Towards more predictive, physiological and animal free in vitro models: Advances in Cell and Tissue Culture 2020 Conference Proceedings

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Abstract

Experimental systems that faithfully replicate human physiology at cellular, tissue and organ level are crucial to the development of efficacious and safe therapies with high success rates and low cost. Development of such systems is challenging and requires the skills, expertise, and inputs from diverse fields including biologists, physicists, engineers, clinicians, regulatory bodies, etc. Kirkstall Limited, a biotechnology company based in York, UK organised the annual conference, Advances in Cell and Tissue Culture, which brought together people from a variety of expertise areas and interest to present and discuss the latest developments in the field of cell and tissue culture and in-vitro modelling. The conference has also been influential in engaging animal welfare organisations in the promotion of research, collaborative projects and funding opportunities. This report describes the proceedings of the latest such conference which was held virtually on 30th September and 1st October 2020 and included sessions on in-vitro models in the following areas: advanced skin and respiratory models, neurological disease, cancer research, advanced models including three-dimensional, fluid flow and co-cultures, diabetes and other age-related disorders, and animal-free research. The round table session on day two was very interactive and drew huge interest with intriguing discussion among all participants on the theme of replacement of animal models of disease.

Introduction

The collaborative efforts by individuals from diverse skill sets and expertise have led to the accomplishment of some of the path-breaking milestones in the field of in vitro biology and tissue engineering over the last few decades. This has established that the many of the most predictive human-relevant in vitro models have the potential to replace animal models and establish an indispensable contribution to drug discovery process [Avila, 2020]. The ACTC conference is an annual conference organised by Kirkstall Ltd, a UK based biotechnology company. The aim of this conference is to bring together a multitude of individuals and institutions from science and technology backgrounds to present and discuss the latest developments in the field of cell and tissue culture and in-vitro modelling. This also helps foster networking and collaborative opportunities utilising a rich and diverse ‘expertise ecosystem’ and ’community of practice’. In addition to the keynote lectures and session presentations, in this conference, a special focus session was organised in the format of a roundtable discussion where scientists from academia and industry, along with representatives from animal welfare organisations participated in the discussion on the challenges, gaps and potential approaches to accelerate the implementation of human-relevant science. This paper reports the proceedings of the latest programme of this conference that took place on a virtual platform (www.kesonline.info) on 30th September and 1st October 2020. 78 delegates from 16 countries across four continents participated in this conference. The geographical distribution of the delegