of human tissue in the industrial production of RhS models are discussed. Finally, innovative approaches to the production of standardised 3D RhS models including automated production, development of more representative 3D RhS models using advanced additive manufacturing tools, microfluidics technologies, and bioprinting are presented. The future outlook for 3D RhS models includes a prevalence of high-quality models which will be fabricated by end-users rather than commercial producers. These will overcome problems with shipments and customs clearance that many users still face when buying RhS from overseas commercial suppliers. Open-source technologies and commercial components for "do-it-yourself" RhS will significantly change the skin model market as well as regulatory acceptance of open-source models during the next decade. All of these developments and improvements will together allow more widespread use of in vitro RhS models for broader application as animal replacements in areas ranging from industrial and regulatory toxicology and pharmacology, to drug development and personalised medicine.

Keywords

3D-printing · Automated production · Quality controls · Reconstructed human skin models · Reproducibility · Validation

1 Introduction

The technologies leading to the three-dimensional (3D) reconstructed skin models have been known for almost 40 years. First models were produced by James Rheinwald and Howard Green on a layer of inactivated mouse 3T3 fibroblasts and were formed of 3–4 layers of keratinocytes (Green et al. 1979; O'Connor et al. 1981). Their primary use was to treat burned patients. One of the essential technological breakthroughs was a method developed by Pruniéras, where human keratinocytes cultivated on a de-epidermised dermis are exposed to the air–liquid interface. This method resulted in a fully-differentiated epidermis featuring characteristics of human epidermis in vivo (Prunieras 1979; Pruniéras et al. 1983).

Several other laboratories have reported a similar approach leading to the well-differentiated three-dimensional epidermis and full-thickness skin that also included dermal compartment (Augustin et al. 1995; Bell et al. 1991; Cannon et al. 1994; Naughton et al. 1989; Regnier et al. 1981; Rosdy and Clauss 1990; Tinois et al. 1991).

Apart from the skin grafting use in burned patients, the first tissue models served mainly for research interests. However, as the technology of tissue reconstruction improved, skin models started to be used for broader purposes. These included safety and efficacy testing of cosmetic ingredients and products, as well as hazard assessment of chemicals and pesticides. The models also found their use in the pre-clinical testing of novel drugs and topically applied pharmaceuticals (Groeber et al. 2011; Hayden et al. 2015, 2016; MacNeil 2007; Marques et al. 2018). Following the standardisation of tissue production by commercial developers, international validation studies and regulatory acceptance, 3D reconstructed human skin models are used now globally in both industrial and academic research laboratories. They have found usability in regulatory as well as non-regulatory testing purposes including:

- safety and efficacy of raw materials and formulations intentionally applied on the skin (cosmetics, pharmaceutical formulations). This process includes an assessment of skin irritation, inflammation, as well as genotoxicity, and phototoxicity,
- hazard and risk assessment of chemicals or formulations with accidental contact with human skin (regulated, e.g. by REACH and other chemical legislations) and help to address occupational safety,
- mechanistic information that can be utilised e.g. to determine whether a molecule or compound can be altered to reduce irritation or toxicity without loss of efficacy,
- prevention or enhancement of the penetration of a substance into the skin.

In medicine, pharmacology-oriented research and drug development, normal (i.e. obtained from healthy donors) as well as disease skin models are considered beneficial for the modelling and understanding of physiological and pathological skin conditions. Their use has been described for:

- skin cancer, psoriasis, atopic dermatitis, scleroderma research,
- burn and non-burn injuries and wound healing,
- infectious skin diseases,
- drug reactions including the relevance of drug metabolism (e.g. phototoxicity or skin rashes, adverse reactions to chemotherapy) as well as
- personalised therapies.

The need for highly standardised models, resembling closely healthy human epidermis (reconstructed human epidermis, RhE) or skin (full-thickness reconstructed human skin, RhS), is evident mainly for the regulatory testing purposes, e.g. in the process of classification and labelling of chemicals and formulations. Thanks to the extensive validation studies conducted in the past

Table 1 Examples of industrial reconstructed 3D skin models adopted into the regulatory toxicity testing and OECD test guidelines

Skin model	Producer	Production sites	Regulatory acceptance in guidelines[a]
EpiDerm	MatTek Life Sciences (formerly MatTek Corporation)	USA and Slovakia	OECD TG 431, 439 ISO-10993:23
EPISKIN	EPISKIN Laboratories	France, Brazil, and China	OECD TG 431, 439
EpiCS	Cell Systems,	Germany	OECD TG 431, 439
SkinEthic RHE	EPISKIN Laboratories	France, Brazil, and China	OECD TG 431, 439 ISO-10993:23
LabCyte	J-TEC	Japan	OECD TG 431
Skin+TM	Sterlab	France	OECD TG 439

[a]Use of the reconstructed skin models is also described in the ICH S10 guideline on photosafety assessment and ISO 10993 on biocompatibility testing of medical devices

(Alépée et al. 2010; Desprez et al. 2015; Fentem et al. 1998; Kandárová et al. 2006a, b, 2009; Liebsch et al. 2000; Spielmann et al. 2007) two OECD Test Guidelines (TGs) refer explicitly to the use of RhE for skin corrosion and irritation testing, i.e. OECD TG 431 and OECD TG 439 (Kandarova and Liebsch 2017; OECD 2004, 2009). These test guidelines also define the essential structural and functional criteria for the tissue models applicable within these TGs. They set several requirements for the tissue model producers as well as their users. These requirements are met in the industrial type of production that follows the rules of Good Manufacturing Practice (GMP), and also have other appropriate quality systems in place. The use of RhE models has also been considered in pharmaceutical regulations (ICH 2013; Kandarova and Liebsch 2017; Liebsch et al. 1999) and medical devices biocompatibility testing procedures as ISO-10993:10 and 23 (De Jong et al. 2018; Kandarova et al. 2018). Recently, validation studies with RhE and RhS have also been completed for genotoxicity testing using the Comet and Micronucleus assays (Hu et al. 2009; Reisinger et al. 2018), allowing their use in a tier-testing strategy. The methods have been accepted into the OECD test guideline development programme (Pfuhler et al. 2020, 2021) (Table 1).

2 Industrial Production of the 3D Skin Models in Standardised Conditions

GMP-compliant production together with ISO 9001 documentation practices are highly useful tools in managing the production process from the initial acquisition of the cells and raw materials, quality control throughout the production until the final distribution to the end-user. The aim of the standardised mass production of 3D tissue models is to ensure batch-to-batch reproducibility and correct prediction of test substances under the standardised or validated protocols. The production of skin models can be still manual (by trained lab technicians), but usually includes some

semi-automated processes or in a few cases already runs under the full automation. In the past decade, there are also promising attempts to produce tissue models using 3D bioprinting technology, but standardisation and validation of these systems are still underway. Regardless of the type of production, many parameters influence the lot-to-lot reproducibility of reconstructed tissue models (Rispin et al. 2006). These are not limited only to the production of skin tissues, but are more general, and include in particular:

(a) Source of the skin cells and cell type
  - Age of the donor (neonatal vs adult cells),
  - Skin site (foreskin vs breast or abdominal skin from surgeries),
  - Ethnic origin (Asian, Caucasian, African skin types),
  - Fibroblasts source (papillary vs reticular dermis),
  - Exposure of the donor to xenobiotics (resulting, e.g. into CYP activity differences),
  - Primary cells vs cell lines or induced Pluripotent Stem cells (iPSc),
  - Passage number of the cells in the production.

(b) Quality and amounts of the supplements
  - Choice of standard culture media,
  - Choice and concentrations of growth factors and additional supplements as, e.g. Ca^{2+}, ascorbic acid, hydrocortisone, etc.,
  - Inclusion/non-inclusion of the bovine sera in the production.

(c) Method of production
  - Intervals of media exchanges (one-time exchange vs continuous or semi-continuous flow),
  - Air–liquid interphase (ALI) approach,
  - Total time of the production (before and after ALI exposure).

(d) A substrate on which the skin model is produced
  - Microporous membrane (for instance, polycarbonate or Teflon) and coating of the membrane (for instance, collagen layer),
  - Scaffold (biological origin or engineered) that mimics the underlying dermis and can also be populated with fibroblasts,
  - Type of the insert.

(e) Training of the production personnel

(f) Quality controls (QC) of the tissues in the production process and main QC of the finalised tissue batch.

2.1 Barrier Properties

A critical feature of the tissue batch release is the ability of the skin model to identify full spectra of responses (from very weak to very strong) to standard test materials and the presence of an effective skin barrier able to resist the enhanced penetration of the cytotoxic materials. A well-established test to address these concerns is the assessment of the ET-50 value (i.e. Effective Time at which toxic material of a

fixed concentration and volume reduces the tissue viability to 50% compared to negative control) or IC-50 value (i.e. Inhibition Concentration of a toxic substance that causes the 50% reduction in cell viability in the skin model). Usually, 1% Triton is used for the ET-50 skin test and SDS in at least three concentrations is used in the IC-50 test.

Every skin model producer has established typical ET-50 or IC-50 ranges into which the standard production must fall (Rispin et al. 2006). For the validated tissue models, these values are part of the OECD TGs 431 and 439, but the skin model producer should provide these values also on the certificate of every tissue batch released. The range is usually established as a mean of ET-50/IC-50 values over a one-year production period and ± 2 standard deviations. It is important to note that the ET-50/IC-50 values may not be directly comparable between the skin models since these values are dependent on the dose/area ratio (i.e. the volume of the material applied and the surface of the tissue model) and concentration of the control material used for the test.

Recently, impedance spectroscopy has been proposed as a non-destructive complementary method and a reliable parameter to measure the barrier properties of the skin models (Groeber et al. 2015). This approach, however, is not yet implemented into the OECD guidelines.

2.2 Histology and Immunohistochemistry

Histology, including immunohistochemistry of the reconstructed skin model, are of critical importance and thus are assessed by the producers regularly or even for every tissue batch. In the case of a new skin model development, a full immunohistochemistry profile should be part of the evaluation process. Immunofluorescent localisation of epidermal markers (such as, e.g. keratins 5, 10, and 14, involucrin, loricrin, filaggrin, transglutaminase, etc.) should reveal normal epidermal differentiation. In the case of full-thickness models (RhS), it is of importance to evaluate dermal-epidermal junction markers (laminin 5, collagen-IV, collagen VII) and dermal fibrillin 1 as well as procollagen I. Cell proliferation marker Ki67 is studied as well. Once a model has been established, haematoxylin-eosin sections are sufficient for the standard control (Bouwstra and Ponec 2006; Boelsma et al. 2000).

The epidermis model should have functional stratum basale and at least 2–3 supra-basal cell layers of the viable epidermis capable of cell metabolism. The other layers typical for the native epidermis, i.e. stratum spinosum, stratum granulosum, and stratum corneum, should be present. The thickness of the well-developed epidermis model ranges 70–140 μm. The thickness of RhS models may differ significantly, depending on the thickness of the reconstructed dermis or biological scaffold used.

In some cases, the thickness of the stratum corneum (SC) may be seen as a measure of good barrier properties of the model. However, only the combination of the correct lipid profile and appropriate organisation of the lipids in the SC will assure that the barrier will resist fast penetration of xenobiotics. Thus, a skin model

with relatively thick SC may exhibit poor barrier properties if it lacks lipid classes essential for the barrier formation (mostly ceramides). The highly resistant barrier structure of the SC is determined by the chemical composition of protein-enriched corneocytes and the surrounding extracellular lipids.

2.3 Lipid Profile Characterisation

The lipid profile of commercially available skin models has been described many times in the literature (Boelsma et al. 2000; Kandarova 2006; Ponec et al. 2002). As mentioned above, lipids in the SC are the critical component to the formation of the barrier properties of the skin model. The establishment of an appropriate lipid profile is mostly influenced by the supplements in the culture media, but can be also are donor-specific (Simard et al. 2019; Bouwstra and Gooris 2010). Alteration of the lipid profile will be immediately visible in the tests for barrier properties (especially in the ET-50 test). Since the characterisation of the lipid profile is a significant time and resource consuming activity, it is not recommended to conduct it as a standard Quality Control (QC) test for every tissue batch. However, lipid profile characterisation is essential in the case of new tissue model development or the occurrence of significant production modifications (Ponec et al. 1997, 2000).

2.4 Tissue Viability and the Effects of Transport

A fourth critical parameter in the industrial tissue model production is tissue viability before and after shipment of the product to the end-user. The skin model producer must ensure that the tissue integrity and viability remain comparable to the tissue viability assessed in the laboratory during the final QC. For that reason, the producers also invest a considerable amount of time into the evaluation of the shipping procedures from the manufacturer's laboratory to the end-users, which may be either local, national or international. Special attention is required for the shipping of the products overseas (Kaluzhny et al. 2015). In these cases, the packaging must ensure that the content will not be exposed to either high or low temperatures and that it remains intact despite the multiple means of transport. For that reason, commercial tissue model producers must conduct shipping studies before the launch of the product to ensure the optimal conditions for the packaging, transportation as well as the shelf-life of the product. These data should be provided at request to the end-users.

3 Industrial Production of 3D Disease Tissue Models

Many skin disorders have been successfully modelled with the use of bioengineered skin, for example, epidermolysis bullosa, psoriasis, skin ulcers or atopic dermatitis (Semlin et al. 2011; Sarkiri et al. 2019; Roy et al. 2018; Jean et al. 2009). However,

large-scale or even automated industrial productions of the above-mentioned 3D skin disease models are not yet feasible. Currently available commercial 3D tissues are usually too simple to mimic the complex skin diseases, the pathogenesis of which results from complex interactions between multiple cellular or molecular components and where even the skin microbiome may play an important role.

There are three main methods of generating skin disease models:

1. reconstruction using patient cells,
2. reconstruction by addition of substances to initially normal bioengineered skin, acting as disease triggers
3. use of genetically modified cells to construct the skin model.

Because of practical drawbacks in the sourcing of skin from donors suffering from various diseases, the production of disease models is mostly in the hands of academic institutions and hospitals with access to the patients. The second and third types of the 3D models are more frequently described in the scientific reports, but the experimental approach varies from laboratory to laboratory. Despite the above mentioned, some of the models of skin diseases are commercially available (Table 2) and can be used in the pre-clinical testing of drug candidates.

Development of 3D human in vitro tissue models, capable of reliably and reproducibly mimicking human diseases will be of great importance for the drug development and also for personalised medicine. For many types of aggressive skin cancers, but also, e.g. for psoriasis research, the existing animal models poorly reflect the situation in patients.

Table 2 Examples of industrial reconstructed 3D skin models mimicking skin disease or photo-ageing

Skin model	Producer and country	Remark
Psoriasis	MatTek Life Sciences, USA	Non-transformed human epidermal keratinocytes (NHEK) from neonatal foreskin and psoriatic human dermal fibroblasts derived from neonatal foreskin and adult psoriatic explants
	Sterlab, France	Human epidermal keratinocytes stimulated with psoriasis inducing substances. Components are added to the media during tissue reconstruction
	Straticell, Belgium	Two models available, psoriatic features are induced by IL-17 and IL-22 exposure
Melanoma	MatTek Life Sciences, USA	The model consists of a human malignant melanoma cell line (A375), NHEK and non-transformed, human dermal fibroblasts
Atopic dermatitis	Straticell, Belgium	Atopic dermatitis pathology is induced by interleukins (IL-4, IL-13, IL-25) exposure
Photo-aged skin	EPISKIN Laboratories, France	NHEK seeded on the photo-aged fibroblasts

On the other hand, even the best 3D skin model is still an isolated system and therefore attempts to incorporate immune cells, and structures mimicking vascularisation into the model/system are of crucial importance for future development in this field (Marino et al. 2014). Standardisation of these models will be significantly more challenging since, e.g. the functional vascularised models will have to be developed and standardised together with microfluidic and perfusion devices. This combined approach will, on the other hand, ensure adequate and reproducible tissue perfusion.

4 Safety and Ethical Concerns in the Industrial Production of Normal and Disease Models

There are three sources of cells commonly used for building of 3D skin models: primary cells, cell lines, and stem cell-derived cells. Primary cells used in skin constructs are isolated from human donor tissue (e.g. neonatal foreskin or breast surgeries). As such, they present a biological risk and must undergo screening for the absence of HIV, hepatitis viruses, Treponema pallidum, and other infectious pathogens (Pedrosa et al. 2017). Particular attention is required for work with samples generated from cells of diseased donors. Donor consent must be in place. If the cells are adequately harvested from the target tissue at the desired health/disease stage, they are the best candidates to recapitulate the specific tissue functions and responses.

A severe problem in the industrial production is microbial contamination that might be coming either from the donor cells or can be introduced in the laboratory by improper culture techniques or by use of non-sterile components. Standard tissue models are usually produced in the culture media containing antibiotics. However, antibiotic concentration is typically low, and thus attention must be paid to maintaining sterility, especially in the mass production of the models. The introduction of contamination into the semi-automated or automated production will result in failure of the whole tissue lot and may also be traced to future production lots if the source cannot be easily and quickly identified and eliminated.

Use of bovine sera or other animal-derived components in the keratinocyte culture and in the production of the 3D models remains a challenge for many developers. Animal-derived components provide a broad spectrum of macromolecules, proteins, nutrients, hormones, and growth factors, that are essential in the culture of keratinocytes or fibroblasts. The serum is, however, the most frequent component leading to the high variability of the 3D cultures as its composition varies from batch to batch. In the last two decades, this knowledge together with the ethical issues related to the use of foetal or new-born bovine sera led into a number of chemically defined serum-free media formulations or even use of human-derived sera in some of the assays. Still, not every laboratory or 3D model developer may be willing to consider these alternatives that might be more expensive, despite the possibilities for improvement in quality and reproducibility of the models (Gstraunthaler 2003).

5 Innovative Approaches to the Production of the Standardised 3D Skin Models

There are two main areas for innovative approaches that are being introduced to the standardised 3D skin models:

- the automation of the technological process that results in the reduction of manual labour, shortening the production time and improvement of the reproducibility of the production influenced by the human factor,
- development of more representative 3D models using advanced additive manufacturing tools, microfluidics technologies, additive manufacturing, and bioprinting.

Every producer of reconstructed human tissue models has at least partly automated some of the production processes once the tissue demand increased. For instance, cell counting, feeding procedures, media exchanges, and overall liquid handling can be semi-automated or fully automated relatively easily. The introduction of automation, however, requires laboratory hardware adjustments as compared to the standard manual production. This includes, for instance, change of the shape or type of the trans-well inserts used to culture tissue models to allow for different levels of media in the feeding trays. Other necessary steps are adjustments of the incubators to accommodate tubing and pumping or even a purchase of robotic production stations that can handle these steps in an aseptic and fully controlled environment. Automation is also present on the site of the users of the 3D technologies, and therefore the tissue model producers need to consider innovative products that could be compatible with the automation in the users' labs. This advancement has led, for instance, into the development of the skin models in 24 or 96 well plates.

The fully automated production of 3D skin tissue models is rare but has been described, e.g. by a Fraunhofer Institute Group. They generated a system called "Skin Factory", in which all the bioengineered skin fabrication steps, ranging from sterilisation of the skin biopsies to the assembly of the cells in the 3D matrix, are carried out by a robotic arm with no participation of humans. The same group has managed to automatically generate human epidermis models with the use of a robot (Sarkiri et al. 2019). Systems such as the "Skin Factory" are, however, costly and require very complex knowledge that includes programming and mechanical engineering. Industrial production would need several of these production units, which at the current price, is not an affordable business concept. Moreover, despite the automation of the production process, it remains time-consuming, as it includes the same steps as the manual fabrication by laboratory technicians. The only benefit seems to be the replacement of human hands by robots, which, however, brings another drawback of this method since robots are barren of human adaptability.

A relatively novel technology that allows for faster semi-automated production of the full skin models under standardised conditions is lyophilisation and electrospinning (Yun et al. 2018; Lee et al. 2014; Powell and Boyce 2009). These

two techniques are of importance for the creation of the scaffolds needed further to generate RhS models. By lyophilisation, a collagen solution converts into a dry porous collagen scaffold, and in electrospinning, collagen fibres are attracted together forming porous collagen structures, too, which give cells space to grow, proliferate, and migrate. The formation of the scaffolds is not manual; however, the seeding of the cells is still manually conducted and hence tedious and not standardised (Sarkiri et al. 2019). Full-thickness models build on the electrospun dermal scaffolds have been recently described (Yun et al. 2018; Lee et al. 2014; Powell and Boyce 2009). In these models, fibroblasts infiltrate into the structure and synthesise extracellular matrix components which self-assemble in situ forming a robust stromal matrix including the basement membrane, without the need for animal-derived collagen typically used for the production of RhS (Derr et al. 2019; Marengo et al. 2019).

3D bioprinting is an emerging bioengineering technique that offers an excellent opportunity to expand output and improve the precision of traditional tissue engineering methods. There are several 3D bioprinting methods, each with its advantages and limitations. The primary types of 3D bioprinting technologies include inkjet-based, extrusion-based, and light-assisted printing. Each of these approaches has the capability to both print scaffolds for cell seeding and encapsulate cells directly within scaffolds to build tissue constructs. However, these platforms differ in various aspects, including their printing mechanisms, resolution, time, and material choice. Based on recent publications, extrusion-based printing seems to be the most used technique followed by light-assisted and inkjet-based printing approaches (Ma et al. 2018).

There have been notable efforts to generate bioprinted skin equivalents in recent years. Bioprinting companies like Organovo, CTIBiotech or Poites also teamed up with, e.g. L'Oréal or BASF to advance the development of bioprinted skin via research projects and collaboration (Russon 2015; Annon 2018; BASF 2019). Bioprinting seems to be a novel, highly disruptive technology that may enable standardised as well as customised production of the reconstructed tissue models in the end-users laboratories. This technology may lead to the creation of highly standardised "Open-source models" that by quality and variability will possibly be same or even better than tissue models manufactured by the industry today.

6 Outlook

The industrial skin model reconstruction technology has achieved over the past 40 years, several important milestones. Multiple validation studies showed that:

- batch-to-batch reproducibility of the skin models, and
- precisely optimised methods addressing particular toxicology concerns,

are the keys to the implementation of the technology into the regulatory framework. The understanding of the reproducibility importance led most of the manufacturers

to several significant improvements in the technology process of model fabrication. Improvements include minimising or complete elimination of the animal components from the culture medium (bovine sera, collagen, etc.) and use and semi-automated production.

The disruptive new technologies of the twenty-first century such as automation and bioprinting, and advances in the material engineering will likely enable in the near future large scale industrial production of at least partly immuno-competent skin models for the use on microfluidic chips. Attempts to include reproducible vascular networks and missing skin appendages in the reconstructed skin models are already described in the literature. Thanks to the implementation of 3D bioprinting technology in the testing laboratories, we will see in the following years more frequently models, that will be fabricated by end-users rather than commercial skin model producers. The quality of these tissues will be probably equal if not better than the variety of skin models produced commercially in the past decade. These advancements will overcome problems with shipments and customs clearance that many countries still face when buying tissues from overseas. Open-source technologies and commercial companies offering components for "do-it-yourself" skin models will probably significantly change the skin model market as well as regulatory acceptance of the "open-source models" during the next decade. All of these developments and improvements will together allow more widespread use of in vitro RhS models for broader application as animal replacements in areas ranging from industrial and regulatory toxicology and pharmacology, to drug development and personalised medicine.

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Part II

Models For Drug Research

In Vitro Models of the Blood-Brain Barrier

Winfried Neuhaus

Contents

Abstract

Knowledge about the transport of active compounds across the blood-brain barrier is of essential importance for drug development. Systemically applied drugs for the central nervous system (CNS) must be able to cross the blood-brain barrier in order to reach their target sites, whereas drugs that are supposed to act in the periphery should not permeate the blood-brain barrier so that they do not trigger any adverse central adverse effects. A number of approaches have been pursued, and manifold in silico, in vitro, and in vivo animal models were developed in order to be able to make a better prediction for humans about the possible penetration of active substances into the CNS. In this particular case, however, in vitro models play a special role, since the data basis for in silico models is usually in need of improvement, and the predictive power of in vivo animal models has to be checked for possible species differences. The blood-brain barrier is a dynamic, highly selective barrier formed by brain capillary endothelial cells. One of its main tasks is the maintenance of homeostasis in the

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_370

CNS. The function of the barrier is regulated by cells of the microenvironment and the shear stress mediated by the blood flow, which makes the model development most complex. In general, one could follow the credo "as easy as possible, as complex as necessary" for the usage of in vitro BBB models for drug development. In addition to the description of the classical cell culture models (transwell, hollow fiber) and guidance how to apply them, the latest developments (spheroids, microfluidic models) will be introduced in this chapter, as it is attempted to get more in vivo-like and to be applicable for high-throughput usage with these models. Moreover, details about the development of models based on stem cells derived from different sources with a special focus on human induced pluripotent stem cells are presented.

Keywords

Astrocyte · hiPSC · Microphysiological systems · Neuron · Oligodendrocyte · Pericyte

1 The Blood-Brain Barrier

The blood-brain barrier (BBB) separates the periphery from the central nervous system (CNS) and is responsible for maintaining homeostasis in the CNS. The BBB can be considered a highly selective, active, bidirectional filtering system that strictly regulates what enters the CNS and what leaves from the CNS into the blood. The BBB protects against biological (pathogens), chemical (xenobiotic, toxins), and physical damage (trauma). Over 95% of the compounds with targets within the brain cannot enter the CNS (Pardridge 2007). In the case of biopharmaceutics, almost none can permeate across the BBB in a reasonable, treatment relevant amount. Therefore, there are great efforts to develop drugs that can overcome the BBB, as there is an insufficient number and consequently a major need for effective therapies for a myriad of CNS-related neurological, neurodegenerative, or neurodevelopmental diseases. On the contrary, in drug development it is also very important to know whether drugs addressing targets in the periphery can overcome the BBB and trigger undesired adverse effects in the CNS.

The original concept of a barrier preventing the movement of substances between blood and the brain stemmed from studies of dye injections made into the circulation. A variety of vital dyes stained almost all animal organs, except for the brain and spinal cord. Originally, these first findings were attributed to the German scientist Ehrlich (1885), although more detailed surveys of the publications showed that the corresponding staining experiments were carried out somewhat later and the idea of a brain barrier was developed by scientists such as Goldmann, Lewandowski, Bouffard, and Franke. The term "barrier" was probably first formulated by the scientist Lina Stern around the year 1920 (Ribatti et al. 2006; Joó 1993; Ehrlich 1885; Saunders et al. 2014). Today we know that the sealing component and the BBB per se are the brain capillary endothelial cells (BCECs) (Fig. 1a: Scheme of the BBB). In contrast to peripheral endothelial cells, BCECs are characterized by a

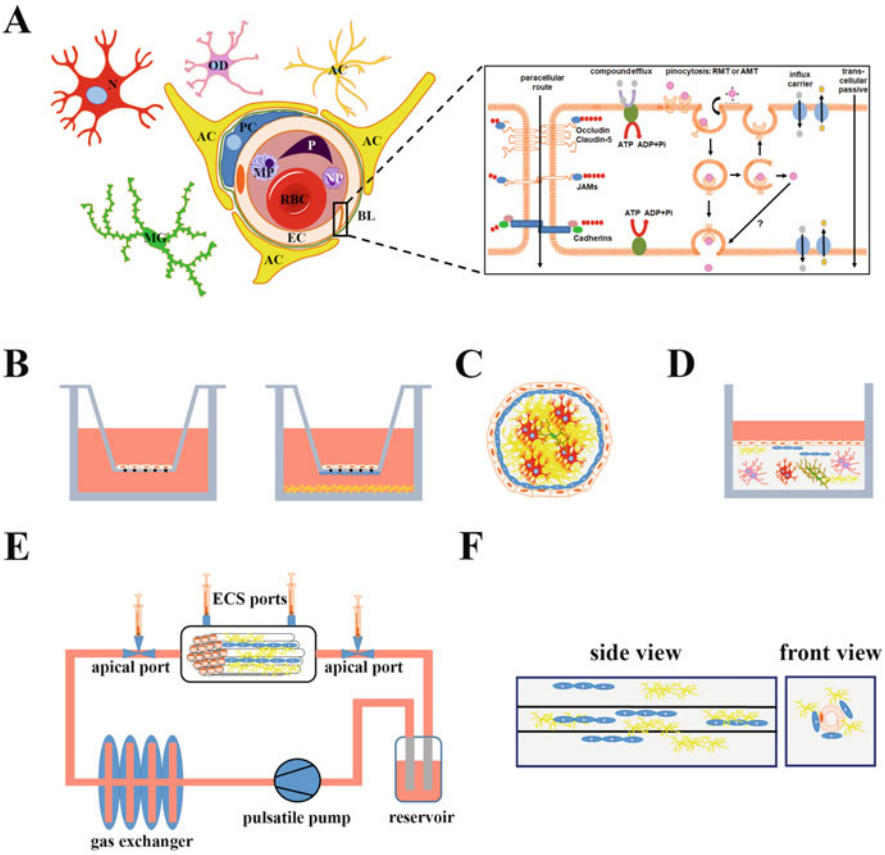

Fig. 1 Schematic cross-section of a brain capillary (**a**) showing the brain capillary endothelial cells (ECs) with a neighbored pericyte (PC) sharing the same extracellular matrix surrounded by the basal lamina and covered by astrocyte (AC) endfeet. Microglia (MG), oligodendrocytes (OD), and neurons (N) in the brain side and erythrocytes (RBC = red blood cells), platelets (P), macrophages (MP), and neutrophils (NP) in the blood are close to the BCECs illustrating the role of the BBB as interface between the periphery and the CNS. The enlarged picture on the right side illustrates the different routes across the BBB including the paracellular pathway restricted by tight (occludin, claudins) and adherens (cadherin) junctions and the transcellular pathways including passive diffusion and the role of efflux (e.g., ABC-transporters such as P-gp or BCRP) as well as influx mechanisms (*RMT* receptor-mediated transcytosis, *AMT* adsorption-mediated transcytosis, carriers for, e.g., glucose or amino acids). Schemes of cell culture models are presented (**b**-**f**), in particular, the transwell model with mono-cultured BCECs and triple-cultured BCECs plus pericytes on the opposite of the membrane and astrocytes on the bottom of the well (**b**); the BBB spheroid model with a core of CNS cells ensheathed with pericytes and covered with the outmost layer of BCECs (**c**); a hydrogel model with CNS cells embedded in a hydrogel covered with a layer of BCECs on the top (**d**); the hollow fiber model consisting of the cartridge with a bundle of capillaries, two extracellular space (ECS) ports and two apical ports, a growth medium reservoir, a pulsatile pump, and as a gas exchanger the silicone tubing (**e**); and a side and a front view of a microfluidic models made of hydrogel cast with a capillary lumen, in which BCECs are cultured surrounded by CNS cells within the hydrogel block (**f**) connected to a tubing and a pump system (not shown in the figure)

tightly sealed cell interspace, the absence of fenestrae, and reduced pinocytosis (Joó 1993). The BBB is not only a physical (paracellular) barrier, which makes the passage of hydrophilic molecules more difficult, but also a transport and metabolic barrier. BCECs possess an arsenal of ABC (ATP-binding cassette) and SLC (solute carrier) transporter proteins, which together regulate the transport of substances across the BBB. Drug development focused on the group of ABC transporters, which were associated with multidrug resistance. In particular, P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2), and the group of multidrug resistance-related proteins (MRPs, ABCCs, especially MRP1-5) were intensively studied (Mahringer and Fricker 2016). These transporters recognize a large number of drugs and prevent the transfer from the blood to the CNS under energy consumption by pumping them back into the blood. A bit less than about 400 SLC transporters are known, including many orphan transporters of which the function has not yet been elucidated (Suhy et al. 2017; Morris et al. 2017; Geier et al. 2013). Some of the SLC transporters are responsible for supplying the CNS with nutrients. They transport endogenous substrates such as hormones, amino acids, or hexoses such as glucose through the BCECs into the CNS and are therefore often referred to as influx transporters. In this context, there are repeated efforts in the field of drug delivery to use the SLC transporters (e.g., SLC7A5 = LAT-1) as an influx shuttle into the CNS or to block the ABC transporters and their efflux function to overcome the BBB (Scalise et al. 2018; Huttunen et al. 2019; Breedveld et al. 2006; Pavan et al. 2014; Abdullahi et al. 2017; Wagner et al. 2009).

In addition to the transport barrier, the BBB also represents a metabolic barrier. BCECs have a significantly higher density of mitochondria compared to peripheral endothelial cells in order to provide the high energy demand required for barrier function. Many enzymes are involved in the barrier function (Kalaria and Hase 2019; Oldendorf et al. 1977; El-Bacha and Minn 1999). These can convert active substances into less effective metabolites or modify them for subsequent excretion. The exact knowledge of the enzyme activities in the BCECs can, however, also be used for prodrug strategies in drug delivery. As an additional barrier component, the glycocalyx at the cell membrane of BCECs recently receives increasing interest (Santa-Maria et al. 2019; Hempel et al. 2014). Basically, a distinction is made between paracellular and transcellular transport routes across cell layers. The paracellular route across the healthy BBB is normally closed by the tight junctions (TJs). There are several ways to get transcellular through the BCECs. Substances that have the appropriate lipophilicity and are not recognized by efflux pumps can permeate by diffusion through the cell membranes and the cytosol. More hydrophilic or very lipophilic substances can overcome the cell membranes with the support of carrier systems (often SLC transporters), whereby the principles can be different (e.g., mono- or co-transporters) (Abbott et al. 2006). Proteins or particulate drug delivery systems (nanoparticles, liposomes) can be endocytosed by receptor-mediated or adsorption-mediated-dependent mechanisms, whereby the cargo of the membrane-derived vesicles can be released within the cell or also transferred to coupled transporting vehicles or be transported directly to the other side of the cell (Abbott et al. 2010).

The barrier functions of the BCECs are strongly regulated by their microenvironment. Both studies about BBB development and in vitro studies have shown that astrocytes, pericytes, and neurons can modulate TJs and transporter systems. At the same time, BCECs can secrete factors that, for example, alter the function of astrocytes (Abbott et al. 2006). The term neuro/glia vascular units (NVUs) were introduced with cope for this essential mutual regulation of functions (Fig. 1a: Scheme of the NVU). It is currently hypothesized that astrocytes are important for the induction of BBB properties (TJs and ABC transporter functions), whereas pericytes are responsible for the suppression of peripheral endothelial cell properties (e.g., reduced pinocytosis rate) (Daneman and Prat 2015). It can also be assumed that microglia and oligodendrocytes play an important role in the communication within the NVUs, although the current number of studies on the interaction between BCECs and microglia or oligodendrocytes is rather low (Kimura et al. 2019).

Another BBB regulating factor is the shear stress exerted by blood flow on the BCECs. It is assumed that the shear stress in the brain capillaries is 5 to 25 dyne/cm^2 (Koutsiaris et al. 2007; Cucullo et al. 2013; Wang et al. 2019). Shear stress significantly reduces proliferation, strengthens the paracellular barrier, increases the expression of BBB relevant transporter proteins, and changes metabolism (Cucullo et al. 2011). In addition, the morphology of BCECs is changed into a more elongated form, and the lifespan of the cells is significantly prolonged (Neuhaus et al. 2006b; Ott et al. 1995). Based on the barrier functions of the BBB, corresponding general markers can be used for in vitro studies to verify and validate cell culture models.

For the estimation of the paracellular barrier of the BCEC layers in in vitro models, the noninvasive measure of transendothelial electrical resistance (TEER) is often determined. This value reflects the permeability of ions. The more ions can permeate, the lower the measured TEER. In vivo data of frog and rat experiments suggested average resistance values of the BBB between 1,500 and 2,000 Ω cm^2 as a benchmark also applicable for in vitro models (Crone and Olesen 1982; Butt et al. 1990). When comparing these numbers with the values of in vitro models, however, the differences in the applied TEER measurement methods (electrode setup, applied voltage/current and frequency, data calculation formula) should be considered in a very careful manner. Furthermore, it should not be overlooked that these values represent an approximation to humans based on ultrastructural similarities of TJs in animals and permeation data. As the author of this chapter, I would particularly recommend the comparison of permeation data from paracellular markers as a benchmark instead of relying on TEER data, especially when it comes to the transport of drugs (which are significantly larger than ions).

For TJ characterization, the TJ-associated molecules zonula occludens-1 and occludin are most often tested. Claudin-5 is considered BBB specific in the brain. Important roles for the TJs at the BBB have also been postulated for claudin-1, claudin-3, and claudin-12, although the function and their presence in the healthy BBB have recently been clearly questioned when studying mouse models. A problem in the analysis of claudins lies in the specificity of the antibodies, since claudins have high sequence homologies, and therefore often cross-reactions occur with claudin-detecting antibodies (Castro Dias et al. 2019a, b). For the assessment of an in vitro BBB model, it is important to know about differences in claudin species-

dependent expression patterns and intensities between, for example, mouse and human (Berndt et al. 2019). In addition, both the modes of tissue sampling (e.g., laser microdissection of brain section versus isolation of capillaries) and cell cultivation alter claudin expression patterns. In principle, almost all of the currently 26 known claudins can be detected in the transcript of human samples. This raises the question of how close an in vitro model could and should mimic the expression patterns of the tissue samples. Currently, the focus is mainly on claudin-5 because it is one of the most abundant claudins and its role for the paracellular barrier is proven both in vivo and in vitro (Nitta et al. 2003; Neuhaus et al. 2018). For the transporters, the expression and function of the ABC transporters P-gP, BCRP, and sometimes MRPs and the SLC transporters Glut-1 and Lat-1 are used as benchmarks; species differences in the relative expressions should be taken into account. For example, in contrast to rodents, BCRP expression significantly exceeds P-gP expression in human capillaries (Shawahna et al. 2011). The transferrin receptor, the insulin receptor, and the LDL receptor-related proteins are regarded as important markers for the BBB. BBB markers also include general endothelial cell markers such as von Willebrand factor (vWF), CD31/PECAM-1, Tie-2 (TEK receptor tyrosine kinase), VEGFR2 (vascular endothelial growth factor receptor 2; = KDR (kinase insert domain receptor)), and VE-cadherin (vascular endothelial cadherin or CDH5 as member of the adherens junctions). These markers should be present to prove endothelial identity especially when investigating endothelial-specific signaling pathways.

Most drugs are developed to treat diseases. Therefore, it is of immense importance to note that the BBB is altered in many diseases. This can affect the physical, the transport, as well as the metabolic barrier. These changes can clearly influence the progression of the disease, and the return of the BBB to its healthy state can have a positive effect on the disease progression. Since the BBB is the interface with the largest surface between the periphery and the CNS, its important role is not surprising, as the BBB also transmits the communication of changes in blood circulation to the CNS and can influence the cells of the CNS such as astrocytes, microglia, and neurons. Therefore, including the BBB in the examination of CNS diseases is often helpful to improve the understanding of disease progression and to develop new therapeutic strategies. Diseases, in which the BBB is altered, include Alzheimer's disease (Zenaro et al. 2017; Montagne et al. 2017), Parkinson's disease, multiple sclerosis (Sweeney et al. 2018), brain tumors (Liebner et al. 2018), pain (Lochhead et al. 2017), hypertension (Setiadi et al. 2018), epilepsy (Swissa et al. 2019), lysosomal storage diseases (Begley et al. 2008), cerebral malaria (Nishanth and Schlüter 2019), stroke (Stamatovic et al. 2019), traumatic brain injury (Shlosberg et al. 2010), amyotrophic lateral sclerosis (Kakaroubas et al. 2019), and many more (Sweeney et al. 2018). There are also diseases in which mutations lead to altered transporter function in BCECs, and these mutations at the BBB are causally involved in these diseases (MCT8-deficiency, role of LAT-1 mutations in autism (Vatine et al. 2017; Tărlungeanu et al. 2016)).

When selecting and using in vitro models for drug development, many factors have to be taken into account that are dependent on the particular topic to be

investigated, such as the following: relevance of the microenvironmental cell types, type of transport route to be studied (relevance of high TEER/low paracellular permeability, expression/localization/functionality of relevant transport proteins), species differences, relevance of disease-specific alterations, relevance of shear stress, source and availability of BCECs (relation between available cell number and growth surface of the model), in vitro model-specific properties (scaffold material – cell interactions (stiffness of the scaffold, pore sizes, porosity)), type of basal lamina (extracellular matrix determines also BBB properties (Grifno et al. 2019; Zobel et al. 2016; Gaston et al. 2017), and the availability of human data as benchmark. Human data are recommended to represent the benchmark, especially functional parameters.

Based on these facts, the chapter is divided in such a way that first a general overview of BBB in vitro models is given, followed by special parts on cell culture models. This starts with the description of possible cell sources as they represent the most important component of the cell culture models; next the variety of cell culture model setups is described. A special focus is put on the latest developments with stem cells and microfluidic models.

2 In Vitro Methods to Study the Blood-Brain Barrier

There are a large number of in vitro methods available that can be used in drug development to estimate the permeability of a substance across the blood-brain barrier. In addition to cell culture models, brain slices or isolated capillaries can be used immediately after brain tissue collection, or plasma vesicles can be obtained by several centrifugation steps to perform drug uptake or transport experiments (Loryan et al. 2013; Krämer et al. 2001; Peterson and Hawkins 1998; Soldner et al. 2019). There are also several cell-free methods, such as PAMPA (parallel artificial membrane permeability assay) or IAM-HPLC (immobilized artificial membrane HPLC). IAM-HPLC or similar techniques are used to estimate the interaction between the substances and the lipophilic part of the cell membrane. IAM-HPLC involves the use of special columns coated with lipophilic scaffolds, e.g., lecithin. The resulting retention times of the active substances are a measure of the interaction with the lipophilic membrane. It can be assumed that the higher the retention time, the stronger the interaction with the membrane is and consequently a higher probability exists that the substance could permeate through the cell layer by passive diffusion (Alvarez et al. 1993).

PAMPA was developed as a cell-free, two-chamber test system for permeability studies of small molecules. A multi-well plate is used as a compartment in which a plate with inserts is inserted. The bottom of the inserts is made of a porous plastic membrane over which a plastic cell defines the volume of the second chamber. The plastic membrane is impregnated with lipids immediately before the experiment. These lipid solutions can be quite simple, e.g., 2% lecithin dissolved in hexane or can consist of special purified lipid membrane components isolated from tissue, which can also be purchased. For the test itself, it is important that these lipids do not

dry out and that the test solutions are applied relatively quickly. A classical test lasts up to 6–24 h and is carried out with concentrations of 10–100 μM substance at RT or 37°C. The test solutions are usually added into the lower chamber, and the permeated amount of substance after incubation is measured from the upper chamber with HPLC/UPLC-UV or MS. The apparent permeability Papp [cm/min], the permeability coefficient [cm/s], the mass balance [%], or the flux [%] can then be calculated. Permeated quantities are related to the amounts applied in the donor chamber, the volume of the acceptor (receiver) fluid, and the surface of the plastic membrane. Particular attention must be paid to the mass balance to ensure that the substance does not adsorb in the membrane or on the plastic too much and that the resulting permeability coefficients are not influenced by this loss of compound in the system. In addition, the so-called unstirred water layer can be a very relevant influencing factor in the PAMPA experiments, which is why an additional stirrer system is often offered in the commercially available models. Here small magnetic stirrers are introduced into each individual well and stir up the unstirred water layer so that it no longer represents a too significant barrier for substance permeation (Kansy et al. 1998, 2004, Di et al. 2003; Avdeef and Tsinman 2006; Bicker et al. 2016). It should also be mentioned that these systems can only be used for estimating the transport by passive diffusion. The data can be correlated with parameters describing the lipophilicity of the molecules, such as the logP value. As the degree of dissociation of certain molecules depends on the pH value, the use of logD values at certain pH values is recommended. In order to investigate the influence of active transport processes, vesicle systems expressing the according transporters within the cell membrane can be used, or one can immediately switch to cellular systems. For the vesicle-based test systems, cell membranes are prepared from various sources and used in biochemical assays based either on measuring ATPase activity of ABC transporters, the translocation of substrates into so-called inside-out vesicles, or the occlusion of the nucleotide during the transport catalytic cycle of ABC transporters (Glavinas et al. 2008). Several test setups are also commercially available. In the special case of testing large molecules such as biopharmaceuticals, cell-based systems are indispensable.

Brain slices could be very useful to measure the binding and uptake of drugs to cells of the CNS. These data in connection with results from animal experiments using the same species as for the brain slice tests and the determination of the free, unbound fraction $F_{u,\ brain}$ of the active substances in the CNS, after they have crossed the blood-brain barrier, can lead to well-founded pharmacokinetic estimates ($K_{p\ u,u,\ brain}$, unbound drug partitioning coefficients) of the truly free and potentially effective permeated amount of the substances in the CNS (Mihajlica et al. 2018). Nevertheless, brain slices are not suitable for measuring the permeability of drugs.

Isolated brain capillaries represent a unique tool to simulate transport processes at the blood-brain barrier. They must be used relatively quickly after the brain tissue has been removed and the capillaries isolated, as the energy balance decreases fast, which can be very relevant for the testing of active transport processes. In most cases, fluorescent substrates are used for readout. In particular, the functional activity of the transporters is recorded by fluorescence microscopy over time by applying the fluorescent substrates from the outside and measuring the enrichment of the

fluorescence in the lumen of the capillaries and then evaluated using image processing software programs. These experiments can be combined with inhibitors, signaling pathway modulators, or disease-specific stimuli (Hartz et al. 2004, 2018). In addition, the capillaries can be used for molecular or protein-chemical investigations to study underlying mechanisms by harvesting the capillaries and subject to, e.g., Western blot analysis. Moreover, capillaries from disease animal models or patients are used to better understand the BBB in health versus disease (Soldner et al. 2019). Limitations of the applicability are that capillaries have to be used very quickly after isolation as mentioned above, that the potential for high throughput is rather low, and that the investigation of transport processes in particular currently depends on fluorescent substrates, since sampling from the lumen of the capillaries is quite difficult. Interestingly, the possible contamination of the capillaries on the outer walls with pericytes or astrocyte endfeet after isolation previously regarded as a disadvantage (Cornford and Hyman 2005) is nowadays discussed within the BBB scientific community as an advantage as it allows a more complete NVU being active during the studies.

In order to study the individual cellular components of the NVU, cell culture models were developed in which the BCECs were cultivated alone or spatially separated from the other cells of the NVU. In addition, the systems should allow for noninvasive characterization of BBB tightness properties by not changing the cell layers' integrity, the molecular investigation of the single cell types, and the accessible sample volumes sufficient for subsequent drug analysis.

3 Cell Sources for Cell Culture Models

In recent decades, various cells have been tested and used for blood-brain barrier models. A basic distinction is made between tumor cells, immortalized cell lines, and primary cells. The latest development in this field is the use of stem cells to generate brain-like endothelial cells. Since BCECs have very specific properties compared to other biological barriers, it is assumed that only BCECs should be used for blood-brain barrier models. Nevertheless, now and then barrier models based on epithelial cells such as Caco-2 or MDCK have been used or are still referred to as blood-brain barrier models even though they are not derived from the brain (Veszelka et al. 2018; Avdeef et al. 2015). Since these epithelial cell models express certain ABC transporters similarly to the blood-brain barrier or derivatives were generated that even overexpress specific ABC transporters, they can be used as general barrier models to test whether certain substances are possible substrates of transporters that are relevant for the blood-brain barrier (Luo et al. 2002; Ozgür et al. 2018). These data can be used to assess whether a substance can permeate across the blood-brain barrier and be correlated to in vivo animal data, but it should not be neglected in interpretation of results that the transporter composition at the blood-brain barrier is different. This means that the tested transporters may occur at a different frequency and other transporters relevant for the both efflux and influx of the test substance may be present as well (Bao et al. 2019).

Another controversial cell line is the human ECV304, of which apparently different clones have been used in different laboratories. Genetic fingerprinting results showed that certain (Drexler et al. 2002) but not all the clones are identical to the bladder carcinoma cell line T24 (Takahashi et al. 1990; Suda et al. 2001; Iartseva and Fedortseva 2008). Moreover, different ECV304 clones reacted differently to astrocyte stimuli. In one clone tight junction function was improved, in the other not. Other ECV304 clones also showed significant differences in cytogenetic investigations compared to cell line T24. In summary, the cell line ECV304 is still used as blood-brain barrier model, especially in Asia. The genesis of this "spontaneously transformed" cell line still seems somewhat unclear; ECV304 can be useful as a barrier model, but validation of the endothelial properties of the applied clone is essential if ECV304 cells are intended to be used for the investigation of endothelial signaling pathways or blood-brain barrier disease modeling, since doubts have been raised about the endothelial origin of ECV304.

For drug development, a human, paracellular very tight cell model of the blood-brain barrier with a similar transporter and enzyme configuration as in vivo is most desirable. This model could be used as a benchmark tool and compared with animal cell culture as well as animal models in order to estimate possible species differences. Unfortunately, human primary brain endothelial cells build up low paracellular tightness; the same is true for several human immortalized cell lines (Eigenmann et al. 2013; Weksler et al. 2005; Megard et al. 2002; Zenker et al. 2003). In addition, primary human cells are difficult to access in quantity required for drug screenings. They are obtained from either autopsies or biopsies, for example, from surgeries on tumors or epilepsy patients. For these reasons (accessibility, weaker barrier), a number of animal models based on both immortalized cell lines and primary cells have also been developed. Several cell lines from mice, rats, and pigs with different barrier properties have been developed, validated, and also used for drug transport studies, but none of them reach the paracellular barrier properties of the models from primary cells from, e.g., cows, rats, or pigs (Deli et al. 2005; Avdeef et al. 2015). Primary cells are not altered like immortalized cells; monolayer cultures achieve significantly higher paracellular tightness and, above all, maintain the signaling pathways closest to the in vivo animal models (Galla 2018; Dehouck et al. 1990; Coisne et al. 2005; Cantrill et al. 2012; Abbott et al. 2012). Nevertheless, it is important to note that primary cells are also subject to dedifferentiation processes during isolation and culture and can change significantly (Pamies et al. 2018). For these reasons, the idea was pursued to differentiate brain-like capillary endothelial cells from human stem cells.

3.1 Criteria for Blood-Brain Barrier In Vitro Models in Drug Development

Before assessing the success of the generation of BCECs from stem cells, one should try to establish criteria for what properties a human blood-brain barrier model should ideally have for use in drug development. Following criteria could be suggested:

1. Sufficient paracellular tightness: Tight cell-cell contacts are a prerequisite to seal the paracellular space in order to prevent hydrophilic molecules from paracellular unregulated permeation and to ensure the likelihood of distinct polarization of cells into apical and basolateral sites. This can be crucial for transporter proteins to ensure a clearly directed transport, for example, ABCB1 should be mainly apically (blood side) localized (Roberts et al. 2008). Functionally, paracellular tightness is determined by the transendothelial electrical resistance (TEER) or by the passage of paracellular markers. The comparison of absolute values of the TEER is difficult, as the values from model to model depend on the measurement setup (number of electrodes), the applied voltage, the distance between the electrodes, the electrode material, the mathematical model behind the calculation, and the practical handling of the devices by the single operators. In addition, very few TEER data from the animal in vivo models have been published. The most cited values are averaged values and range 1,500–2,000 Ohm cm^2, but the data basis for these averaged values was between a few hundreds to 6,000–-8,000 Ohm cm^2 (Crone and Olesen 1982; Butt et al. 1990). Moreover, these values were obtained from electrodes located rather at the pial surface than within the CNS parenchyma, where the BBB is located. Thus, it is also unclear which in vivo values can really be used as a benchmark for the in vitro models. In addition, there are no values available from human brains in vivo. Values for the human BBB are generally assumed similar to the ones of the animals because of the similar tight junction ultrastructure and morphology to the BBB of animals.
   For in vitro models, several exponential relationships between TEER and the permeability of paracellular markers were shown (Gaillard and de Boer 2000; Neuhaus et al. 2006a). These relationships also reveal certain TEER threshold values above which the permeability of paracellular markers does not change significantly. It is assumed that further increases of TEER $>1{,}000$ Ohm cm^2 are no longer regarded as very relevant for drug transport studies and the proportion of paracellular transport for the tested molecules (Mantle et al. 2016). Furthermore, a meaningful comparison of the absolute TEER values is complicated because the values are influenced by the surface of the membrane on which the cells grew, as well as from the porosity and the material, since this can also influence the differentiation of the cells. Therefore, it is recommended to compare the permeability of paracellular marker molecules of similar size. As paracellular marker molecules, for example, fluorescent Lucifer yellow, fluorescein, carboxyfluorescein, radiolabeled mannitol or FITC-dextran of different molecular weights (4, 10, 20, 40, 70 kDa), or inulin can be used. Dextrans have rather elongated molecular structures. In order to measure the permeability of globular structures, proteins such as bovine serum albumin (BSA) or horseradish peroxidase can be used as alternatives. Permeability coefficients of <10 μm/min of smaller markers such as fluorescein or Lucifer yellow are considered sufficient as quality benchmark for in vitro BBB models to be used for in vitro/in vivo correlations of drug transport data (Lundquist et al. 2002; Culot et al. 2008; Mysiorek et al. 2009). Nevertheless, permeability coefficients of small

paracellular markers <2–3 μm/min are the requirement for in vivo similar, high-tightness models.

2. In vivo-like structure of tight junctions: TJs can be investigated with methods of ultrastructural research such as transmission electron microscopy using negative contrast ratio or freeze fracture. Tight junction proteins are visualized by immunofluorescence (IF) microscopy, and their localization is checked at the cell-cell contacts. Although IF is used very often to show whether the proteins are generally located at the cell-cell contacts or whether their localization has changed, for example, due to treatment, the analysis of single tight junction proteins could only be regarded as surrogate markers for the entire TJs. Mostly claudin-5, occludin, and ZO-1 are investigated as BBB markers. In this context, however, it must be remembered that it is still unclear how many and which TJ proteins are really relevant for the human BBB (Berndt et al. 2019). Therefore, a TJ structure check by transmission electron microscopy (TEM) is still a very important and meaningful method to understand how similar the TJ structure in the respective cell culture model is to the in vivo TJ structure in animal or human.
3. The presence and activity of efflux transport processes: Research has paid particular attention to the ABC transporters at the BBB, because ABC transporters have been held responsible for preventing a large proportion of drugs from entering the CNS and thus from reaching their target structure. P-glycoprotein (P-gp, ABCB1) and BCRP (ABCG2) are considered the most important ones at the BBB. Schinkel et al. (1994) described the important role of P-gp in mouse BBB using mdr1a knock-out mice exposed to neurotoxic pesticide ivermectin. Eisenblätter et al. (2003) published the role of the brain multidrug resistance protein (BMDP) – which is the porcine homologous to human BCRP – at the porcine BBB with experiments with daunorubicin. In addition to P-gp and BCRP, a number of multidrug resistance-related proteins (MRPs, ABCCs) are present at the BBB, which also act as efflux transporters for various substrates (Neuhaus and Noe 2009). The expression, but above all the localization and function of these transporters, is regarded as a basic prerequisite and essential feature for BBB cell culture models. In order to test the function, selective substrates are used in uptake or transport experiments (e.g., calcein AM or rhodamine 123 for P-gp, Bodipy-FL-prazosin for BCRP), and the changes in uptake or transport rates are tested in the presence of specific inhibitors of the transporters. In addition, the presence of a directed transport is often tested by comparing the permeability coefficients from the transport direction apical/basolateral versus basolateral/apical (a/b vs. b/a) or by performing so-called equilibration studies in which the same concentration of the transporter substrate is added apically and basolaterally, and it is observed over several hours whether the substances accumulate in one or the other compartment. In regard to testing ABC transporter functionality, it is also important to note that species differences are known. Whereas P-gp was previously classified as the most important ABC transporter at the BBB, proteomics data showed that significantly more BCRP than P-gp was found in human brain capillaries (Shawahna et al. 2011).

4. Presence and activity of influx transporters: A number of influx transporters belonging to the group of solute carriers (SLC) are known at the BBB. Among the most important are GLUT-1 (SLC2A1) and LAT-1 (SLC7A5). Both are responsible for the provision of essential nutrients for the CNS. GLUT-1 is classified as the most important glucose transporter of the BBB, whereas LAT-1 is considered as one of the most important amino acid transporters (Veszelka et al. 2018; Roberts et al. 2008). In addition, both are regulated in diseases, and mutations of these two transporters at the BBB are seen in direct causal relation to the etiology of diseases such as GLUT-1 deficiency syndrome or autism spectrum disorder (Seidner et al. 1998; Cascio et al. 2019). In order to test their function, uptake and transport experiments with specific substrates and inhibitors are carried out. For drug delivery these SLC transporters are also used targets for the transport of pro-drugs. In general, it is important to know both the influx and the efflux mechanisms of a compound in order to finally be able to estimate the net flux across the BBB for the concrete substance.
5. Significant large measurement window: In order to classify substances according to their permeation behavior across the BBB, a large difference between slow- and fast-permeating substances is helpful. Cell models with high paracellular tightness can perform better, especially in the case of hydrophilic substances or compounds that are substrates of active transporter proteins. If lipophilic substances permeating only via passive, transcellular diffusion using the total cellular surface, the paracellular tightness of the cell models is not such an important parameter. In that case, also models with a low paracellular barrier can be very useful for drug transport studies.
6. Robustness of the model: The changes of the model over a longer period of use should be detectable. Data on the use of the model over several years would be best in order to detect drifts in the performance of the models. For this purpose, in routine applications, a set of standard substances should be studied at defined time intervals, e.g., every second week or included into the routine runs of the experiments. For normalizations – also in basic science – within an experiment or between experiments, the use of internal standards, which are simultaneously added to the substance to be investigated, is recommended. Substances that are known to permeate mainly passively across the BBB, such as diazepam for the transcellular route and paracellular markers such as Lucifer yellow, are particularly suitable for this purpose.
7. Presence of endothelial marker molecules: The endothelial cells lining the inner lumen of the brain capillaries are the main component of the BBB in mammals and humans. Especially in studies for which intracellular signaling pathways play a major role, such as toxicity testing or BBB relevant pathways in diseases, it is essential that the cell culture models also have endothelial cell characteristics. This concerns surface markers, receptors, as well as transcription factors. In this context, it should not be forgotten that BCECs are specialized endothelial cells that are not necessarily comparable one-to-one with peripheral endothelial cells. For example, it has often been shown that BCECs have clearly different transcriptomes compared to endothelial cells of peripheral organs such as the

lung or heart (Huntley et al. 2014; Munji et al. 2019). Nevertheless, in addition to BBB-specific markers (TJ proteins, ABC and SLC transporter proteins), general endothelial cell markers found in the brain should also be found in BBB models such as vWF, CD31, Tie-2, or VEGFRs. When assessing these markers, it should also be borne in mind that these are molecules that are also regulated in certain situations and that absolute numbers should not be used as a benchmark in expression.

8. Inducibility of BBB markers by cells from the NVU: Since it is known that, e.g., astrocytes can upregulate several characteristics of the BBB (P-gp, TJ proteins, TEER) or that co-culture with astrocytes in disease models is essential to damage the barrier accordingly (Neuhaus et al. 2014), the influence of cells of the NVU on relevant barrier molecules and function should be demonstrated for the cell culture models. For this purpose, it can also be investigated whether the cells used – as one would expect from endothelial cells – also carry out independent tube formation in hydrogels or react to stimuli such as VEGF with increased migration or barrier collapse.

With the help of these criteria, BCECs and their functionality can now be assessed in a wide variety of models. Depending on the application, further criteria can be added for the evaluation, such as suitability for long-term experiments, direct access for microscopic analysis, or the possibility of using other noninvasive measurement parameters such as TEER, sample volume size for subsequent analysis, complexity, need for direct cell-cell contact between different cell types, separation of cell types into different compartments for subsequent analysis of the individual cell types, the need of hydrogels with adjustable stiffness, or the implementation of shear stress.

3.2 Stem Cell-Derived Brain Capillary Endothelial Cells

The development of protocols for the production of BCECs derived from stem cells was a milestone for BBB research, as high-tightness BCECs of human origin were available for the first time. With the publication of Lippmann et al. (2012), the first protocol for the differentiation of human-induced pluripotent stem cells (hiPSCs) into BCECs was presented, and the starting signal for further protocol developments was fired. In addition to hiPSC, protocols have also been developed that have successfully used other stem cell types (e.g., from cord blood) to develop relevant tight human BBB in vitro models (Cecchelli et al. 2014). However, the hiPSC might be the most interesting possibility at the moment, since they can also be differentiated into other cell types of the NVU due to their pluripotency, and thus isogenic models with several cell types can be produced, which all originate from one and the same hiPSC clone. This is particularly advantageous for disease models and personalized models. On the other hand, the availability of hiPSC is much higher; they are easily expandable, available via hiPSC cell banks, or

reprogrammable from different cell types (skin fibroblasts, epithelial cells isolated from urine samples, B cells from blood, etc.) (Neuhaus 2017).

In the meantime, several protocols for the differentiation of hiPSC into brain-like endothelial cells have been published. Basically, two general approaches have been taken so far. In the first strategy, the hiPSC cells are differentiated into a co-culture of endothelial cells and neural cells, and in further steps, the endothelial cells are separated from the neural cells and specified to brain-like endothelial cells. This co-cultivation should reflect the joint growth of these cell types during BBB development (Appelt-Menzel et al. 2017; Lippmann et al. 2012). In the second strategy, the hiPSC are cultured towards mesodermal precursors and further to endothelial progenitors, which in turn are specialized to brain-like endothelial cells. In recent years, improvements or adaptations of these protocols were published, with the current developments mainly aiming at simplifying and improving the robustness of the protocols. This includes above all the use of defined media and media additives as well as the search for cheaper alternatives compared to the rather expensive media for stem cell culture. The majority of the protocols lead to highly tight, human brain-like endothelial cells with high TEER values of several thousands of $\Omega\,cm^2$, whereas the specialization to brain-like endothelial cells could be achieved by co-cultivation with astrocytes, pericytes, and/or neuronal stem cells or by the addition of retinoic acid (RA) (Lippmann et al. 2012, 2014; Stebbins et al. 2018; Neal et al. 2019). The addition of RA is interesting from two aspects. First, it is known that RA is secreted by astrocytes; thus by adding RA, a part of the astrocyte secretome is reproduced (Mizee et al. 2014). Second, RA can act as anti-inflammatory via a number of activated nuclear receptors, which has to be considered in inflammation or infection studies and limits the possible applicability of the protocols or has to lead to protocol adaptations (Kim et al. 2017). For the analysis of tight junctions, claudin-5, occludin, and ZO-1 are analyzed as surrogate markers in most protocols. Only rarely the TJ structure was examined by TEM and the freeze-fracture technique (Appelt-Menzel et al. 2017, Lippmann et al. 2012). Several times it could be shown that not the total expression of the TJ proteins but the localization was regulated and was decisive for higher TEER values (Canfield et al. 2017). It is also interesting that mostly only claudin-5, occludin, and ZO-1 are investigated, although studies with human brain capillaries as well as with brain-like endothelial cells differentiated from hiPSCs clearly showed that besides claudin-5, at least at the mRNA level, considerably more claudins such as claudin-11 or claudin-25 occur in the human BBB (Berndt et al. 2019). RNA-seq data suggested that there may be many more claudins in the human BBB and hiPSC-derived brain-like endothelial cells (Lim et al. 2017; Vatine et al. 2017; Delsing et al. 2018). With regard to transporter functionality, the focus was set on P-gp and BCRP, which have been detected several times. In addition, a high agreement was found in the expression of TJ, ABC, and SLC transporters between human primary brain endothelial cells and the hiPSC-BCECs (Qian et al. 2017). Furthermore, a very high dynamic range between the permeability coefficients of the slowest and the fastest substances of up to 60–150 times could be detected in the hiPSC-BCEC models (Appelt-Menzel et al. 2017; Lippmann et al. 2012). In this context, the importance of blank value

studies without cells for the transport studies must be pointed out here, since the inclusion of blank values could lead to a significantly increased dynamic measurement window for drug transport studies (Appelt-Menzel et al. 2017). The hiPSC co-culture differentiation protocol and slight adaptions based on Lippmann et al. (2012) were already used to study the influence of the extracellular matrix including electrospun fibers (Patel and Alahmad 2016; Qi et al. 2018; Al-Ahmad et al. 2019); the co-culture with hiPSC-differentiated astrocytes mimicking different brain regions or even isogenic hiPSC-derived pericytes, astrocytes, or neurons (Delsing et al. 2019; Canfield et al. 2019; Bradley et al. 2019); effects of drugs or drug transport (Ohshima et al. 2019; Clark et al. 2016; Albekairi et al. 2019); toxicity (Patel et al. 2018; Martinez and Al-Ahmad 2019); sex differences (Patel et al. 2017); oxygen/glucose deprivation as model for cerebral ischemia (Page et al. 2016, 2019); or infections (Alimonti et al. 2018; Kim et al. 2019; Martins Gomes et al. 2019).

However, one of the biggest weaknesses of the hiPSC-based models at the moment is their minor robustness and lab-to-lab reproducibility; therefore several protocol developments focus on the simplification of protocols and the exact definition of media and media supplements. In general, reproducibility of hiPSC cultivation is very much dependent on the experience of the laboratory staff and who takes which steps and how. It can already make clear differences for the differentiation success how the hiPSCs are subcultured (detachment solutions, duration of detachment, steps of centrifugation, intensity of homogenization, etc.) and with which cell number the differentiations are started. These steps and conditions need to be optimized for every single cell line. Therefore, the comparability of protocols from laboratory to laboratory is still a big challenge, and the comparison of data is difficult due to so many relevant parameters that the importance of internal controls is immense. In the protocol development of the co-cultivation approach, for example, E8 Medium was tried as an alternative to mTeSR^{TM}1 medium for the hiPSC on Matrigel for the first 2–3 days of cultivation. For the unconditioned medium (UM) for the next 6 days for differentiation initiation, E6 was used, and B-27, N2, and ITS were tested instead of platelet-poor plasma-derived bovine serum (PDS), which was added in the endothelial cell-specific medium SFM for the next 2 days to suppress differentiation of pericytes (Hollmann et al. 2017; Neal et al. 2019). Other interesting approaches were to use puromycin to inhibit pericyte growth or to adjust osmolality after endothelial cell specification to generate a slightly longer and more stable barrier (Roux et al. 2019; Ribecco-Lutkiewicz et al. 2018). In the protocols that differentiate hiPSC as a monoculture into brain-like endothelial cells, the mesodermal direction was determined by adding CHIR99021, a glycogen synthase kinase 3b (GSK-3b) inhibitor and canonical Wnt pathway agonist, or with a mixture of BMP4 (bone morphogenetic protein 4), bFGF, SB431542 (TGF inhibitor), and LY294002 (PI3K pathway inhibitor) in E6 medium (Qian et al. 2017; Cader and Chintawar 2019; Grifno et al. 2019). After CHIR99021 induction, the cells are cultured with a medium based on DMEM/F12 with MEM-NEAA, Glutamax, and mercaptoethanol plus B-27 as serum replacement and differentiated into brain-like endothelial cells in a human endothelial serum-free medium (hESFM) with bFGF, RA, and B-27. In the case of mesoderm induction with BMP4, bFGF, SB431542,

and LY294002, after reduction of BMP4 and SB431542, and an intermediate step with human endothelial serum-free medium with VEGFa and forskolin, the cells are fully differentiated in the endothelial cell medium EGM-2 with RA, bFGF, rock inhibitor, Wnt7a, Shh, and Ang1. All these protocols lead not only to high TEER values and expression of TJ proteins but also to the expression of endothelial markers (CD31, vWF, VE-cadherin Tie-2, or VEGFR2) and the ability of VEGFa to enhance tube formation in Matrigel or the uptake of Ac-LDL, another endothelial characteristic. Nevertheless, the endothelial nature of hiPSC brain-like endothelial cells is questioned, and there is a discussion about their neuroectodermal epithelial properties. When comparing the media UM and E6 used for differentiation at the beginning, it is noticeable that both are based on DMEM/F12 but have different additives (20% knock-out serum replacement, MEM-NEAA, β-mercaptoethanol versus ascorbic acid 2-phosphate sesquimagnesium salt hydrate, sodium selenite, sodium bicarbonate, insulin, or holo-transferrin). Anyhow, interestingly the same E6 medium was also used to differentiate hiPSCs into neuroepithelial cells (Pax6, N-cadherin, Otx2, Sox-2 positive; E-cadherin reduced, SOX-17 negative) after 4–6 days (Lippmann et al. 2014a, b). In this context, authors of a recently online accessible but not yet-peer reviewed report claimed that the use of the described protocols developed by Lippmann et al. could also lead to the expression of epithelial markers such as EpCAM and that a number of essential endothelial markers and transcription factors were missing (Lu et al. 2019). In addition, transfection with some of the missing transfection factors should lead to a more endothelial phenotype (Lu et al. 2019). Two further protocols, some of them with a much longer cultivation duration, which focused more on the differentiation towards the endothelial phenotype with the help of precisely defined media and additives, led to significantly leakier cell layers and, despite their great potential, require further improvement with regard to the development of BBB properties (Delsing et al. 2018; Praça et al. 2019).

In summary, some protocols are available that lead to very tight cell barriers with endothelial and BBB properties. These models are excellent as human barrier models to test drug transport across the human BBB and to use personalized and mutation-based disease models of the BBB. Nevertheless, the coming years will have to bring improvements in order to increase the reproducibility and robustness of the models so that they can also be used for industrial, high-throughput applications. Currently, the protocols are still too dependent on the individual laboratories, the single hiPSC clones, and their cultivation (different hiPSC quality control systems, too many minor differences in the protocols and the handling influence the differentiation results); moreover the human BBB has to be understood and characterized in a more comprehensive manner in order to establish a real benchmark for protocol development.

4 Cell Culture Models

The appropriate selection of the cells used in the BBB models is decisive for the success and the significance of the results obtained. The second decisive parameter is the model design. Basically, one can distinguish between the following model types: transwell model, BBB spheroids, hydrogel models, hollow fiber model, and microfluidic models. Each model has advantages and disadvantages, and dependent on the application question, these have to be weighed against each other (Table 1).

The transwell model is the most widely used model (Fig. 1b). So-called inserts are inserted in wells of a well plate by standing on the bottom or hanging on the rims of the wells. The inserts have a porous membrane at the bottom, which is attached to a circular plastic wall leading upwards. The insertion of these inserts into wells leads to a two-compartment system, the space inside the circular plastic wall at the top and the space of the well at the bottom. These compartments are connected via the porous membrane at the bottom of the insert and can thus communicate with each other. In the case of BBB modeling, the BCECs are usually grown on the membrane inside the insert. In addition, other cell types such as astrocytes or pericytes can be grown either on the bottom of the wells or on the opposite side of the porous membrane. If the cells are to adhere to the back of the membrane, the insert is usually first turned upside down; the cells are distributed over the membrane in a sufficiently large drop

Table 1 Classification of BBB cell culture models using parameters for their suitability for different applications, characterization methods, and drug transport studies

Parameter	Transwell	BBB-Spheroids	Hydrogel model	Dynamic hollow-fiber model	Microfluidic models[a]
Complexity	+	++	++	+++	+++
Shear stress	-[b]	-	-	+++	++
Direct cell-cell contact	+/-	+++	++	+/-	+
Longevity	-	+	+/-	+++	++
Direct microscopical assessment	+++	+	++	-	+++
TEER	+++	-	-	+++	+++/-
Permeability studies	+++	+	++	+++	+++
Sample volume for drug analysis	+++	+	++	+++	+
Growth surface area to lyse cells for molecular analysis	+++	-	+++	+++	+
Separation of cell types for molecular analysis	+++	~	++	+++	~

- very difficult or not applicable, ~ difficult, dependent on the specific model, + limited, ++ well applicable, +++ very well applicable

[a]Since various microfluidic models have been developed, the parameter assessment can only be understood as an overall average estimate

[b]Transwell models could be used with millifluidic pump systems

of culture medium and after about 2–4 h turned upside down again for subsequent cultivation or seeding of the BCECs on the other side. Depending on the cells used, the membrane is often coated with protein solutions before seeding. Typical for BCECs would be fibronectin or collagen IV (sometimes Matrigel); for astrocytes and pericytes, poly-L-lysine is often used. Inserts can be obtained from different manufacturers in different formats (6-well, 12-well, 24-well, 96-well), materials (PVDF, PC, PET, etc.), pore sizes (0.4, 1, 3 and 8 μm), and porosities. These parameters can have a significant influence on the results of drug testing by leading to different barrier properties of the cell culture models on the one hand and having a direct influence on the drug test on the other hand. For example, lower pore sizes tend to lead to tighter barriers but also reduce the net flux of active substances. Different well formats have different ratios between total medium volumes and growth surface, which can influence the cell cultivation and differentiation, since in absolute terms, less or more nutrients are available per surface area. Depending on the properties of the substances to be tested, the material of the membranes can also be decisive. For example, lipophilic substances may adsorb the substance in the plastic membrane (or the plastic of the well or the insert walls), which leads to a significantly lower recovery rate and makes the interpretation of the experiment more difficult. In this case, more hydrophilic membranes can be used, or proteins such as BSA in the receiver compartment can be used as substance scavengers. In order to generally test the influence of the plastic membrane of the inserts, blank values should always be performed without cells, both to optimize sampling times, to test the recovery rate, and to include the additional barrier effects of the plastic membrane in the results. Since the plastic membrane is significantly thicker (about 10 μm) than the extracellular matrix in vivo (20–80 nm), it should be measured as an additional barrier for drug transport and included in the calculations.

Two general methods for transport experiments have become established. On the one hand, the samples can be taken at certain times. The volume taken should be replaced by fresh transport medium after each sampling step in order not to generate hydrostatic pressure as additional driving force. With this technique, the substances accumulate in the receiver compartment over time, and thus the concentration gradient of the substance to be tested is reduced. In order to maintain the concentration gradient over the time of the experiment, the inserts can alternatively be transferred into a new well, which has already been filled with fresh transport medium, at the respective sampling time. In order to minimize the unstirred water layer, the well plates can also be moved on the shaker during the experiment. If experiments are carried out in co-culture, the individual cell types must be adapted to each other for the cultivation time (different cell culture media for each cell type or one medium suitable for all cell types; increased nutrient consumption that can negatively influence the barrier formation). Alternatively, the individual cell types can be grown in parallel and assembled together only 1–2 days or on the test day. Since the growth medium is not pumped in the transwell model, it is often referred to as a static model.

In order to incorporate the influence of shear stress, so-called dynamic, hollow fiber-based models or microfluidic models were developed. Alternatively, transwell

models could generally be used in so-called millifluidic models (e.g., from company Kirkstall) for which data from BBB models are not yet available. The advantages would be not only the application of shear stress but also nutrient supply from a medium reservoir which ensures constant concentrations of the nutrients during cultivation. In order to guarantee the missing cell-cell contact in transwell models, models with cells from the NVU in hydrogel-based models or BBB spheroids were developed.

BBB spheroids represent a very novel type of BBB in vitro models (Nzou et al. 2018; Bergmann et al. 2018; Cho et al. 2017; Song et al. 2019). Since the current protocols for the cultivation of brain organoids do not lead to a developed brain vasculature network (Ham et al. 2020), BBB spheroids have been established as an alternative. A core of CNS cells such as astrocytes, pericytes, and/or neurons is surrounded by a layer of BCECs. The pericytes are essential for the self-assembly and correct arrangement of the brain capillary endothelial cells (Fig. 1c). The average diameter of the BBB spheroids is 200–400 μm. Thus, the spheroids for fluorescence studies can be analyzed as a whole mount and do not have to be cut beforehand, which is a distinct advantage for high-throughput applications. The main disadvantages of BBB spheroids are that the analyses are currently based on fluorescence recordings and a significant amount of spheroids have to be pooled both for molecular biological measurements and for the detection of non-fluorescent substances. The BBB spheroids can be produced with cell lines as well as with hiPSC-derived brain-like endothelial cells and thus could be developed to alternatives to conventional disease models.

For BBB models using hydrogels, there are two basic approaches. The hydrogel can be used as scaffold for CNS cells on which the BCECs grow as monolayers (Sreekanthreddy et al. 2015), or the BCECs are introduced into the hydrogels together with the CNS cells to form 3D capillary structures (Al Ahmad et al. 2011). The second variant is not so useful for drug testing, as complex imaging and evaluation procedures are necessary to measure the differences in the capillary structure (number, length and diameter of capillaries, number of branches, etc.). The first variant is much more exciting for drug testing, because it is possible to carry out permeability studies and it may also be possible to determine which cell type in the hydrogel prefers to interact or uptake the test compounds (Fig. 1d, Sreekanthreddy et al. 2015). This can be of particular interest for biopharmaceuticals such as antibodies, nanoparticles, or liposomes. However, small molecules must be extracted from the hydrogel for analysis after the experiment. In addition to the possible cell-cell contact of cells in the hydrogel with the BCECs on top of the hydrogel, the stiffness of the hydrogel is a highly relevant parameter for the formation of barrier properties, as this can also have a positive effect on the localization of TJ proteins (Grifno et al. 2019). In addition to static models, hydrogels are also used in microfluidic models (Faley et al. 2019). In order to incorporate the influence of shear stress in BBB models, hollow fiber models in the millifluidic scale or microfluidic models were developed.

Hollow fiber models consist of a bundle of capillaries consisting of porous ultrafiltration membranes (Fig. 1e). These capillaries are encased in a cylindrical

or ashlar-shaped, transparent housing (made of e.g., polycarbonate), which creates a two-chamber system consisting of the lumen of the capillaries and the outer space around the capillaries, the so-called extracapillary space (ECS). The ends on both sides of the capillaries are fixed in a resin, in that way that the medium coming from the reservoir bottle is only pumped into the capillaries. The capillary cartridges are connected via Luer-lock connections to tubes that close the circuit to the medium reservoir bottle and the pump system. The tubings are often made of silicone, which allows the gas exchange (oxygen vs. CO_2). The pump system enables pulsatile flow with an adjustable flow rate and beat rates simulating the human circulation. By setting the pump rate, the applied shear stress is determined, which can be adjusted in these models in the in vivo relevant range of 5 dyne/cm^2. The capillary cartridges are available from different manufacturers with the corresponding pump systems (FiberCell, Flocel, Cellmax). Depending on the number of cells available, modules with different numbers of capillaries (4–320) and thus different growth surface areas can be selected. Access via connected Luer-lock syringes is available both before and after the capillaries, as well as at two positions of the ECS, allowing direct access to the two compartments to introduce cells or substances and also to draw samples. A disadvantage of these models is that light microscopic control of cell growth and adherence is not possible. Integrated TEER measurement systems or transport studies with paracellular markers are used to monitor the formation of the cellular barrier. In addition, the recovery rates of cell numbers and the consumption of glucose and the production of lactic acid are measured to determine cultivation success and the metabolic state. In addition to the TEER electrodes, flow and pressure sensors could be installed (Cucullo et al. 2013). The hollow fiber models are highly sophisticated and not intended for high-throughput applications. The system can be used for long-term experiments over several months (Neuhaus et al. 2006b). Due to the shear stress and the constant nutrient supply, the expression of TJ and transporter proteins is induced, and the proportion of cytoskeletal proteins significantly increased. The first data of the Damir Janigro's group for BBB hollow fiber models were published in 1996 (Stanness et al. 1996); in the meantime, the models were used for a number of questions including some disease models with co- and multi-cultures (Stanness et al. 1997, 1999; Pekny et al. 1998; Cucullo et al. 2002, 2008; Krizanac-Bengez et al. 2006; Santaguida et al. 2006). For transport studies, the substances can be introduced into the reservoir, and during the study, they can be pumped in a circle at constant concentration over a longer period of time simulating an infusion or injected directly in front of the capillaries via a syringe, and the samples are taken while the substance bolus is pumped through the lumen of the capillaries. Mathematical models were developed for both test designs to calculate the permeability coefficients (Neuhaus et al. 2006b; Stanness et al. 1997). Lipophilic substances can be partially adsorbed by the silicone tubes. This has to be considered at least with the help of blank value tests without cells during infusion transport tests, or less adsorbing tube materials are used. In summary, the hollow fiber models are highly sophisticated models, which lead to more in vivo similar BBB cell layers by the shear stress and are applicable for complex long-term disease models. Furthermore, the volumes of the compartments are large enough to draw sufficient sample

volumes for subsequent analyses. However, they are not suitable for high-throughput analysis and live imaging (latest models include microscopic fields of view). Microfluidic models were developed to improve these points among others.

The number and variety of microfluidic models have been increasing in the last years tremendously. In general, the "BBB-on-a-chip" models were classified into four different categories: (1) 2D microfluidic models, (2) hybrid microfluidic models, (3) 3D templated models, or (4) self-assembly models (DeStefano et al. 2018). Permeable membranes are incorporated in 2D microfluidic models similar to conventional transwell inserts separating two compartments. These models could be applied for, e.g., drug screening. Some of them have included TEER measurement devices (Booth and Kim 2012; Brown et al. 2015; Yeon et al. 2012; Prabhakarpandian et al. 2013). Hybrid microfluidic models are more complex but have no cylindrical geometry and thus are not able to respond to vasodilation/constriction (Adriani et al. 2017; Wevers et al. 2018). 3D templating approaches form single cylindrical microvessels embedded within a hydrogel that can be integrated into a flow system and used for live-cell imaging (Cho et al. 2015; Herland et al. 2016; Katt et al. 2018; Linville et al. 2019). Lastly, self-assembly approaches are developed. They exploit the intrinsic, endothelial property for vasculogenesis and/or angiogenesis and form multicellular models of brain microvascular networks (Al Ahmad et al. 2011; Bang et al. 2017; Campisi et al. 2018).

The complexity of the models, their reproducibility, robustness, and limited practicality currently make it difficult to use them for drug development. Drug transport studies are not easy to implement in several microfluidic models, as the volumes required for drug analysis are hardly available from the apical and basolateral side. In addition, the models, in which the CNS cells are embedded in hydrogels, hardly allow a/b - b/a transport experiments to investigate the directionality of a transport process. Currently, tests based on fluorescent substances or transport substrates that can be detected and evaluated using live imaging have the best chance of implementation. However, this excludes a large group of active substances that either do not fluoresce or are not fluorescent-labeled in order not to fundamentally change their chemical structure and functionality, respectively. Indirect measurements of substances to what extent they can influence the transport of fluorescent substrates should, however, be possible. In addition, many microfluidic models are produced by hand in the chip labs and can therefore hardly be reproduced by other groups, making them unsuitable for drug screening for the other groups unless they do not collaborate with the chip producers. In this context, Fabre et al. (2019) suggested to define analytical performance standards to ease the implementation of microfluidic models for industrial use including throughput capability, biological platform stability, drug-biomaterial interactions, intra- and inter-laboratory reproducibility, integration and compatibility with existing laboratory processes, and feasibility of shipping these delicate systems between vendors and users (Fabre et al. 2019).

To improve reproducibility, there is, for example, an approach to produce models in well plate formats for high-throughput applications (Wevers et al. 2018). These could have the potential for drug screening, especially if sensor technology or

electrodes for TEER measurements will be incorporated. Basically, there is also a clear discrepancy between the data on the role of shear stress in different models, and these often do not allow uniform conclusions to be drawn. There are models in which the barrier function has been improved compared to the static models without medium flow. Then, there are models in which it is claimed that shear stress did not effect on the paracellular barrier. This was justified by the fact that the used hiPSC-BCECs already possess a very high baseline barrier and thus the shear stress does not induce any additional effect. Nevertheless, medium pumping led to a moderate reduction of proliferation and apoptosis (DeStefano et al. 2018). These are effects, which were already known from hollow fiber models (Cucullo et al. 2011). This raises the question to what extent the shear stress or the continuous supply of fresh medium is the decisive factor for these effects. Contrary to the abovementioned publication, in another model a shear-stress dependent, barrier-improving effect for hiPSC-BCEC at up to 0.3 dyne/cm^2 (100 μL/min) was shown. However, at even higher shear stress values of 1 dyne/cm^2, which were still significantly lower than the values in in vivo capillaries, no additional positive effect, even a barrier reducing effect, was found (Faley et al. 2019). An additional point to be considered for the data interpretation is the question to what extent a lower expression of shear stress receptors like CD31/PECAM-1 (Conway et al. 2013; Privratsky and Newman 2014; Russell-Puleri et al. 2017; Meza et al. 2017) in the hiPSC-BCEC might lead to a weaker translation of the shear stress signal. Moreover, in the reported models, hiPSC-BCECs were grown in monoculture without further cells of the NVU, which might provide stabilization/support of the hiPSC-BCECs in the hydrogel-formed capillaries.

A positive development with these models is that more and more it is shown that the cultivation time of some microfluidic models could partly be extended to 6, 9, or even 21 days, whereas static transwell models are normally used only for 2–3 days after the cell barrier establishment (Linville et al. 2019; Wevers et al. 2018; Faley et al. 2019). This reveals the potential for microfluidic models for long-term experiments. Some publications in very high-ranked journals show that the topic and the development of microfluidic or microphysiological models is considered highly relevant in the scientific community (Maoz et al. 2018; Vatine et al. 2019; Park et al. 2019; Shin et al. 2019; Motallebnejad et al. 2019). In one of these publications, a very interesting approach to study the communication between the individual cell types of the NVU separately was to link three individual chips together in such a way that a BBB chip with BCECs, astrocytes, and pericytes could communicate with a brain chip with a mixed population of neurons and glia cells, which in turn was linked to a BBB chip at the other end. In this setup, the changes and mutual influences in the proteome and metabolism of the individual cell types were investigated, and the consequences of barrier breakdowns by amphetamines were analyzed in this primary human brain microvessel endothelial cell-based model, whose paracellular barrier was significantly lower than that of hiPSC models. In addition, the shear stress for the endothelial cells was very low with only 0.02 dyne/cm^2 (Maoz et al. 2018). Despite the optimization possibilities, it

is already a very complex, exciting model, which could be highly relevant for physiological and pathophysiological studies.

In order to achieve the aimed in vivo-like barrier properties, hiPSC-BCECs were used in another model, which uses chips of which 12 can be cultivated simultaneously on the "Emulate platform." The influence of different shear stress rates for 48 h was first investigated (exposed to flow using a peristaltic pump at a rate of 30, 1,500, or 6,500 mL/h equivalent to 0.01, 0.5, or 2.4 dyne/cm^2), and based on RNA-seq data, it was confirmed that the mRNA expression of claudins and endothelial cell markers such as vWF or CD31 was flow-dependent. Interestingly, at the same time, hardly any difference was found comparing samples cultured with 0.5 or 2.4 dyne/cm^2. The data indicated that the mechanical forces and not the medium replenishment were mainly responsible for the effects. The comparison of RNA-seq data showed a close correlation between hiPSC-BCECs and endothelial lineage cells but also some similarities to epithelial cells. Co-culture with human primary astrocytes and pericytes or a cell mixture of astrocytes, neurons, and nestin-positive progenitors (differentiated from seeded neural EZ spheres) led to an increase of the barrier. P-gp activity, restricted permeability for proteins (IgG and albumin), but also transport for transferrin and concentration-dependent barrier-damaging effects of cytokines TNFα, IL-1β, and Il-8 were successfully demonstrated (Vatine et al. 2019). In addition, differences between healthy controls and models based on hiPSC-BCECs of Huntington's disease or MCT8-deficiency patients in the paracellular barrier or the transport of thyroid T3 hormones confirmed previous publications with the same cell lines applied in transwell models (Vatine et al. 2017; Lim et al. 2017). Moreover, hiPSC-BCECs maintained a stable barrier to blood introduced in the "blood channel" of the chip at 5 dyne/cm^2 (60 μL/min). Unfortunately, this model was not suitable for long-term experiments, as the TEER gradually decreased and could only be kept above 500 Ohm cm^2 for 5 days (Vatine et al. 2019).

In another model several important aspects were impressively presented. Hypoxia or hypoxia-inducing compounds such as $CoCl_2$ significantly increased the expression of endothelial and BBB markers during the differentiation of hiPSC to BCECs, which were probably mediated by activation of the transcription factor HIF1α. These improved hiPSC-BCECs were cultured at a flow rate of 100 μL/min (= 6 dyne/cm^2) in a two-chamber microfluidic system with primary human astrocytes and pericytes over 2 weeks leading to a stabilized barrier function over time and significant effects on the expression of barrier markers. The model was suitable for both transporter function and receptor-mediated transport studies. Furthermore, barrier damage by hyperosmolar mannitol used in the clinic to open the BBB could be recapitulated in this model, and the analysis of the drug citalopram by mass spectrometry was applicable (Park et al. 2019).

In summary, the microfluidic systems represent a field of BBB models that has developed enormously in recent years. Great partial successes have already been achieved. Nevertheless, no model has been established until now that optimally resembled the NVU. With regard to drug development, the hiPSC-BCEC models in particular could become very valuable for modeling diseases especially with a

specific genetic background. With regard to the microfluidic models, a clear statement is still missing on cutoff values of minimum shear stress, which should be applied to achieve the maximum induction of the aimed barrier properties. Moreover, more detailed analyses are lacking linking the functional observations with omics data. In general, the success of the models depends very much on the choice of the best suitable cells and in the case of the hiPSC-BCECs also on the appropriate differentiation protocols. Already these two factors are highly variable in the currently developed models and complicate the comparison of the data obtained from the different models. In addition, the conduct of transport experiments needs to be standardized, and blank experiments without cells are needed for control – especially in the case of microfluidic models. Currently, these are not included for permeability coefficient calculations neglecting the barrier formed by the scaffold itself. Finally, since cultured human primary brain endothelial cells are already in clearly dedifferentiated form when used for analysis, it would be a better benchmark to use fresh, human brain tissue for the analysis and evaluation of the cells derived from the in vitro BBB models.

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Neural In Vitro Models for Studying Substances Acting on the Central Nervous System

Ellen Fritsche, Julia Tigges, Julia Hartmann, Julia Kapr, Melania Maria Serafini, and Barbara Viviani

Contents

Abstract

Animal models have been greatly contributing to our understanding of physiology, mechanisms of diseases, and toxicity. Yet, their limitations due to, e.g., interspecies variation are reflected in the high number of drug attrition rates, especially in central nervous system (CNS) diseases. Therefore, human-based neural in vitro models for studying safety and efficacy of substances acting on the CNS are needed. Human iPSC-derived cells offer such a platform with the unique advantage of reproducing the "human context" in vitro by preserving the genetic

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_367

and molecular phenotype of their donors. Guiding the differentiation of hiPSC into cells of the nervous system and combining them in a 2D or 3D format allows to obtain complex models suitable for investigating neurotoxicity or brain-related diseases with patient-derived cells. This chapter will give an overview over stem cell-based human 2D neuronal and mixed neuronal/astrocyte models, in vitro cultures of microglia, as well as CNS disease models and considers new developments in the field, more specifically the use of brain organoids and 3D bioprinted in vitro models for safety and efficacy evaluation.

Keywords

Bioprinted neuronal models · Brain organoids · CNS disease models · Developmental neurotoxicity (DNT) · Human induced pluripotent stem cells (hiPSCs) · Microglia culture · Neurotoxicity (NT)

1 Introduction into In Vitro Neurotoxicity Evaluation

Adult *neurotoxicity* occurs when exposure to natural or human-made toxic substances (*neurotoxicants*) alters the normal activity of the nervous system. It can eventually disrupt or even kill *neurons* or the surrounding *glial* cells, influencing the transmission and processing of signals in the brain and other parts of the *nervous system*. Neurotoxicity can result from exposure to substances used in radiation treatment, chemotherapy, other drug therapies, and organ transplants, as well as exposure to heavy metals such as lead and mercury; certain foods and food additives; pesticides; industrial and/or cleaning solvents; cosmetics, i.e., mercury for skin bleaching or new actives with unknown systemic effects; and some naturally occurring substances (Massaro 2002). Symptoms may appear immediately after exposure or be delayed. They may include limb weakness or numbness; loss of memory or vision; headache; intellect, cognitive, and behavioral problems; and visceral, including sexual dysfunction. Individuals with certain disorders may be especially vulnerable to neurotoxicants (National Institute of Health Neurotoxicity Information 2019).

The recognized test method for evaluating the neurotoxic potential of chemicals is the OECD Guideline 424 (Neurotoxicity studies in rodents). This method uses complex in vivo tests which are often labor-intensive and expensive (Crofton et al. 2012) and might also not well reflect the human situation because of interspecies variation (Leist and Hartung 2013). Such interspecies variation is also thought to be one of the reasons for the high attrition rates in drug development. Before a drug candidate can be taken into human clinical trials, it must be tested for safety and efficacy in animals that display relevant disease characteristics. This poses unique challenges in *central nervous system* (CNS) research, because of the difficulties to induce or quantify, e.g., depression, anxiety, or impairment of social interaction. In addition, pharmacokinetics and pharmacodynamics might differ between species

and thus cause poor prediction for beneficial or adverse effects in humans (Toutain et al. 2010). It stands to reason that diseases with the most complex and least understood etiologies are typically the ones that are the hardest to develop treatments for, which is reflected in the translation failure of CNS drug discovery (Danon et al. 2019; Gribkoff and Kaczmarek 2017).

Understanding compounds' modes of action (*MoA*) and pathophysiology of disease in the human context is of high importance for correct safety and efficacy predictions. This is exemplified by the activation of peroxisome proliferator-activated receptor alpha (PPARα) via PPARα agonists inducing liver tumors in rodents, yet not in humans, probably due to lower PPARα and/or co-activators/co-repressors expression in the latter (Klaunig et al. 2003). Here, animal models overestimate PPARα agonists' hazard for human health. In the case of searching for drugs curing Alzheimer's disease, animal models, which are genetically predisposed to generate Aβ plaques or neurofibrillary tangles of Tau protein, have been used. Yet no results have translated from these animal disease models into effective human medication, probably because they do not represent human AD pathophysiology sufficiently (Danon et al. 2019).

These two examples nicely pin down the issue of model predictivity in compound safety and efficacy evaluation and their translation to human health. One strategy to overcome such translational shortcomings lies in the use of test systems of human origin. Therefore, the biomedical achievement of producing human induced pluripotent stem cells (*hiPSC*) from somatic cells (Takahashi et al. 2007) opened up a whole new arena in the ethically sound production of an unlimited number of human cells, including neurons and glia. In addition, the recent surge in tissue modeling, by culturing such cells in three dimensions (*3D*), is producing a paradigm shift in disease modeling and in pharmacological as well as toxicological testing strategies (Lancaster and Knoblich 2014; Lancaster et al. 2017; Pasca 2018). In vitro cultures are currently also taken to the next level by their growth in bioreactors, which, when connected, can be assembled as organs-on-the-chip and designed to mimic in vivo environments (Park et al. 2019).

Such *new approach methods* (NAMs) cannot be used in an isolated manner, as a cell culture does not represent a whole organism, even if cells grow in 3D. Therefore, frameworks are needed that allow the interpretation of data generated with human 2D or 3D in vitro methods (Fig. 1). One general deficiency of in vitro methods is the lack of picturing pharmacokinetics that is crucial for toxicity and efficacy evaluation. Here, physiology-based pharmacokinetics modeling can be of great help (Paini et al. 2019; Zhuang and Lu 2016) as it provides wet-lab researchers with target tissue concentrations as rationales for their in vitro studies. Finding that human exposure-relevant concentrations is fundamental, yet how to choose and proceed with the readouts of in vitro studies? Here, the "Adverse Outcome Pathway" (AOP) concept is of tremendous help. The AOP is an organizational model that identifies a sequence of biochemical and cellular events (*molecular initiating event*, MIE; *key events*, KE) required to produce a toxic effect (*adverse outcome*, AO) when an organism is exposed to a substance (Fig. 1). Construction of an AOP can (1) organize information about biological interactions and toxicity mechanisms into models that describe

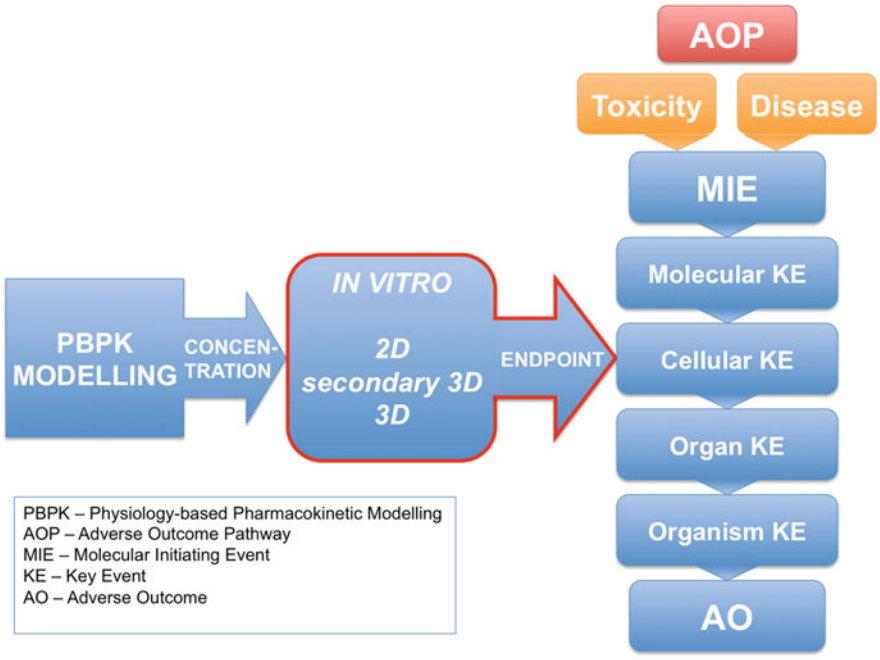

Fig. 1 Framing of data from in vitro models with pharmacokinetics information from PBPK modeling and endpoint judgment according to the AOP concept

how exposure to a substance might cause illness or injury, (2) suggest cell- or biochemical-based tests for pathway elements that could be used to develop testing strategies for targeted toxicity, and (3) identify data gaps in a pathway of toxicity that need more information with the final goal of using fewer resources and experimental animals (Ankley et al. 2010). This concept was soon also applied to neurotoxicity (Bal-Price et al. 2015). Recently it was suggested that the AOP framework is also applicable to understanding disease pathways for prevention, diagnosis, and treatment and in biomedical and clinical research for drug discovery, efficacy, and safety testing (Carusi et al. 2018). Studying cellular effects with in vitro methods in a conceptual framework for toxicity or disease provides drug developers and basic or regulatory scientists with greater confidence in the meaningfulness and thus applicability of generated in vitro data (Fig. 1). In the end, higher human relevance of scientific outcomes will protect society and the individuum and also reduce healthcare costs.

Neurotoxicity can be triggered by a multitude of MoA (Masjosthusmann et al. 2018). In vitro, this MoA can either be measured as specific changes in endpoints, like effects on ion currents, specific receptor activation, or loss in myelin. In addition, MoA can cause neuronal cell death, an endpoint relevant for in vitro and in vivo neurotoxicology. For example, excitatory cell death can occur through stimulation of glutamatergic neurotransmission, or dopaminergic cell death can be

induced via mitochondrial dysfunction. Although neural cell death is indeed a relevant endpoint, it does not inform on the underlying MIE. Moreover, a compound not inducing neural cell death cannot be excluded as a neurotoxicant. When studying neurotoxicity in vitro, knowledge about molecular equipment of cells is crucial for defining the application domain of the respective model.

This chapter now intends to fill the red box in Fig. 1 by summarizing the current state of the art on hiPSC/ESC-based 2D or secondary 3D neuronal and mixed neuronal/glial as well as microglial models, neural organoids, bioprinted neural models, and 2D or 3D neurological disease models. Such find their application in pharma- and toxicological studies by investigating endpoints in vitro that lead to AOs or possibly represent relevant therapeutic targets. A summary of the main toxicological targets is given in Table 1. Although historically most brain-related in vitro data has been derived from rodents (Masjosthusmann et al. 2018), this chapter will focus on published human test systems due to the species specificities discussed above.

1.1 Stem Cell-Based Human 2D Neuronal and Mixed Neuronal/Astrocyte Models

Stem cells (SC) are divided into adult stem cells and *embryonic stem cells* (ESC), depending on their origin and potency (Singh et al. 2015). ESCs are derived from the inner cell mass of the blastocyst and have the ability to self-renew and to generate all cell types of the body except extraembryonic cells (placenta) and are therefore termed pluripotent (Guenther 2011). In 1998 the first human ESC line was isolated from human embryos initially produced for in vitro fertilization (Thomson et al. 1998).

Yamanaka and co-workers created the basis for a new generation of neural in vitro models by developing the Nobel Prize-winning cell system of *human induced pluripotent stem cells* (hiPSC). These cells can be derived from human mature somatic cells by different reprogramming methods (Janabi et al. 1995; Lowry and Plath 2008; Warren et al. 2010; Zhang et al. 2013; Victor et al. 2014) and thus avoid the ethical issues of human ESC (Takahashi et al. 2007; Yu et al. 2007). Human iPSC can be differentiated into cells from all three germ layers (Takahashi et al. 2007; Shi et al. 2017).

Using hiPSC, it is possible to induce a variety of neural cell types. *Neural stem cells* (NSCs) and *neuronal progenitor cells* (NPCs) can be differentiated from hiPSC in large quantities with high reproducibility (Farkhondeh et al. 2019). Cheng and co-workers describe a method to generate NPC from hiPSC using a multistep protocol including embryoid body formation and formation of neural rosettes, followed by multidimensional fluorescence-activated cell sorting (FACS) to purify NPCs by using a set of cell surface markers (Cheng et al. 2017). The authors claim that these cells are suitable for probing human neuroplasticity and mechanisms underlying CNS disorders using high-content, single-cell level automated microscopy assays. Still, a proof-of-concept study remains to be published.

Table 1 Mode of action (MoA) relevant for human neurotoxicity identified within a systematic review investigating 248 individual chemical compounds, 23 compound classes, and 212 natural neurotoxins (Masjosthusmann et al. 2018; modified from Appendix D)

#	Mode of action	MoA related to
1	Stimulation of cholinergic neurotransmission	Neurotransmission
2	Inhibition of cholinergic neurotransmission	
3	Stimulation of GABAergic neurotransmission	
5	Inhibition of glycinergic neurotransmission	
6	Stimulation of glutamatergic neurotransmission	
7	Inhibition of glutamatergic neurotransmission	
8	Stimulation of adrenergic neurotransmission	
9	Inhibition of adrenergic neurotransmission	
10	Stimulation of serotoninergic neurotransmission	
11	Inhibition of serotoninergic neurotransmission	
12	Inhibition of dopaminergic neurotransmission	
13	Neurotransmission in general	
14	Activation of sodium channels	Ion channels/receptors
15	Inhibition of sodium channels	
16	Inhibition of potassium channels	
17	Inhibition of calcium channels	
18	Activation of chloride channels	
19	Inhibition of chloride channels	
20	Effects on other neuronal receptors	
21	Mitochondrial dysfunction/oxidative stress/apoptosis	Cell biology
22	Redox cycling	
23	Altered calcium signaling	
24	Cytoskeletal alterations	
25	Neuroinflammation	
26	Axonopathies	
27	Myelin toxicity	
28	Delayed neuropathy	
29	Enzyme inhibition	
30	Other	Other

Human iPSC-derived neurons can be generated directly from hiPSC or with NSCs/NPCs as an intermediate step (Yu et al. 2014; Ghaffari et al. 2018). The latter protocol takes about 2 weeks and can be used for the evaluation of drug efficacy, although purity and maturity of the cells are in question and need further characterization (Farkhondeh et al. 2019; Dai et al. 2016).

Numerous protocols have been published describing the generation of specific neuronal subtypes as well as glial cells from hiPSC such as *cortical neurons* (Shi et al. 2014; Eiraku et al. 2008; Boissart et al. 2013), *glutamatergic neurons* (Boissart et al. 2013; Cheng et al. 2017; Wang et al. 2017; D'Aiuto et al. 2014; Sanchez-Danes et al. 2012; Yu et al. 2009; Nehme et al. 2018), *GABAergic neurons* (Yang et al. 2017; Liu et al. 2013; Flames et al. 2007; Manabe et al. 2005), *serotonergic and*

dopaminergic neurons (Chambers et al. 2009; Cooper et al. 2012; Kriks et al. 2011; Sanchez-Danes et al. 2012; Li et al. 2017), *motor neurons* (Corti et al. 2012; Sareen et al. 2012, 2013; Kiskinis et al. 2014; Maury et al. 2015), *sensory neurons* (Boisvert et al. 2015; Stacey et al. 2018), *astrocytes* (Lundin et al. 2018; Suga et al. 2019), *oligodendrocytes* (Osaki et al. 2018; Ehrlich et al. 2017; García-León et al. 2018b), and *microglia* (McQuade et al. 2018), just to name a few. In this chapter, we will focus on published in vitro systems that have already been used for screening approaches or are at a state of assay development that will allow substance screening in the near future.

Malik and co-workers established a high-throughput screening platform using hiPSC-derived NSCs and rat cortical cells to screen a compound library of 2,000 chemicals including known drugs (50%), natural products (30%), and bioactive compounds (20%) for their cytotoxic potential (Malik et al. 2014; Efthymiou et al. 2014). In a follow-up study, a subset of 100 compounds was screened in hiPSC, NSC-derived neurons (Efthymiou et al. 2014), and fetal astrocytes. This approach enabled the authors to identify species- and cell type-specific differences in responses to compounds. Specifically, they found that human NSCs were more sensitive to the screened compounds than rodent cultures. In addition, they identified compounds with cell type-specific toxicities. A limitation of the study is the assessment of cytotoxicity as the sole endpoint, which might not be the most sensitive one. Another restriction of this approach is the lack of co-culture of neuronal and glial cells. Moreover, in the species comparison, cells from different maturation stages and single (human) versus co-cultures (rat) were related, making data interpretation difficult.

Another study used small molecule-based NPCs differentiated from three different hiPSC lines, which were then differentiated into neurons and astrocytes within 15 days, using a highly standardized protocol (Seidel et al. 2017). The authors used *multi-microelectrode arrays* (MMEA) for monitoring neuronal network activities via field potential measurements. Such recordings assess multiple endpoints stipulating that different neuronal receptors are expressed by the cells (Table 1). Here, they show reactivity towards dopamine, GABA, serotonin, acetylcholine, and glutamic acid, but not norepinephrine. To date, there are no general guidelines for the analysis and quantification of MMEA measurements. Seidel et al. use single electrodes as the statistical unit, but using different chips or experiments as an individual "n" number would be preferable to assess the reproducibility and standardization between experiments (Masjosthusmann et al. 2018).

In recent years, more and more companies have been offering commercially available hiPSC-based neuronal cells. One study compared different commercially available hiPSC-derived mature neurons (excitatory and inhibitory) from different suppliers, with and without astrocyte co-culture, again utilizing MMEA activity as a functional readout, this time in combination with measurements of calcium signaling (Tukker et al. 2016). Treatment with glutamate and GABA strongly reduced the mean spike rate of the analyzed cultures. Calcium transients of individual neurons were generated upon treatment with glutamate, GABA, and acetylcholine. Here, astrocytes seem to be crucial for *neuronal network* generation because pure neuronal

cultures in the absence of astrocytes lack bursting, a sign for neuronal network maturity. In this study, the statistical unit was chosen as one well of a 48-well plate, not allowing assessment of reproducibility and standardization between experiments (Masjosthusmann et al. 2018). A follow-up study in 2018 also used commercially available hiPSC-derived neurons and astrocytes, this time exploring the effect of the ratio of mixed neurons and astrocytes (Tukker et al. 2018). This study strongly supports the previous observation that the addition of astrocytes to the model in near-physiological proportions of 50% (glia/neuron ratio 1:1; von Bartheld et al. 2017) and a ratio of 1:5 for GABAergic inhibitory neurons and excitatory neurons (Hendry et al. 1987; Sahara et al. 2012) indeed promotes neuronal network formation and maturation best. This study primarily indicates that hiPSC-derived neuronal models must be carefully designed and characterized before their large-scale use in neurotoxicity screenings, as each model exerts different responses to compounds, depending on the composition of the networks. The importance of the presence of astrocytes was also assessed by another study using commercially available cortical neurons on MMEAs (Kayama et al. 2018).

For controlled plating of neuron/astrocyte ratios, cells must be differentiated separately. A recent protocol instructs how to differentiate hiPSC into astroglia (NES-Astro) within 28 days (Lundin et al. 2018). These cells were extensively characterized using transcriptomics, proteomics, glutamate uptake, inflammatory competence, calcium signaling response, and APOE secretion and were compared to primary astrocytes, commercially available hiPSC-derived astrocytes, and an astrocytoma cell line. The data show large diversity among the different analyzed astrocytic models and strongly suggest to take the cellular context into account when studying astrocyte biology. Taking this to the next level, it indicates the importance of choosing the right astrocytic model to combine with hiPSC-derived neurons for the testing of substances acting on the CNS.

One major challenge in the field is the availability of a sufficient number of cells for large-scale screening approaches. Stacey et al. (2018) describe the concept of cryopreserved "near-assay-ready" cells, which decouples complex cell production from assay development and screening. Using this approach, the authors developed a 384-well veratridine-evoked calcium flux assay which assesses neuronal excitability and screened 2,700 compounds to profile the range of target-based mechanisms able to inhibit veratridine-evoked excitability using hiPSC-derived sensory neurons. In order to be able to use this approach for the identification of active compounds with unknown MoA, further secondary assays (e.g., using MMEA-technology) need to be developed to characterize the hits on a mechanistic level (Stacey et al. 2018). In addition, experiments were performed using pure neuronal cultures without the addition of astrocytes, probably leading to different neuronal responses than with astrocytes present.

Along those lines another high-throughput screening using 11 different compound libraries with a total of 4,421 unique substances, all bioactive small molecules, which include approved drugs, well-characterized tool compounds, natural products, and human metabolites, has been described lately (Sherman and Bang 2018). The authors use high-content image analysis, focusing on neurite outgrowth

of commercially available hiPSC-derived neurons, consisting primarily of GABAergic and glutamatergic neurons, but no astrocytes. They identified 108 hit compounds containing 38 approved drugs (outgrowth: erlotinib, clomiphene, tamoxifen, 17β-estradiol, dehydroepiandrosterone-3-acetate (DHEA), alfacalcidol, lynestrenol, benztropine, dibucaine, fluphenazine, perphenazine, prochlorperazine, trifluoperazine, sertindole, quetiapine, ifenprodil, meclizine, alverine, econazole, oxiconazole, letrozole, SAHA (Vorinostat); inhibition: methyltestosterone, thioridazine, methotrimeprazine, colchicine, docetaxel, vincristine, mebendazole, emetine, daunorubicin, doxorubicin, mitoxantrone, topotecan, hexachlorophene, ouabain, digoxin, suramin) which fall into the following categories: kinase inhibitors, steroid hormone receptor modulators, and channel and neurotransmitter system modulators (Sherman and Bang 2018). Inhibition of neurite outgrowth is one key characteristic in developmental neurotoxicity (Fritsche et al. 2018a, b), yet its implication in adult neurotoxicity is not clear.

Using a similar readout, hiPSC-derived peripheral-like neurons were applied to study the effect of chemotherapeutic agents on neuronal cytotoxicity and neurite length, again using high-content image analysis (Rana et al. 2017). This approach identified compounds that cause interference in microtubule dynamics but failed to depict the adverse effects of platinum and anti-angiogenic chemotherapeutics, which are compounds that do not act directly on neuronal processes. Here the addition of astrocytes to the model might lead to a higher predictivity, as the administration of fluorocitrate, an astrocyte-specific metabolic inhibitor, increased the pain tolerance of the animals in a rat model of oxaliplatin-induced neuropathic pain (Di Cesare et al. 2014; Kanat et al. 2017), indicating the role of astrocytes in sustaining platinum-mediated neurotoxicity.

One important cell type for neurotoxicological assessment of substances is myelin-producing oligodendrocytes. Yet publications on hiPSC-derived oligodendrocyte are scarce, and the protocols that are available are very time-consuming and of limited efficiency (Wang et al. 2013; Douvaras et al. 2014; Djelloul et al. 2015). Therefore, they are not suitable for medium- to high-throughput screening approaches. In contrast, the three transcription factors SOX10, OLIG2, and NKX6.2 produced 80% $O4^+$ oligodendrocytes from hiPSC within 28 days and might thus be a promising approach for future neurotoxicological applications (Ehrlich et al. 2017). Another recent study even reports that the overexpression of the transcription factor SOX10 alone is sufficient to generate 60% $O4^+$ and 10% MBP^+ cells in only 22 days (García-León et al. 2018b).

Although this part of the chapter primarily covers the use of hiPSC for the generation of neural in vitro models for studying substances acting on the CNS, the method of direct reprogramming of neuronal cells from somatic cells should not be disregarded. Lee et al. (2015) directly reprogrammed human blood to NPC without the intermediate step of hiPSC generation. These induced neurons (iNs) can be generated by overexpression of a set of transcription factors (Ichida and Kiskinis 2015; Vierbuchen et al. 2010; Ambasudhan et al. 2011; Hu et al. 2015; Wapinski et al. 2017) or miRNAs (Victor et al. 2014; Yoo et al. 2011; Abernathy et al. 2017) that promote chromatin remodeling and drive direct neural lineage

differentiation (Silva and Haggarty 2019). Especially for research regarding age-associated neurodegenerative diseases, like Alzheimer's disease (AD) or Parkinson's disease (PD), this method is of high interest, as bypassing the hiPSC reprogramming process reduces the disruption of epigenetic markers associated with the age of the somatic cell, therefore allowing to create neuronal models at "pathogenic ages" (Mertens et al. 2018). This method preserves multiple age-associated markers, including DNA methylation patterns, transcriptomic and microRNA profiles, oxidative stress, DNA damage (loss of heterochromatin and nuclear organization), and telomere length (Mertens et al. 2018; Silva and Haggarty 2019), and is therefore a promising approach to study substances acting on the aged CNS or screening for pharmaceuticals as a treatment for these conditions.

When working with either of these models, it is of utmost importance to have a well-characterized cell system, which suits the research question in case of basic research or contains a defined application domain for neurotoxic MoA (Table 1) when used for screening applications. Lack of characterization or definition of the application domain might result in false-negative data due to a lack of cellular targets. In addition, as with other in vitro approaches, the multicellular context of cultures seems to be crucial, possibly resulting in false predictions of chemicals when pure neuronal cultures lacking glia are used.

1.2 In Vitro Cultures of Microglia

Microglia constitute 5–10% of total brain cells and represent the resident innate immune cells of the CNS (Arcuri et al. 2017). Microglia discovery dates back to the end of the nineteenth century, but the name was coined in the 1920s by del Rio Hortega who phenotypically characterized the only immune cells resident in the brain parenchyma (Pérez-Cerdá et al. 2015). The function of microglia was for a long time underestimated because of the misconception that the brain is an immune-privileged site; moreover it was initially wrongly thought that this cell type originates from the neuroectoderm. To date, it is known that microglia arise from embryonic yolk sac (YS) precursors (Ginhoux et al. 2010) which give rise to YS macrophages that colonize the embryo, including the brain, to generate all types of tissue-resident macrophages (Li and Barres 2018). In the CNS, microglia maintain their population by self-renewal (Ajami et al. 2007) and by recruiting monocytes from the bloodstream (Hashimoto et al. 2013). The presence of microglia in the brain parenchyma is fundamental because of the variety of functions they perform from early brain development throughout the entire life of the organism, both in brain homeostasis and disease (for an extensive review, see Li and Barres 2018).

Considering the pivotal contribution of microglia to brain functions, it is important to have in vitro models containing microglia when studying the influence of drugs and toxicants on the brain. The majority of published in vitro studies mainly used primary microglia cultures from embryonic/neonatal rodent brain (mouse or rat). Still, fetal microglia seem to be quite different from adult ones. Due to ethical reasons, it is challenging to obtain brain-derived microglia from humans. The few

human microglia cell lines generated, such as HMO6 (Nagai et al. 2005) and HMC3 (Janabi et al. 1995), are not considered as an optimal model because long-term culture and genetic manipulation altered their functions and morphology. Finally, the low number of cells collected from humans does not allow large-scale neurotoxicity in vitro studies.

Starting from these premises, Leone and colleagues set up a monocyte-derived microglia-like cell model by culturing human monocytes with astrocyte-conditioned medium (Leone et al. 2006). This protocol was successively standardized using human peripheral blood mononuclear cells (PBMCs) stimulated with four recombinant human cytokines. The microglia cells obtained display a ramified morphology after 2 weeks in culture and express surface markers typical for the known pattern of microglia (Etemad et al. 2012).

More recently, human microglia-like cells were obtained from hESC and hiPSC. As previously stated, microglia derive from non-monocytic primitive myeloid cells, unlike adult bone marrow-derived macrophages. Thus microglia-like cells derived from PBMCs do not mirror this ontogeny. Muffat and co-workers established a robust protocol that allows the derivation of microglia-like cells from hiPSC, obtained from reprogrammed fibroblasts, using a serum-free medium that mimics the environment of the CNS interstitial milieu and adding interleukin 34 (IL-34), an alternative ligand for colony-stimulating factor 1 receptor. The microglia-like cells obtained with this protocol are highly phagocytic, and their gene expression profile resembles human primary microglia. They progressively adopt a ramified morphology when cultured in isolation, while when co-cultured in the presence of hiPSC-derived neurons, microglia-like cells refine their molecular signature. In terms of activity and response to stressors, unstimulated microglia-like cells secrete detectable levels of various cytokines and chemokines, which were enhanced after stimulation with lipopolysaccharide (Muffat et al. 2016) (Fig. 2).

Similar protocols were published a few months later reprogramming fibroblasts or PBMCs. In the paper of Pandya et al. (2017), hiPSCs were sequentially differentiated into myeloid progenitor-like intermediate cells and then into cells with the phenotypic, transcriptional, and in vitro functional signatures of brain-derived microglia. Abud and co-workers demonstrated that microglia-like cells obtained from hiPSC secrete cytokines in response to inflammatory stimuli, migrate, undergo calcium transients, and phagocytose (Abud et al. 2017). All those protocols require from 30 to about 70 days of time to obtain mature glia (McComish and Caldwell 2018). Taken together, those data suggest that microglia obtained from reprogrammed hiPSC better mirror the developmental stages of microglia maturation and ontogeny, in comparison to microglia-like cells derived from PBMCs stimulated with a cocktail of factors.

The potential applications of hiPSC-derived microglia include drug discovery studies, neurotoxicity screening assays, and use in disease modeling. Microglia-mediated inflammation can negatively impact the brain, and much evidence shows that microglial activation plays a role in neurodegeneration, contributing to the etiology of neurodegenerative disorders (Ransohoff and El Khoury 2016). The availability of robust protocols to generate and maintain microglia from patients with different brain dysfunctions in culture would facilitate the study of the

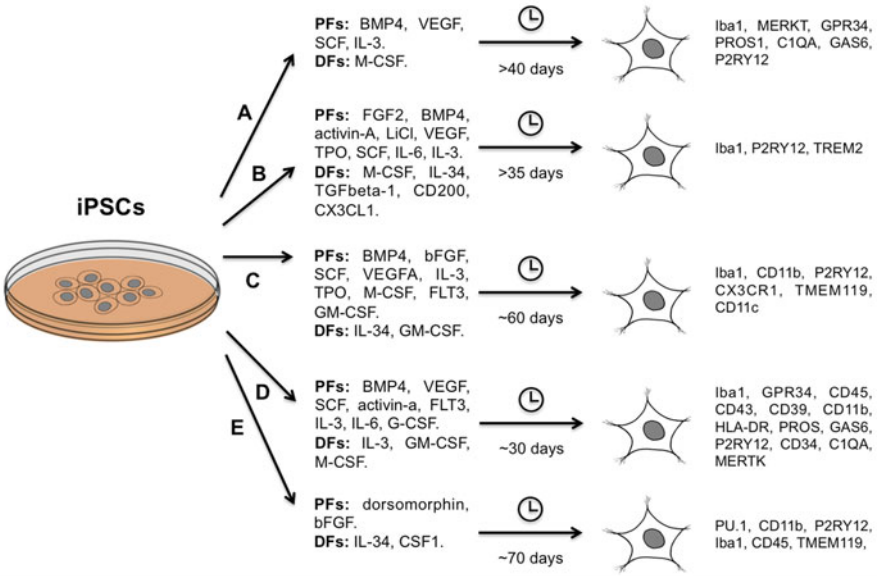

Fig. 2 Human iPSC-derived microglia protocols. (**a**) Haenseler et al. (2017), (**b**) Abud et al. (2017), (**c**) Douvaras et al. (2017), (**d**) Pandya et al. (2017), (**e**) Muffat et al. (2016). *PFs* patterning factors, *DFs* differentiation factors, *BMP4* brain morphogenetic protein 4, *VEGF* vascular endothelial growth factor, *SCF* stem cell factor, *IL-3* interleukin 3, *M-CSF* macrophage colony-stimulating factor, *FGF2 or bFGF* fibroblast growth factor 2, *TPO* thrombopoietin, *IL-6* interleukin 6, *IL-34* interleukin 34, *TGFbeta-1* transforming growth factor beta 1, *CD200* cluster of differentiation 200, *CX3CL1* fractalkine, *VEGF-A* vascular endothelial growth factor A, *FLT-3* fm-like tyrosine kinase 3, *GM-CSF* granulocyte-macrophage colony-stimulating factor, *G-CSF* granulocyte colony-stimulating factor, *CSF1* colony-stimulating factor 1, *Iba1* ionized calcium-binding adapter molecule 1, *MERTK* tyrosine kinase phagocytic receptor, *GPR34* G protein-coupled receptor 34, *PROS1* protein S1, *C1QA* complement C1q subcomponent subunit A, *GAS6* growth arrest-specific 6, *P2RY12* purinergic receptor P2Y, *TREM2* triggering receptor expressed on myeloid cells 2, *CD11b* cluster of differentiation 11b, *CX3CR1* CX3C chemokine receptor 1, *TMEM119* transmembrane protein 119, *CD11c* cluster of differentiation 11c, *CD45* cluster of differentiation 45, *CD43* cluster of differentiation 43, *CD39* cluster of differentiation 39, *HLA-DR* human leukocyte antigen – DR isotype, *CD34* cluster of differentiation 34

pathology and the discovery of new pharmacological approaches. The first evidence of in vitro culturing disease-related microglia cells from patients was in 2012, when Almeida and colleagues generated multiple induced hiPSC lines from subjects with frontotemporal dementia (Almeida et al. 2012). More recently, Ryan and co-workers performed, on human monocyte-derived microglia-like cells, a quantitative expression trait locus study to examine the effects of common genetic variation on the expression of genes found in susceptibility loci for Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Ryan et al. 2017). Microglia-like cells obtained by reprogramming PBMCs were also combined with neural progenitor cells and synaptosomes from hiPSC-derived neurons to create patient-specific cellular models useful to model CNS diseases facilitating high-throughput drug screening and neurotoxicity assays based on microglia function in the future (Sellgren et al. 2017).

In conclusion, different protocols for the derivation of human microglia are available that enable experiments in authentic human in vitro systems. Unlike methods for the derivation of neurons and astrocytes, protocols for microglia lack regionality and do not reflect microglia subtypes found within the brain (Grabert et al. 2016), which is a true challenge for the future.

1.3 Moving In Vitro Cultures into the Third Dimension with Brain Organoids

For investigating possible CNS disease mechanisms or screening drugs or toxins for safety and efficacy, it is thought to be advantageous to use complex 3D systems such as *brain organoids*. The benefits of organoids compared to "conventional" cultures lie in their composition of multiple cell types, which are functional in an in vivo-like manner and display morphological features of the organ to be modeled (Lancaster and Knoblich 2014). Yet, one has to be aware that there are major differences between in vivo embryogenesis or organogenesis and in vitro organoid formation, since even extremely well-controlled in vitro conditions strongly differ from real, regionally defined, physiological in vivo conditions (Bayir et al. 2019).

Different protocols for generating brain organoids have been established. Lancaster et al. (2017) used a floating scaffold out of poly(lactide-co-glycolide) copolymer (PLGA) fiber microfilaments to generate elongated embryoid bodies, called microfilament-engineered cerebral organoids (enCORs). Other groups used shaking platforms (Matsui et al. 2018), self-made spinning bioreactors (Qian et al. 2016), or soft matrices for embedding the cells (Lindborg et al. 2016; Bian et al. 2018) to let them form self-organized brain organoids. While neural organoids mostly mimic the early phases of embryonic development of the human brain, Matsui et al. (2018) cultivated their organoids up to 6 months and showed cell differentiation into functional neurons and myelin basic protein (MBP)-positive oligodendrocytes. The cerebral organoids fabricated by Quadrato et al. (2017) contained mature neurons including dendritic spine-like structures that generated spontaneously active neuronal networks as well as photosensitive cells after 8–9 months. These CNS models that display a later developmental status can now be used for safety and efficacy evaluations in medium to high throughput. One has to note that integration of microglia into organoids will be necessary in the future to better model toxicity and disease. This advanced technology is currently evolving (Ormel et al. 2018). In addition, although seemingly much more complex than 2D models, organoids also need definition of their applicability domains, with regard to the presence of cellular targets mediating neurotoxicity or drug efficacy (Table 1). Because reproducibility of organoid formation is still an issue with high variation making well-to-well comparison difficult, this neurotoxicity/efficacy target characterization has to be performed with great caution focusing on reproducibility of results.

As an example, hiPSC-derived brain organoids were recently employed for drug efficacy screening against ZIKA virus infection (Zhou et al. 2017) indicating that it is

possible to use such complex in vitro systems for medium- to high-throughput applications. The authors' high-content imaging approach identified a compound prohibiting organoid ZIKA virus infection and eliminating virus from infected organoids (Zhou et al. 2017). Other ZIKA virus-infected organoids were also published recently (Cugola et al. 2016; Garcez et al. 2016; Dang et al. 2016; Qian et al. 2016). Two groups (Watanabe et al. 2017; Xu et al. 2016) used these organoids similar to Zhou et al. (2017), for drug screening against ZIKA virus. Since the declaration of a public health emergency of international concern by the Word Health Organization in 2016, a lot of drug candidates against ZIKA virus were tested in in vitro as well as in in vivo systems, but only few of them with anti-ZIKA virus activity in animal models made it to clinical trials (Bernatchez et al. 2019).

Due to the higher throughput of such models, they are logistically superior over the low-throughput mouse models and can thus screen a large number of compounds. Moreover, their translational success to the human in vivo situation might be higher due to the human nature of cells. However, as already pointed out in Fig. 1, kinetic modeling is crucial for correct predictions that has to go hand in hand with the in vitro work.

While brain organoids are good models to examine the effects of disease and genetic aberrances on brain development, they lack to model the blood-brain barrier (BBB) of the adult human cortex. Many drugs for neurologic diseases and disorders fail to pass the BBB; therefore, there is a need for a BBB model that enables the examination of the permeability of these drugs. BBB organoid models can be derived from three (Cho et al. 2017; Bergmann et al. 2018) or from six (Nzou et al. 2018) different cell types that include astrocytes, pericytes, and endothelial cells. Both models are able to reproduce the properties and functions of the BBB through the exhibition of tight/adherent junctions, efflux pumps, and transporters. Nzou et al. (2018) showed in their organoids also the impenetrability of the BBB for specific molecules: by adding MPTP, MPP+, and mercury chloride to the medium they proofed that their models indeed have a charge-selective barrier. Data from such models might also be useful for feeding PBPK models.

In conclusion, there are currently several different organoid models in development, both normal and disease models. However, these are still in the process of establishment and characterization and are not yet in use for substance screenings.

1.4 3D Bioprinted In Vitro Neural Models

The next generation of in vitro models already arising is fabricated by 3D *bioprinting*. The state of the art of 3D bioprinting of brain cells was recently comprehensively reviewed by Antill-O'Brien et al. (2019). 3D bioprinting of brain cells is faced with a variety of challenges needing sophisticated solutions. The first is the choice of biomaterial biomimicking brain tissue from extracellular matrix components as well as the mechanical, structural, biochemical, and diffusive properties of the brain with high cellular biocompatibility. Here, especially, the tremendous softness of brain tissue poses a great challenge for bioengineers.

Biomaterials currently used for neural cell culture are *hydrogels*. These hydrophilic polymers can be reversibly or irreversibly cross-linked via chemical or physical triggers to maintain their structure over a long period of time. Hydrogels are an attractive material for culturing cells in 3D due to their biocompatibility, high water content, and tuneable physical and chemical properties. For neuronal cultures, the pore structure of the hydrogel must be able to support neural cell bodies, which are 10–50 μm in diameter, and allow neurite extension (Antill-O'Brien et al. 2019). Once a suitable biomaterial is identified, the biofabrication strategy has to be defined. Prior to printing, 3D neural tissue can be manufactured via layering. Cell embedding in gels and manual layering thereby allow studying cytocompatibility of hydrogels. Printing of soft materials is often challenging. A major hurdle to 3D biofabrication of such soft structures is their shaping into 3D structures with high spatial resolution to achieve an anisotropically accurate mimic of the brain microstructure. Sacrificial scaffolds, e.g., from gelatine, have previously been used to support soft gels like 0.5% alginate, which otherwise fall below the printable viscosity range. Usage of this sacrificial scaffold improved cell survival in the hydrogel (Naghieh et al. 2019). For scaffold-free 3D bioprinting, extrusion-based printing has mainly been employed due to its economy, ease of use, and capability to print with high cell density with a wide range of materials. Despite a small number of studies using rat or hiPSC-derived neurons for extrusion-based bioprinting, one study should be highlighted. Joung et al. (2018) developed a bioengineered spinal cord combining bioprinting with 3D printed scaffolds in the only example of functional neurons with extensive axon propagation from bioprinted neural precursor cells. Pre-differentiated spinal NPC and oligodendrocyte progenitor cells from hiPSC were bioprinted in precise alternating points in silicon channels. After 4 days, β-III tubulin-positive axons spread throughout the channel, and after 14 days the cells were found to have differentiated into mature glutamate-responsive neurons with synchronous responses to K^+ and glutamate (Joung et al. 2018). This method of a spinal cord model could be applied to CNS neural tissue engineering.

This paragraph is not supposed to give a comprehensive overview of 3D bioprinting of neural structures, yet should touch on the challenges of this rising technique. 3D bioprinting of neural models is still in the early stages of development and offers great potential for exquisite spatial bioink patterning to recapitulate the microarchitecture of brain tissue. Although 3D bioprinted neural disease models have not yet been developed, the potential advantages over animal models include species- and patient-specific disease modeling. For more detailed information on cells, materials, techniques, and readouts, the reader is referred to Antill-O'Brien et al. (Antill-O'Brien et al. 2019).

1.5 CNS Disease Models

Improvement of the hiPSC technology allows to obtain and culture neurological patient-specific hiPSC lines, which recapitulate molecular and cellular phenotypic aspects of the respective disease. These offer a unique opportunity to generate

physiologically relevant in vitro models to understand disease etiology and progression, as well as to support preclinical drug discovery. Genomically unaltered human iPSC-derived neurons and astrocytes have been derived from Alzheimer's disease (AD), Parkinson's disease (PD), *Huntington's disease* (HD), *amyotrophic lateral sclerosis* (ALS), and idiopathic *autistic spectrum disorder* (ASD) patients (Table 2), to provide 2D and, most recently, 3D cultures reproducing features of these neurological diseases. In addition to patient-derived iPSCs, inserting genetic changes manually into iPSCs can also generate disease models. Manipulating gene expression of *LIS1*, the most common gene mutated in patients with *lissencephaly*, and using an on-chip organoid approach as an exciting example, allowed studying the emergence of folding during in vitro development and the physical mechanisms of folding reproducing pathogenesis of lissencephaly using organoids (Karzbrun et al. 2018).

In 2D cultures, patient-derived iPSCs are generally committed to differentiate into neuronal monoculture representative of the affected cell type: in PD research iPSCs are differentiated into dopaminergic neurons (TH-positive) functionally characterized by dopamine decarboxylase and the dopamine transporter (Hartfield et al. 2014), while cortical glutamatergic neuron or motor neurons are derived to best represent AD or ALS features, respectively.

More recently, greater attention has been dedicated to both microglia (Haenseler et al. 2017) and patient-derived astrocytes (Kondo et al. 2013; Qian et al. 2017; Hsiao et al. 2015), due to the recognition of the relevance of glial cells in contributing to disease initiation and progression. Similar to toxicity evaluation described above, the aim here is to develop co-cultures, which take the complexity of neuron-glia interactions into account, hence also considering inflammatory responses, which have a high impact on the course of pathology (Haenseler et al. 2017). In addition, the presence of multiple cell type allows to address neuron-glia cross-talk in drug discovery. The diversity of cell types in a single culture is also retained in organoid models, which add a further step of complexity by respecting brain cytoarchitecture.

A clarifying example on the potentiality of patient-derived stem cell models comes from AD patients (for extensive review, see Arber et al. 2017). Extracellular amyloid plaques composed of amyloid beta peptide (Aβ) and Tau protein intracellular neurofibrillary tangles are considered hallmarks of AD. Aβ is the product of β- and γ-secretase processing of amyloid precursor protein (APP). Autosomal dominant mutations in APP and alternative subunits of γ-secretase presenilin 1 and 2 (PSEN1 and PSEN2) have been detected in AD patients, implicating an altered APP processing and Aβ imbalance in AD pathogenesis. Reprogramming cells derived from patients with genetic predisposition to AD into cortical glutamatergic neurons, cortical interneurons, and cholinergic neurons (Table 2) allowed to reproduce AD features like increased Aβ42:40 ratio and enhanced Tau phosphorylation (Table 2). These models were then used to gain insight into biochemical pathomechanisms of AD (Kondo et al. 2013), contribution of neurons and astrocytes to pathophysiology (Kondo et al. 2013; Oksanen et al. 2017) and identify drug targets potentially relevant in the progression of the disease. In these studies, also

Table 2 Patients' iPSC-derived neurons as disease models for investigating neurological disorders

	Mutations investigated	Cell type	Pathological hallmarks	Reference
Alzheimer's disease				
fAD	PSEN1 A246E PSEN1 N1411	Neurons	Increased Aβ Increased Aβ42:40 ratio	Yagi et al. (2011)
fAD sAD	APP duplication	Neurons Glutamatergic GABAergic Cholinergic	Increased Aβ40 Increased pTau Activated GSK3b	Israel et al. (2012)
fAD sAD	APP E693Δ APP V717L	Cortical neurons[1] or astrocytes[2]	Increased Aβ42:40 ratio[1] Extracellular Aβ oligomers[1,2]	Kondo et al. (2013)
fAD	PSEN1 ΔE9	Neurons	Increased Aβ42:40 ratio	Woodruff et al. (2013)
sAD	ApoE3/E4 (AD-E3/E4)	Cholinergic neurons	Increased Aβ42:40 ratio	Duan et al. (2014)
fAD	APPV717I	Neurons	Increased Aβ42:40 ratio Increased Aβ38:40 ratio Increased total and phosphorylated Tau	Muratore et al. (2014)
fAD	PSEN1 ΔE9	Astrocytes	Accumulation of full-length PS-1 Increased Aβ42:40 ratio Disturbance in ER calcium signaling Development of a pro-inflammatory profile	Oksanen et al. (2017)
fAD sAD	PSEN 1 M146L *ApoE4*$^{+/+}$	Astrocytes		Jones et al. (2017)
fAD	APP duplication PSEN 1 M146I PSEN 1 A264E	3D organoids	Amyloid aggregation Hyperphosphorylated tau protein Endosome abnormalities	Raja et al. (2016)
Down syndrome		Cortical glutamatergic neurons	Increased Aβ40 Increased Aβ42 Aβ aggregates	Shi et al. (2012)
Down syndrome		Neurons with forebrain characteristics	Aβaggregates Tau protein hyperphosphorylation Tau intracellular redistribution	Chang et al. (2015)

(continued)

Table 2 (continued)

	Mutations investigated	Cell type	Pathological hallmarks	Reference
Parkinson's disease				
	PARK2/Parkin V324A PINK1 Q456X	Midbrain DA neurons	Increased α-synuclein expression	Chung et al. (2016)
	SNCA gene triplication	DA neurons	Increased α-synuclein expression	Oliveira et al. (2015)
	a-synuclein A53T	Cortical neurons and glia	α-Synuclein expression	Chung et al. (2013)
Amyotrophic lateral sclerosis				
sALS	Patients presented symptoms relating to motor-neurons degeneration of the spinal cord (not in brainstem or cortex) in the absence of C9ORF72 gene mutations	Astrocytes	Increased GFAP immunofluorescence	Qian et al. (2017)
	GGGGCC repeat expansions in C9ORF72	Motor neurons	Aging increased DNA damage	Lopez-Gonzalez et al. (2016)
	VCP mutations R191Q or R155C	Enriched cultures of motor neurons and astrocytes	Increased percentage of cytosolic TDP-43	Hall et al. (2017)
	SOD1 E100G	Spinal motor neurons	Significant decline in survival compared with the healthy control MNs	Bhinge et al. (2017)
fALS	TDP-43	Motor neurons	Cytosolic aggregates Shorter neurites	Egawa et al. (2012)
Huntington's disease				
HD	43 CAG repeats	Astrocytes	Increased vascular endothelial growth factor A	Hsiao et al. (2015)
HD Juvenile HD	109 CAG repeats		Contain both normal and mutant huntingtin	Szlachcic et al. (2015)
Juvenile HD	60 and 109 CAG repeats	Mixed culture: Neurons, glia, and progenitor cells	Alterations in genes and gene pathways that are associated with a pathological CAG repeat length	Consortium TH iPSC (2017)
HD	180 CAG repeats	Striatal-like cultures	Significantly lower ATP levels than control	Rindt et al. (2017)

fAD familiar AD, *sAD* sporadic AD, *sALS* sporadic ALS, *DA* dopaminergic

novel mechanisms like endoplasmic reticulum and oxidative stress were identified (Kondo et al. 2013; Muratore et al. 2014). In addition, iPSCs derived from patients carrying multiple genetic variants allow investigations of AD risk factors, i.e., linking mutations to increased risk of late onset AD (Duan et al. 2014; Young et al. 2015; Huang et al. 2017; Schröter et al. 2016). The organization of genomically unaltered iPSC-derived neurons in a 3D structure, thereby reproducing brain cytoarchitecture, favors the retention of proteins secreted by cells that are lost in a 2D culture, like Aβ peptides. In 3D their local concentration is increased and pathology better recapitulated (Raja et al. 2016). 2D and 3D models obtained from iPSCs derived from AD patients have been used to investigate drug efficacy and toxicity, so far targeting β- and γ-secretase with specific inhibitors (Yagi et al. 2011; Shi et al. 2012; Kondo et al. 2013; Duan et al. 2014; Raja et al. 2016; Woodruff et al. 2013) and inflammation with nonsteroidal anti-inflammatory drugs (Yahata et al. 2011).

Although the models obtained from patients' hiPSCs exhibit clear advantages, their use to model aging and neurodegenerative diseases poses a relevant challenge due to the fact that differentiation protocols mimic neurodevelopmental processes. Indeed, derived cells retain molecular characteristics closer to the fetal than the adult stage (Patani et al. 2012; Camp et al. 2015), which might limit the full development of an AD model. For example, Tau isoform expression is tightly regulated during development (Bunker et al. 2004), and the lack of formation of aggregated Tau in non-manipulated patients' hiPSCs might reflect the absence of the adult isoforms. In general, this implicates that any disease phenotype has to be discriminated from phenotypes of earlier developmental stages. So far this issue that represents a possible limit in drug discovery has been solved by generating footprint-free triple MAPT-mutant human iPSCs (García-León et al. 2018a), overexpressing mutant PSEN1 (DE9) and APP (K670N/M671L plus V7171; Choi et al. 2014), or could be overcome by direct reprogramming, thus skipping the intermediate step of hiPSC (for details on this, see second to last paragraph of Sect. 1.1 of this chapter).

In the context of CNS pathologies recapitulated by an hiPSC approach, neurodevelopmental and psychiatric diseases are worth a note. CNS cells derived from patients by reprogramming allow to capture a complex genetic architecture of diseases that are highly polygenic in nature, overcoming the difficulty to generate genetically accurate animal models of psychiatric disorders. Patient hiPSC-derived neural cells allow to dissect the gene network associated with features of altered neurodevelopment that controls the phenotypic trait and the signaling pathways involved (Mariani et al. 2015; Haggarty et al. 2016).

As listed in Table 2, patients' hiPSCs replicate disease phenotypes to an extent to represent clinically relevant features of the illness, thus providing human cellular assays that may improve drug preclinical evaluation and translation of the results to clinical trial in the process of drug discovery (Silva and Haggarty 2019). Clinically relevant targets and phenotypes displayed in hiPSC-derived disease models may drive the testing of candidate drugs selected on a hypothesis-driven screening or the screening of large compound libraries for identification of novel molecules for their ability to rescue disease phenotypes. In addition, the generation of large numbers of

patients' cell models representative of the heterogeneity of each disorder may represent a strategy to identify patient subpopulations with specific responsiveness to therapeutic agents. Finally, by interlinking a patient's genetic background with specific disease characteristics, hiPSCs apply to the concept of personalized medicine, possibly allowing the development of personalized drug evaluation in the future (Engle and Puppala 2013).

2 Summary and Conclusion

Animal models have been greatly contributing to our understanding of physiology, mechanisms of diseases, and toxicity. Yet, they have limitations due to interspecies variation, which determines the lack of information of the "human context," and deficiency in pathophysiologically relevant disease models. This deficiency has a tremendous negative impact on the understanding of basic physiology, human disease, mechanisms of toxicity, and the process of successful drug discovery.

Human iPSC-derived cells offer a platform with the unique advantage of reproducing the "human context" missing in animal models, by preserving the genetic and the molecular phenotype of donors. Forcing the differentiation of hiPSC into cells of the nervous system and combining them in a 2D or 3D format allows obtaining complex models suitable to investigate neurodevelopmental processes and to reproduce neurodegenerative diseases with patient-derived cells. This has the potentiality to drive the identification of molecular targets that may be predictive for the evolution of specific human diseases as well as for beneficial and/or adverse drug responses. Thus, with such cell platforms, screening assays can be set up that are based on human-relevant targets and thus are useful for drug testing and discovery with the hope of overcoming the low success rate of CNS drug development due to poor clinical efficacy or elevated toxicity. Cell culture standardization is mandatory in this process. Well-characterized and overall reproducible cell systems that contain neural and immune cells of the CNS, are based on standardized protocols and procedures to generate differentiated and mature cells representative of different brain areas, and are able to address the fundamental unanswered questions of drug discovery and toxicity are urgently needed.

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Engineered Heart Muscle Models in Phenotypic Drug Screens

Wolfram-Hubertus Zimmermann

Contents

Abstract

Classical drug development is compromised by considerable clinical failure of promising drug candidates after decades of costly preclinical work. Failure can be because of previously unrecognized safety concerns or more commonly lack of clinical efficacy. Classical drug discovery and safety pharmacology programs rely heavily on well-established in vitro and preclinical animal models. The availability of human pluripotent stem cells and the possibility to direct them into any somatic cell type suggest that a paradigm shift in drug development may be possible and timely, with the opportunity to test safety and efficacy of candidate drugs on the human target cells and tissue. However, there is considerable uncertainty as to whether human models would only qualify as replacement for well-established tools or add substantially more information to the preclinical data package, to facilitate translation of more promising drug candidates into clinical practice. This chapter provides an overview of tissue-engineered macro-

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_385

scale heart muscle models for applications in drug discovery and safety pharmacology.

Keywords

Cardiomyocytes · Heart · Phenotypic drug screens · Stem cells · Tissue engineering

Abbreviations

ABCF-I	Activin A, BMP4, CHIR, and FGF2 followed by IWP4
ACTN2	Sarcomeric alpha-actinin 2
BMP4	Bone morphogenetic protein 4
CHIR99021	6-((2-((4-(2,4-Dichlorophenyl)-5-(4-methyl-1H-imidazol-2-yl) pyrimidin-2-yl)amino)ethyl)amino)nicotinonitrile
CiPA	Comprehensive in vitro proarrhythmia assay
CSA	Cross-sectional area
DKK1	Dickkopf-related protein 1
EB	Embryoid body
EHM	Engineered heart muscle or engineered human myocardium
EHT	Engineered heart tissue
ESC	Embryonic stem cells
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor-2
FOC	Force of contraction
FT	Force transducer
GiWi	Staged GSK and Wnt inhibition
GSK	Glycogen synthase kinase
hERG	Ether-à-go-go-related gene-related channels
hvCOC	Human ventricular cardiac organoid chambers
hvCTS	Human ventricular cardiac tissue strips
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
iPSC	Induced pluripotent stem cells
IWP2	Inhibitor of Wnt processing and secretion 2 – N-(6-methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-d] pyrimidin-2-yl)thio]-acetamide
IWP4	Inhibitor of Wnt processing and secretion 4 – N-(6-methyl-2-benzothiazolyl)-2-[[3,4,6,7-tetrahydro-3-(2-methoxyphenyl)-4-oxothieno[3,2-d]pyrimidin-2-yl]thio]-acetamide
KLF4	Krüppel-like factor 4 (reprogramming factor)
KY02111	N-(6-Chloro-2-benzothiazolyl)-3,4-dimethoxy-benzenepropanamide
MaPS	Macro-physiological systems
MiPS	Micro-physiological systems

MYC	Myelocytomatosis proto-oncogene (reprogramming factor)
MYH7	Myosin heavy chain beta
NT-proBNP	N-terminal pro-B-type natriuretic peptide
OCT4	Octamer-binding transcription factor 4 (reprogramming factor)
PB	Pole bending
SOX2	Sex-determining region Y-box 2 (reprogramming factor)
TdP	Torsade de pointes
TNNT2	Cardiac muscle troponin T
VEGFA	Vascular endothelial growth factor-A
Wnt	Wingless and Int-1
Wnt-C59	Inhibitor of mammalian porcupine acyltransferase activity – 4-(2-methyl-4-pyridinyl)-N-[4-(3-pyridinyl)phenyl] benzeneacetamide
XAV939	3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano [4,3-d]pyrimidin-4-one

1 Introduction

After typically decades of fundamental academic research, the most promising therapeutic concepts are recognized and taken over by industry for the ultimate translation into clinical practice (Spector et al. 2018). Once at the industry level, drug development and in particular safety pharmacology testing are highly standardized. Cell culture models and animal models expressing the drug target are at the core of preclinical development. After a plausible mode of action has been experimentally confirmed, safety pharmacology testing is the bottleneck for a further advancement of promising drug candidates into clinical testing. Despite a wealth of knowledge as to how to synthesize drug candidates with low clinical risk, it is the safety pharmacology units, which have the final say as to either promoting or rejecting new drug candidates.

In cardiovascular drug development, a key safety concern relates to the blockade of ether-à-go-go-related gene (hERG)-related channels, which can induce torsade de pointes (TdP) arrhythmia and consequently sudden cardiac death. After the introduction of the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) S7B and E14 guidelines, no newly introduced drugs failed in clinical practice because of the risk of hERG-related TdP arrhythmia (Wisniowska et al. 2017). This demonstrates that available test batteries are sufficient to evaluate hERG-related liabilities in preclinical drug development. Aiming at safety pharmacological studies beyond hERG-related issues, the more recent Comprehensive in vitro Proarrhythmia Assay (CiPA) initiative aims at introducing "a new paradigm for assessment of clinical potential of TdP that is not measured exclusively by potency of hERG block and not at all by QT prolongation." Data from multichannel patch clamp studies and in silico modelling as well as confirmatory studies in human primary and pluripotent stem cell-derived

cardiomyocytes are main fields of research of the CiPA consortium (Strauss et al. 2019).

As in vitro heart muscle models, human pluripotent stem cell (PSC)-derived cardiomyocytes in 2D or 3D culture formats are particularly attractive, primarily because they harbor an individual human genome, which encodes for the specific human drug target, and secondarily because of available standardized protocols to grow, maintain, manipulate, and analyze these cultures. A pivotal step toward the introduction of human cardiomyocytes was the adoption of protocols for the derivation of embryonic stem cells (ESCs) from mouse (Evans and Kaufman 1981; Martin 1981) to non-human primate and eventually human (Thomson et al. 1998). This in turn provided the ground for the development of induced pluripotent stem cells (iPSCs), first in mouse (Takahashi and Yamanaka 2006) and then in human (Takahashi et al. 2007), by making use of transcription factors known to be involved in the maintenance of pluripotency, i.e., OCT4, KLF4, SOX2, and MYC or alternatively OCT4, SOX2, NANOG, and LIN28 (Yu et al. 2007). Using appropriate culture conditions, human iPSCs and ESCs have been confirmed as biologically similar and, for the most part, interchangeable in drug screening applications. Given the ethical concerns associated with the use of human ESCs, iPSCs are the most logical choice for applications in drug development. The possibility to derive and create iPSC models from any individual (healthy subject and patient) and apply them as patient-in-the-dish model is another strong argument for the use of iPSCs. In combination with genome editing, a powerful toolbox to individualize drug development in rare disease indications, but also in non-communicable diseases without a primary genetic cause, exists (Soldner and Jaenisch 2018).

After the first introduction of an undirected embryoid body differentiation protocol for the derivation of human cardiomyocytes from human embryonic stem cells (Kehat et al. 2001), numerous serum-free protocols have been developed for the derivation of cell populations highly enriched for ventricular-like cardiomyocytes (Table 1). Subtle, but important, refinements of the protocols are, however, required for every PSC line for optimal results. Purification can be supported by metabolic selection as required, taking advantage of cardiomyocyte survival under glucose-free/lactate-containing culture medium (Tohyama et al. 2013). Importantly, available protocols can be adapted to bioreactor cultures for scalable production of human cardiomyocytes (Chen et al. 2015b). Note that for tissue engineering applications, not cardiomyocyte purity, but the use of carefully optimized mixtures of cardiomyocytes and stromal cells is advantageous (Tiburcy et al. 2017).

Applications of PSC-derived cardiomyocytes can be as (1) unstructured or patterned monolayer cultures (Agarwal et al. 2013; Feinberg et al. 2007; Grosberg et al. 2011; Lind et al. 2017; Ribeiro et al. 2015a, b; Stancescu et al. 2015); (2) aggregate cultures, also known as cardiac bodies (Huebsch et al. 2016; Kelm et al. 2004; Mathur et al. 2015); and (3) tissue-engineered formats, also referred to as organotypic or organoid cultures (Boudou et al. 2012; Breckwoldt et al. 2017; Chen et al. 2015a; Conant et al. 2017; Kensah et al. 2013; Li et al. 2018; Mills et al. 2017; Nunes et al. 2013; Ronaldson-Bouchard et al. 2018; Schaaf et al. 2011; Shadrin et al. 2017; Soong et al. 2012; Tiburcy et al. 2017; Tulloch et al. 2011; Turnbull et al.

Table 1 Overview of protocols for the derivation of cardiomyocytes from human PSCs (all can be applied in tissue engineering with or without addition of stromal cells)

Protocol	Growth factors	Small molecules	Cardiomyocyte yield	Reference
EB differentiation	n/a (20% FBS)	n/a	8% of EBs	Kehat et al. (2001)
Growth factor-directed	Activin A, BMP4	n/a	30% MYH7+	Laflamme et al. (2007)
Stage-specific induction	Activin A, BMP4, FGF2, DKK1, VEGFA	n/a	50% TNNT2+	Yang et al. (2008)
Small molecules (GiWi)	n/a	CHIR + KY or XAV CHIR + IWP4	98% TNNT2+ 85% TNNT2+	Minami et al. (2012), Lian et al. (2012)
Chemically defined	n/a	CHIR + Wnt-C59 CHIR + IWP2	95% TNNT2+ 88–98% TNNT2+	Burridge et al. (2014), Lian et al. (2015)
ABCF-I	Activin A, BMP4, FGF2	CHIR + IWP4	>90% ACTN2 +/TNNT2+	Tiburcy et al. (2017)

EB Embryoid body, *FBS* fetal bovine serum, *GiWi* staged GSK and Wnt inhibition, *ABCF-I* mesoderm induction with activin A, *BMP4*, CHIR, and FGF2 followed by cardiac specification with IWP4. *Growth factors*: *BMP4* bone morphogenetic protein 4, *FGF2* fibroblast growth factor-2, *DKK1* Dickkopf-related protein 1, *VEGFA* vascular endothelial growth factor-A
Small molecules used in GiWi protocol: (1) Wnt activator – CHIR (CHIR99021) – a highly specific glycogen synthase kinase (GSK)-3 inhibitor; (2) Wnt inhibitors, KY (KY02111), XAV (XAV939), IWP2, IWP4, and Wnt-C59. *Muscle-specific protein signatures used for the identification of cardiomyocytes by flow cytometry*: *MYH7* myosin heavy chain beta, *TNNT2* cardiac muscle troponin T, *ACTN2* sarcomeric alpha-actinin 2

2014). Tissue-engineered formats for applications in drug discovery and safety pharmacology can further be distinguished in micro- and macro-physiological systems (MiPS and MaPS (Meyer et al. 2019)).

This overview has a focus on tissue-engineered heart muscle MaPS. We define MaPS by size (>1 mm to centimeter scale) and macroscopically visible contractility, allowing for simple video-optic or organ bath analyses. Automation of MaPS production, culture, and analyses is important to lower costs and facilitate high-throughput applications in drug discovery. Acceptance by regulatory authorities as potency assay would greatly support the consideration of MaPS in safety pharmacology studies. Of additional interest may be the application of iPSC-based and potentially individualized MaPS in studies paralleling clinical trials to gather additional comprehensive organ-specific data (e.g., long-term efficacy and toxicity) and validate or even enhance the knowledge base as to the mode of action associated with drug candidates.

2 Macro-scale Heart Muscle Models for Drug Discovery

Several engineered heart tissue models have been introduced over the past 30 years (selection of human MaPS in Table 2). Hydrogel-based models are most widely used and typically formulated by mixing cardiomyocytes with supporting stromal cells in collagen or fibrin. In the original collagen-hydrogel studies in rodent models, Matrigel™ was essential to support spreading and formation of a functional syncytium of neonatal rat cardiomyocyte preparations (Zimmermann et al. 2000). The first human PSC-based heart muscle engineering studies adopted the use of Matrigel™ (Schaaf et al. 2011; Soong et al. 2012; Tulloch et al. 2011). Further protocol refinements, mainly by applying defined cultures comprising extracellular matrix producing stromal cells in fibrin- (Ronaldson-Bouchard et al. 2018) and collagen- (Tiburcy et al. 2017) based models, allowed for an omission of Matrigel™ without a compromise in function. These models can be scaled up and down according to the respective need (Mills et al. 2019; Tiburcy et al. 2017) and are typically developed under dynamic mechanical strain to simulate the hemodynamic load of the heart. While proper mechanical loading, ideally to support auxotonic contractions, is an essential requirement (Schaaf et al. 2011; Soong et al. 2012; Tulloch et al. 2011), electrical stimulation may be applied in addition to support maturation (Nunes et al. 2013). Advanced cardiac MaPS exhibit a postnatal level of maturation (Ronaldson-

Table 2 Human macro-scale 3D (organoid) heart muscle models for applications in drug development (cardiac MaPS)

Model	Hydrogel	Cardiomyocytes	Max FOC	Reference
Engineered heart tissue (EHT)	Collagen +matrigel	2×10^6/EHT	16 µN (FT)	Tulloch et al. (2011)
Biowire	Collagen +fibrin +matrigel	150,000/ biowire	33 µN (PB)	Conant et al. (2017)
hvCTS (cardiac tissue strips) hvCOC (cardiac organoid chambers)	Collagen +matrigel Collagen +matrigel	1×10^6/hvCTS 1×10^7/hvCOC	0.7 mN (FT) 1.26 mm H_2O	Turnbull et al. (2014) Li et al. (2018)
Engineered heart tissue (EHT)	Fibrin	2×10^6/EHT	0.9 mN (FT)	Ronaldson-Bouchard et al. (2018)
Engineered heart tissue (EHT)	Fibrin +matrigel	1×10^6/EHT	0.15 mN (PB)	Breckwoldt et al. (2017)
Engineered human myocardium (EHM)	Collagen	1×10^6/EHM	2.5 mN (FT)	Tiburcy et al. (2017)

Note that this is a selection of human MaPS with applications in drug development and not a full overview of tissue-engineered heart muscle models. Contractile force was assessed video-optically by pole bending (PB) assays, force of contraction (FOC) measurements using force transducers (FT), or the recording of hydrostatic pressure. Max. FOC (in mN) was either reported as indicated or recalculated according to data (e.g., cross-sectional area in case FOC was presented as mN/CSA) provided in the cited references

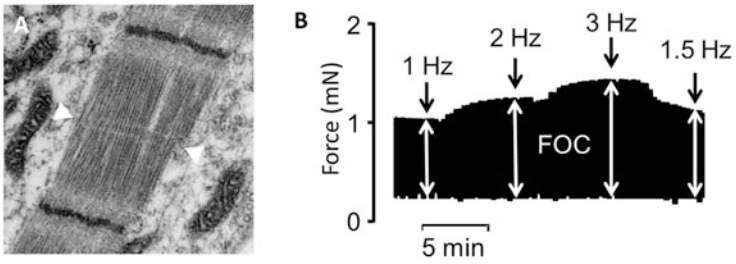

Fig. 1 Properties of postnatal heart muscle in engineered human myocardium. (**a**) Presence of M-bands (indicated by white arrows) and (**b**) a positive force-frequency behavior recorded in engineered human myocardium (Tiburcy et al. 2017)

Bouchard et al. 2018; Tiburcy et al. 2017), which is evident by the documentation of the following structural and functional features: (1) the presence of sarcomeric M-bands (identified by electron microscopy) and (2) a positive force-frequency response (identified under isometric suspension conditions and electrical stimulation; Fig. 1).

The development of T-tubules, often referred to as a hallmark of maturation, appears to be primarily associated with cardiomyocyte volume, which increases with postnatal heart development by hypertrophy in the by then mostly post-mitotic cardiomyocytes. At the first 6 months after birth, cardiomyocyte volume has been reported with ~2,500 μm^3 (Bergmann et al. 2015). Cardiomyocytes in EHM (engineered human myocardium) exhibit a similar length (~92 μm) but smaller width (~13 μm; (Tiburcy et al. 2017)) in comparison with the reported dimensions in the adult human heart (length/width: 60–150/20–35 μm (Severs 2000; Tracy and Sander 2011)). With a volume of ~12,000 μm^3 in EHM (Tiburcy et al. 2017), and an anticipated maximal volume of 35,000 μm^3 at the age of 25, cardiomyocyte in EHM resembles cardiomyocytes in 5–10-year-old humans (Bergmann et al. 2015). This observation is in line with the observed ultrastructural and function parameters (Fig. 1) and further supports the interpretation that PSC-derived cardiomyocytes in EHM formulations exhibit an advanced degree of maturation and "physiological" hypertrophic growth behavior.

Contractile performance in heart muscle MaPS are best assessed by video-optic recordings (Nunes et al. 2013; Schaaf et al. 2011; Thavandiran et al. 2020; Tiburcy et al. 2020) or classical organ bath force of contraction measurements under isometric conditions ((Soong et al. 2012; Tiburcy et al. 2017; Tulloch et al. 2011); Fig. 2). While video-optic recordings allow, in principle, for a high parallelization of data acquisition and multimodal imaging of, for example, motion and fluorescing dyes or genetically encoded reporters (e.g., for calcium and membrane voltage imaging), organ bath assessments under freely adjustable preloading and electrical stimulation remain the gold standard for comprehensive contractile phenotyping. Importantly, engineered in contrast to bona fide heart muscle is not compromised by a significant rundown in function and may be assayed for hours or days without compromise in function, with in addition the opportunity to sample tissues during a screening

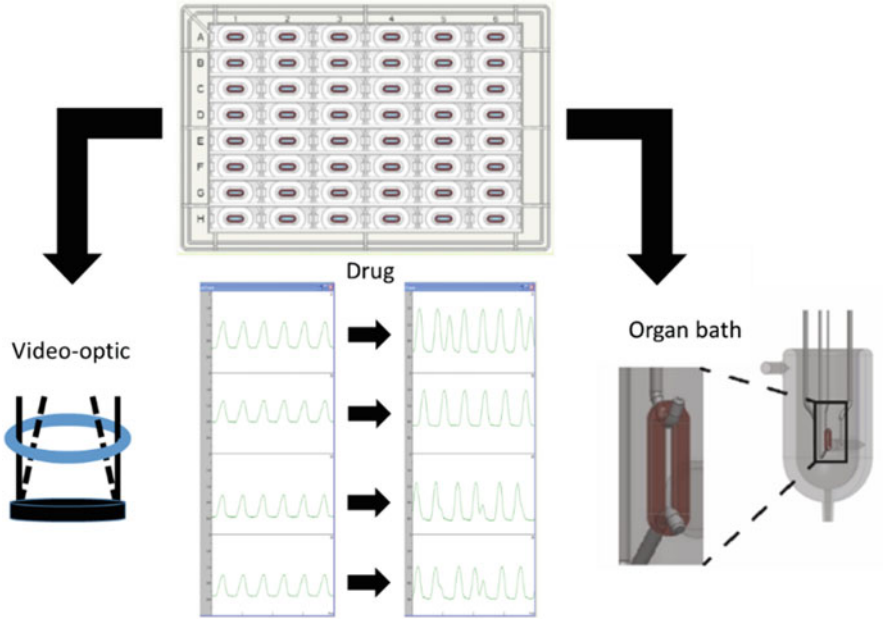

Fig. 2 Automated engineering and culture of heart muscle for applications in drug testing. Tissue production can be automated by the use of dedicated culture dishes and combined with video-optic recordings for phenotypic screens at a high throughput. Alternatively, engineered heart muscle may be transferred into standard organ baths for analyses under defined isometric conditions (i.e., defined preload) and electrical stimulation for deep contractile phenotyping

exercise for supplemental studies of, for example, structure, transcriptome, or proteome. In addition, culture medium supernatant may be used to analyze the effective soluble drug concentration, biomarker release (e.g., NT-proBNP, cardiac troponin), or the metabolic state (e.g., lactate, glucose, fatty acids).

3 Pros and Cons of Tissue-Engineered Myocardium in Drug Screening Applications

Bringing preclinical drug development closer to clinical reality is without doubt an important aspiration. Whether established pluripotent stem cell models with their intrinsic cellular immaturity can indeed bridge this gap and add more information than available through standard models is commonly and rightfully questioned. It is without doubt that advanced monolayer and tissue-engineered (organoid) cultures do not fully resemble the patient heart, not only with respect to maturity but also as to cellular heterogeneity and typical age-acquired deficiencies. However, it is also absolutely clear that animal models, with their often fundamentally different cardiovascular pathophysiology and a common lack of specific drug targets, do not resemble the human heart either. Despite these fundamental limitations associated

with animal models and the availability of human models, as of today there appears to be little motivation to modify established drug development pipelines. A common concern toward the use of iPSC-derived heart muscle models relates to the lack of proof for a successful application in clinical risk assessment and the (additional) costs associated with the introduction of another ex vivo model into classical drug development pipelines. Nevertheless, the following advantages associated with the use of tissue-engineered heart muscle at the MaPS scale should be considered: (1) the possibility to engineer organotypic physiology (e.g., anisotropic syncytia demonstrating positive force-frequency and force-preload as well as physiological drug response (Ronaldson-Bouchard et al. 2018; Tiburcy et al. 2017)); (2) the possibility to simulate clinical pathologies (e.g., induced heart failure phenotypes either by neurohumoral overstimulation with phenylephrine or endothelin-1 (Tiburcy et al. 2017); (3) analyses of contractile parameters using highly standardized and well-established methods (video-optic or even more so isometric force measurements (Schaaf et al. 2011; Thavandiran et al. 2020; Tiburcy et al. 2020); and (4) higher reproducibility and stability as compared to native heart muscle preparations. Obvious disadvantages include (1) limited experience as to applications in industry drug discovery and safety pharmacology programs; (2) consideration as in-between technology, i.e., more complex than cells, but not a human heart; (3) lower degree of maturity as compared to the typically aged patient heart; (4) higher costs apparently inhibitory for high-throughput drug discovery applications; and (4) no agreement on or regulatory acceptance of a reference method.

4 Conclusion

Tissue engineering of heart muscle at the MaPS scale was introduced at the end of the last millennium. Compelling evidence as to an advanced degree of maturation compared to monolayer cultures, the possibility of functional studies, and its use with human pluripotent stem cell-derived cardiomyocytes has resulted in a steep increase of reports (Fig. 3) using tissue-engineered heart muscle in fundamental

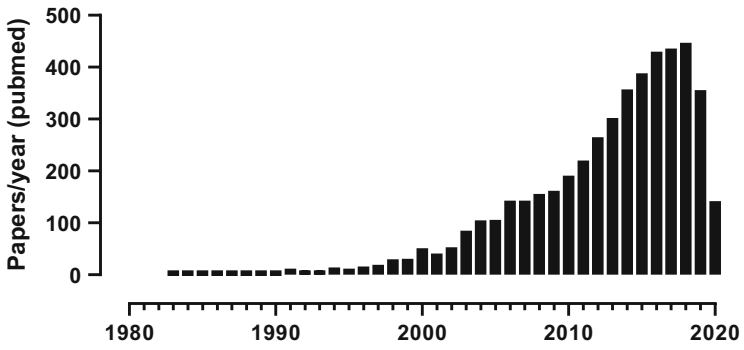

Fig. 3 Overview of papers in PubMed; search term: Tissue Engineering Heart Muscle

studies of heart pathophysiology, target validation, drug screening, and tissue-engineered heart repair. Although not yet broadly applied as standard model in drug discovery and safety studies by pharma industry, there is a clear trend away from flat cultures to 3D cultures not only in academia, and it only seems a matter of time until tissue-engineered heart muscle models will be applied routinely in modern drug development pipelines as advanced patient-in-the-dish models.

Acknowledgment This chapter focusses on studies using human heart MaPS model, and thus references are, with few exceptions to provide historical background, restricted to human models.

Financial Support W.H.Z. is supported by the DZHK (German Center for Cardiovascular Research), the Federal Ministry for Science and Education (BMBF), the German Research Foundation (SFB 1002 TP C04/S01; MBExC), and the Fondation Leducq.

Conflict of Interest W.H.Z. is listed as inventor on several filed and granted patents in the field of stem cell models and tissue engineering. W.H.Z. is founder and advisor of myriamed GmbH, which is offering stem cell technologies and tissue engineering-based drug screening services, and Repairon GmbH, which is developing engineered human myocardium for clinical applications in heart failure repair.

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Disease Models: Lung Models for Testing Drugs Against Inflammation and Infection

Patrick Carius, Justus C. Horstmann, Cristiane de Souza Carvalho-Wodarz, and Claus-Michael Lehr

Contents

Abstract

Lung diseases have increasingly attracted interest in the past years. The all-known fear of failing treatments against severe pulmonary infections and plans of the

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_366

pharmaceutical industry to limit research on anti-infectives to a minimum due to cost reasons makes infections of the lung nowadays a "hot topic." Inhalable antibiotics show promising efficacy while limiting adverse systemic effects to a minimum. Moreover, in times of increased life expectancy in developed countries, the treatment of chronic maladies implicating inflammatory diseases, like bronchial asthma or chronic obstructive pulmonary disease, becomes more and more exigent and still lacks proper treatment.

In this chapter, we address in vitro models as well as necessary in vivo models to help develop new drugs for the treatment of various severe pulmonary diseases with a strong focus on infectious diseases. By first presenting the essential hands-on techniques for the setup of in vitro models, we intend to combine these with already successful and interesting model approaches to serve as some guideline for the development of future models. The overall goal is to maximize time and cost-efficacy and to minimize attrition as well as animal trials when developing novel anti-infective therapeutics.

Keywords

Air-blood barrier · Air-liquid interface (ALI) · Biofilm · Cystic fibrosis · Deposition · *Mycobacterium tuberculosis* · *Pseudomonas aeruginosa* · Transepithelial electrical resistance (TEER) · Tuberculosis

Abbreviations

ALI	Air-liquid interface
AT-I/AT-II	Alveolar type I/II pneumocytes
CF	Cystic fibrosis
CFBE41o-	Cystic fibrosis human bronchial epithelial cells
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
ECM	Extracellular matrix
EMA	European Medicines Agency
FDA	Federal Drug Administration
hAELVi	Human alveolar epithelial lentivirus immortalized
hAEpCs	Human alveolar epithelial cells
IPF	Idiopathic pulmonary fibrosis
LCC	Liquid-covered conditions
LPS	Lipopolysaccharide
NHBE	Normal human bronchial epithelial cells
PQS	*Pseudomonas* quinolone signal
TB	Tuberculosis
TEER	Transepithelial (-endothelial) electrical resistance

TNF	Tumor necrosis factor
WHO	World Health Organization

1 Introduction

The lung is exposed to potential harmful noxae with every taken breath. These agents range from toxic particulate matter that can cause inflammatory disease to pathogenic microorganisms causing infectious respiratory diseases. Although novel inhaled medicines for the treatment of most of the inflammatory conditions are available, they rather serve as a maintenance treatment to delay the progression of the disease but not as a curative treatment (Strong et al. 2018). The recently approved triple combination Trelegy Ellipta (GlaxoSmithKline, London, United Kingdom) comprises an inhaled glucocorticoid, a long-acting muscarinic antagonist, and a long-acting β_2-agonist (fluticasone furoate/umeclidinium/vilanterol) for the treatment of chronic obstructive pulmonary disease (COPD). This combination product was more effective in reducing severe to moderate COPD exacerbations compared to therapy with fluticasone furoate-vilanterol or umeclidinium-vilanterol (Lipson et al. 2018). However, while this triple therapy reduces inhaler use to one inhalation per day as well as the rate of hospitalization due to COPD per patient, it remains symptomatic and still does not cure the underlying disease.

In the case of infectious respiratory disease, the need for more effective treatment options might be even higher. It is estimated that 33,000 deaths in the EU alone are attributed to antimicrobial-resistant infections; on a global scale, the number increases to 700,000 deaths (Cassini et al. 2019; O'Neill 2014). From all the isolates that were reported to the European Antimicrobial Resistance Surveillance Network (EARS-Net), a third of the *Klebsiella pneumoniae* isolates, a respiratory pathogen, showed resistance at least against one antimicrobial group under regular surveillance. Moreover, some countries reported that more than 10% of the *Klebsiella pneumoniae* isolates showed resistance against carbapenems – a critically important class of broad-spectrum antibiotics. The percentage of carbapenem-resistant isolates is even higher for *Pseudomonas aeruginosa* (*P. aeruginosa*), a respiratory pathogen that puts a high burden, especially on cystic fibrosis (CF) patients by forming hard to target biofilms (ECDC 2018). Taken all together, respiratory conditions globally account for four of the top ten causes of death estimated by the World Health Organization (WHO) (World Health Organisation 2019).

To tackle these challenges, the inhalation route seems to have distinct advantages, since pulmonary drug delivery can be utilized for local drug targeting inside the lungs as well as for systemic pulmonary delivery (Newman 2017). For the treatment of pulmonary microbial infections, higher local concentrations can be achieved – that in turn would lead to higher bacterial eradication efficacy – further reducing the risk for resistance development (Flume and VanDevanter 2014; Geller et al. 2002; Ho et al. 2019; Wenzler et al. 2016). The well-perfused respiratory mucosa is characterized by a very thin diffusion barrier (~0.6 μm) that spans over a large surface area, which is in

contact with air (~100 m^2) (Crapo et al. 1982; Gehr et al. 1978). In combination with a low local enzymatic activity that circumvents first-pass metabolism, systemic pulmonary delivery can lead to a rapid onset of action and favorable plasma levels as it was shown for inhaled insulin (Santos Cavaiola and Edelman 2014).

However, contrary to the proposed benefits of inhalation therapy, the success rate for market approval of a compound in the respiratory therapeutic area is one of the lowest in the industry (Mestre-Ferrandiz et al. 2012). Roughly one third out of 33 respiratory projects reported by AstraZeneca that entered clinical trials were canceled due to lung-specific toxicity (Cook et al. 2014). This high attrition rate, also within preclinical drug development and discovery, is one of the two major hurdles slowing down R&D productivity, together with an increasing regulatory demand relating to preclinical testing, to prevent harm to human trial participants (Marx et al. 2016; Rovida et al. 2015). The strategy of employing more advanced in vivo tests on laboratory animals, next to conventional in vitro testing of cell lines, grown on titer plates in the context of preclinical safety evaluation did neither reduce attrition rates nor did it raise predictivity of human toxicity for novel medicines during the last years (Gintant et al. 2016; Leist and Hartung 2013). By now well-known, as well as unknown species-species variations between humans and model organisms (e.g., in lung anatomy, cellular composition, gene expression, or protein synthesis) will further prevent the acquisition of new mechanistic knowledge about human disease. There is a high need for interconnected reliable models of human origin that comprise the different epithelia of the airway as well as the respiratory mucosa together with noncellular barriers, like, e.g., mucus and pulmonary surfactant, for advanced drug testing.

Advanced dynamic in vitro models of human origin are far away from and not even aimed to replicate the full complexity of living organisms. Instead, their purpose is to reflect certain (patho)physiological parameters or conditions that are likely to be important when developing a pharmaceutical product. We want to give an overview of such techniques from already available standard monocultures to more complex co-cultures as well as promising developments, especially in the context of infection research that may lead to innovative disease models for use in preclinical drug development in the near future.

2 Morpho-functional Characteristics of the Lung

Looking at the three major outer human epithelia the skin, the gastrointestinal tract, and the lung, their morphology complements their function. The skin protects the human body from light, injuries, or infections by covering the body's outer surface like a coat. The gastrointestinal tract can be simplified as a tube that selectively absorbs nutrients and eliminates solid waste in a directed motion. The lung, however, better compares to a bucket, i.e., input and output coming from the same opening (mouth/nose). The respiratory tract is designed for effective gas exchange during breathing and thus comprises different sections with distinct morphological and physiological features (Fig. 1).

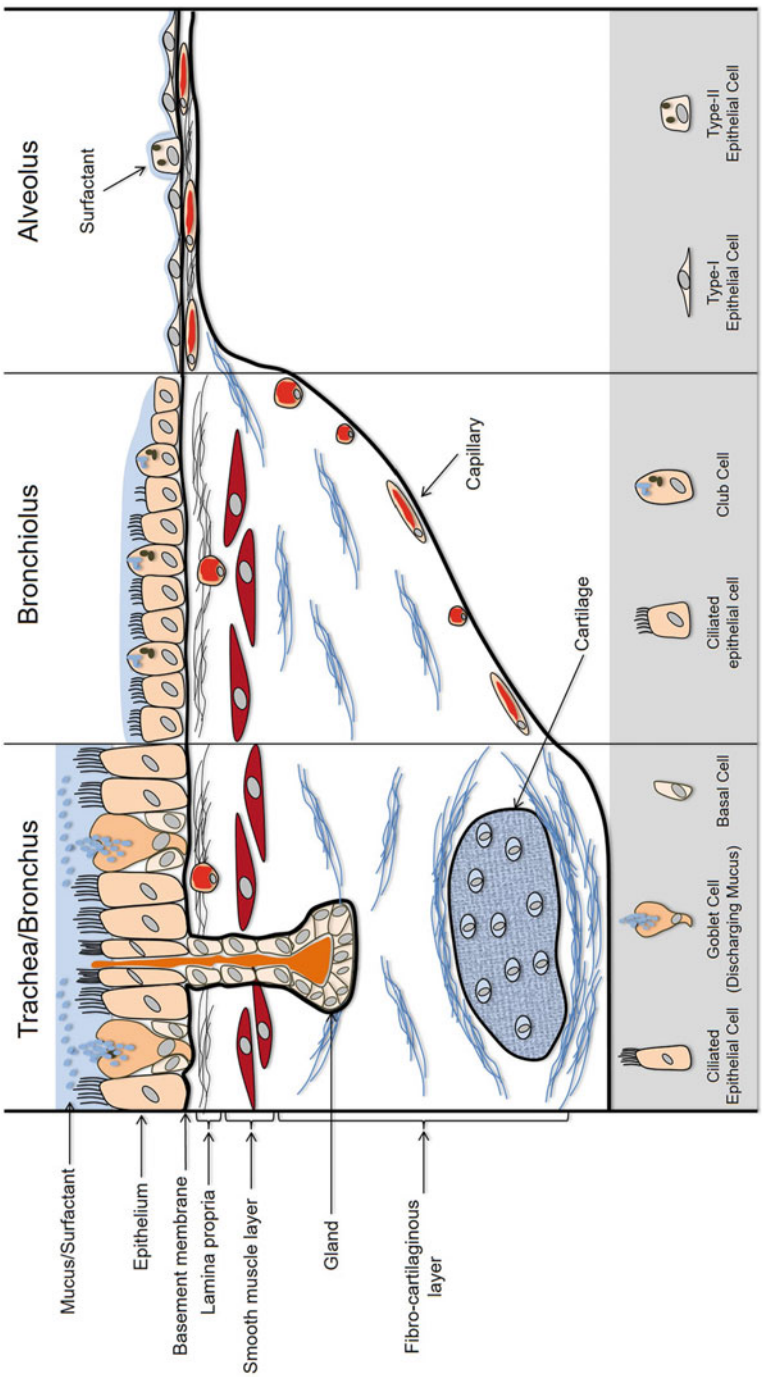

Fig. 1 Tissue organization in the lung. Schematic representation of the different cell types and the mucosal tissue in the different regions of the lung. The pseudostratified epithelium lining the trachea and the main bronchi (left) is gradually changing to cuboidal in the region of the bronchiolus (middle) and finally becomes mostly squamous in the alveolus (right). The epithelial cells in the trachea and the conductive airways are mixed with interspersed secretory cells. They are supported by cartilage in the trachea as well as smooth muscle cells that disperse down to the alveolus. To enable effective gas exchange, the mucosa thins out towards the alveolus. (Adapted from Klein et al. 2011 with permission, © Elsevier 2011)

Starting from the nasal region, continuing along the trachea, the conductive airways of the central lung expand into the terminal respiratory region of the peripheral lung. The trachea and the large bronchi are lined by a pseudostratified epithelium that predominantly consists of columnar epithelial cells next to mucus-secreting goblet cells that are both supported by basal cells (McDowell et al. 1978). The bronchioles of the lower conducting airways are lined by a columnar ciliated epithelium, containing some non-ciliated secretory cells, also known as club cells, and still some individually dispersed goblet cells. With further ramifications of the bronchiolar tree, the bronchioles develop into respiratory bronchioles until they terminate into pulmonary acini. Each pulmonary acinus includes several alveolar ducts that further extend into two to three alveolar sacs. Every alveolar sac contains multiple alveoli, whereby a single alveolus, lined by alveolar epithelial cells, represents the functional unit of gaseous exchange. In the human lung, the average number of alveoli is estimated to be 480 million, thereby extending the surface of the alveolar epithelium to more than 100 m^2 (Gehr et al. 1978; Ochs et al. 2004).

Two morphological and functional distinct types of pneumocytes essentially form the "air-blood barrier" of the alveolar epithelium: the squamous AT-I cells aid gas exchange as well as the diffusion and transport of solutes, while the cuboidal AT-II cells mainly contribute to immune responses, function as progenitors to AT-I pneumocytes, and are responsible for the homeostasis of pulmonary surfactant. AT-I cells are only part of 8% of all lung cells, but as a result of their average cell surface of ~5,000 μm^2, they contribute 95–98% to the internal surface area of the human lung (Crapo et al. 1982). AT-II cells constitute ~18% of all cells in the human alveolar region, but they only cover a surface area of 2–5% (Stone et al. 1992).

As a noncellular element, pulmonary surfactant covers the respiratory epithelium, and particularly the alveoli, as part of the alveolar lining fluid – a thin aqueous layer mainly composed of water and pulmonary surfactant. Consisting of ~90% lipids and ~10% proteins, including the bioactive surfactant proteins (SP-A, -B, -C, and -D), pulmonary surfactant prevents the alveoli from collapsing by reducing surface tension and also demonstrates antimicrobial activity (Goerke 1998).

Another noncellular element of mucosal epithelia is mucus, a mesh-like hydrogel, mainly consisting of water (~95%), the high molecular glycoprotein mucin (~2–5%) as well as smaller amounts of lipids. Mucus is secreted by goblet cells and lines the upper airways (Boegh and Nielsen 2015). Particulate matter or microorganisms are trapped in mucus and subsequently transported towards the pharynx by the so-called mucociliary escalator, as a result of coordinated movement of the cilia located on the apical side of the airway epithelial cells.

In the past few years, scientific interest in mucus, as well as lung surfactant, has much increased. Insights about the complex biochemical as well as biophysical characteristics of mucus led to drug delivery approaches like mucoadhesion or mucopenetration, where utilization of the mucous structure in the case of the former leads to sustained drug release or enhancement of permeability when favoring the latter (Murgia et al. 2018). Pulmonary surfactant is considered to be the first layer of interaction with airborne particles, forming a protein, and lipid-rich corona after the first contact (Raesch et al. 2015). However, its systematic integration into respiratory in vitro models is still erratic (Garcia-Mouton et al. 2019).

Below the basal epithelial membrane, there is a plethora of different other cell types present in the lungs. Interstitial fibroblasts mainly secrete components of the extracellular matrix (ECM). Airway smooth muscle cells, for example, manage airflow into the lung by changing the diameter of the bronchioles. A variety of immune cells like dendritic cells, neutrophils, eosinophils, as well as B and T lymphocytes or tissue and alveolar macrophages are involved in the control of infections and inflammatory reactions. Three-dimensional in vitro co-culture systems of epithelial cells together with endothelial cells and or immune cells have already been developed in order to model some diseases and will be discussed in some more detail below.

3 Requirements to Model the Lung In Vitro

3.1 Technical Prerequisites

The Danish physiologist Hans Henriksen Ussing enabled the first investigations of transport processes across an epithelium with the introduction of the Ussing chamber in the early 1950s, by mounting frog skin epithelium between two fluid-filled chambers (Ussing and Zerahn 1951).

Continuing with excised bullfrog alveolar epithelium, this setting was used among other also mammalian epithelia to study pulmonary transport in the years after (Kim and Crandall 1983; Wall et al. 1993). Although the classic Ussing chamber can still be purchased today, newer devices emerged that led to more precise electrophysiological as well as diffusion measurements. The combination of membrane-based inserts with such devices permitted the study of not only excised but also reconstituted epithelial tissues of primary cells or cell lines cultured on porous supports. Integration of these microporous membranes into multiwell plates via embedded inserts (e.g., Transwell®) enabled the standardized culture of polarized epithelial cells in vitro.

The epithelial division into an apical as well as basolateral compartment inside a standard culture allows not only for convenient well-based permeability assays but also for exposing the cells to air at the apical surface, as it is the case for the pulmonary epithelia in vivo. Thus, when culturing pulmonary epithelial cells in vitro, it is essential to differentiate between air-liquid interface (ALI) conditions and liquid-covered culture (LCC) conditions. Cells cultivated under ALI conditions show enhanced cellular differentiation and enable in vivo-like deposition of aerosolized liquids or dry powders (Hiemstra et al. 2017; de Jong et al. 1994). On the contrary, the critical advantage of cells cultured under LCC conditions is that bioelectrical measurements can be performed inside the well without any changes to the ion equilibrium. ALI cultures need to be transferred to LCC conditions before any electrophysiological measurements across the epithelium are possible. Besides, the time for the re-establishment of a steady ion equilibrium over such epithelia also needs to be taken into account. Measuring the transepithelial electrical resistance

(TEER) can be a predictor of the barrier properties of an epi- or endothelial monolayer before conducting drug transport experiments.

As a widely accepted quantitative method, TEER values (typically: Ω cm^2) are a representative measurement of the tightness of intercellular junctions in the cell monolayer, which develop because of cellular differentiation. The most applied method to measure TEER in order to evaluate pulmonary epithelial integrity is the combination of a chopstick electrode connected to an epithelial Volt-Ohm meter. Based on a plethora of Ussing chamber experiments, the cellular resistances of different gastrointestinal epithelia from multiple species were classified. Epithelia with TEER values of ~2,000 Ω cm^2 were considered to be "tight," whereas "leaky" epithelia displayed TEER values of 50–100 Ω cm^2 (Powell 1981). For example, monolayers of Caco-2 cells, a commonly used in vitro model of the gastrointestinal tract, develop moderate TEER values of 150–400 Ω cm^2 when cultured on permeable growth supports while still generating a barrier that restricts the diffusion of hydrophilic substances (Artursson et al. 1993; Hidalgo et al. 1989).

Conversely, mammalian alveolar epithelia should be considered electrically tight, since they need to develop a certain resistance to the flow of solutes and water pressing from the vasculature. Based on this assumption, TEER values of ~2,000 Ω cm^2 and higher have been first described for monolayers of rat alveolar epithelial cells cultivated on porous supports in vitro and later also for primary human alveolar epithelial cells cultured in vitro (Cheek et al. 1989; Elbert et al. 1999; Kim et al. 1991).

3.2 Sources of Cells

When cultured in vitro, primary cells from human donors most closely resemble the in vivo morphology as well as the physiology of pulmonary epithelia. Depending on the cell type of interest, different isolation protocols have been described.

Bronchial epithelial cells can be isolated following bronchoscopic biopsies or from lung tissue rejected for transplantation, respecting all biosafety and ethical procedures (Bucchieri et al. 2002; Devalia et al. 1999; Galietta et al. 1998). Growing the cells for 14 days in differentiation media supplemented with retinoic acid, on collagen-coated permeable supports under ALI conditions, these cells display the morphology of normal human bronchial airways (Karp et al. 2002). Commercial sources are also available that comprise normal human bronchial epithelial cells (NHBE; Lonza), MucilAir™ (Epithelix), or EpiAirway™ (MatTek) among others.

In case of the alveolar epithelium, procedures for the isolation of human AT-II pneumocytes, as well as methods to support their trans-differentiation into AT-I-like cells, have been established and steadily improved (Elbert et al. 1999; Fuchs et al. 2003). The most recent version of this protocol allows for the reliable isolation of human alveolar epithelial cells (hAEpCs), that when cultured on permeable membrane supports, develop a diffusive and electrically tight barrier characterized by TEER values of ~2,000–2,500 Ω cm^2 after ~7 days of culture (Daum et al. 2012).

Although primary cell cultures are considered the gold standard to mimic pulmonary epithelia in vitro, limitations of these systems include their limited availability,

donor-to-donor variations or potentially unknown pathologies from the donor. A further problem is that primary respiratory epithelial cells can only be sub-cultivated for a limited amount of passages, before they dedifferentiate (Zabner et al. 2003). This restricted proliferative capacity of primary cells fostered the development of continuous cell lines that can be expanded over several passages and conveniently cryopreserved. Bosquillon et al. compared the permeability between two of the best-characterized human bronchial epithelial cell lines, Calu-3 and 16HBE14o-, as well as primary normal human bronchial epithelial (NHBE) cells, showing that all three human bronchial epithelial models compared well with published absorption rates from rat lungs measured in vivo (Bosquillon et al. 2017). They measured the apparent permeability of seven test substances widely varying in size and lipophilicity (imipramine, propranolol, metoprolol, terbutaline, mannitol, dextran, and formoterol) on monolayers of either cell line as well as the absorption of the same substances in ex vivo perfused rat lungs. Because of their widespread availability, the lung cancer cell line Calu-3 is for many groups now the first choice for routine permeability screening studies.

To find a cell line applicable for in vitro permeation and absorption studies that also reflects the characteristics of the human alveolar epithelium was more complicated than for the bronchial epithelium. As a promising candidate, the human A549 cell line, initially isolated from a lung explant of an adenocarcinoma patient, was considered to show AT-II-like properties because the formation of lamellar bodies was initially reported (Giard et al. 1973; Lieber et al. 1976). Although later reports questioned the suitability of A549 cells as a cell line model for primary AT-II cells, a more recent study demonstrated that A549 cells cultured in Ham's F12 culture medium over 25 days showed a gene expression pattern that was similar to primary AT-II cells (Cooper et al. 2016; Corbière et al. 2011; Swain et al. 2010). Unfortunately, A549 cells are functionally deficient in tight junction formation. An elevated permeability towards low molecular weight molecules, compared to Calu-3 or 16HBE14o- monolayers, thus excludes A549 cells from in vitro transport studies at least for small molecules (Winton et al. 1998). Remarkably enough, the human alveolar epithelial lentivirus immortalized (hAELVi) cell line shows the meaningful synthesis of functional tight junction proteins Occludin and ZO-1. Further, these cells develop an electrically tight barrier with more than 1,000 $\Omega\,cm^2$ under LCC and ALI conditions while preserving a permeability barrier for sodium fluorescein at the same time (Kuehn et al. 2016). hAELVi cells seem to be so far a promising cell line for the assessment of permeability and absorption studies of the alveolar epithelium.

A common limitation of the aforementioned cell-based models is that they mostly comprise single epithelial cell types. In the context of inflammatory or infectious disease, the interplay between several cell types and even pathogens is the root cause of the condition. In addition, as described in the paragraphs above, also the culture devices evolve alongside with increasingly advanced cell models (Fig. 2).

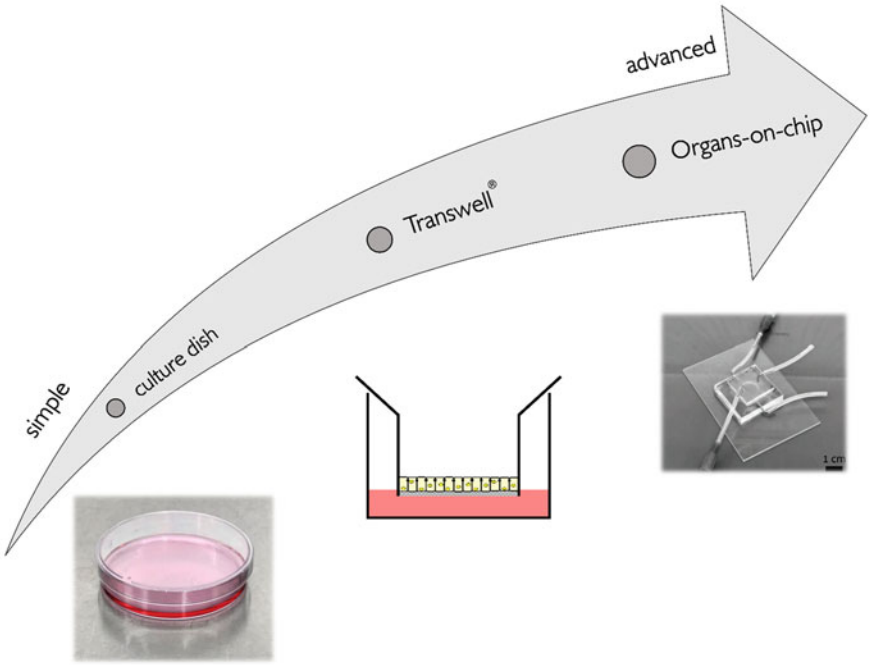

Fig. 2 Technological evolution of different cell culture devices. Standard culture dishes are limited to submerged culture conditions and also prevent epithelial cells from polarization. The introduction of well-based permeable growth supports (e.g., Transwell) not only enabled the growth of polarized epithelial cells in vitro but also allowed permeability assays as well as the establishment of ALI conditions. With the advent of advanced organs-on-chip devices even dynamic breathing motions or fluid flow could be incorporated into epithelial in vitro models (organs-on-chip picture from Artzy-Schnirman et al. 2019)

4 Modeling Pulmonary Diseases In Vitro

4.1 Asthma

The airway epithelium is not only a barrier to inhaled materials or pathogens. It indeed plays an active role in the host defense. Airway epithelial cells collaborate with the immune system, with mucociliary clearance, production of lytic enzymes, and antimicrobial proteins (Parker and Prince 2011). Asthma is an allergic disorder that results from a combination of factors, for instance, a dysregulation of the epithelial response towards environmental antigens and genetic susceptibility. A main characteristic of asthma is the remodeling of bronchial airways, which includes subepithelial fibrosis, hyperplasia of myofibroblasts, and myocytes. Moreover, an increase in smooth muscle fibers, airway inflammation, mucus hypersecretion,

infiltration of eosinophils, and T cells, as well as high angiogenesis, were also observed (Blume and Davies 2013; Elias et al. 2003). Airway remodeling contributes to the deposition of the ECM. The epithelial-mesenchymal transition has been identified as the primary source of fibroblasts (Pain et al. 2014).

Several animal species have been used in asthma studies, for instance, mice, rats, guinea pigs, cats, dogs, horses, and the fruit fly Drosophila (Kirschvink and Reinhold 2008). Even so, those models fail to emulate human lungs, mainly due to the lack of functional homology between the two species (Blume and Davies 2013). In addition, most of the animals do not spontaneously develop the disease (Szelenyi 2000).

In humans, one of the main hallmarks in asthma is the remodeling of the airways, leading to disease chronicity. Human airway remodeling thereby more closely resembles allergic Th2 driven immune responses, whereas studies based on animal models rather predicted a mechanism similar to inflammation (Parker and Prince 2011; Saglani et al. 2007). Therefore, the animal models of asthma have some intrinsic limitations to be considered when translating results from animal models to humans.

Access to human airway tissue has allowed for the development of several experimental models to study human asthma, for instance, ex vivo and in vitro models (Blume and Davies 2013). Ex vivo tissue explants are the simplest human models for studying airway responses in a limited period, which is a limitation to study mechanisms associated with chronicity. Precision-cut slices or bronchial biopsies from asthmatic individuals have contributed to shed light on several signaling pathways related to asthma, with the advantage that these models retain the lung tissue architecture (Wohlsen et al. 2003). However, they have a short period of viability and a compromised epithelial barrier.

In asthma, human cell culture models have mainly been used to study fundamental cellular responses or cell signaling pathways in the airways. The most straightforward and readily accessible cell models are those based on immortalized cell lines. The bronchial airways have been studied through the use of established cell lines. The SV-40 transformed human bronchial epithelial cell line BEAS-2B was highly used in studies aiming to understand signaling pathways like inflammatory responses or mitogen-activated protein kinase (MAPK) (Stokes et al. 2011; Tacon et al. 2012). When it comes to the evaluation of epithelial barrier functionality upon toxicological or drug transport studies, the SV-40 transformed human cell line 16HBE4o- or the human adenocarcinoma cell line Calu-3 has been widely used as a model for the bronchial epithelium in vitro. Although representing an alternative for in vivo studies, these cells fail to reproduce the genetic or epigenetic changes observed in asthma patients. Primary human airway cells from healthy or asthmatic individuals offer a possibility to study the disease-related mechanisms or pathways. However, it is not easy to replicate in vivo conditions for in vitro primary human airway cell cultures due to the lack of sophisticated elements like the basement membrane, smooth muscle cells, and dendritic cells (McLellan et al. 2015).

The use of differentiated cells allows for better recapitulation of the in vivo airways. Culture conditions such as ALI vs. LCC conditions play an essential role

here, to mimic the in vivo situation as closely as possible. For example, once cultured at ALI in the presence of retinoic acid, primary bronchial epithelial cells differentiate into ciliated and mucus-producing goblet cells (McLellan et al. 2015; Pezzulo et al. 2011).

The complex interaction between different cell types remains a challenge. Many efforts have been made in the development of relevant co-culture models and 3D tissue constructs that integrate not only cells but also an ECM. For instance, in vitro co-cultures of primary human airway epithelial cells and human lung fibroblasts showed differential expression of ECM components (e.g., collagen I, collagen III, and hyaluronic acid). These components were higher expressed when human lung fibroblasts were co-cultivated with airway epithelial cells from pediatric asthmatic patients compared to the cultivation with airway epithelial cells from healthy children intubated during anesthesia for elective surgery (Reeves et al. 2014).

4.2 Chronic Obstructive Pulmonary Disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a chronic airway inflammation leading to progressive, non-reversible obstruction. COPD patients suffer from emphysema and chronic bronchitis, too. The latter involves mucus hypersecretion, hyperplasia of goblet cells and submucosal glands, and in the last stage, fibrosis (Puljic and Pahl 2004). The leading causes of COPD are noxious gases and cigarette smoke; lethality is high (Bodas et al. 2016). Although having some aspects in common with asthma, like inflammation and progressive changes, COPD is quite different. It involves different cells, like epithelial cells, macrophages, neutrophils, and CD8 lymphocytes (Puljic and Pahl 2004; Saetta et al. 1999; Tetley 2002). Finally, the epithelial cells, which are a physicochemical barrier to the external environment, are also able to react with several inflammatory mediators (e.g., chemokines) in response to this injury (Puljic and Pahl 2004). Chemokines are essential regulators of leukocyte homeostasis and inflammation and have a wide range of effects, namely, cellular recruitment, activation, and differentiation (Sabroe et al. 2002).

Animal models for COPD include the mouse, rat, and guinea pig. Even as such, they are not ideal since they do not reflect all aspects of the clinical pathology, especially the complex interaction between genetic and environmental stimuli (Bucchieri et al. 2017). Significant species differences, like respiratory anatomy, breathing patterns, and lung protein expression profiles impair the translatability of results from animals to humans (Adamson et al. 2011). Initial studies using monocultures of human alveolar epithelial cells could show that cigarette smoke conditioned medium induces secretion of several important chemokines like IL-8, MCP-1, RANTES, and IL-1 (Puljic and Pahl 2004). Although it was possible to observe such endpoints with relatively simple A549 cultures, the role of immune cells in this response was not yet addressed at that time.

Many efforts have been made towards more physiologically relevant in vitro systems to analyze the effects of aerosols from e-cigarettes to the lung. Neilson et al.

used the commercially available EpiAirway™ model (MatTek Corporation, Boston, MA), which was implemented into an aerosol deposition robot (VITROCELL®) for the controlled exposure to e-cigarette aerosol (Neilson et al. 2015). The authors could show that it is possible to assess parameters like epithelial barrier integrity via TEER measurements and toxicological responses to e-cigarettes, in combination with an appropriate exposure system. Ex vivo explants and lung slices have also been used. Together with primary cell-based models, explants and lung slices have the advantage of carrying relevant genetic and epigenetic information that may contribute to COPD (Bucchieri et al. 2017).

To overcome the limitations of a supporting membrane, epithelial cells and fibroblasts may also be grown as a mixed 3D co-culture in Matrigel® to mimic the epithelial-mesenchymal tropic unit of the bronchial mucosa. Upon exposure to cigarette smoke extract, this bronchial mucosa model showed structural changes similar to those observed in vivo, with the added benefit of allowing long-term experiments (Bucchieri et al. 2017). Another important aspect is the dynamic interstitial flow, which is missing in all in vitro models so far. This would allow assessing, for instance, the impact of aerosol inhalation (e.g., drugs or environment particulate matter) as well as the effect of cytokines and systemic medications in the mesenchymal compartment. Such a model could be used to study not only COPD but also asthma.

As COPD is a chronic disease, reproducible and robust in vitro models that allow for long-term cultures and therefore suitable to assess such chronicity are of need. The interplay between different cell types in COPD reinforces the demand for more complex culture systems taking into account the interaction between different cells. In vitro co-cultures of different cell types to study COPD have been established (Mertens et al. 2017). Clinical and experimental studies have shown that during chronic inflammation in respiratory diseases like asthma and COPD, the remodeling of ECM components plays an important role in the impairment of lung function (Ito et al. 2019). Changes that involve remodeling of different types of collagen fibers or excess of fibronectin deposition are challenging to emulate with the current in vitro models (Fig. 3).

Future models of COPD should reflect not only the inflammatory aspect of the disease but also the infection scenario. Respiratory infections can lead to a higher rate of acute exacerbations in COPD and asthma patients. As epithelial dysfunction is a hallmark of most respiratory diseases, it is a key parameter to identify new targets and thereby to develop therapeutic strategies in asthma and COPD pathogenesis. It is still a challenge to model those epithelial changes in vitro (Mertens et al. 2017). Advanced in vitro models like lung-on-a-chip and precision-cut lung slices allow for mimicking the in vivo situation even closer. However, in the context of asthma and COPD, the complex environmental challenges observed in vivo should be combined with more physiologically relevant in vitro models to allow for a better understanding of these diseases and thereby the development of more effective therapies.

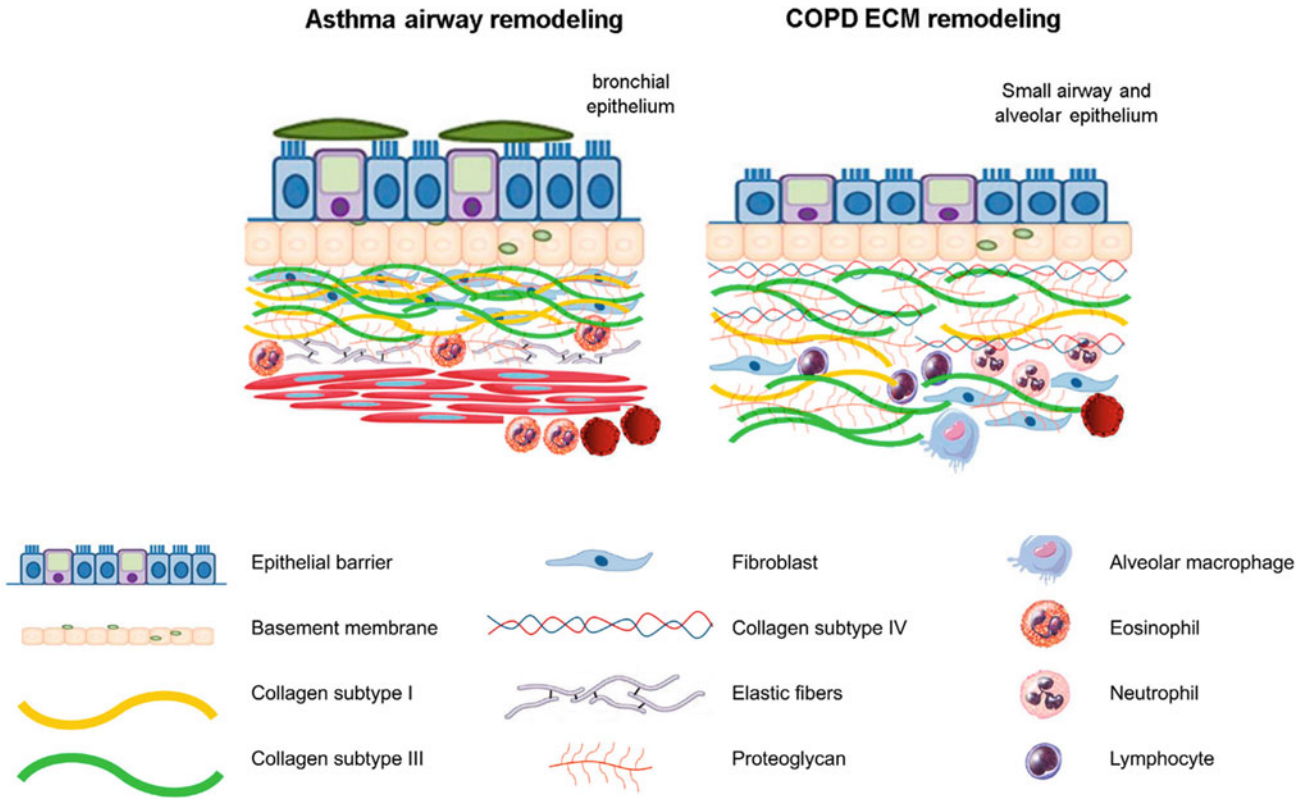

Fig. 3 Scheme of structural and cellular remodeling during asthma and COPD. Inflammation leads to rearrangements of different fibers: in asthma, structural changes are mainly seen in the bronchial epithelium, whereas in COPD, such changes are predominantly observed in small airways and distal areas of parenchyma. ECM remodeling contributes to the loss of lung function in these diseases. (Modified from Ito et al. 2019, with permission)

4.3 Pulmonary Fibrosis

Pulmonary fibrosis is an inflammatory lung disease accompanied by poor quality of life and high mortality rates. Triggered by several noxae and lesions, the bronchial or alveolar epithelium can be inflamed. By still not wholly known mechanisms, lung inflammation becomes unbalanced and chronic. The initial attraction of inflammation factors is followed by the migration of inflammatory cells like macrophages and T cells. These further secrete cytokines like TGF-ß and IL-1ß that trigger fibroblasts to emerge from the bone marrow or transdifferentiate from epithelial cells. Myofibroblasts are releasing ECM proteins like fibronectin and hyaluronic acid for wound closing. In the case of fibrosis, this step is dysregulated and leads to excessive scarring of lung tissue (Wynn 2011). Pulmonary fibrosis can be caused by radiation, medication (e.g., the anticancer drug bleomycin), or environmental noxae but also by genetic disposition like in CF (further illustrated later). Many cases of pulmonary fibrosis do not have a detectable cause and are called "idiopathic pulmonary fibrosis" (IPF) with a life expectancy of about 3 years (Ahluwalia et al. 2014).

To date, only nintedanib (angiokinase inhibitor, which targets the receptors crucially involved in angiogenesis and tumor growth) and pirfenidone (immunomodulatory drug blocking TGF-ß and TNF-α production) are approved as drugs to treat IPF (Maher and Strek 2019). While animal models of bleomycin-induced fibrosis have been instrumental to develop these new drugs based on specific pathophysiological endpoints, the comparison of animal to human lungs still remains limited (Borie et al. 2008; Nagano et al. 2006; Sato et al. 2016). This holds not only true for the varying dimensions but also because of the different cell types involved (Miller and Spence 2017; Sundarakrishnan et al. 2018). The impact of nintedanib and pirfenidone on pulmonary fibrosis was compared between 3D ex vivo precision-cut lung slices and primary murine AT-II cells from mice (bleomycin model) as well as lung slices from human origin (fibrosis induction by a TGF-ß containing cocktail). Collagen1a1 and Fibronectin gene expression declined following treatment with both drugs. Nintedanib additionally upregulated AT-II cell marker expression and restored epithelial gene expression. Such ex vivo models are close to reality but are cost- and time-intensive. Also, ethical questions have to be carefully considered (Lehmann et al. 2018).

Sato and colleagues hypothesized that metformin acting via AMP-activated protein kinase to reduce reactive oxygen species could also be used to lower the TGF-ß-induced myofibroblast differentiation. Lung fibroblasts cultivated under submerged conditions indicated reduced NOX4 expression. In mice, bleomycin-induced fibrosis could be normalized with metformin. These findings demonstrate a distinct in vitro/in vivo correlation (Sato et al. 2016), but so far only within the same species. Moreover, tacrolimus inhibited TGF-ß-induced collagen synthesis in vitro on a human fibroblast cell line derived from fetal lung (TIG-3-20) as well as in mice. Both in vivo and in vitro, the TGF-ß type-1 (TßR-I) receptor was less expressed after treatment (Nagano et al. 2006). Results in clinical studies are to be awaited.

Marinković and colleagues showed an attractive model focusing on the stiffness of lung fibroblasts that originated from healthy humans or IPF diseased patients.

Cells are seeded on polyacrylamide gel substrates with different stiffness grades to analyze differences in contractile function. IPF fibroblasts show resistance to PGE_2, while less stiffness corresponds to a less contractile function of fibroblasts (Marinković et al. 2013). This model could be beneficial also to test new drugs that counteract fibrosis.

4.4 Bacterial Infections in the Context of Cystic Fibrosis and Pneumonia

CF is a genetically inherited disease that affects the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene codes for the CFTR protein, a chloride channel on the apical side of epithelial cells that regulates epithelial ion and water transport. Misfolded CFTR channels cause less water being osmotically attracted to the apical side due to lower chloride concentrations, leading to thicker body fluids in these parts. This causes gastrointestinal problems, for example, pancreas tubes, are blocked, leading to reduced release of pancreatic enzymes to the digestive tract (Saint-Criq and Gray 2017). Most predominantly, thick body fluids affect the bronchial region of the lung. In a healthy state, mucus is easily transported by the mucociliary escalator. With the help of this mechanism, which includes the synchronized beating of the cilia of bronchial epithelial cells in a layer of thin fluid moving the mucus layer towards the throat, humans get rid of excess fluids by coughing or swallowing. In CF, this process is disturbed; cilia cannot move anymore due to the excess of thick mucus physically inhibiting the beating mechanism (Bhagirath et al. 2016). Mucus plugs are the eventual result of it. These inhibit not only normal breathing processes but are also an ideal niche for all sorts of inhaled pathogens landing on it. The mucus is the scaffold for bacteria like *S. aureus* and *P. aeruginosa* that can build biofilms. In CF, those biofilms persist when not consequently being treated with antibiotics. The details for antibiotic resistance in biofilms go beyond the scope of this chapter and can be found in other reviews (Alhede et al. 2014; Høiby et al. 2010).

Antibiotics are usually administered orally, with higher severity grades, intravenous delivery could be beneficial. In CF, most physicians prescribe drug regimens intending to completely eradicate the bug after the first encounter, e.g., *P. aeruginosa*. With bacterial eradication in 70–80% of the cases within 1 month, tobramycin inhalation is an effective treatment option (Elborn 2016).

In contrast to oral or parenteral administration, inhaled tobramycin has the advantage to be more targeted, so more antibiotic reaches the site of infection in the lung. Because systemic exposure is reduced, less adverse drug reactions are induced (Maselli et al. 2017). Currently, tobramycin, aztreonam, colistimethate sodium, levofloxacin, and amikacin are approved either by EMA or FDA or both for anti-infective inhalation therapy in different pharmaceutical forms. Other antibiotics active against *P. aeruginosa* as ciprofloxacin are currently in development (Ho et al. 2019; McKinzie et al. 2019; Velino et al. 2019).

In contrast to the underlying CF, pneumonia has no genetic cause. There are two main types of pneumonia: hospital-acquired pneumonia and community-acquired pneumonia (Torres et al. 2017). Patients suffering from community-acquired pneumonia are infected most commonly by bacteria like *Streptococcus pneumoniae* or *Haemophilus influenzae* or by atypical bacteria like *Mycoplasma pneumonia* (Prina et al. 2015). In severe cases, also *P. aeruginosa* is involved (Knapp et al. 2005). Also, viruses can be causative. There are classic symptoms like cough, fever, and dyspnea. If necessary, oral antibiotics against the most common bacteria are prescribed, which are amoxicillin, tetracycline, or macrolides (Prina et al. 2015).

Tests for new anti-infectives require preclinical evaluation steps regarding the safety and efficacy of the drug. In vitro models could support these studies and may provide additional information, for instance, on the mode of action of the drugs (Castellani et al. 2018). For any model, it is crucial to know the purpose of the investigation. The following part refers to models testing the efficacy of anti-infectives mainly in the context of CF, but also pneumonia. Models addressing pneumonia more specifically are still based on animals.

4.4.1 Modeling the Treatment of Bacterial Infections on Cells In Vitro

There is a particular need for in vitro infection models to monitor the success of a given treatment in an in vivo-like setting. The simple killing of bacteria grown on abiotic surfaces (e.g., petri dishes or well plates) is the first step to identify novel anti-infective drug candidates. Nevertheless, the following findings indicate that the outcome of such tests is more comparable to the processes in vivo if host cells or even secretions like mucus are present. Also, it makes a difference if a model is aiming to better understand the cause and pathophysiological mechanisms of a disease or to evaluate the efficacy and safety of drugs and delivery systems for the treatment of such disease. This chapter deals with the latter case.

Before proceeding to drug testing in models that are more complex, like animals or even in humans, it appears advisable to dissect the host response from antimicrobial efficacy. In this case, also host-bound factors other than cells may be essential and act as barriers that delimit drug action and bioavailability. Müller et al. described a model of human mucus inoculated with *P. aeruginosa* to test susceptibility to tobramycin and colistin. Tobramycin in solution was significantly less effective in mucus compared to buffer. In contrast, there was no difference when using colistin. Tobramycin could not diffuse well through the biofilm formed in mucus, which could explain the different susceptibility in the presence of mucus (Müller et al. 2018). This and other findings in mucus research underscore the importance of this noncellular barrier (Co et al. 2018; Wheeler et al. 2019).

Furthermore, as pulmonary delivery means inhalation of aerosolized medicines, ALI conditions are of interest and often not addressed in pertinent in vitro models. To close this gap, the lab strain *P. aeruginosa* PAO1 was cultivated on Snapwell® inserts for 3 days to form a mature biofilm. At ALI, these biofilms were then treated with nebulized ciprofloxacin, mannitol, and their combination on a special insert

mounted in a modified Andersen Cascade Impactor. The combined treatment with ciprofloxacin and mannitol had better efficiency than the drug alone (Loo et al. 2018). This approach shows the possibility to display mature biofilms under ALI conditions and allows to determine the efficiency of nebulized drug formulations. Still, drug deposition methods have also to be improved for the deposition of dry powder formulations and the inclusion of epithelial cells.

4.4.2 Experimental Conditions to Build an In Vitro Model of CF

First question of a CF model should be "Which factors are most important to include in the model to get the answer to the question I have?" Bowler et al. answered this question by cultivating A549 cells with the *P. aeruginosa* PAO1 bacteria growing in the biofilm mode to show differences to planktonic infection. They used the so-called Calgary biofilm device (Ceri et al. 1999), developed to test antibiotics against biofilms. A549 cells grown on the bottom of multiwell plates in submerse conditions were incubated either with planktonic bacteria or with 24-h-old biofilm covered pegs. The data suggests that *P. aeruginosa*, in the planktonic state, shows a higher internalization into A549 cells, than *P. aeruginosa* grown as a mature biofilm on pegs. Cells that were exposed to planktonic *P. aeruginosa*, however, showed less IL-8 release, than cells exposed to *P. aeruginosa* biofilm grown on pegs. Furthermore, planktonic bacteria caused more cell death compared to biofilms (Bowler et al. 2014). Even though the assay is quite simple, it contains already several essential components: Epithelial lung cells, a biofilm of a well-known bug of CF patients causing death, and, most importantly, the model cover both cell response and bacterial response. The disadvantages are that A549 cells are not bronchial but alveolar epithelial cells and do not feature any of the CFTR mutations. Also, in submerse cultivation in a standard multiwell plate, these cells do not polarize, and typical lung functions are not displayed. Nevertheless, this model could be used as a platform to test drugs as the Calgary biofilm device has already shown.

4.4.3 Testing Drug Efficacy on Infected Cell Lines

Testing antimicrobial activity on bacteria grown on abiotic surfaces (i.e., plastic) might be useful for fast and efficient drug screening, but relevant host factors cannot be analyzed. Therefore, many research groups aim to combine drug testing on microorganisms together with mammalian cells. Modeling host-pathogen-drug interactions becomes possible by testing on infected host cells, which asks for applicable and reasonable culture conditions. The following part gives some examples as well as advice on modeling the infected lung and treatments thereof.

Biofilms form in vivo by assembling microorganisms on specific surfaces, including mammalian cells. The O'Toole laboratory in Dartmouth started to mimic this process using the CF human bronchial epithelial cell line CFBE41o- that contains the ΔF508 deletion to be found in CF. Using submerse cultures in either static mode or in a flow cell chamber, they generated a model to be stable at least for up to 8 h after *P. aeruginosa* infection by supplementation of cell culture medium with arginine (Anderson et al. 2008). Due to the increased iron amount in ΔF508-deleted cells, biofilm formation is enhanced 1,500-fold. Antibiotic resistance

increased by 25-fold compared to biofilms on plastic, and the bactericidal effects of clinically used anti-infectives (tobramycin, ciprofloxacin, and imipenem) in clinically relevant concentrations declined (Moreau-Marquis et al. 2008, 2010).

The model developed by the O'Toole lab was used repeatedly after that to test various drugs. First, Anderson et al. proved that *Pseudomonas* quinolone signal (PQS) biosynthesis and type III secretion system are downregulated following tobramycin treatment. Pre-treated bacteria had lower virulence compared to not pre-treated bacteria in terms of lactate dehydrogenase release of mammalian cells. Yu et al. could show that aztreonam was less active than tobramycin, and no synergy was observed. Nevertheless, results can vary when using clinical isolates instead of the laboratory strain PAO1 (Yu et al. 2012). In the O'Toole model, the novel orphan drug ALX-109 (Alaxia, Lyon, France) composed of iron-binding lactoferrin and bactericidal hypothiocyanite ($OSCN^-$), a component of the innate immune system which is deficient in CF, was proven to be synergistic with tobramycin and aztreonam on PAO1 as well as on clinical isolates (Moreau-Marquis et al. 2015).

Nevertheless, the inclusion of epithelial cells is still the critical factor in such models as nicely shown by Price and colleagues. Co-addition of mannitol and tobramycin showed enhanced killing on an abiotic surface, yet this was not observed in the described biofilm model when exposing the PA14 strain and various clinical isolates (Price et al. 2015). To combat drug resistance, also antimicrobial peptides have attracted interest. Lashua et al. tested engineered cationic antimicrobial peptides on *P. aeruginosa* biofilm grown on CFBE41o-. The substance WLBU2 proved to be synergistic with tobramycin, ciprofloxacin, ceftazidime, and meropenem but not with colistin (Lashua et al. 2016).

In ALI grown cultures of primary human bronchial epithelial cells from CF patients and CFBE41o- cells, nitrite was shown to reduce biofilm amount and worked additionally with colistin (Zemke et al. 2014). The findings from ALI cultures should translate to clinical settings of a developing biofilm, yet the drug efficacy on a mature biofilm remains open.

Because of the relevance of fungal infections in CF, there is the need for testing also antifungal drugs as well in CF models. Seidler et al. published a protocol to transfer *Aspergillus fumigatus* on CFBE41o- and HBE cells and the ability to grow biofilms on it. The authors reported a higher MIC of a variety of antifungal drugs on *A. fumigatus* grown on plastic surfaces than on cells (Seidler et al. 2008).

Taking back the initial idea of building models for drug testing on infected cells, the alveolar A549 cell line is still a valuable tool. In the present example, A549 cells were co-cultured together with U937 human monocytes. On submerged co-cultures with *P. aeruginosa* PAO1, the efficacy of bacteriophages PEV2 and DMS3 was tested. While the release of cytokines IL-6 and TNF-α was increased with incubation of PEV2 only, DMS3 could preserve cell viability after up to 24 h, PEV2 only to a much lesser extent (Shiley et al. 2017). Even though this model does not feature CF cells, it is an example of an easy setup treatment model to test new treatment options like the bacteriophage therapy, which could be a future option to treat lung infections.

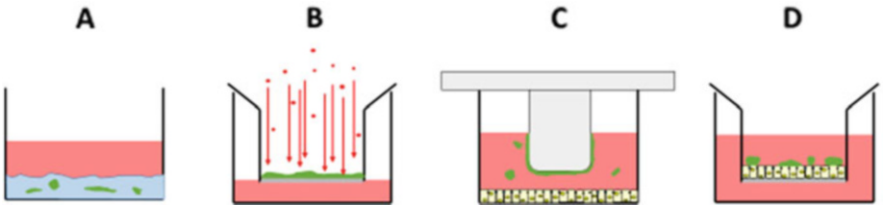

Fig. 4 Graphical representation of different methods to model infection in vitro. (**a**) Infected mucus in a standard well (Müller et al. 2018), (**b**) ALI deposition of anti-infectives on biofilm grown on blank filter inserts (Loo et al. 2018), (**c**) biofilm on PEG infecting indirectly epithelial cells in medium (Bowler et al. 2014), (**d**) early biofilm grown from planktonic infection of epithelial cells on filter inserts (e.g., Moreau-Marquis et al. 2008, and others)

In vitro models featuring mammalian cells give a good insight into host response. Nevertheless, there are also investigations on comparing these models with animal trials. Cory et al. were interested in dissecting the role of azithromycin in improving lung function, e.g., in CF. Azithromycin decreases inflammation by polarizing macrophages. Therefore, the authors build a co-culture model consisting of mouse fibroblast and macrophage cell lines in submerged conditions. They first stimulated their model with IFNγ and then added heat-inactivated *P. aeruginosa* or LPS to simulate bacterial infection. These findings were compared to mice infected and treated with azithromycin. Azithromycin increased fibronectin (mammalian glycoprotein of extracellular matrix) and ECM degrading matrix-metalloproteinase 9 (MMP-9) levels in vitro, whereas both levels were decreased mice (Cory et al. 2013). The human-based in vitro model provides additional insight into the host response and could be beneficial in testing drugs.

A brief overview of the different setups for testing drugs on infected in vitro models gives Fig. 4.

4.5 Tuberculosis

Tuberculosis (TB) is still a significant infectious disease worldwide, killing over 1.8 million people every year. Moreover, over 10.4 million new individuals are infected with *Mycobacterium tuberculosis* per year, and nearly 2 billion people worldwide have the latent form of TB (World Health Organisation 2019). This alarming data reinforce the urgent need for better therapies to cure this disease and to overcome the challenges of bacterial resistance. The latency stage is an essential part in several diseases, in which the individual asymptomatic state is closely correlated to the host immune response (Fonseca et al. 2017). The bacteria can persist in the organism for several years without causing clinical disease. In general, the latent mycobacterium is characterized by slow growth in vitro, a downshift of metabolic pathways, inability to be cultivated on solid media, and resistance to antimycobacterial agents (Alnimr 2015). While immune cells like dendritic cells, T and B cells, and eosinophils can be found in so-called granulomas and participate in the

inflammation, macrophages are still the main protagonist of this enigmatic immunological structure (Pagán and Ramakrishnan 2018). Tuberculous granuloma can undergo necrosis leading to patient morbidity and transmission of the bacteria (Grosset 2003).

Our understanding of TB is largely based on animal models; even so, the translation of findings in those models is restricted due to the species-specific responses, and it is challenging to establish latent TB in animal models (Fonseca et al. 2017). These factors reinforce the need for suitable human-based models for TB that allows us to understand the mechanism of control and disease progression and thereby be able to develop target medicine or vaccines. The animal models used in TB research range from zebra fish to nonhuman primates; between those, the mouse is the leading model (Gupta and Katoch 2005). However, the mouse is not a natural host for *M. tuberculosis* and thereby fails to adequately reflect human immunity to TB, in particular as it does not form typical granuloma, a hallmark of *M. tuberculosis* in the lung (Fonseca et al. 2017). Their role, however, may vary and be both protective and pathogenic (Pagán and Ramakrishnan 2018).

Several efforts have been made to develop cell culture systems of tuberculous granulomas, which are characterized by a caseous center and can become necrotic or fibrotic; such a caseous center is surrounded by myeloid and lymphatic cells (Pagán and Ramakrishnan 2018). Kapoor and colleagues developed a 3D model of *M. tuberculosis* dormancy in a collagen matrix (Kapoor et al. 2013). Human peripheral blood mononuclear cells are embedded into an ECM to allow the formation of 3D structures and granulomatous aggregates in response to virulent *M. tuberculosis*. This model nicely demonstrates the development of *M. tuberculosis* latency and reactivation once immunosuppressed with antitumor necrosis factor-alpha (anti-TNF-α), which makes it a useful tool to understand the host-pathogen interaction during latency. However, the limitation of this model is the low-throughput capability and the challenge to add further cells to allow for a dynamic model. Parasa and colleagues developed a second model by using an existing human in vitro lung tissue model and infected it with *M. tuberculosis* (Parasa et al. 2014). This model is based on a permeable filter membrane containing a collagen matrix with a human fibroblast cell line, primary macrophages, and a human epithelial cell line. The cells are cultured at ALI, and the infection is done with an infected macrophage, which carries *M. tuberculosis* working as "Trojan horses." The limitation of such a model, however, is the absence of immune cells like lymphocytes or neutrophils and the relatively low-throughput capacity. The same restriction is valid for organoid models, which so far have not been used to study lung infection (Fonseca et al. 2017).

The three current challenges in TB are the development of better vaccines, diagnostics tools, and therapies. The models described above still leave considerable room for improvement to address the complexity of chronic host-pathogen interactions. *M. tuberculosis* is predominantly a human pathogen that has a prolonged coevolution period with humans of approximately 70,000 years (Lienhardt et al. 2016). Therefore, an advanced human in vitro model that mimics the main features of human tuberculosis over a more extended time period would be

of high relevance, not only for understanding the fundamental biological processes of the disease but also for testing new diagnostic, therapeutic, and vaccine interventions (Elkington et al. 2019). Strategies like vaccination ask for models that mimic both innate and adaptive immune responses. Effective models as drug development tools are not aimed to be as complex as possible, but instead are aimed to reduce the (patho)physiological complexity to the key events related to TB in vivo.

5 Outlook

Cell and tissue models of the human air-blood barrier for testing orally inhaled drugs against inflammation and infection require air-liquid conditions and must allow for the controlled deposition of aerosol medicines. The use of permeable supports (e.g., Transwell®) allows growing monolayers of polarized epithelial cells with functional tight junctions. Transepithelial electrical resistance (TEER) is a readily measurable, but important biophysical readout for epithelial barrier function, because it rapidly drops upon pathophysiological changes, like inflammation or in the course of bacterial infections. Vice versa, its restoration may be used as an indicator to evaluate some positive drug effects. Including macrophages or other immune cells in more complex 3D co-cultures allows to address additional factors, like e.g., epithelial transmigration, phagocytosis, or cytokine release.

Further expanding these models to additional cell types (e.g., endothelial or smooth muscle cells) may make them particularly useful for testing and evaluating the mechanism of action of novel drugs against COPD or pulmonary fibrosis. Expanding these models to study bacterial or viral infections requires to additionally introduce such pathogens into mammalian host cell cultures. As bacteria grow much differently in absence than in the presence of noncellular host factors, like e.g., mucus or surfactant, it is evident that such elements are highly relevant to be included as well. Although technically demanding, it appears as possible to identify experimental conditions under which, e.g., biofilm-forming bacteria can be grown on top of mucosal cell (co-)cultures for several days. Measuring how the pathogens can be kept under control or eventually eradicated by repeated administration of novel anti-infective modalities (e.g., antibiotics, patho-blockers, or nanocarriers of the same) will provide valuable readouts regarding the efficacy of such new investigational drugs. Monitoring host parameters like epithelial barrier function, cytotoxicity, or cytokine release may complement these data and also provide relevant information regarding the safety of such candidates. Animal models of chronic pulmonary infections are challenging to set up, therefore scarce and moreover, often not truly reflecting the pathophysiological situation in patients at all. Advanced human-based in vitro models would therefore not only be an alternative to animal testing but would also enable scientists to generate relevant preclinical data where adequate animal models are not available or even not existing.

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Skin Disease Models In Vitro and Inflammatory Mechanisms: Predictability for Drug Development

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_428

Abstract

Investigative skin biology, analysis of human skin diseases, and numerous clinical and pharmaceutical applications rely on skin models characterized by reproducibility and predictability. Traditionally, such models include animal models, mainly rodents, and cellular models. While animal models are highly useful in many studies, they are being replaced by human cellular models in more and more approaches amid recent technological development due to ethical considerations. The culture of keratinocytes and fibroblasts has been used in cell biology for many years. However, only the development of co-culture and three-dimensional epidermis and full-skin models have fundamentally contributed to our understanding of cell–cell interaction and cell signalling in the skin, keratinocyte adhesion and differentiation, and mechanisms of skin barrier function. The modelling of skin diseases has highlighted properties of the skin important for its integrity and cutaneous development. Examples of monogenic as well as complex diseases including atopic dermatitis and psoriasis have demonstrated the role of skin models to identify pathomechanisms and drug targets. Recent investigations have indicated that 3D skin models are well suitable for drug testing and preclinical studies of topical therapies. The analysis of skin diseases has recognized the importance of inflammatory mechanisms and immune responses and thus other cell types such as dendritic cells and T cells in the skin. Current developments include the production of more complete skin models comprising a range of different cell types. Organ models and even multi-organ systems are being developed for the analysis of higher levels of cellular interaction and drug responses and are among the most recent innovations in skin modelling. They promise improved robustness and flexibility and aim at a body-on-a-chip solution for comprehensive pharmaceutical in vitro studies.

Keywords

Atopic dermatitis · Congenital ichthyosis · Cytokines · Drug delivery · Fibroblasts · Gene knockdown · Induced pluripotent stem cells · Keratinocytes · Langerhans cells · Macrophages · Protein replacement · Psoriasis · Reconstructed human epidermis · Reconstructed human skin · Skin barrier · Skin equivalent · T cells

Abbreviation

AD	Atopic dermatitis
ARCI	Autosomal recessive congenital ichthyosis
ATRA	All-trans retinoic acid
dPG	Dendritic polyglycerol
ECVAM	European Centre for the Validation of Alternative Methods
EDC	Epidermal differentiation cluster
ETR	Etanercept

H/E	Hematoxylin/eosin
HUVEC	Human umbilical vein endothelial cell
iPS cell	Induced pluripotent stem cell
OECD	Organisation for Economic Cooperation and Development
pNIPAM	Poly(*N*-isopropylacrylamide)
RAMBA	Retinoic acid metabolism blocking agents
RhE	Reconstructed human epidermis
RhS	Reconstructed human skin
TLR	Toll-like receptor
tPG	Thermoresponsive polyglycerol

Gene Symbols

ABCA12	ATP binding cassette subfamily A member 12
ALOX12B	Arachidonate 12-lipoxygenase, 12R type
ALOXE3	Arachidonate lipoxygenase 3
CERS3	Ceramide synthase 3
FLG	Filaggrin
KRT14	Keratin 14
LCE3B	Late cornified envelope 3B
LCE3C	Late cornified envelope 3C
LOR	Loricrin
NIPAL4	NIPA like domain containing 4
PSORS4	Psoriasis susceptibility 4
TGM1	Transglutaminase 1
TMEM45A	Transmembrane protein 45A

1 Introduction

The study of organ development, as well as the investigation of diseases and identification of drug targets, requires the availability of comprehensive model systems that are able to recapitulate reproducibly the developmental and physiological milestones. This has long been done with cells cultured in vitro and with animal experiments. Regarding the skin, which is the largest and among the most exposed human organs, animal models were used, for instance, to analyze irritation from chemical exposure and characteristics of complex diseases affecting the skin since only certain aspects of underlying mechanisms could be analyzed in a monolayer cell culture system. While animal models are still valuable and needed for specific studies, their use has been strictly reduced and partly banned in recent years for technological, ethical, and financial reasons. Skin structure and physiology are rather different between humans and rodents, which limits the suitability of rodent models for the characterization of pathological mechanisms in cutaneous biology. Moreover, the ban of using animal tests for the assessment of cosmetic products and their ingredients, which was established in the UK in 1998 and across the EU in 2013, has

strongly increased the need for alternative methods. Such limitations resulted in the development of co-culture and more and more complex three-dimensional systems that were able to replicate, at least to some extent, the physiological context of the human skin.

2 History

The modelling of skin diseases in vitro is an old perspective for researchers in dermatology. Still, it naturally and progressively took benefit from the long process that led to the more and more sophisticated development of skin models, most usually based on cultured cells and tissue reconstruction. Whilst one of the first steps has been dermal reconstruction based on the immediate and natural organization of collagen components in an extracellular matrix produced by interacting fibroblasts (Bell et al. 1979), next steps soon involved epidermal keratinocytes, which were seeded onto dermal components in order to reconstitute a kind of living skin (Bell et al. 1983). Such models opened almost immediately new ways to study cutaneous diseases by taking advantage of the controlled culture conditions (Saiag et al. 1985).

In the field of toxicology, the emerging availability of innovative tools to reconstruct skin in vitro rapidly became a strong incentive for the development of alternative procedures devoted to replace and reduce the use of laboratory animals. More simple to produce, and thus cheaper than full-thickness living skin associating dermal and epidermal tissues, human epidermis reconstructed solely by cultured keratinocytes grown over a porous membrane or a filter (Rosdy and Clauss 1990) became rapidly recognized as suitable for essential cutaneous toxicology. Indeed, reconstructed human epidermis (RhE) produced in vitro in large numbers provides materials that satisfy the ethical expectations expressed in many countries and represent validated alternatives with strong potentials for prediction of harmful characteristics of chemicals (Alépée et al. 2010). Evaluating corrosive, irritant, and even sensitizing properties of compounds by means of RhE became a reality (Coquette et al. 1999, 2003) that triggered a collaborative open-source dissemination of knowledge in tissue reconstruction and initiated fine analysis of this material (Gazel et al. 2003; Poumay and Coquette 2007; Poumay et al. 2004). RhE models are nowadays available in a ready-to-use format from commercial sources like SkinEthic™ (Episkin) or EpiDerm™ (MatTek), to name a few. They can be also produced rather easily with the help of a do-it-yourself kit offered by CELLnTECH. RhE production is possible using cultured keratinocytes and following published procedures provided as part of the initiative to share protocols and improvements intended to allow in-house autonomy and flexibility. These approaches are appreciated by researchers in skin toxicology, skin pharmacology, and dermatology (De Vuyst et al. 2014; Frankart et al. 2012; Mewes et al. 2016).

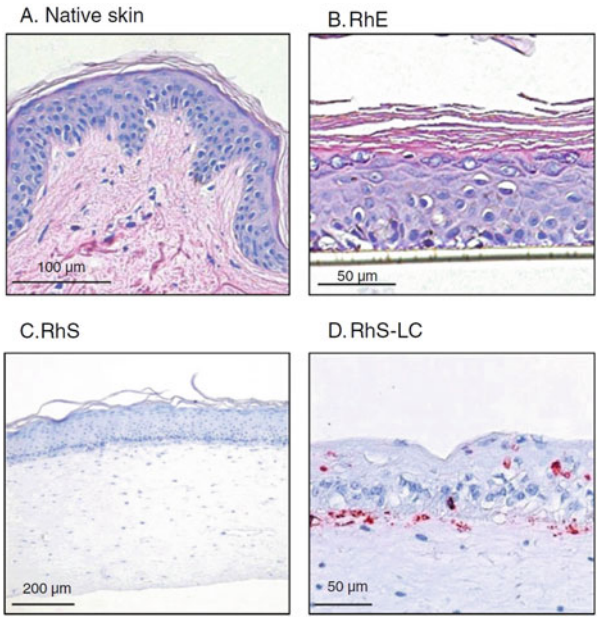

Fig. 1 Examples of skin models characterized with hematoxylin/eosin staining. (**a**) Normal human skin obtained by skin biopsy. (**b**) Reconstructed human epidermis (RhE). (**c**) Full-thickness skin model (reconstructed human skin, RhS) with epidermis equivalent and fibroblast-populated dermis. (**d**) Full-skin model with MUTZ-derived Langerhans cells integrated into the epidermis on a fibroblast-populated collagen matrix. Langerhans cells were stained with HLA-DR immunohistochemistry. Obtained from Rodrigues Neves and Gibbs (2018) under Creative Commons license

RhE is valuable as it displays the stratification of the epidermis with a functional epidermal barrier; it lacks a dermal equivalent and the important interaction between keratinocytes and fibroblasts (Fig. 1). Organotypic co-culture systems have been aimed at combining a dermal extracellular environment, potentially containing fibroblasts, and an epidermal equivalent of differentiating keratinocytes, either HaCaT cells or primary human keratinocytes (Stark et al. 1999, 2004). Characterization with hematoxylin/eosin (H/E) and antibody staining identified the replication of layered epidermis in these models. Approaches with collagen matrices and de-epidermized dermis demonstrated the importance of stabilized dermal structures and interaction between fibroblasts and keratinocytes for the development of regular epidermal stratification, barrier function, and improved lipid synthesis (El Ghalbzouri et al. 2002a, b; Stark et al. 2006). Three-dimensional full-thickness skin models with a dermis equivalent populated with fibroblasts and an epidermal equivalent made of keratinocytes (Fig. 1) are now available for a range of applications including studies of epidermal homeostasis, skin absorption and penetration, corrosion, irritation, wound healing, and drug testing. Studies have shown that epidermal barrier function is diminished in these models compared to human or pig skin but self-consistent and well suitable for the investigation of pathogenic changes of barrier activity (Ackermann et al. 2010; Batheja et al. 2009; Schäfer-Korting et al. 2006; Thakoersing et al. 2012). Several full-skin models have now been marketed and are commercially available, such as EpiDermFT™ (MatTek), T-Skin™ (Episkin), and Phenion® FT (Henkel). Commercial systems have been validated for several applications (Pfuhler et al. 2020), including skin absorption (OECD TG 428), corrosion (TG 431), and irritation (TG 439) following guidelines

of the Organisation for Economic Cooperation and Development (OECD). Model systems have been optimized for reproducibility and predictability; however, they still show some differences between themselves regarding morphology and differentiation and they might have to be tested and compared for their use for more specific applications.

3 Monogenic Diseases

3.1 Monolayer Versus Three-Dimensional Cultures

An early interest and a major application of in vitro skin models is the investigation of skin diseases. Cell cultures have been used to study cellular features and responses to drugs. Monogenic diseases that affect primarily a single cell type seem to be straightforward for the analysis in vitro. Intriguingly, culturing a cell type underlying the clinical phenotype, namely keratinocytes or fibroblasts, would provide a fast and elegant technique to obtain a culture model of the genetic disease; initial experiments, however, have demonstrated a number of limitations for this idea. First and foremost, only 3D models can reproduce the cellular microenvironment of stratifying keratinocytes or even an epidermis combined with a fibroblast-populated dermis. Such models would be able to represent the epidermal differentiation and thus most characteristic features of cutaneous pathologies (Ojeh et al. 2001; Stark et al. 2004, 2006). Second, the availability of primary keratinocytes is often very limited and they can be passaged only few times. Third, the behavior of fibroblasts in a dermal matrix can strongly differ depending on age, properties of the cultured environment, and the disease phenotype (Eisen et al. 1987; Oakley and Priestley 1985). Finally, the mechanistic complexity of human skin with various levels of cellular interaction is still not fully represented even in advanced, recent models.

When keratinocytes from a child born with a congenital Darier lesion on the scalp, for instance, became available for culture after a surgical resection of the affected area, cultures were initially performed as monolayers in order to expand the cell population; they did not show any peculiarities (Lambert de Rouvroit et al. 2013). Similarly, monolayers were not suitable to provide interesting information regarding proteins involved in 3D structural aspects of the epidermis (Arnette et al. 2016). Conversely, once 3D cultures were established, the connection between desmosomal components and their function in tissue organization could be analyzed and decrypted (Arnette et al. 2016). The importance of interactions between cell types has been shown in attempts to model forms of epidermolysis bullosa in vitro. In an attempt to model the rare form of recessive epidermolysis bullosa simplex caused by inactivating mutations in the gene *KRT14* (El Ghalbzouri et al. 2003), patient skin biopsies were cultured on a collagen matrix with patient fibroblasts. These experiments reproduced the bullous phenotype but not the epidermolytic hyperkeratosis of recessive epidermolysis bullosa simplex. The vacuolar phenotype was reverted when patient biopsies were cultured on a matrix with normal fibroblasts, showing a compensatory mechanism involving keratin 17 and thus

indicating the importance of fibroblasts for the integrity and pathological destabilization of the epidermal layers.

3.2 Patient-Derived 3D Models

Many human genodermatoses affect properties of the skin that can be modelled in vitro. Co-culture systems and 3D approaches have significantly contributed to the understanding of the importance of microenvironment and cellular interaction between fibroblasts and keratinocytes for pathologies. When keratinocytes can be collected from donors suffering from a monogenic disease, using biopsies or autopsies, or following surgical interventions, cultured cells from these patients can then be employed for tissue reconstruction and even for skin barrier formation and analysis of barrier function in vitro. In this case, cells can be expected to reproduce anomalies encountered in the patient skin while being involved in the cultured tissue. For instance, keratinocytes isolated from the skin of patients suffering from ichthyosis vulgaris can be used to recreate in vitro this pathological condition of the epidermis (Niehues et al. 2017). Similarly, studies dealing with two genetic conditions that affect calcium pumps and exhibit acantholytic patterns in diseased epidermis have been using 3D epidermal reconstruction. Both for Darier disease (Lambert de Rouvroit et al. 2013) and for Hailey-Hailey disease (Matsuda et al. 2015), reconstruction of the epidermis on polycarbonate filters using patient keratinocytes produced epidermis that exhibited a disruption of cell–cell adhesion, thereby mimicking morphological alterations such as acantholysis typically reported in corresponding skin biopsies. Moreover, *corps ronds* and *grains*, which characterize Darier disease rather than Hailey-Hailey disease, were absent in monolayers and appeared solely in reconstructed Darier epidermis (De Vuyst et al. 2014; Lambert de Rouvroit et al. 2013), pledging confidence in the reproducible representation of pathological features in such models.

Epidermal differentiation, cell adhesion, lipid synthesis, and barrier function are successful topics for the characterization of genetic diseases using skin models. Lipid metabolism of reconstructed skin has long been studied and shown to contribute critically to the barrier activity of models, even though some differences in lipid composition were described between native and reconstructed human skin (Ponec et al. 1997, 2000). Critical changes of lipid metabolism associated with disease have been revealed using skin equivalents; this is true for complex diseases such as filaggrin-deficient atopic dermatitis (AD) (Wallmeyer et al. 2017) as well as monogenic skin diseases. Congenital ichthyoses are characteristically associated with disturbed epidermal barrier function. The majority of mutant genes underlying forms of congenital ichthyosis have been assigned to a role in epidermal lipid synthesis. Harlequin ichthyosis, the most severe form of autosomal recessive congenital ichthyosis (ARCI), is caused by loss of a member of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters. Modelling of *ABCA12* mutations identified the characteristic premature epidermal differentiation and an abnormal lipid content with a reduction in nonpolar lipids (Thomas et al. 2009).

Most forms of ARCI are associated with alterations in the ceramide metabolism. Ceramides play a crucial role in the formation of the cornified lipid envelope and the activity of the skin barrier (Feingold and Jiang 2011; Janssens et al. 2011; Janušová et al. 2011; Rabionet et al. 2014). Ceramide synthase genes are expressed in a strictly cell-type specific manner; accordingly, ARCI can be caused by mutations in the epidermal ceramide synthase gene *CERS3* (Eckl et al. 2013; Radner et al. 2013). The importance of ultra-long acyl chain ceramides for regular cornified lipid envelope formation and barrier function has been revealed using 3D skin models (Eckl et al. 2013), which is in line with the projected barrier organization of stacked lipid bilayers shown with cryo-EM of vitreous skin sections and EM simulation (Iwai et al. 2012). Full-skin models were also able to replicate the importance of cell–cell adhesion for epidermal integrity and barrier activity. Using a range of methods, including the application of mechanical stress on modelled skin, disturbed adhesion was shown to be associated with the loss of adhesion proteins such as corneodesmosin, underlying peeling skin disease (Oji et al. 2010), or epidermal protease inhibitors cystatin A and serpin B8, a member of the serpin B family of serine protease inhibitors, which can cause exfoliative ichthyosis (Blaydon et al. 2011; Pigors et al. 2016).

3.3 Knockdown and Knockout Models

The availability of keratinocytes, which would normally be isolated in small numbers from skin biopsy specimens, and their inaptitude to proliferate over more than few passages, are major bottlenecks for the generation of patient-based models for genetic skin diseases. The inactivation of single genes using RNA interference (RNAi) is a useful strategy to overcome this limitation. RNAi can be applied using transfection of siRNA (small interfering RNA) or viral transduction of shRNA (short hairpin RNA). Using this approach, a gene can be specifically switched off to produce a knockdown model of keratinocytes. The cells can be used for short-term (siRNA) or long-term (shRNA) studies and a number of downstream applications. In particular, they are similarly suitable for the generation of full-skin models that can be analyzed for keratinocyte adhesion and differentiation (Thomas et al. 2009) and the formation of cornified envelopes, other structural components and aspects of immune responses (Hönzke et al. 2016). Epidermal barrier function, which is disturbed in monogenic epidermal diseases such as ARCI as well as in complex diseases such as AD, is of particular interest for such studies. Genes mutant in cases of ARCI, including *TGM1, ALOX12B, ALOXE3, NIPAL4,* and *CERS3*, have been knocked down in normal keratinocytes. The same approach has been used for *FLG,* the gene for filaggrin, which can be mutated in ichthyosis vulgaris and is a major susceptibility factor for AD. These keratinocytes were used for the generation of full-skin models, which demonstrated a critical impairment of barrier activity using the analysis of skin absorption and penetration (Eckl et al. 2011, 2013; Mildner et al. 2010). Such studies have opened an avenue for the identification of novel drug targets and offered important tools for the in vitro

assessment of innovative treatments for skin diseases. There are still limitations; RNAi is sometimes not sufficiently specific for a single target gene and the transfection or transduction are significant manipulations that might alter keratinocyte behavior.

A highly flexible approach to the manipulation of target genes in keratinocytes, both for corrections and the generation of knockout lines, is the use of CRISPR/Cas9 technology. This was demonstrated for Harlequin ichthyosis using CRISPR/Cas9 mediated inactivation of *ABCA12* (Enjalbert et al. 2020). Skin equivalents made with these cells recapitulated the Harlequin ichthyosis phenotype and were suitable for the analysis of differential gene expression, revealing dysregulated pathways and potential targets for more specific therapies.

4 Atopic Dermatitis

4.1 Barrier Function

Atopic dermatitis, most likely because of its complexity, is a common disease that has been and is still studied by means of skin disease models. Whereas the clinical initiation of AD is still undetermined, one can be sure that the process involves alterations in the epidermal barrier and some over-activation of the immune system, mainly in the form of a Th2 immune response (Guttman-Yassky et al. 2019). In AD, a weakened epidermal barrier might open a gate for the entry of foreign substances into the skin and thereby be the initial event responsible for the occurrence of the pathological process. However, a multitude of mechanisms have been considered as responsible for the barrier failing its crucial role. Absence of filaggrin is found in ichthyosis vulgaris and directly involved in the observed scaly phenotype. Expression levels of *FLG* are correlated with the risk for eczema and variants in *FLG* are thus a major predisposing factor for AD and can also strongly alter the proper formation of the cornified most superficial layer in the epidermis (McLean 2016). However, such mutations of filaggrin do not explain all cases of AD. Indeed, several other genes may be linked to the development of the disease, and activation of an immune response has been found to create signal-dependent responses that resulted in reduced expression levels for filaggrin (as well as for other proteins) and an accompanying altered barrier.

4.2 Monolayer Cultures

Investigating the epidermal processes involved in AD by solely studying epidermal keratinocytes can hardly reach satisfaction since the interaction with immune components is major in this disease. Several kinds of in vitro models are available and their main characteristics were part of a previous review (De Vuyst et al. 2017). However, certain culture conditions imposed on epidermal keratinocytes grown as monolayers on a plastic Petri dish can trigger alterations in the phenotype of these

cells; these alterations closely mimic the diseased status identified as the epidermal phenotype of keratinocytes inside AD lesions (Mathay et al. 2011). Exposure of keratinocyte monolayers to Th2 cytokines like IL-4 and IL-13 indicates as well that such culture conditions can alter the phenotype of keratinocytes in a way that hampers the expression of genes involved in differentiation, or involved in the establishment and maintenance of the epidermal barrier (Omori-Miyake et al. 2014). Of special interest, the expression of filaggrin in keratinocytes cultured as monolayers is also highly regulated by the presence of Th2 cytokines like IL-4 and IL-13 (Howell et al. 2009).

4.3 Knockdown and Knockout Models

4.3.1 Filaggrin

Once filaggrin had been identified as a frequently deficient protein in the cornified layer of AD patients (Brown and McLean 2012), numerous attempts were made to downregulate its expression in keratinocytes and to investigate its missing role in the pathological epidermis. For instance, filaggrin expression has been targeted with siRNA introduced into keratinocytes before creating an organotypic skin model (Küchler et al. 2011; Mildner et al. 2010). Alterations in the diffusion barrier were demonstrated in the model but also enhanced UV sensitivity was reported since an increased apoptotic response was observed in tissues with reduced filaggrin expression in comparison with tissues expressing filaggrin at normal levels. Lentiviral transduction of shRNA was used to knock down filaggrin expression in normal keratinocytes before reconstructing the human epidermis on a polycarbonate filter in order to investigate the roles of this protein in the epidermal barrier function (Dang et al. 2015; Pendaries et al. 2014). Indeed, the authors observed that hypogranulosis was the result of the missing profilaggrin in the granular layer. An increased permeability of the barrier accompanied the downregulation of filaggrin, as well as reduced levels of filaggrin-2 and loricrin, two filaggrin-related proteins, and caspase-14 and bleomycin hydrolase, two proteases involved in filaggrin degradation, together with reduction in caspase 14 activation and suppressed natural moisturizing factor components. Intriguingly enough, while studying cells from patients with ichthyosis vulgaris fully lacking filaggrin, Niehues et al. (2017) did not find alterations of the barrier, suggesting that possibly a compensatory mechanism could work in those cases. In addition, in another study, Van Drongelen et al. (2013) achieved the stable expression of shRNA targeting the coding sequence of FLG in N/TERT keratinocytes originally prepared by Dickson et al. (2000) and successfully used these cells to reconstruct skin equivalents with a functional barrier. Again, the reduced expression of filaggrin in the epidermal cells was insufficient to significantly alter epidermal differentiation markers in the knockdown tissue according to that study and was neither responsible for modified lipid organization nor barrier function (Van Drongelen et al. 2013).

4.3.2 TMEM45A

In a screening experiment intending to identify proteins induced during hypoxic conditions that might bring some chemoresistance to cancer cells, the protein TMEM45A, which contains a putative transmembrane domain, was identified in breast and liver cancer cells (Flamant et al. 2012; Schmit and Michiels 2018). *TMEM45A* expression is particularly elevated in growth-arrested keratinocytes, especially in the upper epidermis where its association with the trans-Golgi has been confirmed in concomitance with keratinization (Hayez et al. 2014). In an attempt to understand the epidermal roles of the protein TMEM45A in vivo and in vitro, knockout mice and reconstructed epidermis made with knockdown keratinocytes were produced. Whereas the significance of this protein has been largely confirmed in several cell types during the development of malignant tumors (Schmit and Michiels 2018) and although its expression is particularly inducible in keratinocytes, suppression of the protein TMEM45A did not give any clue to its epidermal function (Hayez et al. 2014, 2016). Indeed, neither the epidermal morphogenesis nor its keratinization was altered when the protein was absent. Similarly, the epidermal barrier was as efficient in knockout and knockdown tissues as in wild-type epidermis, both in vivo and in vitro.

4.4 The Role of Cytokines

The role of Th2 cytokines, especially IL-4 and IL-13, in the onset of atopic dermatitis is rather complex. Therapies targeting signalling through IL-4 and IL-13 receptors, e.g. using dupilumab to impede access of the interleukin ligands to their receptors, have proven efficient to clear AD lesions (Guttman-Yassky et al. 2019). Epidermal keratinocytes naturally express such receptors and are thereby reactive to the presence of their ligands in the epidermal environment. A clear effect of keratinocyte exposure to IL-4 and IL-13 is the suppressed expression of filaggrin but other components involved in the normal epidermal keratinization and epidermal barrier formation are simultaneously reduced by these cytokines (De Vuyst et al. 2017; Smits et al. 2017), reinforcing the weakening effect on the epidermal barrier, and of course opening the tissue for the penetration of foreign substances, allergens, and microorganisms as a direct consequence. Such a tissue entry of exogenous compounds further contributes to the activation of the immune response and thus reinforces the claimed vicious circle suspected in the etiology of AD that often hides the cause by which the process has initially started (De Vuyst et al. 2018).

In reconstructed tissues based on either normal human epidermal keratinocytes or on immortalized keratinocytes like N/TERT keratinocytes, incubation with IL-4 and IL-13 produces phenotypic alterations that mimic the incomplete differentiation observed in lesions of AD. Indeed, some variable alterations in gene expression levels are observed when monitoring several different barrier components. The reduction of filaggrin expression, for instance, results in significant disappearance of the keratohyalin granules usually found in the granular layer and hence can be considered responsible for the absence of this layer. Simultaneously, a reduced

function of the epidermal barrier is observed, together with some intercellular swelling that recalls the so-called spongiosis phenomenon, observed between keratinocytes in the skin of AD patients (De Vuyst et al. 2018; Hubaux et al. 2018; Smits et al. 2017).

5 Psoriasis

Psoriasis is a common inflammatory disease with a complex etiology. Several large studies into the genetics of psoriasis, mainly using genome-wide association studies followed by meta-analyses, have found several susceptibility loci associated with types of psoriasis. However, only few genes involved in mechanisms contributing to the development of the disease were identified so far (Capon 2017; Hwang et al. 2017; Ogawa and Okada 2020). Genetic studies have demonstrated that different mechanisms can contribute to different subtypes, where psoriasis vulgaris is clearly the most common form. Psoriatic skin shows morphological features, including acanthosis and parakeratosis, and is characterized by hyperproliferative keratinocytes, as detected, for instance, by strong Ki-67 staining and keratin 16 expression. The immunological profile is mediated by CD4+ T cells but several other types of immune cells, including macrophages and neutrophils, are found in psoriatic skin. Th1 and Th17 are predominantly involved in the immunological mechanisms of the disease, characterized by overrepresentation of TNF-α, IFN-γ, IL-17, and IL-23 among other cytokines. Members of these pathways are major drug targets in current therapeutic strategies for psoriasis.

5.1 Barrier Function

Like many genetic skin diseases, psoriasis can be associated with epidermal barrier dysfunction. In accordance with other monogenic and complex skin diseases, altered transepidermal water loss has been associated with changes in the ceramide composition in the stratum corneum of psoriatic plaques (Motta et al. 1994). Though the molecular background of the compromised barrier function is not so clear, it could be partly attributed to genetic mechanisms. The gene cluster of the so-called epidermal differentiation complex (EDC), which comprises several genes mainly expressed in terminally differentiating keratinocytes, includes the filaggrin gene *FLG* and lies within *PSORS4*, one of the early identified psoriasis loci (Bhalerao and Bowcock 1998; Capon et al. 2001). A deletion of two genes in the cluster, *LCE3B* and *LCE3C*, was associated with psoriasis vulgaris (de Cid et al. 2009; Riveira-Munoz et al. 2011). The late cornified envelope proteins encoded by these two genes are supposed to contribute directly to the disturbance of terminal keratinocyte differentiation and epidermal barrier function. Moreover, other genetic variants associated with psoriasis might affect cystatins, proteinase inhibitors important for epidermal integrity such as cystatin A and cystatin M/E (Cheng et al. 2009; Samuelsson et al. 2004; Vasilopoulos et al. 2008). Synthesis of further proteins of

terminally differentiating keratinocytes, namely filaggrin, loricrin and involucrin, is disturbed in psoriatic skin, with an upregulation of involucrin and a downregulation of filaggrin and loricrin. These proteins are important for the function of the epidermal barrier and null mutations in *FLG* are associated with ichthyosis vulgaris and atopic dermatitis. Secondary genetic effects are thus also involved in the barrier dysfunction seen in psoriasis, as TNF-α can modulate the expression of *FLG* and *LOR* (Kim et al. 2011).

5.2 Cell Signalling

With this complex make-up of alterations in psoriasis in mind, in vitro models for the analysis of psoriatic skin and the assessment of drugs are necessarily limited and various approaches have been chosen for psoriasis models. Both RhE and full-skin models have been used for psoriasis investigations and proven suitable for drug delivery studies. RhE are relatively cheap and easy to use, which makes them useful for certain defined applications. Considering the importance of cell–cell and cell–matrix interaction and skin barrier function, psoriasis full-skin models have clear advantages. Full-skin models can be successfully generated using keratinocytes and fibroblasts obtained from lesional psoriatic skin (Barker et al. 2004). These models showed typical changes in cytokine levels and hyperproliferation but not full morphological hallmarks of the disease. Similar characteristics, with additional macroscopic features, were seen in models made with various combinations of a normal and psoriatic fibroblast-derived matrix, and normal and psoriatic keratinocytes (Jean et al. 2009); only models that contained psoriatic keratinocytes showed upregulation of involucrin and downregulation of filaggrin and loricrin, which are supposed to contribute to the disturbed barrier function in psoriasis. The importance of cell–cell interaction and lesional fibroblasts for the phenotype, however, is also demonstrated in one of the few commercially available psoriasis skin models (MatTek), which uses normal human epidermal keratinocytes and psoriatic, lesional fibroblasts and represents typical morphological features and cytokine profiles of psoriasis skin. Other models, in particular those without psoriatic cells, did not exhibit any hyperproliferation of keratinocytes. In another approach, the pathological phenotype can be triggered with psoriasis-associated cytokines such as TNF-α, IL-1α, IL-6, and IL-22 in models made of normal keratinocytes cultured on a de-epidermized dermis (Tjabringa et al. 2008). These models demonstrated expression patterns of psoriasis including upregulation of SKALP/elafin, keratin 16 and CXCL8, which were responsive to retinoic acid.

5.3 Immune Cells

For a more integrated view of the disease, significant improvements of in vitro skin models for psoriasis would involve the addition of immune cells, which is an option rendered possible when using full-thickness skin models. Considering the pathology

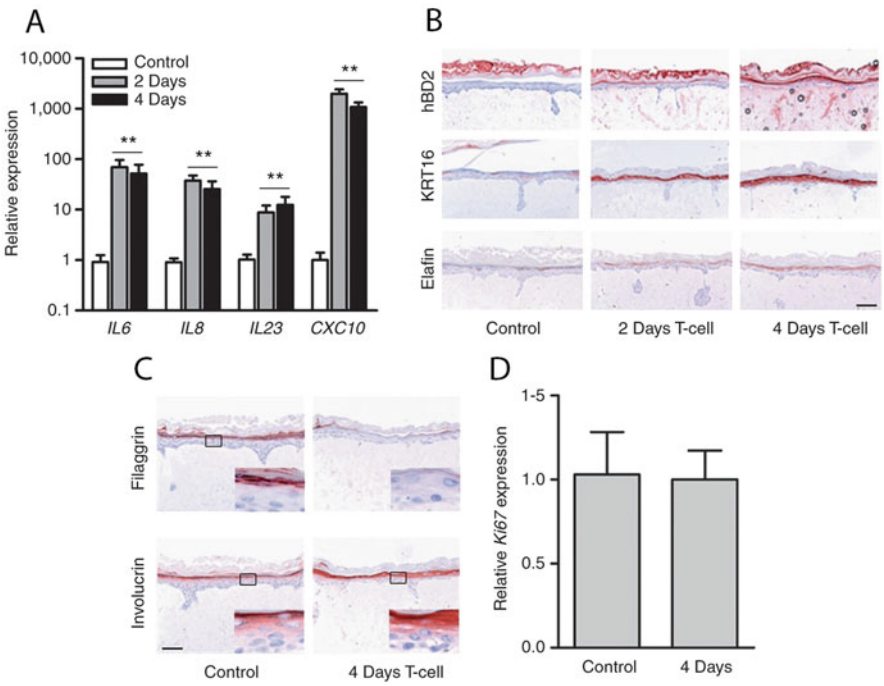

Fig. 2 Epidermal response to T cells represents a psoriasiform phenotype. (**a**) mRNA expression levels of proinflammatory cytokines and chemokines by keratinocytes after 2 days and 4 days of T-cell migration. (**b**) Immunostaining of psoriasis-associated proteins hBD2, keratin 16, and SKALP/elafin after 2 and 4 days of T-cell migration. Bar = 100 μm. (**c**) Epidermal differentiation proteins filaggrin and involucrin demonstrated by immunostaining. Bar = 100 μm. (**d**) Ki-67 mRNA levels of control and T cell-populated skin equivalents. $^{**}p < 0.01$ relative to control skin equivalents. Obtained with permission from Van den Bogaard et al. (2014)

of psoriasis, a primary choice is the addition of T cells. A model of normal keratinocytes grown on de-epidermized dermis and populated with CD4+ T cells showed typical inflammatory signs of psoriasis after 4 days (Van den Bogaard et al. 2014), which were responsive to anti-inflammatory drugs such as all-trans retinoic acid. These models also showed the typical downregulation of filaggrin but not the morphological signs of psoriasis or keratinocyte hyperproliferation (Fig. 2). A further psoriasis model combined a full-skin model produced with lesional psoriatic skin cells with the addition of activated T cells. These models demonstrated the spectrum of psoriasis features of disturbed epidermal differentiation and keratinocyte hyperproliferation, together with typical cytokine patterns (Lorthois et al. 2019). The models, also illustrating their suitability for drug studies, showed a clear response to the immunosuppressant methotrexate.

6 Infection and Immunity

Regarding skin fungal infections with dermatophytes or yeasts of the *Malassezia* genus, several models of interaction between in vitro reconstructed skin and pathological species have become available for investigation. At least three models of dermatophytosis describe how human tissues prepared in vitro by cell culture can be exposed to several species of anthropophilic and zoophilic dermatophytes (Achterman et al. 2015; Faway et al. 2017; Liang et al. 2016). Studies performed this way have shown morphological analysis of the progressive steps of infection; they were characterized for kinetics and analyzed to monitor multiple parameters that identified the kind of biological responses provided by the infected human tissue when dermatophytes penetrate into the epidermis. Colonization of the human epidermis by *Malassezia* yeasts and their interactions with cells composing the tissue were also recently described through studies that used RhE (Pedrosa et al. 2019a, b). In all cases of fungal infection, the role of toll-like receptors (TLR) in detecting infectious agents can be investigated with those experimental tools, and the initiation of an alerting epidermal response analyzed in this context.

7 Immunocompetent Skin Models

The example of psoriasis has demonstrated the importance of immune cells for analyzing skin pathology in vitro and especially for identifying drug targets and assessing drug responses. Psoriasis is a complex disease involving major (auto)-immune pathological mechanisms. The activation of T cells is accompanied by an activation of innate immune cells; accordingly, the cathelicidin LL37 plays a crucial role to cause the secretion of interferon-α, which is associated with psoriasis as well as other autoimmune diseases. The activation of macrophages and secretion of proinflammatory cytokines and chemokines then leads to the inflammatory mechanisms involved in psoriasis.

The introduction of T cells has played an important role in developing skin models for psoriasis, identifying features of psoriatic lesions and assessing genetic factors that might underlie the susceptibility to psoriasis. However, the study of psoriasis showed that also macrophages, dendritic cells, and neutrophils are critically involved in the development of the phenotype. Considering the limitations of using animal models and the fact that several, in particular common skin diseases have an immunological component, there is a need for the development of skin equivalents with compounds of the immune system. Significant progress has been made initially with regard to single types of immune cells. CD34+ hematopoietic progenitor cells were integrated into an endothelialized full-skin model, consisting of an epidermis of differentiating keratinocytes and a dermal equivalent comprising fibroblasts and human umbilical vein endothelial cells (HUVECs). This integration resulted in a complex model with epidermal Langerhans cells and dermal dendritic cells (Dezutter-Dambuyant et al. 2006). These models would thus allow the study of dendritic cell differentiation in an environment with vascular components. Similar

mechanisms are important for age-related alterations. Full-skin models populated with CD14+ monocytes demonstrated the importance of ageing-associated glycation. The use of glycation-modified collagen showed an increase in monocyte-derived dendritic cells and macrophages and the presence of receptors for advanced glycation end products such as class A scavenger receptor on these cells (Pageon et al. 2017).

In a full-skin sensitization model, monocyte-derived dendritic cells were incorporated into an agarose/fibronectin gel to form an "immune layer" on top of a microfiber scaffold populated with fibroblasts (Chau et al. 2013). The dendritic cells were able to migrate and remained responsive to stimulation with skin sensitizers. Monocyte-derived macrophages can be incorporated into a collagen/chitosan/chondroitin sulfate scaffold populated with fibroblasts (Bechetoille et al. 2011). The macrophages retained their surface markers and produced IL-10 in response to lipopolysaccharide. Similarly, full-skin models with Langerhans cells could be constructed using CD34+ human acute myeloid leukemia cell line MUTZ-3 for the generation of Langerhans cells (Masterson et al. 2002). MUTZ-Langerhans cells were able to migrate into the epidermis and were attracted by chemokines CCL5 and CCL20 (Ouwehand et al. 2012). Their migration upon skin irritation could be blocked with antibodies against CCL5. Irritant exposure led to a switch of MUTZ-Langerhans cells to a macrophage-like phenotype in the dermis, which was blocked by anti-IL-10 antibody (Kosten et al. 2015). Strong contact sensitizers such as dinitrochlorobenzene induced secretion of IL-6 and CXCL8 in full-skin models containing either MUTZ 3 or monocyte-derived Langerhans cells and demonstrated enhanced migration of Langerhans-like cells from epidermal to dermal equivalent (Bock et al. 2018). These studies using skin models have strongly contributed to the understanding of Langerhans cells and macrophages and have given insight into the mobility of dendritic cells in the skin. They have demonstrated that skin sensitization and immunological aspects of autoimmune diseases can well be analyzed using advanced in vitro skin models. Many of these systems, however, are experimental models and not readily available for systematic approaches and testing purposes. Moreover, they still do not fully cover the immunocompetence of the skin and represent the complexity of responses to environmental or genetic challenges. Future developments will have to demonstrate to which extent access to automation and approaches to extend the spectrum of cellular differentiation in vitro, for instance using induced pluripotent stem (iPS) cells, will be able to provide the tools for a comprehensive analysis of immune responses and cell–cell and cell–environment interactions in 3D skin modelling.

8 Drug Development and Testing

RhE and full-skin models have been used in a vast range of applications for the assessment of chemicals and other components, for instance regarding skin irritation, corrosion, absorption, and penetration. Such procedures have been validated and standardized by the European Centre for the Validation of Alternative Methods

(ECVAM) and the OECD in their guidelines. In addition to these applications, skin models have gained major interest as they can play a critical role in the evaluation of drug targets and the development of topical therapies against skin diseases. While these approaches rely on the generation of more comprehensive and long-lasting skin models, present models have already contributed to significant progress in drug development and a reduced need for animal experiments. Skin equivalents have been used to study properties of skin barrier function and its disturbance in disease; they are also helpful to assess the permeability of the stratum corneum that is needed for effective epidermal drug delivery. Its ability to prevent the penetration of larger compounds and preference for lipophilic substances represents a major hindrance for transdermal drug delivery (Choy and Prausnitz 2011). Physical or chemical disruptions and various nanotransporters can be used to overcome the barrier activity but most approaches are not suitable for the transport of large and active compounds and their targeted delivery. Because in vitro skin models can be used to mimic barrier function and pathological conditions, their availability opens up multiple and interesting use in the search, testing, and validation of drugs.

8.1 Retinoic Acid Metabolism Blocking Agents (RAMBA)

Skin tissue models allowed evaluation of the potential benefits of different retinoids through precise analysis of regulated gene expression, studying differentiation markers, junction components, production of cytokines and growth factors, and through the monitoring of their secretion (Bernard et al. 2002). A confirmation of well-known effects produced by this class of biologically active molecules in vivo in such in vitro models was initially proving that reconstructed epidermis might be considered as a highly predictive model regarding the efficacy of drugs targeting the human skin. Furthermore, because the sensitivity of the assays studying retinoids is elevated and since serum is excluded from culture procedures devoted to reconstruct human epidermis (De Vuyst et al. 2014), such models were tested in the evaluation of retinoic acid metabolism blocking agents (RAMBA), such as liarozole or talarozole compounds. For instance, the analysis of RhE treated with RAMBA combined with tiny amounts of 10^{-9} M retinoic acid demonstrated that the effects produced were similar to the treatment performed with more elevated concentrations (10^{-6} M) of retinoic acid alone (Giltaire et al. 2009). Recently, novel candidate CYP26B1-selective inhibitors were tested using skin models and their evaluation allowed one of them to be considered as a potentially new therapeutic for keratinization disorders (Veit et al. 2020).

8.2 Antifungal Compounds

In the context of fungal infection of the skin, in vitro models are available to study the interaction between dermatophytes and the human epidermis (Achterman et al. 2015; Liang et al. 2016). Besides investigation of infectious mechanisms themselves

in the epidermal context, such models open up interesting new conditions to evaluate whether antifungal compounds exhibit sufficient efficacy. For instance, the well-known antifungal compound miconazole stopped the infection in RhE previously inoculated with pathological fungi. Miconazole was also able to prevent infection by its use as a pre-treatment before the RhE was exposed to dermatophytes (Faway et al. 2017). The infection of RhE with dermatophytes and the study of responding signalling pathways created additional opportunities for the identification of novel potentially antifungal compounds (Faway et al. 2019).

8.3 Protein Replacement

Targeted epidermal drug delivery can be achieved by making use of the temperature change upon entry into the epidermis (Fig. 3a) (Cuggino et al. 2011; Witting et al.

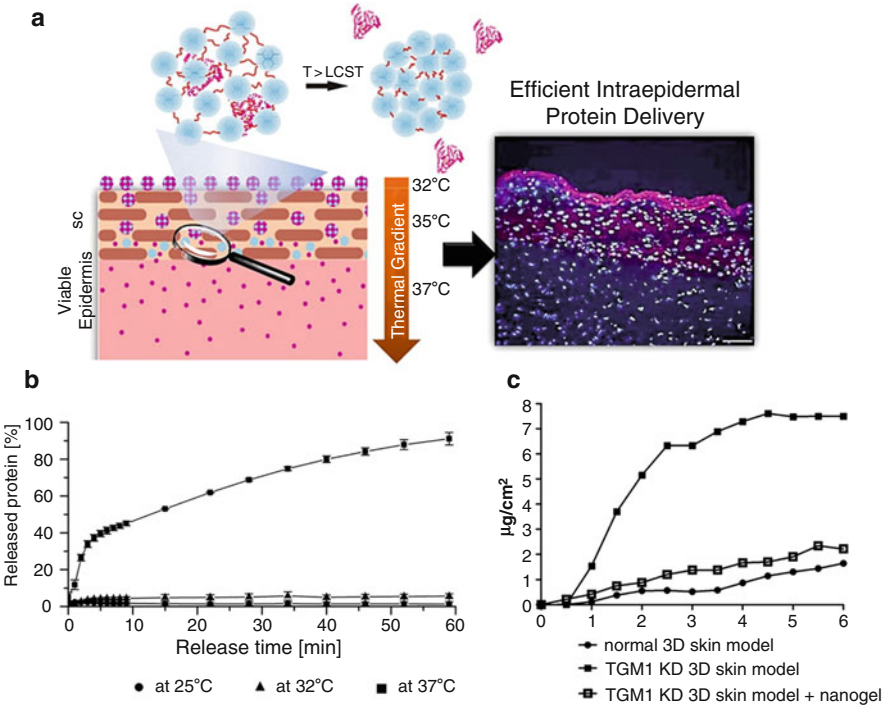

Fig. 3 (**a**) Thermoresponsive dendritic polyglycerol-based nanogels were used for protein loading and targeted release in the epidermal equivalent, preserving enzyme structure and bioactivity. LCST, lower critical solution temperature. (**b**) Release of bovine serum albumin from dPG nanogels over 1 h at 25°C (●), 32°C (▲), and 37°C (■) measured with UV-vis spectroscopy. (**c**) Permeation of ^{3}H-testosterone through normal skin models (●), TGase-1 deficient skin models (■), and TGase-1-deficient skin models treated with TGase-1-loaded nanogels (□). Compiled with permission from Witting et al. (2015)

2015). Thermoresponsive nanotransporters were generated by combining dendritic polyglycerol (dPG) with thermosensitive poly(*N*-isopropylacrylamide) (pNIPAM). These dPG nanogels undergo a phase transition at ~33°C; if the critical temperature is exceeded, the nanogels (~200 nm diameter) shrink and release water and drugs loaded onto them. Efficient release at lower temperatures was shown for bovine serum albumin (BSA) (~68 kDa; Fig. 3b) (Witting et al. 2015). The clinical relevance was demonstrated for ARCI, which is most frequently caused by inactivating mutations in *TGM1*, the gene for transglutaminase 1. ARCI is associated with epidermal barrier impairment, which is replicated in full-skin models with knockdown of *TGM1* (Eckl et al. 2011). Disturbed barrier function was detected by increased permeability for testosterone in the model (Alnasif et al. 2014; Witting et al. 2015). Targeted dPG nanogel-mediated delivery of recombinant human transglutaminase 1 (~89 kDa) resulted in restoration of barrier function almost to the normal level (Fig. 3c). In a similar approach, normal RhE models (EpiDerm™, MatTek) were treated with single filaggrin units (Stout et al. 2014). Epidermal penetration was facilitated by using a fusion construct consisting of a murine filaggrin monomer (mFLG) and a cell-penetrating peptide (RMR) protein motif. The filaggrin unit penetrated to the stratum granulosum of RhE; internalization, processing and restoration of the normal phenotype after treatment with tagged mFLG-RMR (~50 kDa) were then shown in the skin of filaggrin-deficient flaky tail mice.

The efficacy of nanogel-mediated protein delivery was further demonstrated with etanercept (ETR), an anti-TNF-α fusion protein used for the treatment of psoriasis (~150 kDa). Thermoresponsive polyglycerol (tPG) or pNIPAM-based nanogels, both shown to be biocompatible in human keratinocytes (Gerecke et al. 2017), were loaded with ETR. Application to full-skin models showed the delivery through the stratum corneum into the viable epidermis (Giulbudagian et al. 2018). Supplementation of skin equivalents with TNF-α caused an inflammation-like state that was suitable to visualize the success of the ETR treatment, even though these models did not contain any immune models. Application of ETR to stimulated skin models resulted in reduced levels of TNF-α, ICAM-1, and TSLP (Fig. 4).

Using skin models made with keratinocytes and fibroblasts from ARCI patients with *TGM1* mutations, the restoration of the impaired skin barrier function, the major pathophysiological feature of ARCI, was demonstrated in vitro after treatment with recombinant transglutaminase 1 (Plank et al. 2019). These approaches showed delivery of enzyme through the stratum corneum into the viable epidermis and, importantly, its preserved activity during maturation of the models (Fig. 5). The enzyme activity resulted in formation of an efficient barrier function, as demonstrated using several barrier tests such as testosterone permeability, Lucifer yellow permeation, and *N*-hydroxy-sulfosuccinimide-LC-biotin permeability. These experiments clearly showed that full-skin models were not only suitable for the analysis of morphological and physiological features of skin diseases, but also helpful to assess treatments and test drugs. Barrier function is critical in several

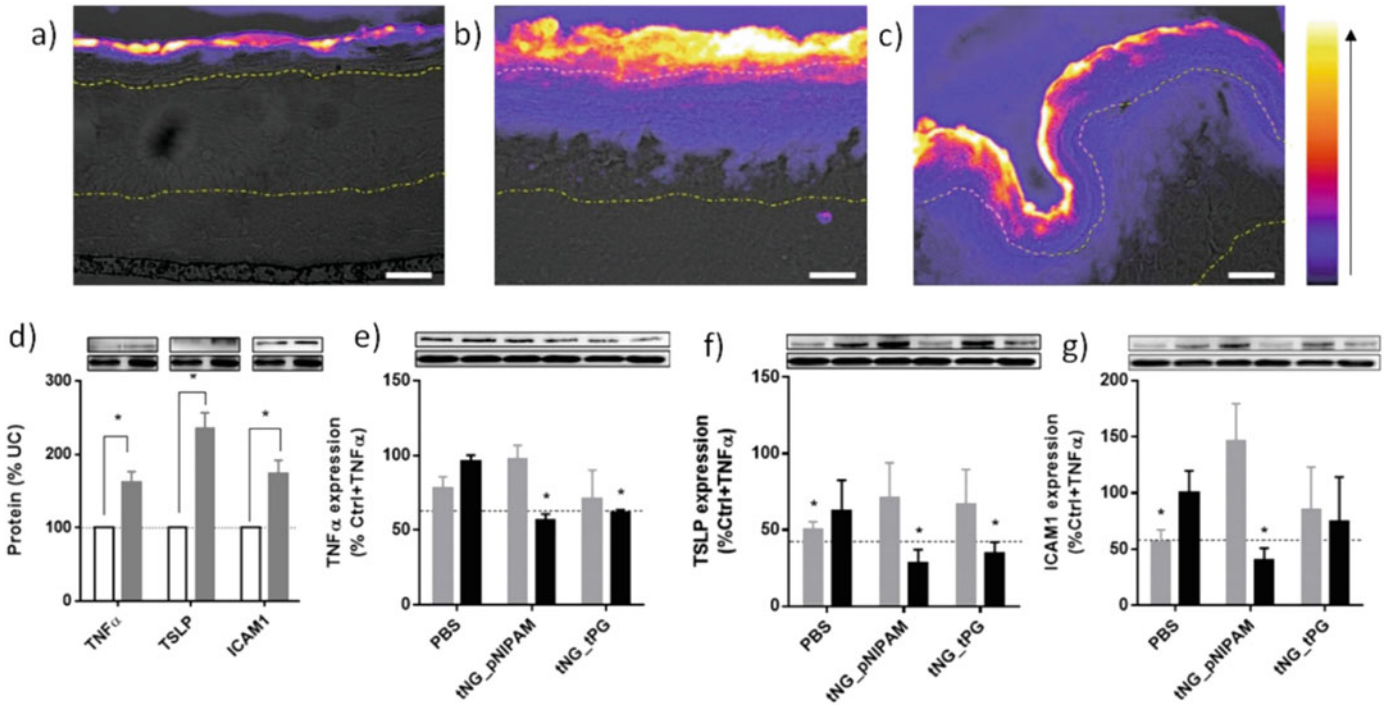

Fig. 4 Immunohistochemical staining against ETR in skin equivalents topically treated with ETR dissolved in PBS (**a**) or encapsulated in pNIPAM (**b**) or tPG-based (**c**) nanogels. Images show pseudo-colored ETR temperature staining (scale and direction of increasing signal intensity shown on the right) overlaid onto differential interference contrast (DIC) images. Boundaries between stratum corneum and viable epidermis (upper) and viable epidermis and dermis (lower) are marked (scale bar = 50 μm). (**d**) Protein levels of TNF-α, TSLP, and ICAM-1 in TNF-α stimulated skin equivalents (black) calculated as a percentage of normal skin equivalents (gray). Protein levels of TNF-α (**e**), TSLP (**f**), and ICAM-1 (**g**) in TNF-α stimulated skin equivalents following treatment with empty (gray) or ETR-loaded nanogels or PBS (black). Values are expressed as a percentage of TNF-α treated controls. Insets show representative western blots from the protein of interest, above, and β-actin, below. Dashed lines indicate mean protein levels found in untreated, normal skin equivalents. Obtained from Giulbudagian et al. (2018) under Creative Commons license

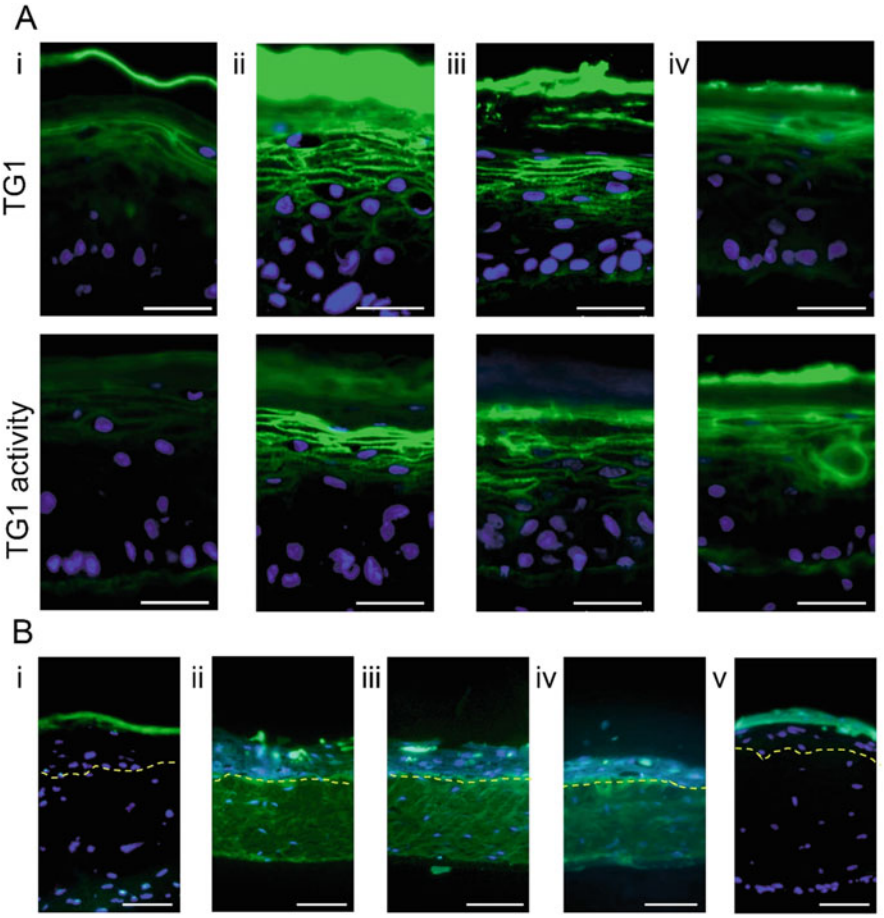

Fig. 5 (**a**) Transglutaminase 1 (TG1) staining and activity in ARCI patient skin equivalents treated with TG1/PBS (i), normal equivalents treated with TG1/tNG (ii), TG1 knockdown equivalent treated with TG1/tNG (iii), and ARCI patient equivalent treated with TG1/tNG (blue = DAPI, green = TG1 protein and activity staining, respectively). Scale bars = 50 μm. (**b**) Lucifer yellow permeation into normal skin equivalent (i), ARCI patient skin equivalent (ii), patient skin equivalent treated with unloaded tNGs (iii), patient skin equivalent treated with TG1 in PBS (iv), and patient skin equivalent treated with TG1/tNG (v) (blue = DAPI, green = Lucifer yellow). Scale bars = 75 μm. Obtained with permission from Plank et al. (2019)

dermatological diseases affecting keratinocytes. The ability of skin equivalents to form an active barrier with properties similar to human skin in vivo and to respond to treatments with active proteins makes them suitable for drug delivery tests, for the development of topical therapies, as well as for studies of the skin barrier and its changes in disease, during ageing, or under certain environmental conditions (Weinmüllner et al. 2020).

9 Future Directions

Recent developments in the composition of skin models have proven their usefulness in a broad range of applications. Models are versatile, robust, and adjustable. Several commercial models are readily available, which can be used off the shelf, including a number of disease models. Yet, there are still limitations. Improvements and sophistication are necessary to render models adequate for further reduction of the number of animals needed for biological and pharmacological investigations and for the development of a definitive in vitro model that will fully represent the human skin. Such models will have to override the biological constraints of animal models due to the differences between human skin and skin of rodents and other model animals with regard to structural and protective properties, developmental processes, and many other aspects. Several skin diseases involve a compromised barrier function, which can be due to altered lipid metabolism or other mechanisms. As discussed herein, existing models are already able to replicate skin barrier function and suitable for some analysis of drug targets, reasonably taking into account the modulation of barrier function during chronic diseases or ageing.

Most human skin diseases have an immunological origin and/or impact. The development of skin models populated with immune cells has already improved their suitability for the study of autoimmune diseases and immune responses; however, many models are still under experimental design and still quite complicated to produce. They do not yet represent the full complexity of integrated immune responses and do not contain the full variety of immune cells, which interact with each other and with the cutaneous tissues, and remain mobile in the skin. Another issue with currently available skin models is still their longer-term use. In fact, many processes will have to be studied over time; we can analyze barrier formation, changing expression profiles or lipid synthesis over a period of days, or a few weeks, but to date, the investigation of ageing-associated mechanisms is, for instance, not well feasible. This problem involves the renewal of the epidermal equivalent and the structure of the dermis. The latter has been addressed with variations of scaffolds for the dermal matrix and the introduction of novel materials. A silk-collagen composite system has been developed to reduce the loss of dermal matrix strength and make it more sustainable. This approach allowed the introduction of another layer, a hypodermis equivalent containing adipocyte-like cells generated from adipose-derived stem cells, which also further adds to the complexity of the model (Bellas et al. 2012). Such skin models become suitable for the addition of other cellular components such as those from the nervous system (Vidal et al. 2019). Considering the importance of the nerve system to understand and solve itching, but also for immune responses and to investigate the interaction between neurons and immune cells, this kind of development can be crucial for the actual immunocompetence of skin models (Vidal Yucha et al. 2019).

Another important development regarding the complexity of the skin is the use of induced pluripotent stem (iPS) cells. They can be generated from fibroblasts or other adult cells and may provide a source for a large variety of cells; basically, they will allow the generation of all cell types needed for organ and disease modelling on an

individual basis, thus they are able to create a lasting patient-based resource. iPS cells have been differentiated into keratinocytes and fibroblasts and used for the generation of iPS cell-derived skin equivalents (Guenou et al. 2009; Itoh et al. 2013; Petrova et al. 2014). Further cell types were added to iPS cell-based skin models, such as melanocytes (Gledhill et al. 2015) and endothelial cells (Abaci et al. 2016). These iPS cell-based models promise a great deal of innovation and a cellular versatility that is impossible with conventional approaches to isolate and culture cells. On the other hand, the targeted differentiation of iPS cells is still complicated and the generation of a variety of different immune cells for the incorporation into series of skin models might not be realistic.

The role of iPS cells for the future of skin modelling is important in combination with another promising development, the generation of skin-on-chip or more generally organ-on-chip models. These models are based on microfluidics technology and provide flexible systems for various test systems. They benefit from a defined environment and allow highly controlled conditions and exchange of cells and compounds. Few systems were established that are suitable for immunocompetence, such as a microfluidic co-culture system comprising HaCaT cells and a human leukemic monocyte lymphoma cell line (Ramadan and Ting 2016). Skin-on-chip systems were used for the introduction of vascularization (Abaci et al. 2016; Mori et al. 2017) and a co-culture system using a commercial full-thickness skin equivalent (EpiDermFT™) with hair follicles (Ataç et al. 2013). In an attempt to model the organ environment and interaction between cell types composing different organs, microfluidics systems can be extended to a multiple-organ-on-chip strategy. Examples include the combination of skin and liver culture (Maschmeyer et al. 2015a; Wagner et al. 2013) or a four-organ chip for interconnected long-term co-culture of human intestine, liver, skin, and kidney equivalents (Maschmeyer et al. 2015b). Though not yet there to take the full complexity of human skin into account, in particular with regard to immune responses, the microfluidics systems are already suitable for drug testing and the analysis of transdermal delivery (Abaci et al. 2015). Safety and efficacy tests could be extended to repeat dose studies such as an anti-EGFR cetuximab analysis in a microfluidic co-culture system of human H292 lung cancer microtissues and human full-thickness skin equivalents (Hübner et al. 2018). The study allowed the simultaneous evaluation of anti-EGFR-induced tumor effects and potential adverse skin effects, such as the loss of proliferative keratinocytes in the basal layer of the skin microtissue and the antibody-associated modulation of CXCL8 and CXCL10 release. These findings clearly indicate the potential of these systems for pharmacological studies and, possibly in combination with the progress in our understanding of iPS cell differentiation, promising directions in the use of in vitro model systems to further replace animal studies and provide advanced research tools for cutaneous biology.

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Immunocompetent Human Intestinal Models in Preclinical Drug Development

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Contents

Abstract

The intestinal epithelial barrier, together with the microbiome and local immune system, is a critical component that maintains intestinal homeostasis. Dysfunction may lead to chronic inflammation, as observed in inflammatory bowel diseases. Animal models have historically been used in preclinical research to identify and validate new drug targets in intestinal inflammatory diseases. Yet, limitations about their biological relevance to humans and advances in tissue engineering have forced the development of more complex three-dimensional reconstructed intestinal epithelium. By introducing immune and commensal microbial cells, these models more accurately mimic the gut's physiology and the pathophysiological changes occurring in vivo in the inflamed intestine. Specific advantages and limitations of two-dimensional (2D) and three-dimensional (3D) intestinal models such as coculture systems, organoids, and microfluidic devices to study

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_429

inflammatory and immune-related responses are highlighted. While current cell culture models lack the cellular and molecular complexity observed in vivo, the emphasis is put on how these models can be used to improve preclinical drug development for inflammatory diseases of the intestine.

Keywords

Animal model replacement · Cell culture · Immune cells · Immunocompetent · In vitro · Inflammatory disease · Microbiome · Small intestine · Tissue engineering

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
E. coli	*Escherichia coli*
EMI	Epithelial-microbiome-immune
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharides
PBMC	Peripheral blood mononuclear cell
TNF	Tumor necrosis factor

1 Introduction

In tissue engineering, substantial progress has been made over the last years leading to improved pathophysiological relevance and predictivity of in vitro models. Human in vitro models of normal and diseased tissues have gained much attention since they can deliver important information about human-specific pathophysiology, the mode of action of drugs and drug toxicity prior to animal studies in vivo and clinical studies (Alepee et al. 2014; Basketter et al. 2012; Leist et al. 2014). Ultimately, such models may accelerate drug discovery by reducing wrong predictions from drug candidate screening and failure rates in late development stages.

Providing a physiological environment, reconstructed human tissues display highly relevant test systems in biomedical research and toxicological risk assessment and adhere to the principles of the 3Rs (Replacement, Refinement, and Reduction), defined by Russell and Burch in 1959. Advances in tissue engineering and microfabrication have led to novel in vitro models of diseases of many different organs as well as models of cancer and infectious diseases (Benam et al. 2015). Simulating inflammatory and immunological processes by in vitro models is still challenging, yet with the increasing complexity of multicellular models and the introduction of immune cells, the models more closely mimic pathophysiological

changes related to inflammatory diseases. The incorporation of immune cells into, e.g., mucous membranes is one of the most advanced examples that allows the assessment of molecular key events in the pathogenesis of localized fungal infections (Weindl et al. 2007; Schaller and Weindl 2009). Important advances in modeling the pathophysiology of inflammatory disorders have been also made for other epithelial barriers including the intestine.

In this book chapter, the recent progress made in the development of in vitro models of the intestine to study inflammatory and immune-related effects in preclinical drug development is highlighted. Furthermore, not only the potential of the models but also their limitations in replacing animals in inflammatory-related gut research are presented. Since the focus is on immunocompetent models, the reader is referred to other recent reviews on healthy intestinal in vitro models (Billat et al. 2017; Lee et al. 2019; Yin et al. 2019; Bein et al. 2018).

2 Inflammatory Responses in the Intestine

The human intestine is one of the most complex organs in the body and has a wide range of physiological functions, including digestion and absorption of nutrients and secretion. It contains an enteric nervous system and is an essential organ of defense, providing a barrier against pathogens, toxins, and antigens. As the major site for absorption of oral drugs, the intestine is of great interest to study oral bioavailability which is also influenced by intestinal metabolism. Importantly, the intestine is the largest compartment of the immune system and intestinal epithelial cells (IECs), myofibroblasts, stromal cells, myeloid cells, T and B cells, innate lymphoid cells, and the microbiota play a central role in health and disease (Round and Mazmanian 2009; Garrett et al. 2010). The three major players in intestinal homeostasis are also referred to as the epithelial-microbiome-immune (EMI) axis.

The intestine is known for specific immune-related diseases such as inflammatory bowel diseases, celiac disease, and cancer as well as bacterial infections but has been implicated also in the development of non-enteric diseases. It is now well established that changes in the composition and function of the human gut microbiome have been associated with many chronic diseases (Durack and Lynch 2019; Arrieta et al. 2014).

The two main phenotypes of human inflammatory bowel diseases (IBDs), Crohn's disease and ulcerative colitis, are characterized by chronic inflammation of the intestinal tract caused by environmental, genetic, as well as commensal microbial factors (De Souza and Fiocchi 2016; Friedrich et al. 2019) (Fig. 1).

The immunopathology of IBD is still not fully understood, however, in recent years novel therapeutic agents such as biologics against integrin $\alpha_4\beta_7$ (vedolizumab) and p40 subunit of both interleukin (IL)-12 and IL-23 (ustekinumab), as well as Janus kinase inhibitors targeting multiple cytokine signaling pathways are successfully used to treat severe corticosteroid-refractory IBD (Chudy-Onwugaje et al. 2019). The rapidly increasing incidence and prevalence of this disease (GBD Inflammatory Bowel Disease Collaborators 2020), together with high failure rates

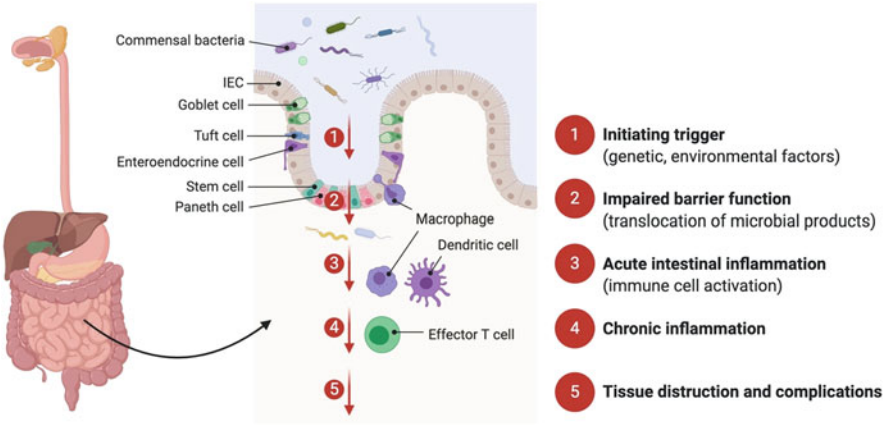

Fig. 1 Key events in the pathogenesis of IBD. Image created with BioRender

in IBD drug development (Harris et al. 2019), highlight that continued efforts are needed to optimize preclinical test systems. Traditionally, the study of intestinal inflammatory diseases has been empowered by mouse models and, more recently, by single-cell analysis of samples from patients, which has revealed new potential drug targets; however, drug development in IBD faces specific challenges (Danese et al. 2020). In addition, animal models of IBD that are modified genetically, chemically, or immunologically (Kiesler et al. 2015) may not be representative of human disease with respect to the genetic heterogeneity and the microbiome, among others, eventually leading to species-specific pharmacological responses. These technical challenges have encouraged the development of advanced human-based in vitro models to simulate and study intestinal inflammatory responses.

3 Human In Vitro Models for Intestinal Inflammation

In vitro models facilitate the study of complex in vivo phenomena in a simplified context and under well-controlled conditions. The focus of intestinal in vitro models during the last decades has been put on drug absorption studies. The development of more complex tissue models has paved their way for broader preclinical drug development in the search for new drugs in inflammatory diseases of the gut.

Alternative methods for gut research include different models, from two-dimensional (2D) to highly complex three-dimensional (3D) culture systems. At present, the most promising tool for preclinical drug development and screening of drug candidates is represented by cocultured systems that have evolved from simple models containing a monolayer of IECs to more complex cell architecture systems with incorporated immune cells and microbiome to reconstruct the polarized EMI axis (Fig. 2). Intestinal organoids, microfluidic devices, and 3D bioprinting represent an improvement towards recapitulating the intestine's structural,

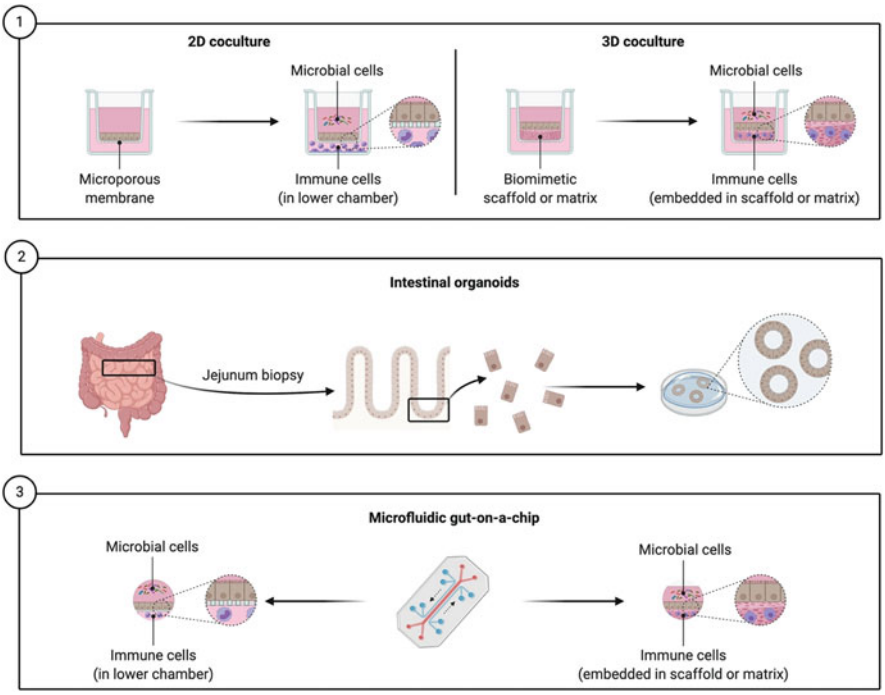

Fig. 2 Human (immunocompetent) in vitro models of the intestine. Image created with BioRender

functional, and molecular aspects to more accurately simulate physiological and pathophysiological processes.

An ideal human in vitro model of the inflamed intestine should (1) contain all representative cell types of the epithelium under inflammatory conditions that retain the most important functional properties during cell culture, (2) permit adequate oxygenation and nutrition of the tissue for long-term studies, and (3) allow crosstalk between epithelium, immune cells, and microbiota. However, the anatomical and physiological complexity of the intestine which is further complicated during inflammatory processes prevents the general applicability of a single model in preclinical drug development. Some of the challenges have recently been overcome by more advanced in vitro models of the inflamed intestine as outlined below.

3.1 Coculture Models

Generally, it is still challenging to differentiate normal human intestinal epithelial cells in culture, despite the progress made in tissue engineering. The human colon carcinoma cell line Caco-2 on a microporous membrane (i.e., transwell insert) still remains the most used 2D model of the intestinal epithelial barrier since its introduction in the late 1980s (Hidalgo et al. 1989), although alternatives such as models

derived from induced pluripotent stem cell lines are emerging (Akazawa et al. 2018). Caco-2 cells form a differentiated and polarized confluent monolayer and can be readily used to study drug absorption and permeability, but its limitations in terms of villi formation, mucus production, or drug metabolism compared to normal intestinal epithelium are well documented (Hidalgo et al. 1989; Sun et al. 2008).

Conventional coculture systems with intestinal epithelial cells, mostly Caco-2 cells, provide apical and basolateral compartments to incorporate microbes and immune cells, respectively, either derived from primary immune cells or cell lines to mimic inflammatory conditions. A simple coculture system of Caco-2 monolayer cells, grown on microporous membranes, and peripheral blood mononuclear cells (PBMCs) (Haller et al. 2000; Parlesak et al. 2004), THP-1 monocytes (Kanzato et al. 2001), or macrophages (Watanabe et al. 2004; Satsu et al. 2006; Moyes et al. 2010; Ishimoto et al. 2011; Kämpfer et al. 2017, 2020) in the lower chamber is used. Coculture of Caco-2 monolayer with whole blood seems possible too (Schmohl et al. 2012). In these settings, the presence of immune cells allows analysis of the crosstalk between both cell types. It mimics an inflamed intestine when cells are stimulated with pro-inflammatory cytokines or lipopolysaccharides (LPS). Standard read-out parameters such as barrier integrity of the intestinal epithelial cells, release of cytokines and chemokines by epithelial and immune cells and cytotoxic effects are used to characterize the inflammatory responses in the coculture system; however, only a few anti-inflammatory drugs such as ibuprofen and prednisolone (Schmohl et al. 2012) and the tumor necrosis factor (TNF) inhibitor etanercept (Satsu et al. 2006) have been tested in these models.

Apart from Caco-2 cells, also coculture models based on HT29 cells are developed and supplemented with U937 macrophages and PBMCs to study enteric host–pathogen interactions and inflammatory responses mediated by activation of pattern recognition receptors, respectively (Barrila et al. 2017; de Kivit et al. 2011).

To further improve the coculture system and mimic an inflamed intestine more closely, macrophages and dendritic cells have been incorporated, resulting in a triple coculture model. In contrast to the above-mentioned studies, immune cells are embedded in a collagen matrix in a transwell insert and IECs are seeded on top. Among the tested IEC lines Caco-2, HT29 and T84, only Caco-2 cells respond to pro-inflammatory stimuli such as LPS, IL-1β or interferon (IFN)-γ by upregulating pro-inflammatory cytokines and decreasing epithelial barrier function. In the presence of immune cells, the inflammatory response in the model is more pronounced compared to inflamed Caco-2 monocultures. Noteworthy, Caco-2 cells have been cultured with macrophages and dendritic cells derived from the immune cell lines THP-1 and MUTZ-3, respectively, (Susewind et al. 2016) but also from PBMCs (Leonard et al. 2010). Thus, this model can be supplemented both with primary immune cells and cell lines, depending on the scope of the study. These models are successfully used to characterize and evaluate the safety of engineered nanoparticles in the context of inflammation (Leonard et al. 2010; Susewind et al. 2016) and to differentiate the therapeutic efficacy of different formulations of budesonide (Leonard et al. 2012, 2020), a glucocorticoid which is clinically relevant in IBD therapy. Alternative approaches aim to better simulate the intestinal barrier by

establishing a transwell-like setting with Caco-2 cells on the apical and endothelial cells attached to the basolateral surface of a collagen scaffold, respectively (Schulte et al. 2020). Peripheral blood leukocytes are integrated into the culture compartment. This model is proposed to mimic human gastroenteritis when infected with *Salmonella* and reveals human-specific local immune responses that are different from the situation in infected mice.

Instead of increasing the number of different immune cell types, other approaches focus on improving in vitro models for drug absorption by integrating additional cells into the intestinal epithelium. The most studied coculture of Caco-2 and mucus-producing HT29 cells better mimics the intestinal drug transport than simple Caco-2 monolayers (Lozoya-Agullo et al. 2017). Using a hydrogel scaffold, this coculture model can be maintained up to 12 weeks and following stimulation with pro-inflammatory cytokines, inflammatory responses and their long-term influence on intestinal permeability can be monitored (Dosh et al. 2019). The integration of THP-1 macrophages into the lower chamber and stimulation with LPS leads to increased release of several pro-inflammatory mediators and chemokines and reduced barrier function that is reversed in the presence of budesonide (Weber et al. 2020). In conventional transwell cultures with Caco-2 and HT29 cells, human blood monocyte-derived macrophages and dendritic cells are incorporated to monitor inflammatory responses of ingested microplastics (Lehner et al. 2020).

A further step towards more complex coculture systems is the triple coculture of epithelial (Caco-2) and goblet (HT29) cells together with THP-1 cells embedded in a collagen matrix which is combined with a synthetic microbiome of the small intestine (Calatayud et al. 2019). Stimulation with LPS from pathogenic *E. coli* reduces epithelial barrier function and increases cytokine and chemokine production, thus resembling inflamed intestinal epithelium in the microbiome model. The integration of different commensal strains may foster studies on host–microbiome interactions using coculture systems.

To overcome the limitations of models derived from immortalized cell lines, a macrophage-enteroid coculture model consisting of primary human cells has been developed to study interactions and signaling between epithelial cells and macrophages. Acute bacterial infections can be followed under controlled conditions and the modular model may be further improved by the addition of other cell lineages including immune cells (Noel et al. 2017). The use of sponge scaffolds instead of membranes further improves the physiological relevance of models with non-transformed cells. Monocyte-derived macrophages embedded in the subepithelial 3D layer enhance responses to inflammatory stimuli indicating the applicability as an experimental model for inflammatory bowel disease (Roh et al. 2019).

3.2 Intestinal Organoids

Intestinal organoids are miniaturized organs that contain various cell types of the intestine in a 3D environment. First developed a decade ago, it is now widely

acknowledged that organoids better mimic native intestinal epithelium compared to other intestinal in vitro models (Wells and Spence 2014; In et al. 2016). Human organoid cultures are derived either from intestinal crypts or from induced pluripotent stem cells that differentiate into self-organizing intestinal stem cells that maintain themselves as stem cell niches and recapitulate tissue turnover (Date and Sato 2015). These organoids are an excellent tool to investigate and analyze stem cell differentiation and intestinal histogenesis; however, organoids are difficult to standardize, and they are limited when it comes to studying pharmacological agents able to modulate inflammatory and immune responses since current models lack immune cells. Yet, organoids have a great potential as preclinical models to study gut disorders and the addition of immune cells or a functional immune system, among other improvements, will make organoids suitable for personalized drug testing in IBD (Angus et al. 2019).

3.3 Microfluidic Gut-on-a-Chip Models

The organ-on-a-chip technology has emerged as a novel in vitro alternative to simulate complex interactions within the tissue and more importantly between different organs. By placing multiple organs on a single chip, the dynamics of a human organism can be monitored under more physiological conditions compared to a static model of a single organ. This approach seems highly promising in academia and industry to screen drug candidates (Prantil-Baun et al. 2018). It may accelerate drug discovery by reducing the notoriously high failure rates in preclinical drug development. The organ-on-a-chip technology is successfully applied to develop intestinal models based on IEC lines or primary cells (Bein et al. 2018). Over the last years, gut-on-a-chip models with increasing complexity have been engineered including the integration of endothelial cells to form a capillary endothelium, immune cells, and commensal as well as pathogenic bacteria.

The microfluidic cell-based system NutriChip allows studying the response of immune cells to pro-inflammatory stimuli or immunomodulators in food (Ramadan et al. 2013; Ramadan and Jing 2016). The dynamic in vitro model consists of a confluent monolayer of epithelial cells (Caco-2) that interact through a permeable membrane with immune cells (U937-derived macrophages) cultured in the lower compartment. Barrier permeability can be monitored in the presence of LPS or TNF, however, it remains to be demonstrated whether this test system may serve as a simple and reliable tool for screening of drug candidates.

A more sophisticated microfluidic gut-on-a-chip device has been developed with peristalsis-like motions to model small intestinal bacterial overgrowth and inflammation over a period of weeks (Kim et al. 2016). To reconstitute intestinal inflammation and injury, the model is cocultured with pathogenic bacteria, commensal microbes, and primary immune cells (i.e., PMBCs) and the inflammatory responses are recapitulated at the organ level. In the presence of enteroinvasive *Escherichia coli*, secretion of pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and TNF is increased. Furthermore, intestinal injury is evident by villus blunting and impaired

intestinal barrier function. Probiotic bacteria and antibiotics largely protect from injury induced by *E. coli*. Of note, bacterial overgrowth is observed when peristalsis-like mechanical motions were ceased, which closely mimics the situation in patients with ileus. This model is further developed as "human gut inflammation-on-a-chip" to study host–microbiome crosstalk during chemically (i.e., dextran sodium sulfate) induced inflammation (Shin and Kim 2018). In this model, impaired barrier function was identified as one of the most critical factors that drives intestinal inflammation. Overall, this modular platform has the potential not only to analyze interactions between the intestine and microbiome and the development of intestinal inflammation but also for testing of antibacterial and anti-inflammatory drug candidates. Since PBMCs are a source of T, B, and natural killer (NK) cells and monocytes and can be easily differentiated into macrophages and dendritic cells, it seems feasible to integrate immune cells of the gut-associated lymphoid tissue as well. The same group reported a radiation injury model of the gut to evaluate protective effects of a potential radiation countermeasure drug (Jalili-Firoozinezhad et al. 2018), however, immune cells that are involved in radiation toxicity are not included in these studies.

More recently, Caco-2 cells have been cultured with vascular endothelial cells (i.e., HUVECs) on a peristaltic microfluidic chip (Jing et al. 2020). To induce intestinal inflammation, *E. coli* and U937-derived macrophages are added to the epithelial cells or incorporated into the vascular cavity. *E. coli*-induced intestinal damage, as documented by a disrupted intestinal barrier and injured villi, as well as inflammatory responses is suppressed by the probiotic *Lactobacillus casei* or the antibiotics penicillin and streptomycin confirming the suitability of this test system for drug screening.

In a modular, microfluidics-based model, termed HuMiX (human–microbial crosstalk), the Caco-2 monolayer is physically separated from microbial cells by a nanoporous membrane which allows coculture with commensal bacteria under anaerobic conditions (Shah et al. 2016). Supplementation of a perfusion microchamber with primary $CD4^+$ T cells seems feasible which underlines the potential of this model to study host–microbe interactions in the presence of immune cells. However, this model is currently limited to short-term exposure ($<$24 h) and mechanical deformations of the IEC monolayer have not been considered. To simulate immune tolerance in the gut, tissue resident innate immune cells representing mucosal macrophages and dendritic cells are included in an intestine-on-chip model that allows functional studies on the interaction between microbes, IECs, and immune cells (Maurer et al. 2019).

Microfluidic devices with intestinal tissue ex vivo are emerging as alternative models to study pathophysiological processes and drug transport, metabolism, and toxicity. Recent proof-of-concept studies demonstrate that whole intestinal segments from mice can be used for up to 24 h to dissect complex inflammatory and immune responses to microbes (Yissachar et al. 2017). Viability and integrity of human intestinal tissues can be maintained for an increased period time, too, although studies are less advanced and the ex vivo models seem not suitable for preclinical drug testing (Dawson et al. 2016; Baydoun et al. 2020; Richardson et al. 2020).

4 Limitations of Current In Vitro Models of the Inflamed Intestine

As outlined, different approaches to obtain 3D immunocompetent models of the intestine have several drawbacks similar to tissue models without immune cells (Hewes et al. 2020). Conventional static coculture models normally fail to create a physiologically relevant culture environment, they lack specialized epithelial cell types and artificial membranes are not representative for the complexity observed in vivo. Furthermore, physiological biomechanics cannot be simulated, and long-term studies to simulate chronic disease are difficult to accomplish. Intestinal organoids cannot be easily modified to have additional tissue components such as vasculature and immune cells, whereas microfluidic gut-on-a-chip models remain challenging to scale and the limited amount of biomaterial restricts downstream analyses.

Although most in vitro models of the inflamed intestine have a limited complexity regarding cell types and signaling pathways, they provide a cost-effective approach for pharmacological screening and mechanistic evaluation of treatment strategies in inflammatory diseases under standardized and reproducible experimental conditions.

5 Conclusions and Outlook

By harnessing in vitro models much progress has been made, however, currently only a few immunocompetent intestinal models are applied in preclinical drug development in inflammatory and immune-related diseases of the gut. This may be partially explained by an as yet incomplete understanding of disease pathology compared to a more advanced knowledge in risk assessment such as skin irritation and sensitization (Almeida et al. 2017; Bock et al. 2018). The establishment of key events in adverse outcome pathways (AOPs) and hypothesis-driven Integrated Approaches to Testing and Assessment (IATA) provided a major step forward in the development of specific in vitro test systems in toxicology (Leist et al. 2017; Tollefsen et al. 2014). By defining advanced test methods for key events in early phases of drug development, drug candidates may be better prioritized. However, for complex chronic inflammatory diseases it will be challenging to define inflammation-related key events and in vitro test systems for a specific disease (Villeneuve et al. 2018).

Other state-of-the-art approaches in tissue engineering such as 3D bioprinting of human tissues and organs using primary cells may have superior physiological relevance and improved performance in ADME (absorption, distribution, metabolism, and excretion) and toxicity studies compared to conventional in vitro models (Murphy and Atala 2014). Indeed, bioprinting allows deposition of primary human intestinal cells and biomaterials to build a structure similar to the native human intestine (Madden et al. 2018). By integration of immune cells and a living microbiome, these models may serve as flexible platform for future preclinical drug discovery and the development of personalized, precision medicine. Further

development of intestinal in vitro models will be a key component to obtain more accurate simulations of the processes observed in vivo and to accelerate drug discovery and development using test systems with enhanced scientific rigor and reproducibility.

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Tissue Engineering for Musculoskeletal Regeneration and Disease Modeling

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_377

Abstract

Musculoskeletal injuries and associated conditions are the leading cause of physical disability worldwide. The concept of tissue engineering has opened up novel approaches to repair musculoskeletal defects in a fast and/or efficient manner. Biomaterials, cells, and signaling molecules constitute the tissue engineering triad. In the past 40 years, significant progress has been made in developing and optimizing all three components, but only a very limited number of technologies have been successfully translated into clinical applications. A major limiting factor of this barrier to translation is the insufficiency of two-dimensional cell cultures and traditional animal models in informing the safety and efficacy of in-human applications. In recent years, microphysiological systems, often referred to as organ or tissue chips, generated according to tissue engineering principles, have been proposed as the next-generation drug testing models. This chapter aims to first review the current tissue engineering-based approaches that are being applied to fabricate and develop the individual critical elements involved in musculoskeletal organ/tissue chips. We next highlight the general strategy of generating musculoskeletal tissue chips and their potential in future regenerative medicine research. Exemplary microphysiological systems mimicking musculoskeletal tissues are described. With sufficient physiological accuracy and relevance, the human cell-derived, three-dimensional, multi-tissue systems have been used to model a number of orthopedic disorders and to test new treatments. We anticipate that the novel emerging tissue chip technology will continually reshape and improve our understanding of human musculoskeletal pathophysiology, ultimately accelerating the development of advanced pharmaceutics and regenerative therapies.

Keywords

Biological induction · Biomaterial · Disease modeling · Drug testing · Growth factors · Microphysiological system · Organoid · Regenerative medicine · Stem cells · Tissue chip

1 Introduction

The human musculoskeletal system, also known as the locomotor system, provides the human body with structural support and load-bearing capacity and offers protection to the delicate internal organs. The musculoskeletal system enables mechanical functions that subject the tissues to wear and tear as well as injuries over a lifetime of use, leading to debilitating pain and weakening or even loss of functions. In fact, musculoskeletal conditions are the leading cause of physical disability worldwide (James et al. 2018). As musculoskeletal disorders are most prevalent in the elderly, the world's aging population represent the major contributing factor to the increasing

medical and socioeconomic burdens of treating musculoskeletal impairments. Conventional treatments include the use of autografts, allograft, and xenografts, but have several drawbacks such as disease transmission, limited availability and reproducibility, donor scarcity, sterilization-induced alteration in natural matrix properties, and immune rejection. Tissue engineering represents a promising strategy of regenerative medicine to restore musculoskeletal structures and functions.

The field of tissue engineering interfaces engineering, life science, and medicine, with the capability of creating living tissues outside the human body (Langer and Vacanti 1993). Traditionally, these in vitro grown tissues are intended to be implanted into the human body to restore, maintain, augment, or replace diseased, injured, or degenerated tissues. Regenerative medicine, as the term indicates, aims to enable and enhance the body's natural repair mechanisms to restore the function of otherwise irreparable tissues or organs in situ.

The tissue engineering triad consists of three key elements: scaffolds, cells, and signaling molecules (O'Brien 2011). In the past few decades, considerable progress has been made in the tissue engineering and regenerative medicine (TERM) field, which continues to evolve rapidly. In particular, new biomaterials are being designed, and novel approaches are emerging to fabricate scaffolds with existing biomaterials; the identification of new stem cell sources and functions and the establishment of more biologically accurate organotypic models promise to broaden the applications of TERM technologies; and innovative delivery and administration strategies are being proposed for growth factors and other biologically active molecules. These exciting developments underscore the importance of comprehensive evaluation of the three TERM components to gain an in-depth understanding of their safety, efficacy, and optimal use. In this chapter we focus specifically on the essential TERM elements of relevance to orthopedic applications.

Successful translation of TERM techniques and products requires evidence-based safety and efficacy assessment prior to the clinical trial. Currently, animal models are an essential component in preclinical studies and have been used to assess various biomaterials, cells, and signaling molecules for musculoskeletal regeneration. Small rodents, especially mice and rats, are the most commonly used animal models in musculoskeletal research. The past few decades have witnessed a continuous increase in the use of mice in orthopedic research, while the use of rats remains relatively unchanged due to the difficulties in genetic manipulations (Ericsson et al. 2013). However, in recent years, concerns have risen about the benefit of animal research to humans. A number of studies have shown that most animal experiments are unable to predict the observations in human trials (Mak et al. 2014; Pound and Bracken 2014). In fact, among the 76 highly influential animal studies (each with $>$500 citations) published on 7 prominent scientific journals, only 37% could be replicated in human randomized trials (Hackam and Redelmeier 2006). Therefore, the benefits of animal models still need to be supported by more systematic evaluations.

In addition to the many commendable achievements in tissue repair, TERM applications have also been successfully extended to disease modeling and drug development over the past decade. Exemplary in vitro models have been established

using TERM principles for mimicking musculoskeletal tissues, including bone and cartilage (Lin et al. 2014; Occhetta et al. 2019; Arrigoni et al. 2020), and skeletal muscle (Truskey 2018), and other tissue/organ systems, such as lung (Huh et al. 2010), blood-brain barrier (Phan et al. 2017), gut (Kim et al. 2012), kidney (Jang et al. 2013), liver (Ribeiro et al. 2019), pancreas (Shik Mun et al. 2019), and heart (Nunes et al. 2013). These exciting studies have suggested that in vitro organotypic models and microphysiological systems (MPS), generated with tissue engineering principles and technologies, may serve as convenient and versatile platforms for the comprehensive evaluation of multiple TERM elements. Once the clinical relevance is validated, in vitro models like MPS will be a powerful alternative to current animal models.

We begin this chapter by describing the properties and performances – both in vitro and in vivo – of the scaffolds, cells, and signaling molecules in musculoskeletal tissue engineering. The derivation of organoids from stem cell aggregates and their potential applications are introduced. We then describe the optimal use of the three TERM elements in representative MPS established thus far for modeling musculoskeletal tissues. Finally, we summarize the advantages and disadvantages of different models and envision their utility in the development of drugs/treatments in the future.

2 Key Elements of Musculoskeletal Tissue Models

Scaffolds, cells, and signaling molecules are considered the three key, enabling components of tissue engineering. For musculoskeletal regeneration, the structure and function of native bone, cartilage, skeletal muscle, tendon, and ligament tissues have significant implications on the selection of the three elements mentioned above. Best reparative and regenerative outcomes are achieved when the three elements act synergistically, where the appropriate cell types are seeded into a scaffold that possesses mechanical, structural, and biochemical characteristics akin to those of the native tissue and are given the biochemical and physical signals that induce anabolic and regenerative responses.

2.1 Biomaterials and Scaffolds

Enormous progress in biomaterials research has been made in the past decades. Many different types of natural and synthetic biomaterials have been utilized to fabricate tissue engineering scaffolds, which can be cell-laden or cell-free when implanted. The safety and efficacy of a new biomaterial have to be rigorously assessed prior to its clinical application. This evaluation process routinely includes both in vitro and in vivo tests. We recognize the myriad biomaterial types that have been utilized to regenerate musculoskeletal tissues. This section thus does not intend to provide a thorough description of existing biomaterials, but rather focuses on an

analysis of the assessment and performances of the representative, novel biomaterials and scaffolds developed in the past decade.

Bone Biomaterial preparation for bone regeneration has been facing a persistent challenge to recapitulate the mechanical, structural, and functional characteristics of native bone. Natural biomaterials such as acellular bone matrix and extracellular matrix (ECM) generated by in vitro cultured mesenchymal stem cells (MSCs) have led to robust osteogenesis (Ni et al. 2014; Liu et al. 2019; Rothrauff and Tuan 2020). A myriad of synthetic biomaterials, including metals, ceramics, polymers, and composites, have been developed to address the unmet medical need of bone grafts. Metallic biomaterials still dominate the market of load-bearing bone substitute materials (Chen and Thouas 2015). One of the most commonly used biometals is Ti-6Al-4V alloys. Human MSCs were cultured on additive manufactured Ti-6Al-4V scaffolds, and surface anodization was found to increase the alkaline phosphatase (ALP) production, osteocalcin (OCN) and type I collagen expression, and mineral deposition (Li et al. 2020a; Groessner-Schreiber and Tuan 1992). A major drawback of most metallic implants is the associated "stress shielding effect." Biodegradable metals such as magnesium alloys have thus been proposed to address this issue. Fracture repair with magnesium-containing orthopedic implants has shown considerable efficacy. Magnesium intramedullary rods were implanted in rat femur, leading to abundant new bone formation through an osteogenic mechanism that involves calcitonin gene-related polypeptide-α (CGRP) (Zhang et al. 2016). It has been noted that there exists a large gap in mechanical properties between existing biodegradable materials and traditional metallic implants. Biodegradable zinc alloys have therefore been developed, tested in vitro with MC3T3-E1 mouse preosteoblast cells and human umbilical vein endothelial cells (HUVECs), and implanted in rat femur to evaluate their degradability (Yang et al. 2020). This group of newly designed zinc alloys was found to show excellent promise in load-bearing orthopedic applications.

Bioceramics mostly possess high chemical stability and biocompatibility; bioactive ceramics are usually osteoconductive and/or osteoinductive. Osteoconductivity is the ability of a surface to support the attachment, proliferation, and migration of bone-forming osteoblasts, while osteoinductivity implies the recruitment and induction of undifferentiated stem cells to become osteoprogenitors (Albrektsson and Johansson 2001). Hydroxyapatite and Bioglass®, as two extensively studies bioactive ceramics, can form direct chemical bonds with surrounding tissues. Because of the close compositional resemblance of calcium phosphates (CaP, minerals containing Ca^{2+} and phosphate anions) to the inorganic phase of natural bone, CaP ceramics are among the most frequently used bone biomaterials (Bose and Tarafder 2012). The suitability of bioceramics for bone repair is typically assessed by culturing osteoblast-like cells and MSCs on bioceramic surfaces or in bioceramic-conditioned medium (Li et al. 2017a, b, 2020b). As a relatively new bioceramic, 2D nanosilicates have become a topic of increasing interest in bone tissue engineering because of their outstanding osteoconductivity and osteoinductivity. Nanosilicate platelets can be conveniently mixed in three-dimensional (3D) hydrogels to form

nanocomposites. A 2D in vitro culture model has been used to assess the osteogenic responses of the MC3T3 E1 subclone 4 cells to nanosilicate-containing scaffolds (Xavier et al. 2015). The cells showed higher quantitative ALP activity and calcium deposition induced by nanosilicate addition. In another study, both in vitro and in vivo models were employed to examine silicate/methacrylated glycol chitosan scaffolds. The in vitro osteoinductivity tests showed markedly increased ALP activity and mineralization as well as upregulated osteogenic gene expression by the encapsulated MSCs in the presence of silicate. In a mouse nonunion calvarial defect model, the silicate-containing, cell-free composites were found to overtly promote native cell infiltration as well as cause remarkably higher bone volume density, bone growth surface area, and trabecular number than either constituent material (Cui et al. 2019).

A main limitation of many bioceramics is their low fracture toughness. To deal with this issue, bioceramics have been combined with biopolymers to obtain composites with improved ductility and toughness (Rezwan et al. 2006). A broader application of biopolymers, especially hydrogels, for musculoskeletal repair is seen in cell- or drug-delivery therapies (Nöth et al. 2010), as will be discussed in Sects. 2.2 and 2.3.

Cartilage Various natural and synthetic biopolymers have been utilized to maintain or enhance the chondro-phenotype of chondrocytes or chondrogenic differentiation of stem cells, a process where new cartilage ECM is synthesized and deposited. Cartilage scaffolds come in various forms such as porous sponges (Wang et al. 2005), fibers/meshes (Li et al. 2005), and hydrogels (Deng et al. 2019). As a natural biomaterial, MSC-derived ECM was shown in our previous study to be a robust substrate for enhancing the chondrogenesis of articular chondrocytes (Yang et al. 2018). A composite hydrogel, consisting of gelatin, fibrinogen, hyaluronan (HA), and glycerol, was used to 3D print scaffolds containing rabbit articular chondrocytes (Kang et al. 2016). In vitro culture and in vivo implantation in mouse dorsal subcutaneous pockets both showed abundant new cartilage matrix deposition in the 3D printed structure. Sharma et al. (2013) designed a poly(ethylene glycol) diacrylate hydrogel and used it in conjunction with a bioadhesive to repair focal cartilage defects in a caprine model and in human patients (Fig. 1a–c). This soft hydrogel could augment the microfracture treatment and promote cartilage regeneration. Electrospun polymer fiber meshes are usually too dense for cell infiltration. To tackle this issue, cryoelectrospinning, with the mandrel kept at −78 °C, was employed to create an ultraporous nanofiber network that permits the infiltration of cell-laden hydrogels (Formica et al. 2016). The scaffolds were assessed with both bovine and human chondrocytes and led to robust production of type II collagen and sulfated glycosaminoglycans (GAGs), characteristic markers of cartilage, in vitro.

Native cartilage undergoes substantial mechanical shear, wear, and compression during a lifetime. The recapitulation of the mechanical and frictional properties has become a key consideration in the design of cartilage biomaterials and scaffolds (Liao et al. 2013). By virtue of its high biocompatibility and safety (and with FDA

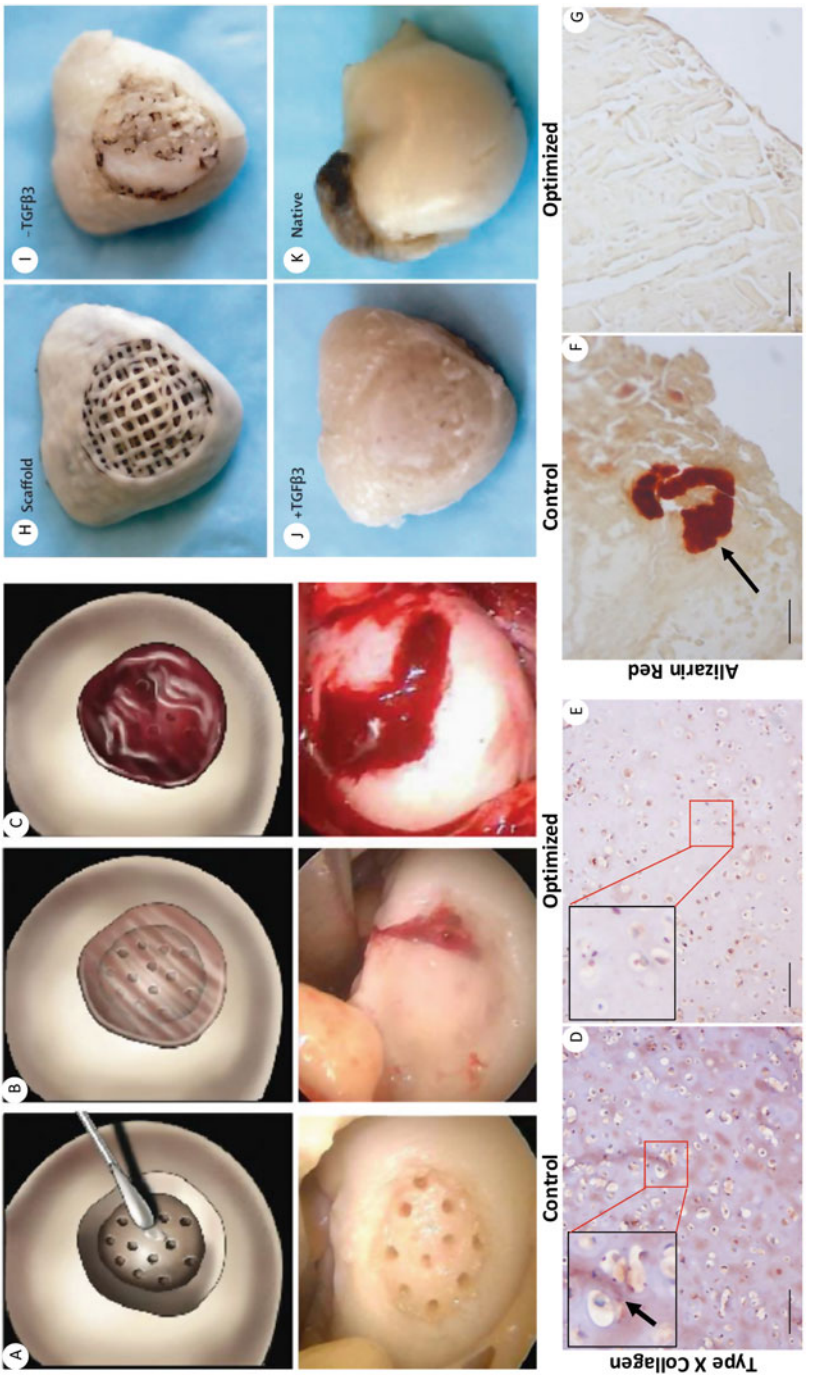

Fig. 1 Representative cartilage regeneration strategies harnessing adhesive-hydrogel composites (**a–c**), MSC chondrogenesis (**d–g**), and growth factor-induced endogenous cell homing (**h–k**). In a pilot clinical study, focal cartilage defects in human patients were applied with an adhesive and microfractured (**a**), filled

approval), poly(ethylene glycol) (PEG) has been widely utilized, despite their low mechanical strength, to prepare various hydrogel scaffolds (Benoit et al. 2008; Brandl et al. 2010). An injectable hydrogel derived from 4-arm star PEG showed a local maximum strength of ~20 MPa. This high-strength PEG-based hydrogel supported the proliferation and phenotype maintenance of encapsulated murine chondrocytes, and the injected chondrocyte-laden hydrogels resulted in formation of new hyaline cartilage integrated with the host tissue in a murine osteochondral defect model (Wang et al. 2017). We recently developed a photo-crosslinkable poly-D,L-lactide acid/PEG hydrogel and assessed its chondrogenic potential with human MSCs (Sun et al. 2017). This hydrogel possesses a compressive modulus in the physiological range of native cartilage and supports the differentiation and maintenance of human MSCs and thus holds promise in point-of-care treatment of cartilage defect.

Skeletal Muscle Generally there are two approaches to skeletal muscle tissue engineering: (1) transplantation of biomaterial scaffolds seeded with muscle cells and other supporting cells and (2) delivery of biomaterial scaffolds, with or without paracrine signaling cells, growth factors, or cytokines, to induce in situ muscle tissue engineering (Kwee and Mooney 2017). In the design of biomaterials for skeletal muscle tissue engineering, it is important to consider the microstructural, physical, and biochemical properties, such as porosity (Hill et al. 2006), degradability (Hong et al. 2011), 2D and 3D patterns (Ku et al. 2012), injectability (Rossi et al. 2011), and native biochemical cues (Perniconi et al. 2011). Electrospinning is suited to a wide variety of natural and synthetic biomaterials, capable of fabricating fibers with varying diameters and orientations and generating scalable, ECM-mimicking scaffolds with desired degree of anisotropy. An alginate-based bioink containing HUVECs has been electrospun onto uniaxially micropatterned PCL/collagen struts, generating scaffolds that provide both topographical and biochemical cues that facilitated the alignment and differentiation of subsequently seeded myocytes (Yeo and Kim 2020). Hydrogels are also commonly used biomaterials for muscle repair. Interestingly, 3D free-standing skeletal muscle fibers engineered from muscle cell-laden Matrigel were found to be able to support the differentiation of neural stem cells into neurons to form neuromuscular junctions (NMJs) (Morimoto et al. 2013).

Fig. 1 (continued) with injectable, photo-crosslinkable hydrogel (**b**), which trapped bleeding from the microfracture holes (**c**). In MSC-based cartilage tissue engineering, an optimized differentiation protocol with exposure to Wnt/β-catenin inhibitor and shorter TGF-β treatment time was found to significantly inhibit chondrocyte hypertrophy both in vitro (**d**, **e**) and in a mouse intramuscular implantation model (**f**, **g**). Scale bars: 100 μm. A 3D printed scaffold (**h**) infused with collagen hydrogel and with/without TGF-β3 loading was used to replace a rabbit proximal humeral joint. After 4 months' implantation, TGFβ3-infused scaffolds showed full articular surface coverage by newly formed cartilage (**i**) similar to the native tissue (**k**), while TGFβ3-free scaffolds had only isolated tissue formation (**j**). Reproduced with permission from Sharma et al. (2013), Deng et al. (2019), and Lee et al. (2010)

Cell-free scaffolds used in regenerative medicine eliminate the stringent cell harvest and administration process and obviate the associated regulatory hurdles and possible immune responses. A biologic-free ferrogel, without the incorporation of any bioreagents or cells, could generate externally actuated mechanical suppressions to reduce fibrosis and inflammation and heal myotoxin-induced severe muscle injuries (Cezar et al. 2016). To regenerate volumetric muscle loss (VML), the self-regeneration capability of native muscle does not suffice. Decellularized bladder ECM was used to repair VML in both mice and human patients and resulted in de novo muscle remodeling linked to the recruitment of perivascular stem cells (Sicari et al. 2014).

Tendon and Ligament Tendons are tough connective tissues that bind muscles to bone, while ligaments connect bones to other bones. A number of design factors, including native tissue anatomy, physical and chemical properties of the materials, and material interactions with native cells, need to be considered to select an optimal biomaterial in tendon and ligament tissue engineering (Kuo et al. 2010).

Our previous study explored the potential application of decellularized tendon-derived, solubilized extracellular matrix (tECM) in adipose stem cell (ASC)-based tendon tissue engineering (Yang et al. 2017). The tECM-supplemented 3D scaffolds not only enhanced the tenogenic differentiation of ASCs and the scaffolds' mechanical properties but also downregulated the expression of osteogenic markers and matrix metalloproteinases. Decellularized tendon ECM combined with stem cells has also been researched for tendon repair, and the natural, decellularized scaffolds were found to provide an inductive environment for the tenogenic differentiation of MSCs (Youngstrom et al. 2013; Ning et al. 2015). Using the synthetic, degradable biopolymer poly(ε-caprolactone), Wang et al. (2018) fabricated a 3D scaffold with tendon-like mechanical properties and microstructural and hierarchical anisotropy. This scaffold supported tenogenic matrix production by human tenocytes, and the acellular scaffolds showed robust pro-tenogenic properties in a micropig model.

Ligament and tendon have very similar structure in spite of differences in collagen and water content in their ECM. Many biomaterials have, therefore, been designed to engineer both tissues and tested with similar methods (Barber et al. 2013). While tenocytes are the major cellular component of tendons, the cells residing in ligaments are mostly fibroblasts. In in vitro studies, ligament biomaterials and scaffolds are typically evaluated with fibroblasts and MSCs (Correia Pinto et al. 2017; Chang et al. 2020), and in vivo models for ligament tissue engineering are mostly created in small-scale animals.

Tendons and ligaments attach to bone at junctions known as entheses. Biomaterials for enthesis tissue engineering are expected to facilitate integration and smooth load transfer between tendon/ligament and bone. Such biomaterials have been investigated in a number of previous studies and are not detailed here (Font Tellado et al. 2015; Tang et al. 2020).

2.2 Cells

The common cell sources employed to engineer musculoskeletal tissues are shown in Fig. 2a. Because of their multipotency, high proliferative capacity, and relative ease of isolation and expansion, MSCs remain the most promising cell source in musculoskeletal regeneration and in general TERM applications. Although embryonic stem cells (ESCs) have wide differentiation potential and extensive expansion properties, for TERM applications, the hurdles include not only ethical issues related to sourcing but also the risks of tumorigenicity and immune rejection as well as the absence of a standardized protocol for ESC differentiation into musculoskeletal tissues (Jukes et al. 2010). Induced pluripotent stem cells (iPSCs) have received much attention for their pluripotency and virtually unlimited supplies. Since iPSCs can be reprogrammed from somatic cells, they not only obviate the need for embryos but can be made individual-specific, thus avoiding immune rejection issues (Takahashi and Yamanaka 2006). Primary cells from native tissues have also been employed for musculoskeletal regeneration, but they usually have lower availability, are more difficult to culture, and show larger patient-to-patient variability.

Bone Human/animal primary osteoblastic cells and stabilized osteoblastic cell lines have both been used in in vitro investigations. Osteoblastic cell lines, including SaOs-2, MG-63, and MC3T3-E1, have been compared with primary human osteoblasts (HOBs) in terms of their proliferation ability, mineralization behavior, and gene expression profile. It was found previously that HOBs only share some of the characteristics with each of the cell lines (Czekanska et al. 2014). Although the uses of HOBs are more clinically relevant than cell lines, their applications are limited by the complicated isolation procedures and heterogeneous phenotypes (Czekanska et al. 2012). As a multipotent stem cell, MSCs can be readily differentiated into osteoblasts and have been applied in various scaffolds to induce osteogenesis. Tissue-engineered bone scaffolds are designed to mimic bone ECM and recruit surrounding cells, forming a bone tissue to repair the bone defect (Amini et al. 2012). Recent studies show that MSCs possess robust osteogenic ability when seeded in collagen scaffolds, polymer-mineral scaffolds, fibrous nanocomposite scaffolds, silica-coated scaffolds, as well as their own ECM (Kuttappan et al. 2020; Duan et al. 2018; Gandhimathi et al. 2019; Harvestine et al. 2018; Meinel et al. 2003). However, autologous MSCs have limited availability. Due to patient heterogeneity, allogeneic MSCs show donor age-dependent proliferation rates and raise immunogenic concerns; MSC isolation involves invasive harvesting procedures in the case of bone marrow MSCs. iPSCs have emerged as a novel alternative to MSCs. Osteoblasts derived from iPSC were seeded on a gelatin scaffold and were found to secret calcium, OCN, and bone sialoprotein in vitro and in vivo (Bilousova et al. 2011). Retinoic acid was used to induce human iPSCs to differentiate into osteoblast-like and osteocyte-like cells, and these cells could generate human bone tissues in mouse calvarial defects (Kawai et al. 2019). Nevertheless, with current differentiation protocols, the multi-lineage differentiation

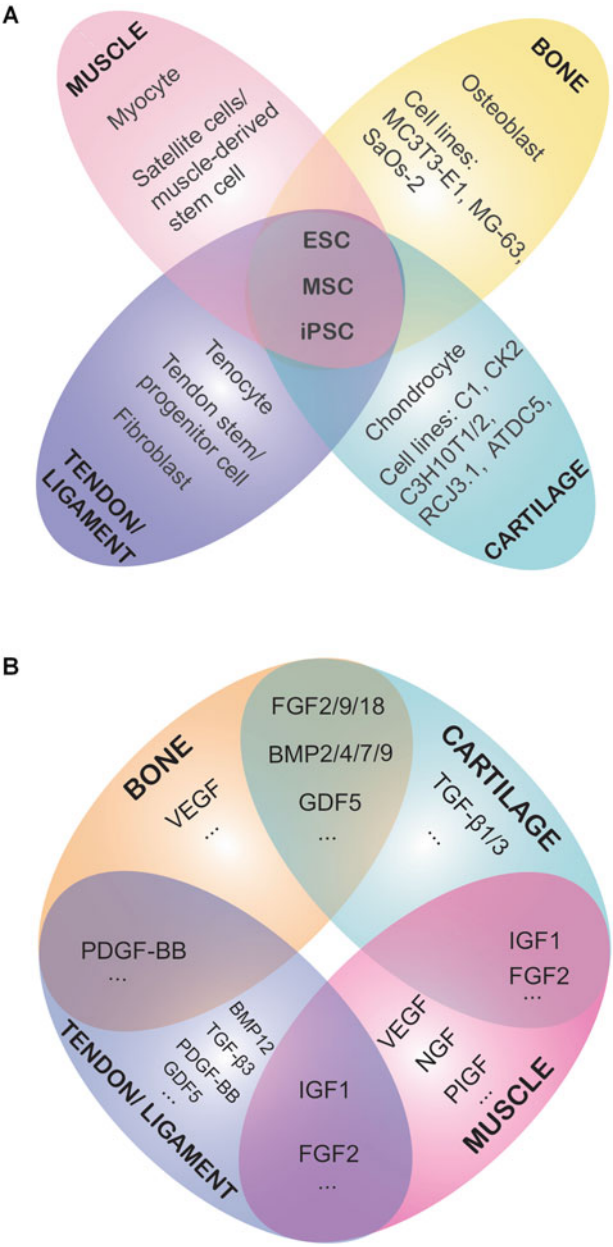

Fig. 2 Commonly used cell sources (**a**) and growth factors (**b**) for musculoskeletal tissue engineering

capability of iPSCs generally does not match that of MSCs (Diederichs and Tuan 2014), and the optimization of differentiation protocols is warranted in future research.

Cartilage Chondrocytes are the only cell type present in articular cartilage and exist within abundant ECM that is neither vascularized nor innervated. The presence and functional state of chondrocytes are of significant orthopedic clinical importance in the case of degenerative joint diseases, such as osteoarthritis (OA), where the articular cartilage suffers from extensive degeneration, resulting in serious physical debilitation. In order to understand OA progression, it is important to examine the changes in chondrocytes within the cartilage. There has been a relentless pursuit of an optimal chondrocyte culture method to reduce their dedifferentiation during in vitro expansion (Caron et al. 2012), and a number of chondrogenic cell lines from mouse or rat, including C1, C3H10T1/2, ATDC5, CK2, and RCJ3.1, have been generated and widely used (Brown et al. 2014). It is now accepted that abnormal biomechanical and genetic factors target chondrocytes and alter their normal functions (Goldring 2000). Dudek et al. (2016) reported that the chondrocyte clock gene BMAL1 plays a key role in the normal function of articular chondrocytes and hence in cartilage integrity. Besides chondrocytes, MSCs, iPSCs, and ESCs have also been utilized to form cartilage tissues. One of the key challenges in the long-term culture of chondrocytes, either native or derived from chondrogenically differentiated stem or progenitor cells, is the process of hypertrophy, whereby the cells enlarge and enter terminal differentiation, accompanied by apoptosis and calcification. While this differentiation and maturation of chondrocytes is a normal process of long bone development from transitional cartilage as part of the process of endochondral ossification (Saito et al. 2010), the hyaline cartilage of the articular joint surface is a permanent cartilage, and the appearance of hypertrophic chondrocytes is in fact part of the degenerative process in OA (Wang et al. 2004). Researchers have thus explored different methods to generate non-hypertrophic, hyaline cartilage. We found in our previous study that inhibiting the WNT signaling pathway during the differentiation process can promote the MSC chondrogenesis and suppress chondrogenic ossification in vitro and in vivo (Fig. 1d–g) (Deng et al. 2019; Narcisi et al. 2015). Different protocols have been proposed for iPSC chondrogenic differentiation (Dicks et al. 2020; Lach et al. 2019; Hu et al. 2020), and further research is needed for protocol optimization and standardization.

Skeletal Muscle Regeneration of skeletal muscle is a complex process orchestrated by heterogeneous cell populations. Satellite cells/muscle-derived stem cells (MDSCs) and MSCs are among the most frequently used cell types for repairing defective muscles (Sacco et al. 2008; Montarras et al. 2005; Koponen et al. 2007; Usas and Huard 2007). Human pluripotent stem cells can be successfully differentiated into induced myogenic progenitor cells, which can readily form 3D contractile multinucleated myotubes (Rao et al. 2018). Other less commonly used cell types in muscle regeneration include adipose-derived stem cells and pericytes (Dunn et al. 2019). Satellite cells are positive for PAX7 and PAX3 (Relaix et al.

2005) and generally considered to stay dormant when there is no injury to the muscle. Interestingly, Keefe et al. (2015) demonstrated that satellite cells contribute to myofibers in both injured and healthy adult mouse muscles. Collins et al. (2005) transplanted as few as seven satellite cells in a single intact myofiber into radiation-ablated muscles, where the satellite cells vigorously self-renewed and expanded, generating clusters of new, compact myofibers. Satellite cells have also been delivered in HA hydrogels to a muscle ablation model in mouse, resulting in both structural and functional recovery (Rossi et al. 2011). Multipotent MDSCs have been employed to regenerate muscle and other musculoskeletal tissues (Usas and Huard 2007). A preplate technique was introduced to isolate MDSCs. In this procedure, the minced muscle tissues first undergo enzymatic digestion, and the resultant slurry is plated on collagen-coated flasks. Non-adhering cells in the culture medium are transferred and plated in new collagen-coated flasks, and this process is repeated ~5 times to obtain a slowly adhering cell population that contains MDSCs (Gharaibeh et al. 2008). The maintenance of MDSC potency and quiescence is challenging in in vitro culture. Quarta et al. (2016) created collagen-based artificial muscle fibers that extended the quiescence of mouse and human MDSCs. Bone marrow-derived MSCs, as another promising cell source for muscle repair, have been seeded onto 3D, porous alginate scaffolds loaded with vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF1), where the local growth factor stimulation could enhance MSC paracrine signaling and support endogenous muscle regeneration in a rat muscle injury (blunt crush) model (Pumberger et al. 2016). The trophic actions of MSCs have been proposed to be a major mechanism underlying the enhanced endogenous muscle repair and regeneration (Sassoli et al. 2012).

Tendon and Ligament Both terminally differentiated cells and stem cells have been utilized for tendon/ligament regeneration. Tenocytes are characterized by specific markers, such as Scleraxis, Mohawk, and early growth response factor (Asahara et al. 2017). To maintain tenocyte phenotype, many collagen-based scaffolds have been engineered. It was reported that collagen-GAG scaffolds were helpful for the long-term maintenance of tenocyte transcriptomic stability (Caliari et al. 2012). In addition, supplementation of growth/differentiation factor 5 (GDF5) and IGF1 together can rescue the tenocyte phenotype and drive cell proliferation (Caliari and Harley 2013). Tendon stem/progenitor cells (TSPCs) possessing the universal characteristics of stem cells were first identified by Bi et al. (2007). TSPCs reside in a biglycan- and fibromodulin-rich niche and can regenerate tendon-like tissues both in vitro and in vivo. Many studies have explored MSC-based tendon tissue engineering strategies, where MSCs were successfully induced to express tenocyte phenotypes (Kryger et al. 2007; Lee et al. 2011; Li et al. 2015; Tokunaga et al. 2015). It has also been shown that co-culture with stem cells derived from bone marrow or adipose tissue can promote tenocyte proliferation (Chen et al. 2018; Kraus et al. 2013). Recently, Komura et al. (2020) generated a tenocyte induction protocol to differentiate murine iPSCs into tenocyte-like cells, which could significantly reduce scar formation and promote tendon regeneration when implanted in

injured mouse tendons. In ligament tissue engineering, ligament-derived fibroblasts (Chang et al. 2020), human dermal fibroblasts (Correia Pinto et al. 2017), and human MSCs have all been employed.

3D Organoids ESCs, adult stem cells (including MSCs), and iPSCs have been used to engineer almost every musculoskeletal tissue (Fig. 2a), because these cells possess extensive proliferation potential and multi-differentiation potency. Aggregates of stem cells can form 3D structures that recapitulate certain architectural and functional characteristics of some native tissues in vitro, and such 3D cell structures are termed organoids (Takahashi 2019). Various ESC- and iPSC-derived organoids have been derived by employing developmental biology principles, and a number of other organoids were generated by subjecting adult stem cells to conditions that mimic tissue renewal or repair processes in vivo (Clevers 2016). For example, organoids derived from pluripotent stem cells have been studied previously to model the brain (Lancaster et al. 2013), retina (Eiraku et al. 2011), adenohypophysis (Suga et al. 2011), stomach (McCracken et al. 2014), liver (Takebe et al. 2013), lung (Dye et al. 2015), and kidney (Takasato et al. 2014); adult stem cells, which can also undergo extensive proliferation and differentiation to form organoids, have been utilized to mimic stomach (Stange Daniel et al. 2013), prostate (Chua et al. 2014), lung (Desai et al. 2014), salivary gland (Maimets et al. 2016), and esophagus (DeWard et al. 2014), just to name a few. Organoid cultures can often proliferate extensively and thus generate sufficient cells to replace damaged or diseased tissues (Yui et al. 2012). In addition, organoids offer several advantages over animal models and conventional cell culture systems in studying human development, physiology, and pathobiology. Organoids possess multiple cell types as well as 3D structural and morphological characteristics similar to native tissues; shorter duration and lower cost can generally be expected for experimentation with organoid models than with animal models; using patient-derived iPSCs and gene manipulation techniques such as the CRISPR/Cas9 system, patient- and disease-specific organoids with edited genome can greatly advance precision medicine research (Ran et al. 2013; Takebe and Wells 2019). In the history of musculoskeletal research, in 1929, Fell and Robison (1929) cultured skeletal tissue fragments from fowl embryos to model the skeletal development process in vitro. However, a relatively small number of musculoskeletal organoids have been established thus far (Mori et al. 2019). To engineer skeletal muscle tissues, a biomaterial scaffold is usually required to induce myofiber alignment under tension (Maffioletti et al. 2018). Recently, mouse iPSCs were used to fabricate 3D spherical bone/cartilage complex by micro-space culture followed by mechanical shaking (Limraksasin et al. 2020). We recently showed that after brief trypsinization, MSC-impregnated ECM experienced mesenchymal condensation and robust chondrogenesis (Yang et al. 2019).

2.3 Signaling Molecules

Cells residing in native musculoskeletal tissues receive a broad array of signals which can be chemically transmitted via growth factors and other molecules (Fig. 2b) or physically exerted through the immediate ECM. The application of such complex signals in TERM has considerable influences on the outcome. This section focuses on the signaling molecule-conveyed biochemical signals for musculoskeletal regeneration. The effects of some other signals are described in Sect. 3.

Bone Bone is a highly vascularized tissue that undergoes constant remodeling throughout our lifetime, and signaling molecules play an important role in the complex bone remodeling process. Bone morphogenetic proteins (BMPs), particularly BMP2 and BMP7, show outstanding osteoinductivity and have therefore been widely incorporated in bone scaffolds (Yilgor et al. 2009). BMP2 was previously co-spun with hydroxyapatite-containing silk fibroin/poly(ethylene oxide) solution to fabricate fibrous bone scaffolds (Li et al. 2006; Lee et al. 2013). The presence of BMP2 markedly enhanced mineralization and upregulated osteogenic gene expression. Platelet-derived growth factor-BB (PDGF-BB), one of the five isomeric forms of PDGF, was found to enhance osteogenesis of adipose-derived MSCs and promote bone formation in a distraction osteogenesis rat model (Hung et al. 2015; Moore et al. 2009).

Vascularization plays a critical role in sustaining transplanted cells and/or scaffolds. The use of angiogenic growth factors, especially VEGF, in bone repair has led to encouraging outcomes (Murphy et al. 2000). Poly(lactic-co-glycolic acid) scaffolds containing human bone marrow-derived MSCs and condensed plasmid DNA encoding for BMP-4 and for VEGF were implanted into SCID mice, and the scaffolds containing all three components produced robust bone regeneration (Huang et al. 2005). Besides signaling molecules that act locally, systemic agents such as human growth hormone and parathyroid hormone are also important for bone regeneration (Dimitriou et al. 2011).

Cartilage Unlike bone, articular cartilage is avascular and recalcitrant to repair and regeneration. Despite the relatively simple tissue composition of cartilage, i.e., chondrocytes embedded in a dense ECM, clinically successful cartilage tissue engineering remains a challenge. The survival and efficacy of transplanted cells in cartilage scaffolds remain controversial. Many approaches utilize biologically active molecules, such as growth factors, to enhance the recruitment of endogenous cells for expedited tissue regeneration. Transforming growth factor beta (TGF-β) plays a critical role in the maintenance of both articular cartilage and subchondral bone homeostasis (Zhen et al. 2013; Zhen and Cao 2014). TGF-β1 promotes MSC chondrogenesis (Miura et al. 1994); TGF-β1 releasing alginate-sulfate scaffold was shown to promote chondrogenesis both in vivo and in vitro (Re'em et al. 2012). Another member in the TGF-β family, TGF-β3, also possesses strong chondrogenic effects (Barry et al. 2001). It was reported that TGF-β3 incorporated

within collagen-hyaluronic acid scaffolds could support the chondrogenesis of MSCs and produce cartilage-like ECM (Matsiko et al. 2015). Lee et al. (2010) 3D printed an anatomically correct scaffold and infused it with TGF-β3-loaded collagen hydrogel. It was found that these scaffolds led to regeneration of hyaline cartilage with superior compressive and shear properties and recruited cells significantly more than the TGF-β3-free scaffolds in a rabbit humeral head defect model (Fig. 1h–k). The use of biologically active molecules, therefore, holds promising potential in facilitating cell homing without cell delivery.

BMPs and the growth differentiation factors (GDFs), as members of the TGF-β superfamily, are also able to enhance cartilage regeneration. BMP2, BMP4, and BMP7 have been incorporated in various controlled delivery strategies for cartilage repair (Lam et al. 2015). Sun et al. (2019) found that GDF5 enhanced the migration and chondrogenesis of MSCs in vitro; furthermore, implanting a 3D-bioprinted GDF5-conjugated MSC-laden scaffold led to robust cartilage regeneration in a rabbit knee defect site.

Muscle Several growth factors, including IGF1, fibroblast growth factor 2 (FGF2), nerve growth factor (NGF), placenta-derived growth factor (PIGF), and VEGF, contribute to muscle repair through different mechanisms (Wei and Huard 2008). IGF is capable of mediating the proliferation and differentiation of muscle stem cells (Adams 2000). Gel delivery of VEGF to ischemic muscle tissue increased the expression of neurotrophic factors and promoted the regrowth and maintenance of damaged axons via NGF/GDNF (nerve growth factor/glial-derived neurotrophic factor) signaling (Shvartsman et al. 2014). The co-delivery of the myogenic factor IGF1 and the angiogenic factor VEGF via an injectable alginate gel has been shown to recover muscle functions from ischemic injuries in a mouse model (Borselli et al. 2010). Wang et al. (2014) engineered a degradable, shape-memory alginate scaffold that delivers myoblasts, IGF, and VEGF through a minimally invasive approach to injured mouse muscle. The implant led to reduced muscle fibrosis, enhanced vascularization, and enhanced functional recovery. A 3D PEG-fibrinogen hydrogel incorporating mouse mesoangioblasts transduced with a PIGF lentivirus was found to attract host vessels and nerves and generate newly formed tissues histologically similar to native muscle in an ablated muscle injury model (Fuoco et al. 2015).

Tendon and Ligament No consensus has been reached on the optimal tenogenic induction protocol. Previous studies have proposed the use of growth factors such as IGF1, FGF2, PDGF-BB, GDF-5, TGF-β3, connective tissue growth factor (CTGF), and BMP12 to promote tendon cell proliferation and enhance tendon regeneration (Raghavan et al. 2012; Rossi et al. 2011; Wolfman et al. 1997). Many of these growth factors were similarly used for ligament repair (Pauly et al. 2017; Hee et al. 2012). It was previously shown that a 12 h BMP-12 (10 ng/ml) treatment could significantly enhance MSC tenogenic marker expression, and this phenotype could be sustained in vivo in rat tendon defects (Lee et al. 2011). Barsby et al. (2014) found

that by loading TGF-β3 into an ESC-seeded 3D collagen scaffold, tendon-associated gene and protein expression by the ESCs could be significantly upregulated.

3 Musculoskeletal Microphysiological Systems

As an in vitro experimental research platform, MPS mimic a tissue or organ by providing living cells with a microenvironment in which the cells can display tissue- or organ-specific phenotypic, structural, and functional characteristics. Successful MPS have been shown to exhibit characteristics that bear high structural and biologic fidelity to tissues that are difficult to achieve in conventional, static 2D, or 3D cultures (Grayson et al. 2010). Namely, MPS-derived musculoskeletal tissues aim to present tissue maturation and complexity as seen in in vivo models, where diverse cellular composition, structurally complex ECM, and interactive signals from biochemical and physical stimuli co-exist. More importantly, the application of MPS not only contributes to achieving musculoskeletal regeneration via the technology of fabricating tissue implants but may also impact the field by establishing disease models that facilitate understanding of musculoskeletal pathogenesis and expediting the development of potential therapies and therapeutic agents. Over the past decade, MPS have been utilized to model various musculoskeletal diseases such as OA (Lin et al. 2019), bone metastasis (Marturano-Kruik et al. 2018), and muscle injury (Agrawal et al. 2017). Examples of pharmacological agents that were tested in musculoskeletal MPS for treating OA include the glucocorticoids dexamethasone and triamcinolone, celecoxib, rapamycin, and HYADD®4 (hyaluronic acid alkylamide) (Lin et al. 2019; Occhetta et al. 2019; Rosser et al. 2019). The physiological relevance and clinical applicability of the MPS depends critically on optimal combination of biomaterial scaffold, appropriate cells, and biochemical and physical signals. Table 1 summarizes the biomaterials, cells, and signals utilized in representative MPS developed to recapitulate musculoskeletal tissues.

Selection of Biomaterials Most MPS employ hydrogels as the cell carrier to create a 3D culture environment. In addition to their biocompatibility and high water content, a major advantage of hydrogels in MPS is their injectability, allowing the cell-laden structure to crosslink in situ and conform to a desired geometry. Among the frequently used hydrogels are collagen (Sakar et al. 2012), fibrin (Rosser et al. 2019), and gelatin (Lin et al. 2014). Devitalized bone, as a natural biomaterial, possesses the microstructural, biochemical, and mechanical properties of native bone, provides an inductive niche for mineralization and angiogenesis, and has been successfully used in bone-on-a-chip systems (Marturano-Kruik et al. 2018; Grayson et al. 2010).

Cell Types As multiple cell types are found in the majority of human tissues, MPS must also replicate this characteristic. Both stem cells and terminally differentiated cells have been utilized to construct MPS. Previous research has shown the

Table 1 Representative MPS that capitulate musculoskeletal tissues/organs, the target tissues/organs, and the biomaterials, cells, and type of stimuli employed

Target tissues	Biomaterials	Cell types	Signals/stimuli	Physiological/clinical relevance	Ref.
Cartilage	PEG-based hydrogel	Human chondrocytes	Mechanical compression; dexamethasone, IL-1Ra, rapamycin, celecoxib treatment	Mechanical overload induced OA phenotype in the tissue	Occhetta et al. (2019)
Cartilage	Fibrin	Equine chondrocytes	Exposure to TNF-α and IL-1β and steroid (triamcinolone) treatment	The microtissues emulate basic properties of native cartilage and respond to biochemical insults and concurrent steroid treatment	Rosser et al. (2019)
Cartilage and synovium	Fibrin	Human articular chondrocytes and synovial fibroblasts	Synovial fluid from OA patients	Synovitis is characterized by the increased infiltrating monocytes and macrophages in synovium (to be verified in model)	Mondadori et al. (2018)
Bone	Devitalized bovine trabecular bone	Human MSCs	Interstitial flow of medium	Geometrically complex temporomandibular joint condylar bone structures were created with high structural and biologic fidelity	Grayson et al. (2010)
Bone	N.A. (cells directly seeded in PDMS mold)	Murine preosteoblast MC3T3-E1	Breast cancer cells MDA-MB-231-BRMS1^{GFP} and MDA-MB-231^{GFP}	Formation of mineralized collagen was seen in the chip and hallmarks of breast cancer bone colonization observed	Hao et al. (2018)
Bone	Fibrin	Human MSCs, osteo-differentiated MSCs and HUVECs	Breast cancer cell MDA-MB-231	A vascularized bone-mimic was generated to study breast cancer cell extravasation and test drugs	Jeon et al. (2015)
Bone	Decellularized bovine bone	Human MSCs and HUVECs	Breast cancer cell MDA-MB-231	Drug resistance and progression of breast cancer cells colonizing the bone were observed in the bone perivascular niche-on-a-chip	Marturano-Kruik et al. (2018)

Osteochondral tissue	Gelatin	Human MSCs	Interleukin (IL)-1β stimulation	IL-1β treatment of either bone or cartilage induced degenerative responses in the other tissue, suggesting their crosstalk	Lin et al. (2014)
Osteochondral tissue	Gelatin	Human iPSCs	IL-1β stimulation on cartilage; celecoxib treatment	The presence of bone aggravated cartilage degeneration	Lin et al. (2019)
Skeletal muscle	Collagen type I	Murine skeletal muscle cell line C2C12	Electrical stimulation	The engineered muscle microtissues contracted when electrically stimulated	Shimizu et al. (2015)
Skeletal muscle	Collagen type I with Matrigel™	Optogenetically encoded myoblasts C2C12 cell expressing Channelrhodopsin-2	Optical stimulation	The device allows convenient testing of the effects of various factors on muscle maturation, structure, and function	Sakar et al. (2012)
Skeletal muscle	Gelatin	C2C12 cell	Cardiotoxin treatment	Cardiotoxin induced structural destruction and reduced passive tension in engineered muscle tissue	Agrawal et al. (2017)
Neuromuscular junction	N.A. (scaffold-free culture in a PDMS chip)	Human skeletal muscle myocytes and human iPSC-derived motoneuron	Electrical stimulation and treatment with 3 NMJ toxins	Muscle contraction induced by motoneuron activation declined or ceased with toxin treatment	Santhanam et al. (2018)

feasibility of generating heterogeneous tissues from a single stem cell source through the perfusion of separate induction medium streams (Lin et al. 2014). Cells isolated from diseased tissues can bring with them "memories" of the disorders, thus displaying different phenotypes in vitro compared to those harvested from healthy tissues. For example, chondrocytes from healthy and OA joints were found to possess altered chondrogenic potential (Yang et al. 2006). Many musculoskeletal diseases are patient-specific, with varying stages, genetics, etiology, and drug sensitivity in different individuals. Using individual-specific cells, MPS offer a personalizable approach to understanding disease mechanisms and testing treatment options. Although the use of primary patient cells best recapitulates patient specificity, it is impractical when large cell numbers are needed. iPSCs, with an almost unlimited proliferative capacity, overcome this limitation. Although there is still a long way to go to realize wide clinical applications of iPSCs due to their possible immunogenicity and tumorigenicity and the presence of genetic and epigenetic aberrations, the use of patient-specific iPSCs has contributed significantly to the advancement of precision medicine (Tabar and Studer 2014; Sayed et al. 2016). For example, MPS engineered from iPSCs can serve as an individualized platform for various preclinical tests, thus facilitating the identification of potentially efficacious therapies tailored to individual patients.

Stem cell-derived organoids have been extensively researched to study human physiology and build disease model-in-a-dish (Lancaster et al. 2013). As tissue analogs generated in vitro, organoids are recognized for their higher biological fidelity and have been used in MPS to generate organoid-on-a-chip systems (Skardal et al. 2016). MPS create a dynamic microenvironment mimicking in vivo conditions such as continuous fluidic flow and varying oxygen concentrations, promoting communications between multiple cell/tissue components, and generating potentially more clinically relevant organoid responses to toxins, drugs, and potential medications. In the organoid-on-a-chip research community, increasing attention has been drawn to the use of custom-designed MPS that allow multi-organoid integration to ultimately generate body-on-a-chip systems with sufficient physiological relevance for accurate prediction of native tissue/organ responses to selected treatments (Skardal et al. 2016).

Biochemical and Physical Stimulations Cells and tissues grown in musculoskeletal MPS can be subjected to various forms of environmental signals, including biochemical (Lin et al. 2014), mechanical (Occhetta et al. 2019), optical (Sakar et al. 2012), and electrical (Santhanam et al. 2018) stimulations, and the elicited responses constitute the MPS readouts. Generally, the application of MPS cultures involves three distinct stages: (1) tissue maturation, (2) disease modeling, and (3) therapeutic drug testing. In the first stage, biochemical signals provided by signaling molecules such as growth factors are usually required if the differentiation of stem cells or progenitors is involved. Other stimulations, including mechanical loading or stretching, interstitial fluid flow, and electromagnetic field, have been shown to be beneficial for enhancing matrix deposition and/or functions of

musculoskeletal tissues (Langelaan et al. 2011; Mauck et al. 2000; Grayson et al. 2010). The most common strategy for generating musculoskeletal disease models in MPS is through the introduction of biochemical insults by proinflammatory cytokines or toxins (Lin et al. 2014; Agrawal et al. 2017). Given the high prevalence of mechanical injury-caused musculoskeletal disorders, hyperphysiological mechanical stress-induced pathogenesis is also believed to be of high clinical relevance (Occhetta et al. 2019). Once tissue abnormalities are observed, potential therapeutics targeting the corresponding mode of tissue injury can be introduced to test efficacy in disease modification as well as to evaluate potential toxicity. In some studies disease modeling and drug testing steps occur simultaneously (Rosser et al. 2019). When therapeutics are introduced prior to and/or along with disease causative agents, MPS may provide valuable information on the drugs' efficacy in not only disease treatment but also disease prevention.

Generally, in the design of the MPS, there exists a tradeoff between device complexity and throughput in the process of balancing the level of biological accuracy and the required technological elements, and it is highly dependent on the needs of the application (Arrigoni et al. 2020). In basic, mechanistic studies, much higher levels of physiological accuracy are desired than in drug screening applications, where throughput and access to resources are important considerations.

As an example, MPS modeling of human synovial joints can serve different purposes. For investigating the pathogenesis and etiology of joint disorders like OA, it is critical to take into account the crosstalk and communication among different joint tissues because OA has long been considered a whole-joint disease (Loeser et al. 2012). The incorporation of multiple tissue components, including cartilage, bone, synovium, and infrapatellar fat pad, in the MPS would be critical to create sufficient physiological relevance. A particular challenge posed by the highly prevalent and debilitating disease of OA is the absence of an effective, FDA-approved disease-modifying medication. A number of candidate disease-modifying OA drugs have been proposed for OA treatment, and there is thus an urgent need of a convenient, efficient, and reliable model for high-throughput drug screening. For this purpose, MPS with low technical sophistication and high cost-effectiveness would be more appropriate. Nevertheless, we believe that investigations using a physiologically relevant and complex MPS, after extensive evaluation and validation, can offer useful guidance, e.g., identification of key readouts, to inform the design of practical and high-throughput systems.

4 Summary and Future Perspectives

It has become clear that intimate crosstalk between different cell types and cell-matrix interactions significantly influence cell proliferation, migration, and differentiation during the regeneration process (Kuraitis et al. 2012; Marsell and Einhorn 2011). Therefore, the traditional 2D culture using one cell type is less informative in predicting future clinical outcomes of potential regenerative treatments. However,

2D cultures are relatively fast and inexpensive and have lower technical requirements compared to animal models and MPS. Thus, conventional 2D dish culture should serve as the first and high-throughput platform to exclude the drugs that display obvious cytotoxicity or inefficacy for further tests. In addition, a 2D dish model allows the easy manipulation of cells, such as gain- or loss-of-function assessment, which enables mechanistic study. The use of animal models is able to partially mimic the local and global physiological changes in humans, recapturing the cell-cell and tissue-tissue crosstalk in tissue injury and repairing processes. In addition, by targeting different mechanisms, animal models are able to simulate different injuries in humans. Therefore, animal models, in particular those using clinically relevant animals, still represent the most powerful models in predicting treatment outcomes in humans. However, as has been recognized for a long time, due to the inherit difference in physiology and anatomic structure, the translation from animal models to human is challenging and not straightforward (Muschler et al. 2010). For example, autologous chondrocyte implantation, a clinically used regenerative method to treat chondral defects, showed robust cartilage repair in the first animal study in rabbits (Grande et al. 1989). However, this technology is mostly only applicable for the treatment of focal defects, and the clinical benefits over older techniques such as microfracture are sometimes questioned (Mollon et al. 2013). Such potential outcome discrepancies between animal models and human patients must be considered when translating regenerative therapies from animal studies to human clinical applications.

As mentioned above, the MPS and organoids have emerged as the next generation of models to assess the utility of regenerative medicine in treating musculoskeletal diseases. The advantages and disadvantages of these technologies, however, are both obvious. With the use of human cells and connective tissues in the manner that is observed in vivo, we may be able to recapitulate the tissue crosstalk and repair processes in humans. The major limitation of current MPS is the insufficient fidelity in replicating native tissues on the anatomical structure, phenotype, or function. In addition, the simulation of some physiological activities, such as mechanical loading, still presents significant technical challenges. Kaarj and Yoon (2019) recently reviewed the methods of delivering mechanical stimuli to MPS. With the progress in scaffold fabrication technologies, in particular 3D printing, as well as the tissue-specific differentiation of iPSCs, the rapid evolution of MPS is expected in the near future. It is noteworthy that immune cells, such as macrophages, which have been shown to play a major role in tissue regeneration (Wynn and Vannella 2016), are often neglected in current MPS. Therefore, successful development and application of MPS requires collaboration among experts from different disciplines and research fields.

The different etiologies and pathogenesis of musculoskeletal injuries and diseases further amplify the challenges in developing generally accepted regenerative treatments. Additional heterogeneity of musculoskeletal injuries and responses to treatments also arise from the patient's genetic background; for example, single nucleotide polymorphisms between the major and minor alleles of expressed genes have recently reported to display significantly different regulatory activities (Klein

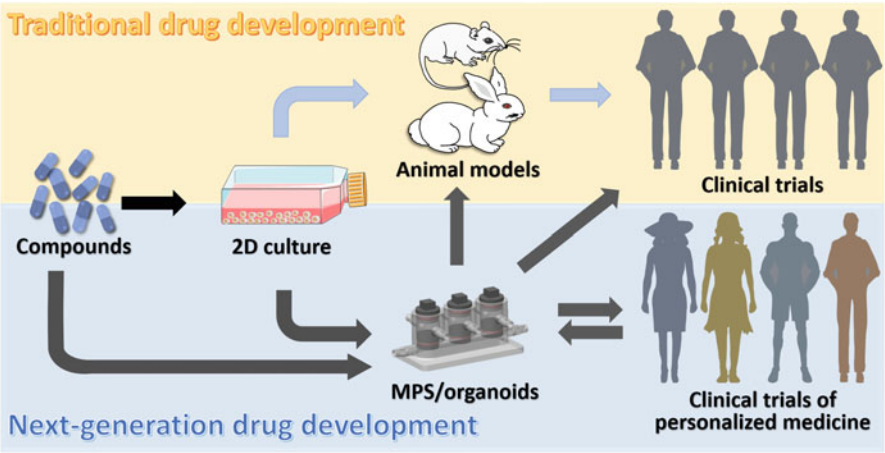

Fig. 3 Schematic of traditional and next-generation drug development pipelines

et al. 2019). Therefore, future models must have the capacity to model patient-specific physiology and pathology, in order to allow the development of personalized regenerative treatments. MPS will be the most promising models for such applications. In particular, given that iPSCs have theoretically unlimited expansion capacity as well as potential to generate all human tissues/organs (Shi et al. 2017), MPS derived from patient-specific iPSCs should possess unique advantages over animal models in developing personalized medicine (Fig. 3). However, significant technological advances in stem cell biology to achieve controlled differentiation of iPSCs are clearly needed.

The key requirement for a clinically relevant model is the capability to replicate the human physiology, under both normal and disease conditions, as well as to precisely predict the human response to a treatment. Achieving this relies on extensive validation. In particular, the model should be able to replicate the success or failure of known treatments that have been used clinically. Owing to the limited number and relatively short history of regenerative medicine products that have been used in clinical applications, the validation of new regenerative medicine products with known treatment is often not feasible. Thus, the establishment of a "gold standard" translation pathway is urgently needed, which will require the collaboration among practitioners of life science, pathology, engineering, and clinical medicine.

Acknowledgments The authors thank Ms. Yuchen He for her assistance with creating Fig. 3. This work was supported by funding from the National Institutes of Health (UG3/UH3TR002136).

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Tumor Models and Cancer Systems Biology for the Investigation of Anticancer Drugs and Resistance Development

Érica Aparecida de Oliveira, Colin R. Goding, and Silvya Stuchi Maria-Engler

Contents

Abstract

The landscape of cancer treatment has improved over the past decades, aiming to reduce systemic toxicity and enhance compatibility with the quality of life of the patient. However, at the therapeutic level, metastatic cancer remains hugely challenging, based on the almost inevitable emergence of therapy resistance. A small subpopulation of cells able to survive drug treatment termed the minimal residual disease may either harbor resistance-associated mutations or be phenotypically resistant, allowing them to regrow and become the dominant population in the therapy-resistant tumor. Characterization of the profile of minimal residual disease represents the key to the identification of resistance drivers that underpin cancer evolution. Therapeutic regimens must, therefore, be dynamic and tailored

The title of this chapter has been changed to Tumor Models and Cancer Systems Biology for the Investigation of Anticancer Drugs and Resistance Development

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_369

to take into account the emergence of resistance as tumors evolve within a complex microenvironment in vivo. This requires the adoption of new technologies based on the culture of cancer cells in ways that more accurately reflect the intratumor microenvironment, and their analysis using omics and system-based technologies to enable a new era in the diagnostics, classification, and treatment of many cancer types by applying the concept "from the cell plate to the patient." In this chapter, we will present and discuss 3D model building and use, and provide comprehensive information on new genomic techniques that are increasing our understanding of drug action and the emergence of resistance.

Keywords

2D- and 3D-culture models · Cancer drug resistance · Cancer system biology · Drug-testing platform

Abbreviations

2D	Two-dimensional space
3D	Three-dimensional space
ABL	Abelson murine leukemia viral oncogene homolog 1
APE1	Apurinic/apyrimidinic endonuclease 1
BCR	The breakpoint cluster region protein
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRAFi	BRAF inhibitor
BRCA	Breast cancer gene
CAF	Cancer-associated fibroblasts
CancerDR	Cancer drug resistance database
CCLE	Cancer Cell Line Encyclopedia
ChIP-seq	Chromatin immunoprecipitation sequencing
CML	Chronic myeloid leukemia
CRISPR	Clustered regularly interspaced short palindromic repeats
CSC	Cancer stem cells
CTRP	The Cancer Therapeutics Response Portal
DNA	Deoxyribonucleic acid
DNA-seq	DNA sequencing
EC	Endothelial cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
FDA	Food and Drug Administration
GDSC	Genomics of Drug Sensitivity in Cancer
GEO	Gene Expression Omnibus
HER2	Human epidermal growth factor receptor-type 2
HFL-1	Human lung fibroblast cell line
Hh	Hedgehog signaling pathway
HMVEC	Human dermal blood microvascular endothelial cxells

IC_{50}	The half maximal inhibitory concentration
ICGC	International Cancer Genomics Consortium
IL	Interleukin
LMTK3	Lemur tyrosine kinase 3
lncRNA	Long non-coding RNA
MEK	Mitogen-activated protein kinase kinase
MicroRNA	Small non-coding RNA molecule
MIO	Gold and magnetic iron oxide
miRNA-seq	MicroRNA sequencing
MITF	Microphthalmia-associated transcription factor
MMP	Matrix metallopeptidase
MSC	Mesenchymal stem cells
multi-OMICS	Multiple omics
NCBI	National Center for Biotechnology Information
NIH	National Institute of Health
NLM	National Library of Medicine
NRAS	Neuroblastoma RAS viral oncogene
p53	Tumor protein p53
p-AMPKa	Phospho-5′ adenosine monophosphate-activated protein kinase
Panc-1	Human pancreatic cancer cell line
Par-4	Prostate apoptosis response 4
PARP	Poly ADP-ribose polymerase
PC9	Non-small cell lung cancer
PCR	Polymerase chain reaction
PDMS	Porous polydimethylsiloxane
PDO	Patient-derived organoids
p-ERK	Phospho-extracellular signal-regulated kinase
PKM2/PKM1	Pyruvate kinase isozymes M1/M2
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RPPA	Reverse-phase protein array
SC	Physiological stem cells
scRNA-seq	Single-cell RNA sequencing
sFRP2	Secreted frizzled-related protein 2
SNP	Single nucleotide polymorphisms
SPCA-1	Human non-small cell lung cancer cell line
TALEN	Transcription activator-like effector nuclease
TAM	Tumor-associated macrophages
TCGA	The Cancer Genome Atlas
TKI	Tyrosine kinase inhibitors
UK	United Kingdom
USA	United States of America
WNT	Wingless-related integration site

1 Introduction

In the last decades, tumor therapies have changed from an era of a majority of nonspecific chemotherapeutics to one characterized by therapies targeted against specific molecules crucial for tumor survival and growth. This breakthrough was only possible with the discovery of specific molecules that could be pharmacologically targeted to improve the success rate in treatment, with the added benefit of reducing toxicity in patients (Peeper 2014). Initial achievements included drugs targeting the tyrosine kinase BCR-ABL for chronic myeloid leukemia (CML), HER2 (human epidermal growth factor receptor-type 2) in breast cancer, PARP (Poly ADP-ribose polymerase) inhibitors for breast cancer gene BRCA-positive, ovarian and prostate cancer, and BRAF (v-Raf murine sarcoma viral oncogene homolog B) and MEK (Mitogen-activated protein kinase kinase) inhibitors for BRAF melanomas (Afghahi and Sledge 2015; Curtin and Szabo 2013).

Unfortunately, although such highly targeted drugs can exhibit substantial initial success, drug resistance leading to relapse is a frequent and sometimes almost inevitable undesirable reality. Resistance can present as an acquired resistance after an initial and successful response or as intrinsic resistance, in which resistance is present since the beginning of the treatment (Schmidt and Efferth 2016; Peeper 2014). For example, in a study with melanoma patients harboring an activating BRAF mutation, around 15% were observed to present intrinsic resistance to BRAF inhibitors, and the rest, despite the initial response, became resistant and relapsed in the first year (Girotti et al. 2014).

Drug resistance, the primary cause for therapy failure in cancer patients, arises from intratumor heterogeneity that can be subdivided into genetic and non-genetic or phenotypic heterogeneity. While genetic heterogeneity can arise through a variety of mechanisms, including replication errors, therapy-induced DNA damage, and repair defects, phenotypic heterogeneity occurs as a consequence of the impact of the intratumor microenvironment (Rambow et al. 2019).

While irreversible genetic lesions acquired in tumor progression may provide a selective advantage in some circumstances, leading to clonal selection, once the selective pressure is removed, the same lesions may confer a disadvantage. An example is tumor cells in a proliferative state demanding more nutrient uptake. To solve this, the tumor grows in a chaotic way through abnormal vessels generating nutritional and hypoxic stress due to the poor blood flow. In that way, distant cells will be even more exposed to nutritional stress than before (Keibler et al. 2016; Nagy et al. 2009; Vaupel et al. 2004).

By contrast, phenotypic heterogeneity imposed by a dynamic microenvironment is not fixed but may be reversible, allowing cells to exploit the plasticity of the cancer genome to promote growth and survival in the face of different microenvironmental stressors. These might include hypoxia, nutrient limitation, inflammatory signaling, and interactions with components of the stroma (García-Jiménez and Goding 2019). Most notably, some phenotypic states are tolerant to both targeted and immunotherapies and can therefore act as a therapy-resistant reservoir from which

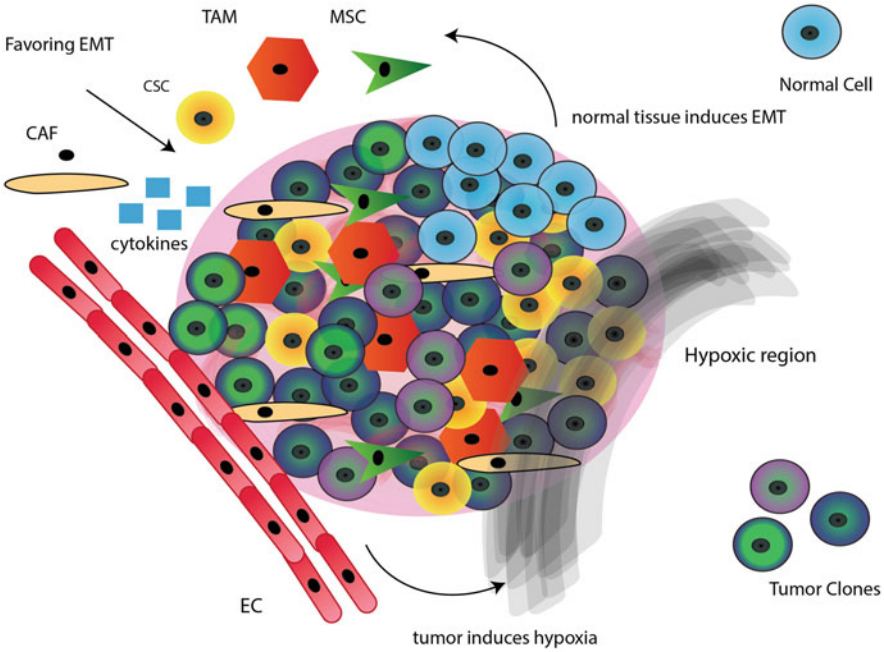

Fig. 1 Cancer stem cells (CSC) inside the heterogeneous tumor niche, enclosed by cancer-associated fibroblasts (CAF), tumor-associated macrophages (TAM), endothelial cells (EC), mesenchymal stem cells (MSC), and extracellular matrix (ECM). The CSCs are positioned in a hypoxic region and are getting stimuli from adjacent cells that increase drug resistance

cells may emerge that are genetic or epigenetically therapy-resistant (Rambow et al. 2019).

Although tumors usually have a monoclonal origin and substantial genome-wide data from bulk tumors is available from genomic and transcriptomic databases, including the The Cancer Genome Atlas (TCGA) and the International Cancer Genomics Consortium (ICGC), recent advances in lineage tracing, genomic sequencing (Burrell and Swanton 2014), as well as single-cell RNA sequencing are beginning to dissect the contribution of genetic and phenotypic heterogeneity to tumor progression. For example, in melanoma, single-cell RNA sequencing evidence suggests that within tumors there coexist multiple phenotypic states, each underpinned by a distinct gene expression program, some of which may be induced by therapy and exhibit drug tolerance. Among the drug-tolerant populations, increasing evidence appears to suggest that for many, and perhaps most cancer types, there exist so-called cancer stem cells (CSCs) that resemble like physiological stem cells (SCs) that preserve the ability for self-renewal and differentiation leading to heterogeneous clones. CSCs also retain high tumorigenicity capacity and are resistant to radiation, chemo-, and immunotherapies, allowing them to fuel relapse and tumor expansion after treatment (Fig. 1) (Al-Hajj et al. 2003).

Although the origin of CSCs has been the topic of debate, it seems likely that they arise as an adaptive pro-survival response to exposure to prolonged stresses within the intratumor environment where cells less able to adapt undergo cell death. Frequently, CSCs reside in specific niches that consist of different types of stromal cells, including endothelial cells, mesenchymal cells, immune cells, and fibroblasts (Li and Xie 2005; Liu et al. 2013) that secrete signals that may affect the probability of CSCs remaining dormant, or may boost an imbalance between CSC self-renewal and differentiation, leading to the proliferation of tumor cells and subsequently invasion and metastasis (Ishimoto et al. 2014; Daverey et al. 2015). Notably, while the process of metastasis is notoriously inefficient, those cells that survive dissemination may frequently stay in a dormant state for long periods of time and reawaken at a later resulting in disease relapse. Since the disease arising as a consequence of reawakening of dormant cells will recapitulate heterogeneity, dormant cancer cells may also represent a form of CSC (Aguirre-Ghiso 2007; Rambow et al. 2019).

Thus while therapies based on targeting specific driver mutations can be successful, the emergence of resistance means that alternative therapeutic strategies may be needed. Most notably, therapeutic vulnerabilities arising as consequence of the stresses present in the tumor microenvironment may be exploited by targeting the adaptive responses that promote survival. Equally, directed phenotype switching by which phenotypically therapy-resistant subpopulations may be induced to adopt a sensitive phenotype may also provide significant patient benefit. Consider that, contrasting to drug resistance, drug tolerance associates to a state in which tumor cells can survive, but not proliferate during therapy (Chisholm et al. 2015). To put in another way, oncogenic mutations are driven cancer progression (Bernards and Weinberg 2002) create an opening in which a phenotypic transition may occur (Hoek and Goding 2010). In fact, the phenotypic plasticity can also be used as a target for pharmacologic intervention aiming to stimulate cells to a state that is drug-sensitive (Sáez-Ayala et al. 2013) or by targeting the changes that lead to the specific phenotypic states (Gupta et al. 2009; Rambow et al. 2018).

Although it is increasingly apparent that phenotypic heterogeneity plays a key role in metastatic dissemination, resistance to therapy, and disease relapse, it is also evident that cell monolayer culture systems are inadequate to address key outstanding questions regarding the origins of cancer cell quiescence, drug-detoxifying capability, resistance to DNA damage, and tolerance to therapy (Viale and Draetta 2016).

In this sense, multi-OMICS data may help to improve drug efficacy and resensitize resistant cells to targeted therapies in a way to improve the quality of life of patients (Chakraborty et al. 2018). This chapter therefore focuses on the different models available that have been employed to elucidate microenvironmental regulation of tumor cell progression and resistance. In particular, we discuss alternatives to animal testing, such as 2D and 3D models, microfluidic-based models, and system biology approaches. Collectively, such microenvironment-mimicking model

systems provide new opportunities and tools to probe therapy-resistant niches and to elucidate the molecular mechanisms regulating tumor progression.

2 Methods for Modeling and Overcoming Resistant Tumors

2.1 2D Models

Although 2D cell culture was developed in the early nineteenth century, it persists as the most frequently used in vitro method for drug discovery owing to its simplicity, reproducibility, and low cost (Breslin and O'Driscoll 2013; Chatzinikolaidou 2016; Niu and Wang 2015; Zips et al. 2005). In 2D models, cells are usually cultured and grown as a monolayer on flat surfaces such as in cell culture flasks or wells. For nutrients like glucose, the medium acts as a finite source and needs to be replaced periodically (Kieninger et al. 2018). In this sense, cultures of only one cell type are intrinsically limited in terms of generating a suitable environment for the study of tumors. This can be improved by co-culture with different cell types, resulting in cell–cell communication and interactions that cannot be assessed in a monoculture of a single-cell type (Mao et al. 2013; Kapałczyńska et al. 2016).

The main advantage of the 2D co-culture model is that it allows an easy and simple method to study specific conditions of the tumor microenvironment (Horvath et al. 2016; Imamura et al. 2015). Although conventional 2D culture can be used to perform a vast range of experiments such as protein expression, cytotoxicity, migration, and adhesion assays, all aspects of cancer progression in vivo, cancer cells grow in 3D and interact with the stroma, something that cannot readily be mimicked with a 2D model. For instance, melanoma cancer cells grown in 2D cultures can easily be killed by low doses of chemotherapeutic drugs or low doses of radiation, while in vivo their activity can be affected by key factors such as restricted access of tumor cells to nutrients and oxygen, the metabolic conversion of drugs within the cells, or the effect of the immune response (Cruz Rodríguez et al. 2019).

It is not an overstatement to say that all work aimed at studying drug resistance will rely at an early stage on traditional cell culture. As an example, Stebbing et al. (2018) studied the effects of LMTK3 (Lemur tyrosine kinase 3) expression in doxorubicin resistance. In this paper, they began by comparing the cytotoxicity in both 2D and 3D models, before moving to RNA-seq, animal models, and immunohistochemistry of patient-derived tissue. Lovitt et al. (2018) showed that 3D cultures demonstrated a higher resistance to therapies than 2D cell cultures, showing the importance of cell-to-extracellular matrix interaction and the different environment of cells in 3D in resistance.

2.2 3D Models

Traditional in vitro culture techniques can also create three-dimensional tumor arrangements which are more physiologically relevant than 2D models and more cost-efficient and ethically acceptable than animal models (Asghar et al. 2015). Three-dimensional (3D) cell cultures are attractive because they mimic the in vivo microenvironment more closely and show more organotypic features than 2D cultures. These properties make them promising models for drug and toxicity screening (Griffith and Swartz 2006; Pampaloni et al. 2007; Simian and Bissell 2017).

Today, spheroids are one of the most used 3D cell culture models. Tumor spheroids resemble avascular in vivo tumors and present similar diffusional aspects of oxygen transfer, nutrients, and waste through cell–cell interactions and cell–extracellular matrix interactions. With appropriate culture conditions, it is possible to achieve layers of cells within the tumorsphere with normoxic and hypoxic states, which reflect the in vivo microenvironment encountered by many cells within a tumor (Grimes et al. 2014; Raghavan et al. 2016). 3D cell culture systems do not require specialized equipment and remain a very useful tool to understand specific aspects of each tumor and to develop and test drugs and appropriate drug delivery systems in a more physiological relevant situation (Vörsmann et al. 2013; Huanga and Gao 2018). Their more accurate recapitulation of the intratumor microenvironment makes 3D cultures attractive in the study of several aspects of basic tumor biology, such as tumor growth kinetics, metabolism, invasion and migration, cancer stem cells and their niche, microenvironment signaling crosstalk, and mechanisms of therapy evasion (Nunes et al. 2019; Nath and Devi 2016).

3D culture methods can be divided into those which are scaffold-free, such as suspension cultures as well as non-adherent surface or hanging drop methods and scaffold-based cultures. Scaffold-free 3D cell cultures self-assemble with no artificial platforms being used to promoting normal or tumoral cell growth (Fig. 2) and can be used to promote the formation of 3D microtissues as cellular aggregates like spheroids or multicellular tumor spheroids in which cells release their own extracellular matrix (Knight and Przyborski 2015).

Hanging droplets are a method for scaffold-free culture of non-adherent microtissues. Cells aggregate and form spheroids at the bottom of the hanging drop under the influence of gravity, where only a liquid/gas interface exists. Droplet size can be constantly maintained through balancing inflow and outflow by active pumping, or surface tension-driven flow (Frey et al. 2014; Kim et al. 2015; de Groot et al. 2016). Importantly, there are commercial plates specially designed to generate hanging drops in a reproducible way using an automated robot to achieve high-throughput drug screening (Tung et al. 2011; Penfornis et al. 2017).

To generate spheroids by suspension culture, cells are suspended in swirling liquid medium using a rotational motion to prevent cell attachment to the culture surface. The swirling cells then form spheroids through collisions and subsequent agglomeration (Carpenedo et al. 2007; Yagi et al. 1993; Mehta et al. 2012).

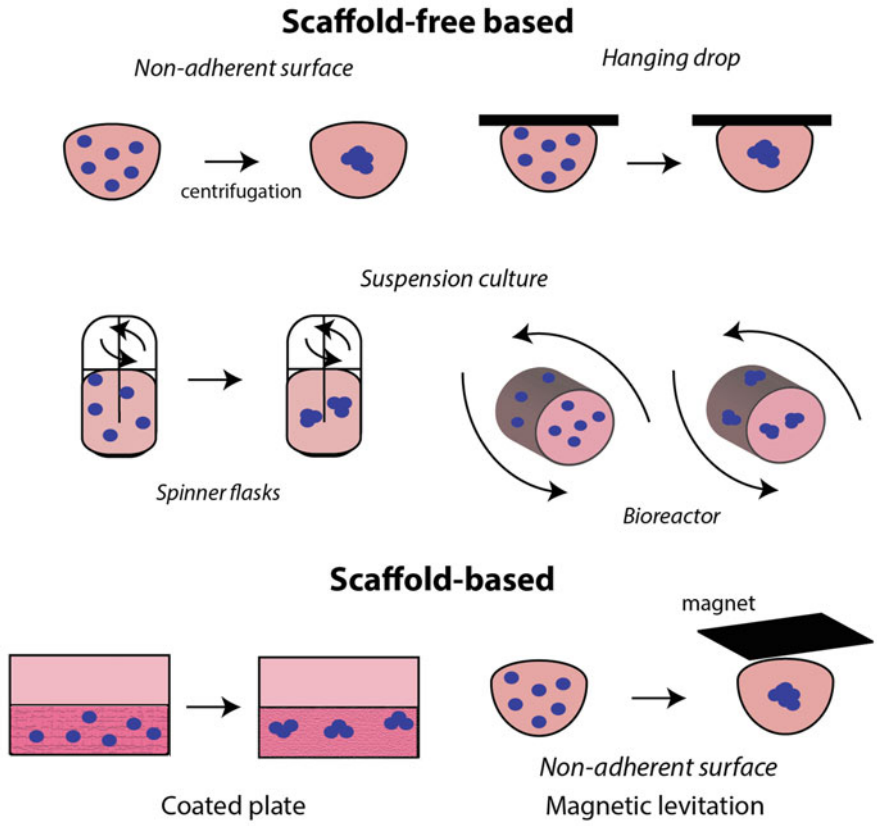

Fig. 2 Scaffold-Free and Scaffold-Based methods for 3D cell cultures

Instead of agitating medium, the non-adherent surface method avoids cell adhesion by using adhesion-free surfaces and facilitates spheroid formation directly (Fennema et al. 2013). It is also possible to use extracellular matrix (ECM) and protein-based hydrogel coatings to create spheroids (Ekert et al. 2014; Ivanov et al. 2014). For instance, in vitro 3D mesenchymal stem cells/stromal cells (MSC) culture using spinner flasks and a rotating wall vessel bioreactor can be beneficial for retaining MSC properties for a prolonged period (Bellotti et al. 2016; Cha et al. 2015; Frith et al. 2010). This technique has been used for large-scale production of tumor spheroids to test the efficacy of chemotherapy or immunotherapeutic drugs (Kloss et al. 2015; Phung et al. 2011; Youn et al. 2005). It has high reliability and reproducibility, but the fluid volume is limited and the method is not good for long-term culture due to difficulty in changing the culture medium (Mehta et al. 2012; Breslin and O'Driscoll 2013).

These 3D culture methodologies have been widely used to investigate several anticancer treatment approaches, such as chemotherapy and combination chemotherapy (LaBarbera et al. 2012; Patel et al. 2015), drug delivery systems (Kim et al.

2010; Mehta et al. 2012; Patel et al. 2015), gene and oncolytic virus therapy (Lamfers and Hemminki 2004), immunotherapy (Herter et al. 2017), photodynamic therapy (Dubessy et al. 2000; Evans 2015), phototherapy (Xiao et al. 2005), photothermal therapy (Moreira et al. 2016), and radiotherapy (Dubessy et al. 2000; Schwachöfer 1990). Especially regarding resistance, Lee et al. (2012), using 3D cell culture models, demonstrated that the efficacy of the chemotherapeutic response was strictly dependent on cell and tissue polarity and architecture. The combination reverted the phenotype and led to the development of a phenotype-based functional genetic screen that allowed the identification of novel genes associated with EGFR-TKI resistance (Lee et al. 2012).

In accordance, Ryabaya et al. (2019) proposed a combination of metformin and the selective MEK inhibitor binimetinib as a promising therapy against BRAF and NRAS mutant melanoma cells in vitro using both 2D and 3D human melanoma cells. They showed the combined treatment led to cell cycle arrest, reduced the number of melanoma-formed colonies, and inhibited cell invasion and migration associated with p-AMPKα upregulation and p-ERK downregulation, suggesting that this could be promising for clinical applications.

Tiago et al. (2014) compared melanoma cell growth in 2D culture and in a dermal equivalent model consisting of fibroblasts embedded in type I collagen matrix. They showed that the 3D model presented decreased expression of p53 after doxorubicin treatment, and this outcome was accompanied by induction of interleukin, IL-6, IL-8, and matrix metalloproteinases 2 and 9, suggesting that this dermal model more accurately reflects drug responses by recapitulating important pro-survival features of the tumor microenvironment.

In scaffold-based approaches, 3D cell cultures are maintained by cell growth on artificial 3D structures, where the cells attach, migrate, and fill the interstices within the structure resembling a microtissue (Knight and Przyborski 2015). The artificial structure could act as a simple mechanical support, but their properties are usually improved to contribute to interactions within the microenvironment, such as moieties, polarity, hydrophobicity, porosity, surface area, stiffness, and porous interconnectivity, and enable the exchange of nutrients, gases, and waste material in a similar way to that seen in vivo (Carletti et al. 2011; Knight and Przyborski 2015; Langhans 2018).

Patient-derived organoids (PDOs) have recently emerged as robust 3D models where a matrix is used as a scaffold to cultivate the cell from patients without any cell isolation. Vlachogiannis et al. (2018) published a model of metastatic gastrointestinal organoids and found 100% sensitivity, 93% specificity, 88% positive predictive value, and 100% negative predictive value in forecasting tumor response to targeted agents or chemotherapy in patients. Thus this “tumor-in-a-dish” allows to compare, and in the future to predict drug responses of patients. Thus, functional genomics tools and molecular pathology can guide the decision-making process of early phase clinical trials.

Artificial 3D structures can be produced with materials including ceramics, glass, polymers, and metals. Among these, natural or synthetic polymers are the most used because they allow their chemical and structural properties to be controlled (Carletti

et al. 2011). To recreate the 3D structure in a fashion much more mimetic to the tissue, it is possible to make use of resources such as 3D printing, electrospinning, foaming, freeze-drying, leaching, lithography, particulate molding, selective laser sintering, and solvent evaporation (Lu et al. 2013; Sultana et al. 2015). Natural biomaterials are attractive for in vitro applications because they are biocompatible and allow cell adhesion sites and exposure to endogenous chemokines and growth factors, which contribute to enhancing the viability and growth of the cells (Nath and Devi 2016). However, there are some disadvantages of scaffold-based 3D cell culture models, for instance, for drug toxicity studies suggest that interactions between the materials and drugs might affect their absorption and adhesion. Besides that, it is not trivial to isolate the cells, protein, and nucleic acids for further molecular investigation (Asghar et al. 2015; Gupta et al. 2016).

The generation and improvements of in vitro 3D skin tissue over the past decades provide models that can replace the need for animal experimentation and advance the ability for more personalized therapies. This is a more complex structure that uses primary skin-derived cells cultured in 3D that also allows for personalized mechanistic and translational studies. The 3D skin model is composed of fibroblasts and keratinocytes, but might also include melanocytes and cancer cells such as melanoma cells. Novel technologies can facilitate the automated production of the skin tissue in a reproducible and functional way. Currently, there are commercially available constructs for applications in humans and also for large-scale production, for example, pigmented skin transplants in the range of 100 cm^2 (Randall et al. 2018; Min et al. 2018).

The overlap of different biofabrication approaches, such as bioprinting and electrospinning, will address many important challenges, such as the incorporation of immune cells and other cell types to increase the ability to develop a physiological skin for drug testing and even personalized medicine and other pre-clinical applications. The incorporation of a microbiome to these physiologically relevant skin components is possible with novel bioengineering technologies and will provide cost-effective and reproducible generation of physiological skin in vitro (Randall et al. 2018).

Magnetic levitation is another technique that uses hydrogels containing gold and magnetic iron oxide (MIO) nanoparticles plus filamentous bacteriophages. Cells are treated with MIO-containing hydrogels, incubated overnight, and then plated into an ultra-low attachment plate with a lid with a neodymium magnet. Spheroids start forming within a few hours at the air–liquid interface due to levitation toward the magnet, and the speed of growth is high when compared to other methods. The size is in a range of mm^2 and is ideal for reproducing necrotic and hypoxic areas. Unfortunately, these beads are expensive and can be toxic to cells at a high concentration (Souza et al. 2010; Nath and Devi 2016; Tseng et al. 2013; Hoarau-Véchot et al. 2018).

Sandri et al. 2016 used different 2D and 3D models to investigate drug resistance in melanoma cells. They applied a 2D transwell invasion assay, a 3D spheroid model, and a reconstructed skin model to show that resistance to vemurafenib induces significant changes in the tumor microenvironment, mainly by upregulation

of MMP-2, with a corresponding increase in cell invasiveness. In a similar approach, Faião-Flores et al. (2017) suggested that targeting the Hh pathway in BRAFi-resistant melanoma may represent a viable therapeutic strategy to restore vemurafenib sensitivity, reducing or even inhibiting the acquired chemoresistance in melanoma patients. Also, the same group improved the human skin reconstruct model by mimicking aging skin, as described in Pennacchi et al. (2015). In this context, Kaur et al. (2016) used a similar model to evaluate whether age-related changes in dermal fibroblasts could drive melanoma metastasis and response to targeted therapy. They found that aged fibroblasts secrete a Wnt antagonist, sFRP2, which activates a multi-step signaling cascade in melanoma cells that result in a decrease in β-catenin and microphthalmia-associated transcription factor (MITF) and ultimately the loss of a key redox effector, APE1. Loss of APE1 attenuated the response of melanoma cells to DNA damage induced by reactive oxygen species, rendering the cells more resistant to targeted therapy (vemurafenib).

In summary, the use of organotypic cultures, such as artificial human skin, enables cell interactions with the ECM and with other cell types to be reproduced with greater fidelity. For these reasons, studies exploring the effects of drugs on tumor cells cultured in a biomimetic environment in vitro can better assist in unraveling the therapeutic action on tumor cells in a situation more closely resembling that which occurs in vivo.

2.3 Microfluidic Devices

Microfluidic devices have been used as in vivo-like physiological models that recapitulate cellular interactions and metabolism in health and disease. The inclusion of 3D matrices into microfluidics devices has allowed the evolution from 2D culture to 3D and multi-organ models. More recently, advances in bioengineering have culminated in highly complex 3D models that mimic several types of cancer, such as breast, lung, liver, or bone cancer. Importantly, the main application areas are in screening for anticancer drugs and basic research into cancer metastasis (Rothbauer et al. 2018; Whitesides 2006).

The main disadvantage of most on-chip models used for drug screening is that these microdevices usually have limited chambers that don't support the high-throughput needed to be an effective screening tool. In order to improve that, Xu et al. (2013) developed a system comprising hydrogel culture chambers in parallel for anticancer drug screening using co-culture of a human non-small cell lung cancer cell line (SPCA-1), a human lung fibroblast cell line (HFL-1), and patient-derived lung cancer cells. The model enabled detailed screening for the sensitivities of eight samples of patient-derived lung cancer cells to different anti-tumor therapies at the same time, enabling the correct doses and single as well as multi-drug chemotherapy schemes to be established. Another 3D microfluidic system containing a concentration gradient generator, called SpheroChip, enabled the on-chip development of spheroids of liver and colon cancer for drug sensitivity testing (Kwapiszewska et al. 2014). A different but promising method was established by Imura et al. (2010,

2012), who developed a bioassay system that can determine intestinal absorption, hepatic metabolism, and bioactivity of ingested molecules, including anticancer therapeutic drugs. This system assesses the output following administration of an anticancer drug to a microsystem that includes or excludes digestion processes. If drug activity was lost after passing through the system, the result indicates whether the drug was degraded by synthetic gastric juices.

Microfluidic systems represent more complex models because they capture important features of the tumor microenvironment such as vasculature, co-culture of multiple cell types, shear stress, pressure, and chemical and oxygen gradients (Fig. 3) (Shang et al. 2019).

The use of an endothelial cell monolayer is the most used approach to mimic blood vessel function in a microfluidic chip. The monolayer design also has the advantage of easily generating shear stress. Shear stress makes cells organize themselves to create a barrier function, and the level of complexity improves with the use of the endothelial cell monolayer to generate a circular endothelial cell tube and ultimately a functional vascular network (Bogorad et al. 2015). As an example, a device was developed to integrate a monolayer of human dermal blood microvascular endothelial cells (HMVECs) onto a porous polydimethylsiloxane (PDMS) layer to mimic blood vessels in a drug screening model (Dereli-Korkut et al. 2014). It was reported that human PC9 non-small cell lung cancer cells cultured in these devices had greater drug resistance to four different apoptotic inducers than two-dimensional cultured PC9 cells (Bai et al. 2015; Riahi et al. 2014). Similarly, Agarwal et al. (2017) created an approach to develop 3D vascularized human tumors with a complex 3D vascular network and used this model to investigate the effect of vascularization on cancer drug resistance using a high-throughput non-planar microfluidic encapsulation technology.

With the knowledge that tumors exhibit interstitial flow, there is also a corresponding fluid shear stress that, while very small compared with the intravascular one, could stimulate some oncogenic signaling pathways on cancer cells and favor vascular angiogenesis against the direction of the interstitial flow (Mitchel and King 2013; Avvisato et al. 2007; Swartz and Lund 2012; Song and Munn 2011). Elevated extracellular stresses increase tumor cell growth and generate a transport barrier to drug delivery, lowering the efficacy of therapies. Atypical solid stress is developed by the deregulated growth of tumor cells and increases drug resistance that could influence tumor cell gene expression, rearrange the ability of immune cells to kill cancerous cells, or even turn macrophages into tumor-promoting cells, enhancing the invasive potential of cancer cells (Stylianopoulos et al. 2012; Demou 2010; Facciabene et al. 2011; Goel et al. 2011; Wilson and Hay 2011).

Another feature addressed by microfluidic systems is the generation of chemical or oxygen gradients either by diffusion or convection that also can affect cancer progression and therapeutic efficacy (Somaweera et al. 2016; Brennan et al. 2014). Most of the microfluidic systems control the oxygen levels in culture chambers and can work by either inserting oxygen scavenging chemicals, gas supply channels near to the cell chamber, or oxygen impermeable materials (Byrne et al. 2014; Chang et al. 2014; Khan et al. 2017). Although the current devices can control chemicals

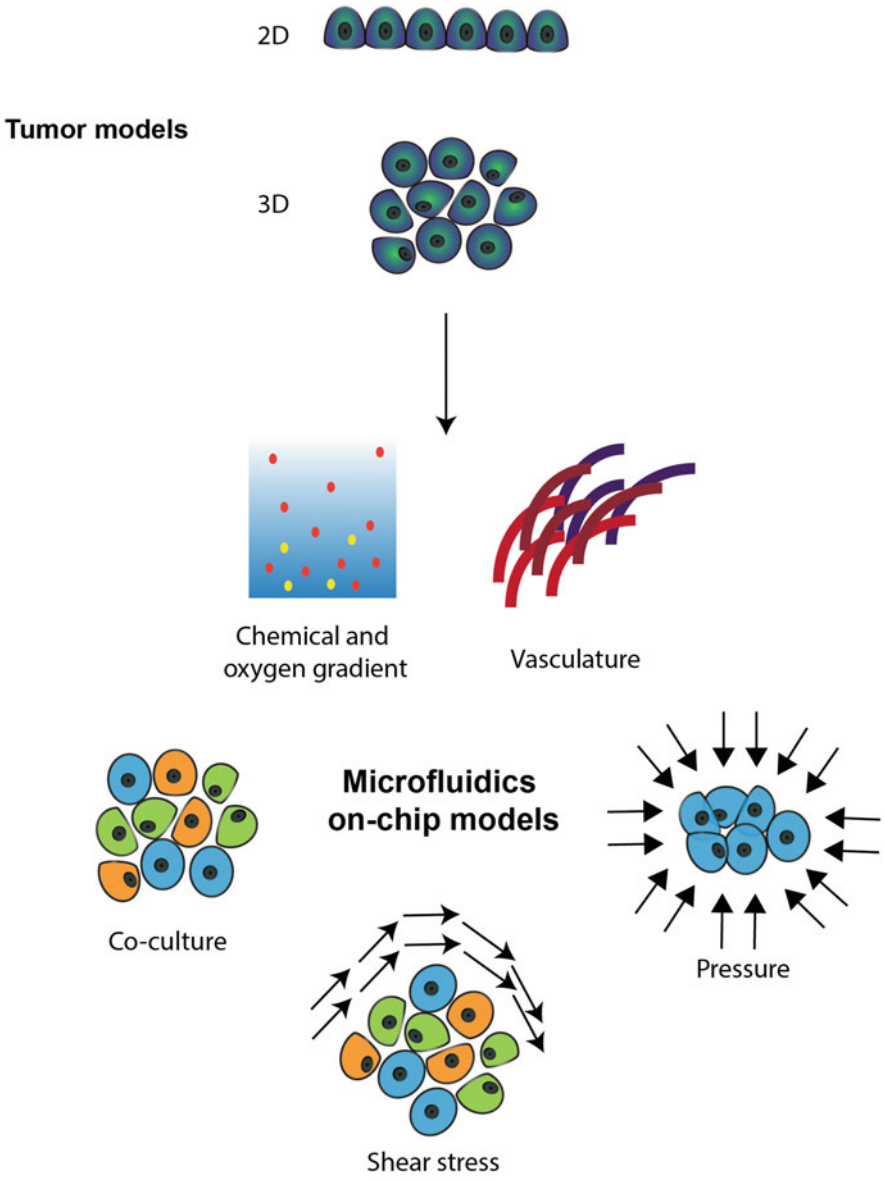

Fig. 3 Microfluids can play a critical role in mimicking the aspects of the tumor niche, including vasculature architecture, co-culture, shear stress, pressure, and chemical and oxygen gradients that tumors are exposed

and oxygen gradients independently, in order to better mimic the tumor microenvironment, they should be coordinated (Chang et al. 2014; Rodenhizer et al. 2016).

Finally, Pandya et al. (2017) described a 3D microfluidic device that can be applied for a real-time analysis of the success rate of a chemotherapeutic drug. The platform is integrated with microsensors that measure the change of electrical response in cancer cells in a 3D extracellular matrix when a chemotherapeutic drug is flowed next to the matrix. The authors showed that the device was able to delineate drug-susceptible, drug-tolerant, and drug-resistant cells in <12 h.

2.4 Bioinformatics/System Biology

Recent advances in biotechnology and bioinformatics have led to a dramatic increase in large-scale data acquisition, genomic and metagenomic data, and their corresponding related databases (Tang et al. 2015; Tatusova 2016). This has led to attempts using computer and mathematical modeling to understand how biological systems operate as a whole. Cancer systems biology methods can be used at different scales, from analysis of the behavior of an individual cell to a patient with a tumor. The most attractive feature of a systems biology approach is the possibility to combine the molecular aspects of tumors at different levels (DNA, RNA, protein, epigenetics, imaging), different intervals (seconds, days), and different conditions (before and after treatment) with integrative analysis. The major challenges are represented both by consequences of tumor heterogeneity and by the problems associated with the acquisition of high-quality data describing clinical attributes, pathology, and treatment response and consolidating the data into accurate predictive models (Werner et al. 2014).

A combination of advances in high-throughput, cost-effective, tissue-sparing technologies and high-speed computing resources that can analyze tumors at multiple levels has enabled a shift toward an integrated systems biology approach (Cancer Genome Atlas Network 2012; Gentles and Gallahan 2011; Basu et al. 2013). A system investigation of the molecular aspects of non-responsive or therapy-resistant tumors is appealing both in terms of unveiling mechanisms underlying resistance and the identification of approaches to overcome resistance (Fig. 4) (Werner et al. 2014).

The ability to access terabytes of data generated by large-scale "omics" approaches and online resources containing well-annotated high-throughput datasets including thousands of "omics" data collected from sensitive and resistant tumors provides an unparalleled opportunity for a systems biology approach to cancer management (Fornecker et al. 2019; Guang et al. 2018; Huang et al. 2019; Sadanandam et al. 2013; Nieman et al. 2011).

Integration of multi-OMICS data provides a platform that can link genomic and epigenomic alterations to associated transcriptomic, proteomic, and metabolomic profiles in response to a specific disease and/or treatment (Chakraborty et al. 2018). The multi-OMICS models underlying the investigation of cancer cells have the promise to reveal the complex molecular mechanisms underlying the expression of

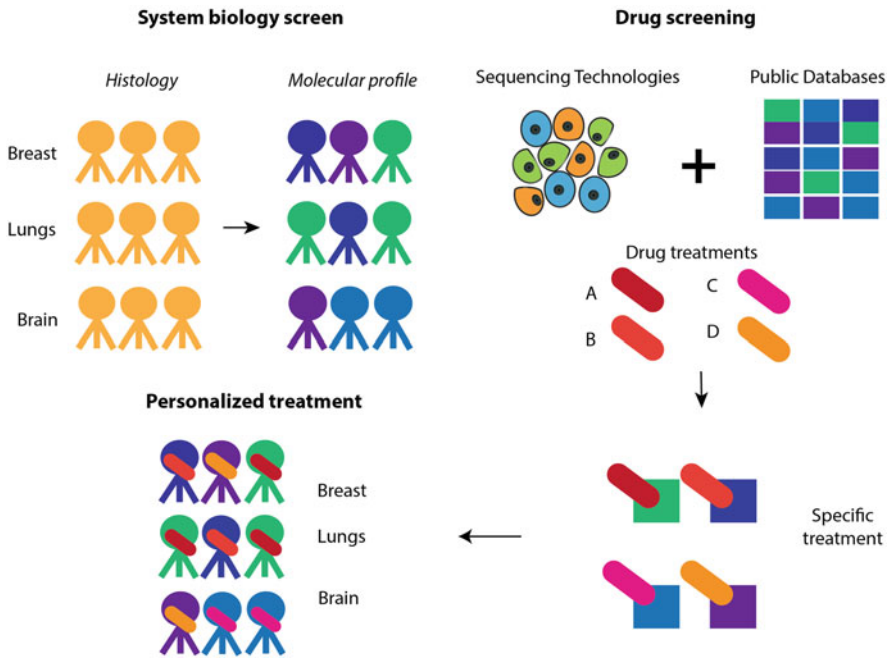

Fig. 4 Combining tumor molecular profiles with pharmacogenomics for personalized treatments. Cancer system biology can connect the broad amount of molecular characteristics of the tumors with pharmacogenomics to provide personalized therapy. Using systems biology models to combine patient-specific datasets with drug response profiles can allow the prognosis of successful patient-specific treatments

specific cancer hallmarks such as metastasis and angiogenesis. Additionally, multi-OMICS approaches can be used to explore the cellular response to chemo- or immunotherapy as well as recognizing molecular features with diagnostic and prognostic value (Chakraborty et al. 2018).

Mathematical modeling is a convenient approach to help understand the cellular dynamics system, by hypothesizing the phenomena affecting cellular dynamics. These computational models are increasingly used to help interpret biomedical data produced by high-throughput genomics and proteomics projects. The application of advanced computer models enabling the simulation of complex biological processes generates hypotheses and suggests experiments. Appropriately interfaced with biomedical databases, models are necessary for rapid access to and sharing of knowledge through data mining and knowledge discovery approaches (Motta and Pappalardo 2013; Hagiwara and Koh 2020). The lack of reliable experimental model systems that mimic patient heterogeneity and clonal evolution is an important limit for bringing adaptive therapies to the clinic (Acar et al. 2020). 3D tissue models and disease models can provide reliable data that consider different phenotypes, clonal evolution, microenvironment, and response to treatments. Computational models, in

vice versa, are also providing valuable data about changes at DNA, RNA, and protein level that help the improving of experimental models as a result.

2.4.1 Sequencing Technologies

RNA Sequencing

RNA sequencing (RNA-seq) is a high-throughput technology for transcriptome (total RNA) analysis, acquiring strand information with very high precision. Using RNA-seq analysis, it is possible to promptly recognize and quantify transcripts, either rare or common, among a large number of samples, including low-quality samples, and provide information on sequence variants that can inform on genomic mutations, gene expression, RNA start and termination sites, and differential splicing (Kukurba and Montgomery 2015; Wang et al. 2009).

MicroRNA sequencing (miRNA-seq) uses material enriched in small RNAs that enables the detection of specific sets of short, noncoding RNAs (miRNAs) that are each capable of controlling a range of genes by affecting mRNA stability and translation. miRNA sequencing can determine tissue-specific miRNA expression profiles, isoforms, and can link miRNA expression with disease and phenotypic state within a specific lineage or cancer type (Bartel 2009; Farazi et al. 2011; Sandhu and Garzon 2011; Gunaratne et al. 2012). The power of transcriptome analysis is exemplified by the study from Rathe et al. (2014), where gene expression analysis was combined with CRISPR/TALEN-based knockouts to recognize and verify the involvement of specific genes related to drug resistance in acute myeloid leukemia cell lines. Sciarrillo et al. (2016) investigated the transcriptomes from many in vitro models of solid tumors and hematological cancers through RNA-seq and showed a higher PKM2/PKM1 ratio, which was not found in the Panc-1 gemcitabine-resistant counterpart, thereby suggesting a different mechanism of drug resistance induced by gemcitabine treatment.

Single-Cell Sequencing

RNA-seq has in general been applied to bulk samples containing many cells and consequently generates an average gene expression across the population. More recently, single-cell sequencing methods have considerably upgraded our ability to examine tumor heterogeneity and recognize even the contribution of rare cells to tumor complexity and drug resistance, particularly in patients and animal models (Seth et al. 2019). Unlike techniques based on population analysis, as its name suggests, single-cell RNA sequencing (scRNA-seq) is focused on transcriptomic analysis of an individual cell. Combining high-throughput sequencing and bioinformatic pipelines, scRNA-seq is able to detect more than 10,000 transcripts in one cell to discriminate individual gene expression programs underpinning specific cell states. Using pseudotime bioinformatic analysis, this approach can be used to infer trajectories of specific cell populations over time, for example, in response to drug treatments. scRNA-seq can provide full-length mRNA information as well as in situ sequencing that allows transcripts to be quantified in their native spatial contexts in single cells.

In cancer research, scRNA-seq technologies promise to improve prognosis and allow more precise targeted therapy by identifying druggable subclones underpinned by distinct gene regulatory networks. To date scRNA-seq of tumor samples has revealed more complex intratumoral heterogeneity than previously anticipated using standard bulk RNA-seq, provided correlations between signaling pathways, stemness, drug resistance, and the tumor architecture shaping the microenvironment, and importantly has also revealed the repertoire of tumor-associated stromal and immune cells. Moreover, scRNA-seq studies of circulating tumor cells showed that several genes cooperate to provide a propensity toward stemness and the epithelial–mesenchymal transition, to improve anchoring and adhesion, and to be involved in mechanisms of anoikis resistance and drug resistance (Xiao and Guo 2018; Zhu et al. 2017). Efforts to understand therapy resistance at the single-cell level were made by Shaffer et al. (2017) in a study showing that melanoma cells can present deep transcriptional variability at the single-cell level that anticipates which cells will become resistant to the treatment. Rambow et al. (2018) applied single-cell RNA sequencing to cells isolated from BRAF mutant patient-derived xenograft melanoma cohorts exposed to concurrent RAF/MEK inhibition. They identified distinct drug-tolerant transcriptional states; one of these exhibited a neural crest stem cell transcriptional program identified as key drivers of resistance and illustrated the therapeutic potential of minimal residual disease-directed therapy.

Very recently, Acar et al. (2020) described a novel approach for evolutionary herding based on a combination of single-cell barcoding, very large populations of cells grown without replating, and mathematical modeling of tumor evolution. They showed that herding allows shifting the clonal composition of a tumor, leading to collateral drug sensitivity and proliferative fitness costs. Through genomic analysis and single-cell sequencing, they were also able to determine the mechanisms that drive such evolved sensitivity.

ChIP Sequencing

Chromatin immunoprecipitation sequencing (ChIP-seq) is a combination of the chromatin immunoprecipitation method followed by heavily parallel sequencing. Chromatin immunoprecipitation allows the identification of specific DNA sequences within the genomes that are recognized by proteins of interest and provide a powerful tool to investigate gene regulation in multiple conditions at remarkable resolution and scale. Proactive quality-control and appropriate data analysis techniques are of crucial importance to obtain the most meaningful results from the data (de Santiago and Carroll 2018). This technique is based on the fixation of chromatin with formaldehyde by covalent linkages between DNA-binding proteins and DNA, cell lysis, and subsequent fragmentation of DNA, where specific DNA–protein complexes are separated by immunoprecipitation with protein-specific antibodies. After reversal of the crosslink, the DNA isolated is amplified by PCR and sequenced using new generation sequence technologies. The reads resulting from sequencing are then aligned to the genome, and the location of the isolated DNA may be identified (Shin et al. 2012; Sá et al. 2018). Studies employing ChIP-Seq have allowed the understanding of the functional interactions between the

binding sites of transcription factors and the DNA sequence elements regulating their target genes (Shin et al. 2012).

This technique has been employed in several studies of drug resistance in tumors, for example, the potential correlation of tubulin alterations and taxane resistance in breast cancer (Nami and Wang 2018) and the pre-existing histone modifications at genes in a poised chromatin state that lead to epigenetic silencing during acquired drug resistance in ovarian carcinomas (Curry et al. 2018). Similarly, estrogen-mediated downregulation of the Par-4 tumor suppressor in hormone-dependent cells could reinstate Par-4 apoptosis-inducing abilities in resistant gynecological cancers by directly binding to its DNA regulatory elements to inhibit estrogen signaling (Brasseur et al. 2017).

Reverse-Phase Protein Array

Reverse-phase protein array (RPPA) is a highly sensitive method that is able to recognize nanograms of proteins. Its advantages rely on reproducibility, high-throughput, functional, and quantitative proteomic analysis for large-scale protein expression profiling, biomarker discovery, and cancer diagnostics. RPPA is an antibody-based technique that provides analysis of >1,000 samples with up to 500 different antibodies at the same time and results in data on protein expression and concentration and, if an appropriate antibody is available, also protein post-translational modification (Spurrier et al. 2008; Stanislaus et al. 2008; Akbani et al. 2014). As an example of the utility of this technique, Li et al. (2017) studied the expression levels of around 230 key cancer-related proteins in more than 650 independent cell lines by RPPA, many of which have publicly available genomic, transcriptomic, and drug screening data. This study outlined the effects of mutated pathways on protein expression observed in patient samples and showed that proteins and particularly phosphoproteins provide information for predicting drug sensitivity that is not available from the corresponding mRNAs.

2.4.2 Public Genomic Databases

A new era of genome-wide sequencing and bioinformatics has shed new light on the cancer genome with the launching of a range of public databases (Stratton et al. 2009; Samur et al. 2013). Large-scale projects such as The Cancer Genome Atlas (TCGA) and the International Cancer Genomics Consortium (ICGC) that started after the success of the Human Genome Project are supporting the "parts list" of cancer, while cancer systems biology will provide the regulatory logic (TCGA 2019; ICGC 2019; Lefebvre et al. 2012). Diversified data categories are used and integrated, including clinical data. Although the range of input cancer biology data is broad, so are the computational methods applied in cancer systems biology, including mathematical and computational algorithms that reflect the dynamic interplay between experimental biology and the quantitative sciences. Here, we highlight the main public databases available that can be used to investigate drug profiles and the interaction with different tumor tissues.

The Cancer Genome Atlas

The aim of the National Institute of Health (NIH) was to make available a comprehensive "atlas" of cancer genomic profiles. As a consequence, in 2005, The Cancer Genome Atlas (TCGA) was created to help the understanding of cancer through genome analysis technologies and has been used for the development of new cancer treatments, diagnostic, and preventive strategies (TCGA 2019, Chin et al. 2011). TCGA is a public funded project that aims to catalogue cancer-causing genome alterations in large cohorts of 33 human cancer types using patient-derived tissue through large-scale genome sequencing and integrated multidimensional analyses by RNA-seq, miRNA-seq, DNA-seq, SNP-based platforms, array-based DNA methylation sequencing, and reverse-phase protein arrays (Wang et al. 2016). TCGA has contributed to the generation of information concerning drug resistance. The culmination of this effort has been a series of manuscripts published over recent years, including drug resistance studies. Most use TCGA data to confirm and give more weight to a hypothesis is already seen using other approaches, such as cell culture and 3D models. Most publications in this context reveal patterns of genes expression among patients that can be correlated with drug response/resistance and tumor staging that can contribute to new personalized therapy strategies (Han and Puri 2018; Corre et al. 2018; Yu et al. 2018; Oliveira et al. 2017). For example, the study of Nabavi (2016) compared sensitive and resistant tumors to generate possible resistance biomarkers, while Yu et al. (2018) published a lncRNA pharmacogenomics landscape after integrating data of cancer cell lines and drug response of anti-tumor drugs.

In 2015, ICGC and TCGA worked together for a combined analysis of over 800 terabytes of data from 1,350 cancer whole genomes. They used infrastructure and platforms developed by Sage Bionetworks to integrate distribution of data and analytical results of the more than 400 researchers involved in the project (ICGC 2019). This new TCGA-atlas called the "Pan-Cancer initiative" has been created and is focused on the genomic, epigenomic, and transcriptomic landscapes of many tumor types (Cancer 2013). The Pan-Cancer analysis associating multi-OMICS data in combination with potent bioinformatics and statistical methods allows a single platform to recognize frequent molecular signatures for the classification of patients with different cancer types and reveal common molecular pathologies of different tumors with the aim of developing targeted therapies. This concept provides an opportunity to understand cellular responses to therapies on a system level but at the same time is also a challenge for systems biology-driven modeling. The ultimate aim is to determine the best therapy for each patient (Chakraborty et al. 2018; Guhathakurta et al. 2013).

In summary, these multi-OMICS datasets may help to improve drug efficacy and defeat the chemo/immunotherapy resistance phenotypes of cancer cells making them sensitive to targeted therapies and finally improving the quality of life of patients (Chakraborty et al. 2018).

The International Cancer Genomics Consortium

The International Cancer Genomics Consortium (ICGC) was created to initiate and organize an extensive number of research projects with the common aim of deciphering the genomic alterations present in cancer that are responsible for cancer in people throughout the world. Currently, there are over 20,000 tumor genomes available worldwide. This enables the generation of a global archive of genomic alterations associated with cancer, a compilation of data that is already helping to reveal the list of mutations that cause this disease and that helps define clinically relevant subtypes of cancer (ICGC 2019).

The main goal is to make genomic alteration data from many cancer subtypes available to the research community, as well all information about the methodology necessary to analyze and integrate the different datasets. The ICGC intends to ease communication among the members and promote forums for discussions, with the objective of maximizing efficiency among the scientists working to understand, treat, and prevent these diseases (ICGC 2019).

An outstanding contribution of this project is the possibility to correlate cancer with differences in the environment of the patient and consequently evaluate how a geographic area influences the prevalence of a determined mutation or response. We can see this kind of approach as a successful tool for understanding, for example, the molecular profiles that favor tumor relapse in Asian women and premenopausal breast cancer association, as described by Yap et al. (2018) and Gröbner et al. (2018) that published studies analyzing genetic alterations in a Pan-Cancer cohort between children and adults.

The Gene Expression Omnibus

The Gene Expression Omnibus (GEO) is an international public functional genomics data repository supported by the National Center for Biotechnology Information (NCBI) at the National Library of Medicine (NLM) that stores and disseminates data from high-throughput gene expression and genomics analyses, facilitating the use of such databases and software by the research and medical community (Tatusova 2016; Clough and Barrett 2016; GEO 2019).

The GEO database is already 18 years old and focused on making the data easily accessible, as the leading public repository for direct deposits of high-throughput gene expression and other functional genomics datasets. In that sense, the GEO has developed different tools for data query, visualization, and analysis that can be performed directly on the GEO website and do not require the download or manipulation of the data files. Several thousand studies have been already published with GEO data used to develop and test new hypotheses. It is notable that researchers are using GEO data to investigate problems far beyond those the initial studies which were intended to tackle (Clough and Barrett 2016). As an example of an application related to drug resistance, Cato et al. (2019) defined a set of potential biomarkers for resistant-prostate cancer using different GEO databases containing data of tumor metastases samples and genome-wide expression profiling of radical prostatectomies. Moreover, they deposited the RNA-seq and ChIP-seq datasets that they generated, contributing to the expansion of the repository.

Drug Response Databases

The Genomics of Drug Sensitivity in Cancer (GDSC) database is the largest public platform for data on drug sensitivity in cancer cells and molecular markers of drug response. The Genomics of Drug Sensitivity in Cancer Project is part of a Wellcome Trust funded collaboration between The Cancer Genome Project at the Wellcome Sanger Institute (UK) and the Center for Molecular Therapeutics, Massachusetts General Hospital Cancer Center (USA). The GDSC consists of drug sensitivity data from around 75,000 different studies, describing the response to 138 anticancer drugs in almost 700 tumor cell lines. This data is associated with genomic datasets available from the Catalogue of Somatic Mutations in Cancer database in order to help to identify molecular markers of drug response, including information on somatic mutations in cancer genes, gene amplification and deletion, tissue type, and transcriptional data (Yang et al. 2013).

Cokelaer et al. (2018) developed GDSC Tools that allow users to reproduce published results from GDSC and to implement new analytical methods. Pozdeyev et al. (2016) also developed a new drug sensitivity metric, the area under the dose-response curve adjusted for the range of tested drug concentrations, which allows the integration of heterogeneous drug sensitivity data from the Cancer Cell Line Encyclopedia (CCLE), the GDSC, and the Cancer Therapeutics Response Portal (CTRP). The results of the largest cancer cell line drug sensitivity data analysis to date are accessible through the online portal, which serves as a platform for high power pharmacogenomics analysis.

A deep cascaded forest model, Deep-Resp-Forest, was created by Su et al. (2019) to classify the anticancer drug response as "sensitive" or "resistant." They evaluated the proposed method on the Cancer Cell Line Encyclopedia (CCLE) and the Genomics of Drug Sensitivity in Cancer (GDSC) datasets and then compared with the support vector machine. The proposed Deep-Resp-Forest has demonstrated the promising use of deep learning and deep forest approaches to drug response prediction tasks. The molecular profiles and the pharmacological information are integrated, allowing the investigation of the response of individual cell lines to anticancer drugs and the relevant biomarkers.

Among the public databases, the initiatives of smaller groups have been published, for example, in the CancerDR (cancer drug resistance database) (Kumar et al. 2013). This group developed a database which contains information about 148 anticancer drugs (36 FDA-approved drugs, 48 drugs in clinical trials, and 64 experimental drugs) for different cancer cell lines. The aim of CancerDR is to make available pharmacological profiling data of anticancer drugs, which will improve the understanding of the effects of mutations in drug targets on acquired drug resistance. In general, the pharmacological profiling data of each drug is available with a clustering module based on IC_{50} values that can be changed to tissue or drug. Also included are gene sequences making it possible to link mutations in the drug targets with resistance (Kumar et al. 2013).

With the future prospect of easy access to whole genome sequences from cancer patients, platforms such as this will become increasingly useful to identify anticancer drugs that will be effective for defined patient subsets, improving the rate of success.

This tool can potentially facilitate personalized medicine. However, the information is based in cell lines that lack the microenvironment context present within tumors, and as such it is yet unclear how relevant the information will be for use in a clinical setting. Nevertheless, the outline of these databases and the information available does provide a foundation for future studies in which drug sensitivities are established in a more complex setting in which 3D architecture is maintained (Gautam et al. 2016).

3 Challenges and Future Directions

To understand the mechanisms underlying therapy resistance, better models that mimic the microenvironment and its signaling are needed. Despite the increase in the application of 3D models in the in vitro evaluation of the efficacy of several drugs, 2D cell cultures remain the most used cell culture model for initial drug screening and validation. Yet, it is already of common knowledge that the behaviors of cells when cultured as monolayers diverge substantially from how they would behave in patients. The 3D models highlighted here will enable the pursuit of a reductionist approach and thereby an understanding how individual microenvironmental signals interact with the genomic plasticity of cancer cells to drive the phenotypic heterogeneity that is key to cancer progression and therapy resistance. A summary of the advantages and disadvantages of the models described here is shown in Table 1.

Unfortunately, several issues influence the adoption of 3D culture models in the pharmaceutical industry. Methodologies must be enhanced to reduce cost and allow production on a large scale, under controlled conditions and in a way that accurately reflects tumors. Moreover, the protocols and techniques that can be used for analyzing the effects of drugs in 3D cell cultures are limited leading to issues with the standardization of the output data analysis. The outputs from 3D culture systems and animal models need to be integrated with the information available from

Table 1 Main advantages and disadvantages of models to study drug resistance

Method	Advantages	Disadvantages
2D	Easy, simple and cheap Often used as preliminary tests before using more elaborated models	Limited in generate suitable environment
3D	Mimic microenvironment, organotypic features, do not require specialized equipment	High cost, interactions between materials
Microfluidic devices	Multi-organ model, capture important essences of the tumor microenvironment	High cost
Sequencing technologies	High-quality data, combination of different molecular aspects of tumors at different levels, cost-effective, tissue-sparing, high-throughput data	High cost
Public databases	Diversified data, population data	Complexity, limited time frame

publicly available genomic and gene expression databases including understanding the complex relationships between specific phenotypic states, expression of drug-metabolizing enzymes and transport proteins, mutations in drug targets, and epigenomics. By focusing on a personalized medicine approach to improve the benefit to patients, 3D models along with cancer systems biology will in the near future be regarded as the gold standard for translational medicine, although extremely costly, decreasing the quantity of pre-clinical in vivo studies to be performed as well as accelerating the drug discovery process.

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The CAM Assay as an Alternative In Vivo Model for Drug Testing

Regine Schneider-Stock and Domenico Ribatti

Contents

Abstract

In the last decade, the chicken chorioallantoic membrane (CAM) assay has been re-discovered in cancer research to study the molecular mechanisms of anti-cancer drug effects. Literature about the CAM assay as an alternative in vivo

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M. Schäfer-Korting et al. (eds.), *Organotypic Models in Drug Development*,
Handbook of Experimental Pharmacology 265, https://doi.org/10.1007/164_2020_375

cancer xenograft model according to the 3R principles has exploded in the last 3 years. Following a summary of the basic knowledge about the chicken embryo, we compare advantages and disadvantages with the classical mouse xenograft model, exemplify established and innovative imaging techniques that are used in the CAM model, and give examples of its successful utilization for studying major hallmarks of cancer such as angiogenesis, proliferation, invasion, and metastasis.

Keywords

3R model · Angiogenesis · Anti-angiogenesis · CAM model · CAM xenografts · Chorioallantoic membrane · Drug delivery · In vivo model · Metastasis · Tumor growth

Abbreviations

4-MU	4-methyllumbelliferone
5-FU	5-fluoruracil
CAM	Chorioallantoic membrane
Cas	CRISPR-associated
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats
CT	Computer tomography
DAPK1	Death associated protein kinase
DZNep	Deazaneplanocin
ECT	Electrochemotherapy
EMT	Epithelial to mesenchymal transition
EZH2	Zeste homolog 2
GFP	Green fluorescent protein
HET-CAM assay	Hen's egg test on the CAM
MGUS	Monoclonal gammopathies of undermined significance
MM	Multiple myeloma
MMP	Matrix metalloproteinases
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MTT	Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OECD	Organisation for Economic Co-operation and Development
PDcE	Patient derived chicken egg tumor
PDGFR	Platelet derived growth factor receptor
PDX	Patient derived tumor xenograft
PET	Position electron tomography
RB	Retinoblastoma
SAHA	Suberanilohydroxamic acid
VEGFR-2	Vascular endothelial growth factor receptor-2
ZEB1	Zinc finger E-box-binding homeobox 1

1 General Remarks: Development and Structure of the Chick Chorioallantoic Membrane (CAM)

The chorioallantoic membrane (CAM) is a highly vascularized membrane that serves as the primitive respiratory organ of the chicken embryo and is rich in growth factors and oxygen. The CAM forms on days 3–4 of embryonal development (Fig. 1a) by the fusion of the chorion and the allantois. It consists of three layers, ectoderm (from the chorion), mesoderm (fused somatic mesoderm from the chorion), and splanchnic mesoderm (from the allantois) (Fig. 1b) (Romanoff 1960). The CAM has a rich vascular system with arteries, capillaries, and veins. Immature blood vessels scattering in the mesoderm (fused somatic mesoderm from the chorion and splanchnic mesoderm from the allantois), and endoderm (from the allanotis) instead of mesoderm (fused somatic mesoderm from the chorion), and splanchnic mesoderm (from the allantois) grow very rapidly until day 8. Capillary proliferation continues until day 10 and attains its final arrangement on day 12 (Ausprunk et al. 1974).

Being naturally immunodeficient, the chick embryo accepts transplantation from various tissues and species without immune response. The chick immune system does not begin to function until about 2 weeks (Jankovic et al. 1975; Weber and Mausner 1977). As other vertebrates, chickens are protected by a dual immune system comprised of B and T cells, controlling the antibody- and cell-mediated immunity, respectively. T cells can be first detected at day 11 and B cells at day 12 (Janse and Jeurissen 1991), and by day 18 chicken embryos become immunocompetent (Jankovic et al. 1975; Weber and Mausner 1977).

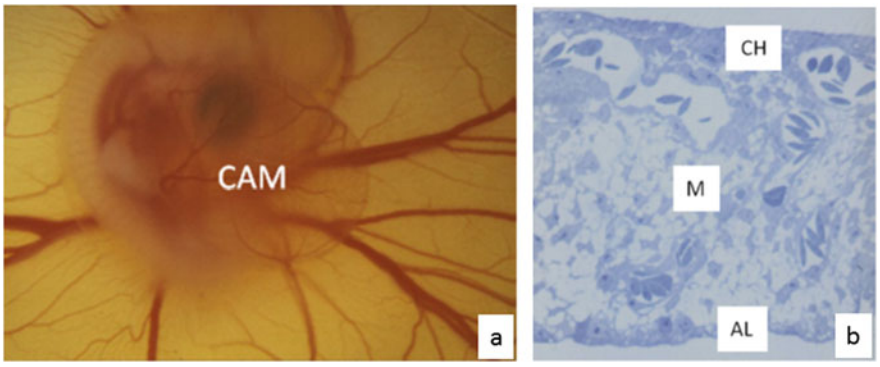

Fig. 1 (**a**) Macroscopic *in ovo* features of the chick chorioallantoic membrane (CAM) at day 5 of incubation. Original magnification, 30×. Reproduced with permission from Ribatti (2014). (**b**) A semi-thin section of the CAM, showing the chorionic epithelium (CH), the intermediate vascularized mesenchyme (M), and the deep allantoic epithelium (AL). Original magnification, 160. Reproduced with permission from Ribatti (2014)

2 CAM Model: History and Regularities

Originally the CAM model was developed for *teratological studies* (Goerttler 1962). Later it was considered as an alternative system used to evaluate the potential ocular irritancy of a test substance as measured by its ability to induce toxicity in the CAM of a chicken. It was proposed as a substitutive to the classical Draize rabbit eye test which effects are measured by the onset of (1) hemorrhage; (2) coagulation; and (3) vessel lysis. These assessments are considered individually and then combined to derive a score, which is used to classify the irritancy level of the test substance (Leighton et al. 1985; Steiling et al. 1999). Meanwhile, the HET-CAM assay is used by cosmetics industry to identify potential irritating materials in in-house testing (ec.europa.eu), but it is still in validation discussion by OECD to be included as a guideline. Nevertheless, the CAM model has received important attention also in cancer research as a "non-animal" experiment. Embryos and fetus are not mentioned in the German Animal Right Law (https://www.gesetze-im-internet.de/tierschg/BJNR012770972.html). In the official gazette of the European Union 1986 Art. 2a, they are not defined as an animal (https://eur-lex.europa.eu/legal-content/DE). According to the actual Directive 2010/63/EU of the European parliament and of the Council, experiments with avian embryos are considered as "no animal" experiments until the hatching (http://data.europa.eu/eli/dir/2010/63/oj).

3 CAM Model in Cancer Research

All studies of mammalian tumors in the CAM model have utilized solid biopsy specimens, tumor cell suspensions, or, more traditionally, tumor cell lines (Tables 1 and 2) (Ribatti 2010). Tumor cells mixed with Matrigel as support material are transplanted onto the CAM between days 6 and 9 of embryonal development. Two to five days after tumor cell inoculation, the tumor xenografts become visible, supplied with vessels of CAM origin, and begin a phase of rapid growth. Tumor

Table 1 Human tumor samples (biopsies) implanted onto the CAM

• Adenocarcinoma of the endometrium	Palczak and Splawinski (1989)
• Ovarian endometrioma	Ria et al. (2002)
• Glioblastoma	Klagsbrun et al. (1976)
• Head and neck squamous cell carcinoma	Petruzzelli et al. (1993)
• Hepatocellular carcinoma	Marzullo et al. (1998)
• Lipoma	Lucarelli et al. (1999)
• Lymphoma	Mostafa et al. (1980)
• B-cell non-Hodgkin's lymphoma	Ribatti et al. (1990)
• Meningioma	Klagsbrun et al. (1976)
• Neuroblastoma	Ribatti et al. (2002)
• Vascular anomalies	Jedelska et al. (2013)

Table 2 Tumor cell lines tested onto the CAM

• Chinese hamster ovary cells transfected with endothelin-1 (CHO-ET-1)	Cruz et al. (2001)
• Colorectal carcinoma cells	Böhm et al. (2019), Ndreshkjana et al. (2019), Steinmann et al. (2019), Lindner et al. (2020), Maiuthed et al. (2018)
• Plasma cells isolated from patients with multiple myeloma	Ribatti et al. (2003)
• Endothelial cells isolated from patients with multiple myeloma	Vacca et al. (2003)
• Friend erythroleukemia cells	Pacini et al. (2008)
• GM7373 endothelial cells overexpressing uPA	Ribatti et al. (1999)
• Gynecologic tumor cell lines	Ishiwata et al. (1988)
• Hepatocellular carcinoma (HepG2) cells	Muenzner et al. (2018)
• Lymphoblastoid cells	Vacca et al. (1998)
• Mammary tumor cells transfected with int-2 oncogene	Costa et al. (1994)
• Mammary tumor cells transfected with VEGF	Ribatti et al. (2001b)
• Melanoma cells – mouse	Auerbach et al. (1976)
• Melanoma cells – human, primary	Monteiro et al. (2019)
• Neuroblastoma cells	Ribatti et al. (2002)
• Neurofibroma Schwann cells	Sheela et al. (1990)
• Ovarian carcinoma cells	Lokman et al. (2012)
• Osteosarcoma cells	Kunz et al. (2019)
• Pancreatic duct cells	Rovithi et al. (2017)
• Retinoblastoma cells	Busch et al. (2017, 2018)
• Urothelial carcinoma cells with low MKP-1 expression	Shimada et al. (2007)
• Walker carcinoma 256 cells	Klagsbrun et al. (1976)

cells can be applied also intravenously to study invasion and metastasis. Dependent on their potential to metastasis, they will colonize in embryonic organs.

Already in 1911, Rous and Murphy demonstrated historically the growth of the Rous 45 chicken sarcoma transplanted onto the chick embryo CAM (Rous and Murphy 1911). Knighton et al. investigated the time course of rat Walker 256 carcinoma specimens transplanted on the CAM surface. Tumors did not exceed a mean diameter of 0.93 $\pm$ 0.29 mm during the pre-vascular phase (approximately 72 h). Rapid growth started 24 h after vascularization, and tumors reached a mean diameter of 8.0 $\pm$ 2.5 mm by 7 days. When tumor grafts from 1 to 4 mm were implanted on the 9-day CAM, grafts larger than 1 mm undergo necrosis and autolysis during the pre-vascular phase (Knighton et al. 1977).

4 The CAM Model Compared to the Mouse Xenograft Model

The CAM model has the potential to replace mouse xenograft experiments in order to study *hallmarks of cancer* as well as *anti-cancer drug effects*. Table 3 summarizes the *advantages* and *disadvantages* of the CAM assay in comparison with mouse xenografts. Being naturally immunodeficient, the chick embryo may receive transplantations from different tissues and species, without immune responses. Moreover, CAM allows a rapid vascularization of the tumors placed on its surface. Dependent on the aggressiveness of tumor cells, CAM vessels are attracted to grow into the developing tumor. Then tumor cells might intravasate into the vasculature. In contrast to standard mouse models, most cancer cells arrested in the CAM microcirculation survive without cell damage, and a large number of them complete

Table 3 Comparison between CAM model and mouse xenograft model

Advantages of the CAM model
• Short experimental time
• Easy handling, easy accessibility
• High reproducibility
• Low costs
• High-throughput – more than one tumor on the CAM (*ex ovo* system)
• Biology and physiology well known
• In vivo imaging, real-time visualization, monitoring over time
• Natural immune-deficient model
• Fast vascular growth, natural bioreactor (full with growth factors, oxygen, and electrolytes) with connection to blood vessels
• Reproducibility and reliability
• Analysis of growth characteristics/hallmarks of cancer such as proliferation, angiogenesis, invasion, metastasis, and drug response
• Accepted model for drug testing, rapid screening platform in scientific community
• No application necessary for approval of an animal test
• High flexibility for planning of experiments
• No pain perception before day 12
• Need more than 1,000 times less drug
Disadvantages
• Limited availability of reagents, antibodies, etc.
• Limited amount of tissue for analysis
• Relative immunodeficiency
• Rapid changes of the CAM during embryogenesis (vessel density, morphology)
• Sensitive to environmental changes (shell dust)
• Different in drug metabolism compared to humans
• Low standardization of protocols
• No oral drug administration possible
• Non-specific inflammatory reactions
• Short post-treatment observation period

extravasation within 24 h after injections (Koop et al. 1994). Compared with mammalian models, where tumor growth takes between 3 and 6 weeks, chick CAM is faster: between 2 and 5 days after tumor cell transplantation, microtumors become visible. Finally, the simplicity and low cost strengthen the use of this assay. The major *drawback of the CAM model* is the fact that most tumor cells cannot form macroscopic visible colonies in secondary organs, due to short time (8–10 days) between the implantation and chick hatching. Another disadvantage is that the CAM already contains a well-developed vascular network and neovascularization is only hardly distinguishable from vasodilation and rearrangement of pre-existing vessels. Moreover, despite the immaturity of the chick immune system, non-specific inflammatory reactions are observable. In view of these limitations, two different assays (sprouting assays, stimulation of HUVEC cells, etc.) should ideally be performed in parallel to confirm the pro-angiogenic or anti-angiogenic activity of a drug.

5 Analysis of CAM Xenografts

The in vivo growing CAM tumors are easily accessible for modern imaging techniques such as magnetic resonance (MR) imaging (MRI; Zuo et al. 2017). The biodistribution of MR contrast agent-labeled compounds has been successfully imaged in different chicken organs as well as in transplanted mammary tumors (Zuo et al. 2017). High-resolution MRI of tumors grown on the CAM delivered excellent anatomical structures, and tumor growth could be monitored over time (Zuo et al. 2015). Thus, the CAM model could be used as a promising initial test model for novel radio-labeled substances. Moreover, PET imaging demonstrated successfully tumor glucose metabolism and protein synthesis in human glioblastoma cells that were transplanted and grown on the CAM. The authors consider the CAM model to have the potential for replacing the mouse model for many in vivo oncology research especially when novel *PET* tracers have to be tested (Warnock et al. 2013).

Different post-experimental analysis techniques are available when analyzing CAM xenografts. Tumor size can be measured. The tumors can be harvested as fresh material for RNA and protein preparation. The tumors can also be fixed in formalin with subsequent embedding in paraffin for immunohistochemical studies. Because the human genome is uniquely enriched in Alu sequences, it is possible to detect disseminating tumor cells by *Alu-PCR* (Alu-specific human repetitive DNA sequences) when harvesting the embryonic organs. When cells have been pre-labeled, they can be detected in the body of the chicken embryo by in vivo *imaging*. *Tumor blocks* can be easily archived, and for further analysis, they will be cut into 4 μm paraffin sections and stained with hematoxylin-eosin (H&E) to make tumor cells visible. Although the nude mouse is still the gold standard as an in vivo xenograft model, also the H&E staining of CAM xenografts allows to evaluate angiogenic effects (chicken embryo vessels can be differentiated by their nucleated erythrocytes), tumor budding at invasion front, proliferation and tumor growth

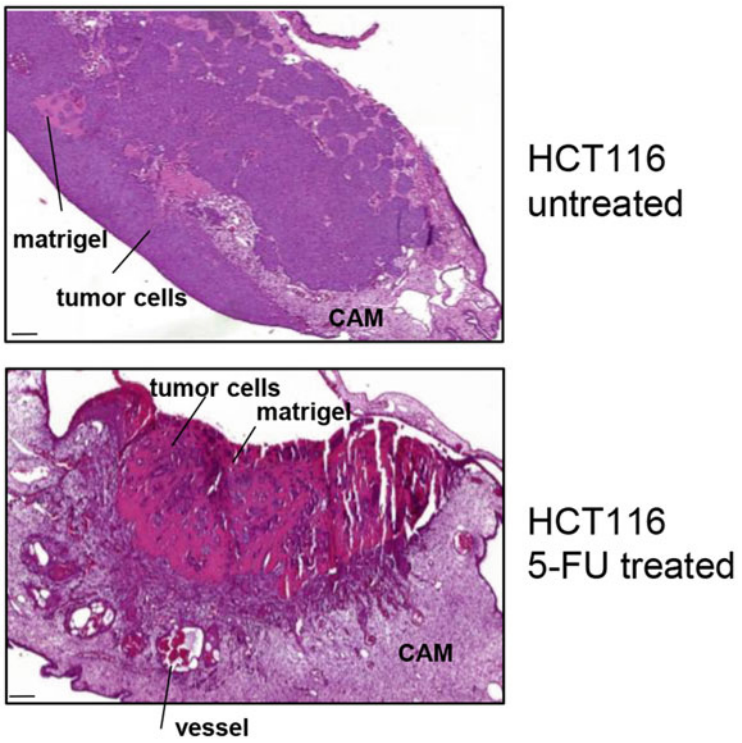

Fig. 2 H&E staining of CAM xenografts grown from untreated or 5-Fluorouracil (15 μM)-treated colorectal cancer cells. 5-FU induces massive bleeding in the surrounding CAM (acknowledged to Dr. Benardina Ndreshkjana, Experimental Tumor Pathology University Hospital, Erlangen)

characteristics, cell density, and cellular phenotype (spindle or cobble stone morphology) as described for mouse xenotransplants.

Drug effects are evaluated microscopically as desmoplasia, necrosis, and presence of pycnotic nuclei or massive bleeding (Fig. 2) and by *immunohistochemical staining* of cell death markers, *PCR* and *Western blotting* of specific apoptosis markers.

6 The CAM and Study of Hallmarks of Cancer

6.1 Angiogenesis

Angiogenesis-associated effects can be studied by directly applying substances onto the CAM using gelatin sponges as a carrier. Patient-derived multiple myeloma plasma cells pre-soaked to gelatin sponges and placed on the CAM from day 8 to day 12 induced a remarkable vaso-proliferative response. This angiogenic response was significantly higher with plasma cells from patients with active multiple

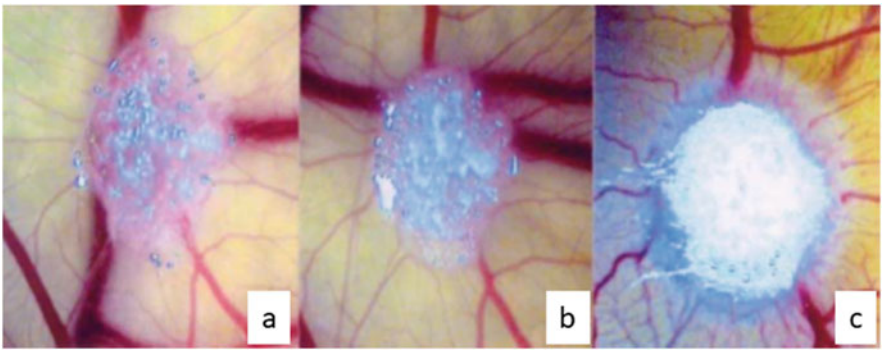

Fig. 3 Time course of the macroscopic appearance of a CAM implanted at day 8 (**a**), with a sponge loaded with 18,000 plasma cells of an active multiple myeloma patient. Note that whereas on day 9 (**b**), no vascular reaction is detectable, on day 12 (**c**), numerous allantoic vessels develop radially toward the implant in a "spoked-wheel" pattern. Reproduced with permission from Ribatti et al. (2003)

myeloma as from those with non-active multiple myeloma (Fig. 3; Ribatti et al. 2003). Implants made from chick embryo developmental day 8 to day 10 were strongly angiogenic, while those made from day 11 to day 12 were not. This might be due to the fact that the high mitotic rate of the endothelium of the CAM declines rapidly after day 10 (Ausprunk et al. 1974).

When implanting *multiple myeloma* cells using Matrigel as carrier, the mRNA expression of *endostatin* is significantly lower compared to the control CAM, suggesting that the angiogenic switch in multiple myeloma may involve the loss of an endogenous angiogenesis inhibitor, such as *endostatin* (Fig. 4), (Mangieri et al. 2008). By implanting human tumor biopsy tissues on the CAM surface, the tumor graft disintegrated within 24 h, and proliferating host vessels penetrated into the tumor tissue mainly due to angiogenic factors released from the tumor cells (Ausprunk and Folkman 1976). Moreover, the authors compared these rat tumor grafts to grafts of normal adult and embryo rat tissues. In grafts from embryonic tissue, pre-existing rat vessels disintegrated and anastomosed to the CAM vessels, while in adult human tissues, this ability was lost, and pre-existing rat graft vessels disintegrated and did not further stimulate capillary proliferation of the CAM (Ausprunk et al. 1975).

Steinmann et al. investigated the invasive potential of CAM-transplanted *colorectal tumor* cells that have lost the cytoskeleton-associated protein kinase *Death-associated protein kinase* (DAPK1) by CRISPR/Cas technology. A significant increase in tumor budding and vasculature of CAM xenografts under DAPK1 loss indicated metastasis inhibitory and anti-angiogenic roles of DAPK1, respectively. Moreover, a DAPK1-dependent altered collagen structure in the chicken extracellular matrix was shown by two-photon microscopy (Steinmann et al. 2019).

Recently the CAM model has been used for studying in vivo alterations of epigenetic players under induction of epithelial-to-mesenchymal transition (EMT)

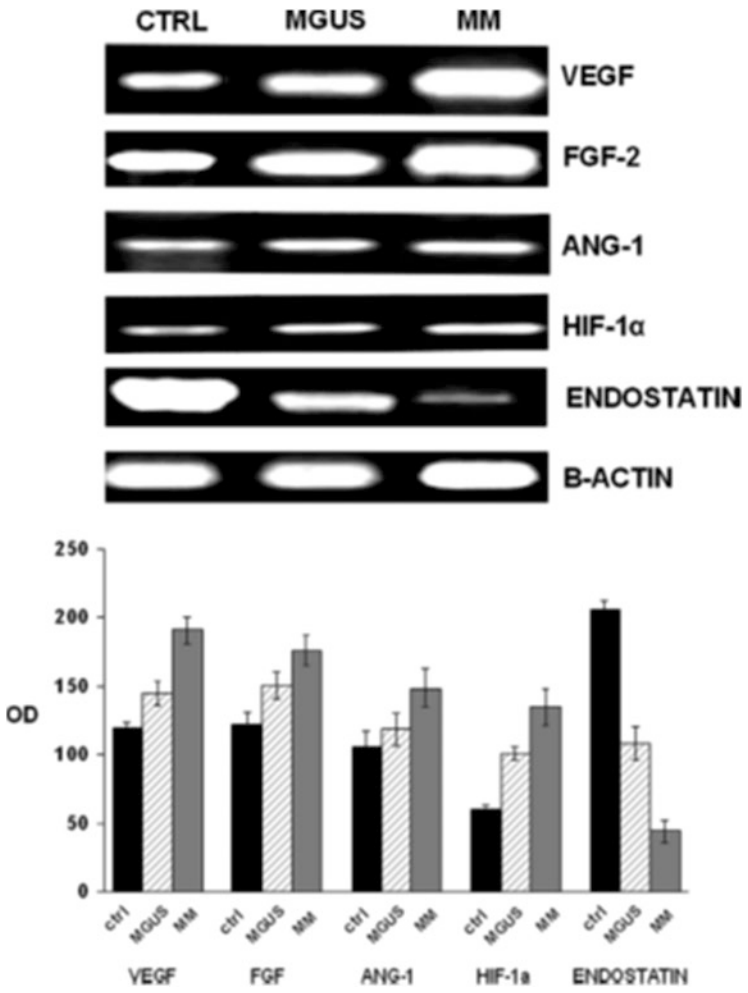

Fig. 4 Expression levels of mRNA coding for human *vascular endothelial growth factor* (VEGF), *fibroblast growth factor 2* (FGF-2), *angiopoietin 1* (Ang-1), *hypoxia-inducible factor 1α* (HIF1-α), and endostatin evaluated by semiquantitative RT-PCR. Transcript levels from the CAM assay are referred to endothelial cells obtained from six multiple myelomas (MM) and six monoclonal gammopathies of undetermined significance (MGUS) patients, and the error band represents the standard deviation of six experiments. Reproduced with permission from Mangieri et al. (2008)

in *colorectal cancer cells*. EMT leads to a spindle cell like more mesenchymal cell shape promoting migration and invasion of tumor cells. The in vivo findings on CAM tumors verified that an increase in the protein expression of the EMT transcription factor *Zinc finger E-box-binding homeobox 1* (ZEB1) was associated with higher expression of the methyltransferase *SET Domain Containing 1B* (SETD1B) that activates gene expression by acetylation of histone H3 (Lindner et al. 2020).

6.2 Tumor Invasion and Metastasis

The metastasis chick embryo model, based on the grafting of human tumor cells on the CAM, has provided valuable information regarding tumor cell penetration of the chorionic epithelium, invasion of the mesenchyme, survival of tumor cells in the circulation, their arrest in the vasculature, extravasation, and proliferation in the distant organs, including the lung, liver, and brain (Armstrong et al. 1982; Kim et al. 1998; Lugassy and Barnhill 2007; Ribatti et al. 2013; Scher et al. 1976). The model of *spontaneous metastasis* is based on grafting human tumor cells on the CAM surface. Tumor cells disseminate throughout the vascular system of the chick. Specific serine proteases and *matrix metalloproteinases* (MMPs), including tumor-derived MMP-9, are involved in this invasive process (Kim et al. 1998). Vice versa, invasion of tumor cells can be blocked by the inhibition of *urokinase plasminogen activator* (uPA) activity or uPA production suggesting that invasion is associated with cell surface-associated proteolytic activity (Ossowski 1988a, b). More than 80% of injected cells survive in the microcirculation and have extravasated by 1–3 days later. Cancer cells migrate through the mesenchyme, attach to arterioles, and accumulate in the vicinity of pre-existing vessels (Koop et al. 1994). The changes in morphology of cancer cells arrested in the CAM microcirculation can be readily observed by in vivo microscopy, and also after extravasation, most of them survived without significant cell damage.

Within few days after inoculation of human *melanoma cells* on the CAM, tumor cells can be identified in CAM areas distant from the inoculation site, as well as in internal organs, including chick lung, liver, and brain (Chambers et al. 1992). mRNA levels of several metastasis-related genes in cancer cells increased temporarily during the early phases of this process, and the levels of *vascular endothelial growth factor receptor-2* (VEGFR-2) mRNA increased 6 h after injection by triggering both, angiogenesis and an increase in vascular permeability (Shioda et al. 1997). A remarkable increase in invasive capabilities of stem cell-enriched human *hepatocellular carcinoma* cells has been shown by in vitro 3D spheroid model and in vivo imaging of CAM xenografts. Cell sub-clones with highest invasive characteristics in 3D spheroids showed the highest tumor cell budding and a significant higher frequency of disseminating tumor cells in the embryonic organs of the chicken (Muenzner et al. 2018).

When comparing the pattern of *experimental metastasis* in chick and mouse after intravenous injection of murine melanoma cell lines, differences between the two models became obvious: (1) the number of developed tumors for a given number of cells injected is much higher in the chick than in the mouse; (2) B16-F1 tumors grew in all embryonic chick organs, while growth was restricted primarily to the lungs in the mouse; and (3) in the chick B16-F1 and B16-F10 cells formed a comparable number of tumors in embryonic organs after intravenous injection, whereas B16-F10 cells formed more tumors in the lung than B16-F1 cells after intravenous injections into mice (Chambers et al. 1992).

Table 4 Most common techniques to visualize and detect tumor cells in the CAM assay

• In vivo video microscopy	Ossowski and Reich (1980)
• Detection of human urokinase plasminogen activator	Ossowski and Reich (1980)
• Green fluorescent protein (GFP)-labeled tumor cells	Bobek et al. (2004)
• PCR-mediated amplification of human-specific Alu sequences	Muenzner et al. (2018)
• Viral nanoparticles	Cho et al. (2014)
• MRI, high-resolution MRI	Zuo et al. (2015), Zuo et al. (2017)
• PET/CT imaging	Warnock et al. (2013)

6.3 Visualization and Detection of Tumor Cells (Table 4)

Several methods for semiquantitative analysis of disseminating tumor cells in the chick embryo have been developed including morphometric quantitation of individual fluorescent-labeled metastasized cells by video microscopy (Ossowski and Reich 1980), detection of microscopic tumor colonies (MacDonald et al. 1992), detection of human urokinase plasminogen activator within secondary organs of the embryo (Ossowski and Reich 1980), the use of green fluorescent protein (GFP), and in vivo video microscopy (Khokha et al. 1992; Koop et al. 1996). PCR-mediated amplification of human-specific Alu sequences was used for semiquantitative detection of disseminating cells arrested in specific embryonic organs (Kim et al. 1998), followed by sensitive real-time Alu-PCR assay (Mira et al. 2002; van der Horst et al. 2004; Zijlstra et al. 2002). Bobek et al. (2004) developed a GFP-labeled tumor cell assay transplanted to the chick embryo for screening anti-metastatic agents. Yet, extravasation or real metastasis formation cannot be differentiated from accumulation of tumor cells in highly vascularized embryonic organs.

7 The CAM Model and Molecular Pathways

In the last 2 years, the number of publications that implemented the CAM assay as an alternative in vivo test system for validating molecular pathways and functional aspects remarkably rose. Here we give some recent examples how the CAM model supported findings from pharmacological functional in vitro studies and helped for interpretation of the findings.

7.1 Pharmacological Inhibition of Angiogenesis

The inhibition of angiogenesis seems to be a promising anti-tumor strategy particularly in the adjuvant setting. In this regard the CAM assay can be utilized as a powerful model to measure drug effects on vasculature. In accordance, human *U87*

glioblastoma cells implanted on the CAM formed avascular tumors within 2 days, which progressed in the next days through VEGFR-2-dependent angiogenesis. Blocking of VEGFR-2 and *platelet-derived growth factor receptor* (PDGFR) signaling pathways with small molecule receptor tyrosine kinase inhibitors such as PTK787/ZK 222845 and imatinib, respectively, suppressed vessel density and tumor growth. Indeed, gene expression analysis during the angiogenic switch identified genes associated with tumor vascularization and growth (Hagedorn et al. 2005).

There are several reports on the effects of anti-cancer drugs on angiogenesis in the CAM model. As an example, the angiogenic response induced by neuroblastoma cell-derived conditioned medium, *neuroblastoma tumor* xenografts, and human neuroblastoma biopsy specimens could be effectively blocked by the proteasome inhibitor bortezomib. These anti-tumor effects have been also shown in parallel in mouse models (Brignole et al. 2006). Treating neuroblastoma cells with combination of bortezomib and the synthetic retinoid fenretinide induced a marked reduction in intra-tumoral vessel density in mouse tumor grafts. Transplanting these tumors on the CAM and treating them according to the combination protocol confirmed a clear anti-angiogenic response when morphometrically assessing the micro-vessel area (Di Paolo et al. 2009). Exposing neuroblastoma cells to the chemotherapeutics vinblastine and rapamycin alone as well as in combination significantly reduced the growth of tumor and endothelial cells as observed in vitro and in the CAM model. Interestingly, also the conditioned supernatant of treated tumor cells had a reduced capacity to induce angiogenesis (Marimpietri et al. 2005, 2007).

When pre-treating *melanoma* cells with the angiogenesis inhibitor axitinib, smaller tumor masses were detected, and tumor cells showed a remarkably reduced capability to invade the CAM. Different antibodies have been used for immunohistochemical staining to evaluate the proliferation index or mitotic counts. Using the CAM model, this study linked angiogenesis with tumor aggressiveness in melanoma (Monteiro et al. 2019). For aggressive highly vascularized *uveal melanoma* grown on the CAM, efficacy of bleomycin chemotherapy increased when coupled with electroporation (electrochemotherapy – ECT) due to enhanced membrane permeability for the drug. Cytotoxic effects were measured by evaluating the tumor size, viability with MTT assay, and Ki67 proliferation index in histological slices. Tumor necrosis was induced in the bleomycin only and the ECT group, yet a significant reduction of tumor growth was seen only following additional ECT treatment (Fiorentzis et al. 2019).

7.2 Pharmacological Inhibition of Tumor Growth

Primary ovarian cancer cells grown on the CAM and treated with the hyaluronan inhibitor 4-methylumbelliferone (4-MU) showed a significant decrease of invasion accompanied by a reduction of CD44 stem cell marker expression determined by immunohistochemistry. 4-MU when combined with the chemotherapeutic drug carboplatin even increased the response of chemoresistant primary cells (Lokman et al. 2019).

When exposing tumor cells to anti-cancer drugs before transplanting them on the CAM, it is possible to analyze drug effects against cancer stem cells since only cancer stem cells should be able to self-renew and to rebuild the tumor. Here a novel hybrid linking 5-fluoruracil (5-FU) and the plant-derived compound thymoquinone showed high efficacy against the *colorectal* CD133+ *tumor* population while inhibiting the Wnt signaling pathway. Immunohistochemically it was observed a strong nuclear staining pattern for ß-catenin and E-cadherin in agreement with a decreased ß-catenin activity in vitro. High vessel density in CAM xenografts was observed under 5-FU, whereas vasculature was strongly inhibited under hybrid exposure (Fig. 2) (Ndreshkjana et al. 2019). The accumulation of cytoplasmic p21WAF1, a cell cycle inhibitor, has been identified as a possible mechanism for 5-FU resistance in colon cancer cells and was confirmed in vivo using the CAM model (Maiuthed et al. 2018).

In addition, the CAM model served to investigate the response of a *colon tumor cell line* to 3-deazaneplanocin A (DZNep), an inhibitor of the histone methyltransferase enhancer of zeste homolog 2 (EZH2). The Ki67 proliferation marker confirmed the drug-induced growth arrest as shown in monolayer cultures of the tumor cell line. The reduction of EZH2 expression at the tumor invasion front as observed in human primary tumor samples was also visible in the CAM xenografts grown from a colon tumor cell line (Böhm et al. 2019).

When hypoxia pre-conditioned GFP-labeled *neuroblastoma* cells were transplanted on the CAM, inhibitors for cyclin-dependent kinases, palbociclib and RO-3306, reduced the metastatic capability of these tumor cells (Swadi et al. 2019). Retinoid-induced effects on the differentiation of myc-activated neuroblastoma cells were monitored by changes in morphology, Ki67 proliferation index, and gene expression of different stem cell markers. Interestingly, retinoid treatment had higher anti-tumor efficacy than the treatment with the Aurora kinase A inhibitor MLN8237 (Swadi et al. 2018).

Busch et al. characterized different etoposide- and cisplatin-resistant *retinoblastoma (Rb)* cell lines for their morphological changes compared to their chemosensitive counterparts using 3D imaging for tumor size and different staining on cryo-sectioned material (Busch et al. 2018). Trifoil factor 1-overexpressing Rb tumor cells showed a remarkably reduced tumor formation and migratory potential when injected intravenously or transplanted on the CAM (Busch et al. 2017).

In CAM xenografts grown from *urothelial carcinoma cell lines*, topical application of the combination of cisplatin and the histone deacetylase inhibitor suberanilohydroxamic acid (SAHA) (day 11 of embryonal development) led to an additive anti-tumor effect. The drug dose was adjusted to the estimated blood volume of the corresponding embryo developmental day. Tumor size was evaluated by bio-imaging and immunohistochemistry (Skowron et al. 2017).

8 CAM Use in Drug Delivery Studies

Importantly the CAM model is also suitable for monitoring drug delivery to the tumor, including targeted delivery of diagnostics when loaded to nanoparticles. Recently, nanoparticles reinforced the medical imaging technology having a high potential for early detection of cancer. Indeed, viral nanoparticles injected into the bloodstream of the chicken embryo can be imaged for several hours or days in the newly formed xenografts showing that the CAM model is very suitable for intravital and tumor imaging studies (Cho et al. 2014). Viral nanoparticles are also suitable to visualize newly formed vasculature in expanding tumors (Leong et al. 2010). High-resolution imaging of human tumors grown on the CAM reveals fluid and small molecule dynamics within tumors (Cho et al. 2011).

When different doses of biodegradable organosilica nanoparticles loaded with doxorubicin were intravenously injected into the chicken egg, it was possible to monitor drug effects in CAM tumors grown from human ovarian cancer. Doxorubicin led to an effective and selective tumor elimination without major side effects, whereas the treatment with free doxorubicin induced intensive damage of chicken embryonic organs (Vu et al. 2018). When treating transplanted feline fibrosarcoma cells with doxorubicin-conjugated glutathione-stabilized gold nanoparticles tumor growth reduction could be successfully monitored (Zabielska-Koczywas et al. 2017), too.

9 The CAM Model and Personalized Medicine

The *p*atient-*d*erived tumor *x*enograft (PDX) model is well established for transplanting patient tumor samples to immunocompromised mice, and hence, by principle, personalized therapies can be approved for every single patient. As an example, the Epo GmbH (Berlin, Germany) has meanwhile more than 500 well-characterized PDX, xenografts, and syngeneic and humanized tumor models available for research and preclinical testing of novel compounds (https://www.epo-berlin.com/). The major limitation of this model with respect to personalized therapy is the long cultivation time with sequential harvest/splitting/re-transplantation cycles that need about several months before being ready for drug testing, a time frame that many patients do not survive. Moreover, during the propagation of tumors, the human stroma is replaced stepwise by mouse stroma components changing the tumor over time. Besides that, costs are not covered by health insurance so far, and the maintenance and feed of animals are cost- and time-intensive (DeBord et al. 2018).

The *p*atient-*d*erived *c*hicken *e*gg tumor (PDcE) model has been developed as an alternative system to overcome the shortcomings of the mouse PDX model using ovarian tumors (Vu et al. 2018; Komatsu et al. 2019). Since tumors are formed in 3–4 days after transplantation of tumor cells, a library of 10–20 CAM tumors could be produced in short time to screen patient-specific anti-cancer drugs. Successful grafting or re-grafting of biopsies from bladder tumor, laryngeal squamous cell

carcinoma, neuroblastoma, endometrial adenocarcinoma, and vascular anomalies was already reported. Tumor biopsies survived and showed the typical morphological and proliferative characteristics of the original tumor (Dohle et al. 2009; Uloza et al. 2015; Ribatti et al. 1997, 2001a; Balciuniene et al. 2009). PDcE like PDX retain the characteristic tumor features, tumor heterogeneity, and pathophysiology of the donor tumor (reviewed by DeBord et al. 2018). Yet, so far there are only a few success studies about therapeutic intervention on PDcE. Freshly transplanted biopsies of malignant ovarian tumors have been treated by photodynamic therapy on the CAM, and remarkable anti-cancer effects could be observed and even reinforced when tumors have been serially re-grafted (Ismail et al. 1999). There is a recent paper showing that the CAM model has been successfully integrated into the clinical workflow for oral squamous cell carcinoma (Kauffmann et al. 2018).

10 Outlook

Besides very promising data, there is still a lack of standardization and general acceptance among the researcher's community for the CAM model. Thus, there is an urgent need for more publicity about this promising alternative in vivo model. We need to establish standard operation procedures for this model corresponding to the respective research question. For drug testing this would involve the definition of the best application window for the drugs, the most suitable imaging technique, as well as the exact handling of the transplanted specimens. The future will show, if this might improve the selection of drug candidates and reduce the high failure rate of anti-cancer drugs in clinical studies (Wong et al. 2019), not less important, the model might contribute to the 3R concept being an experimental alternative to a classical animal test.

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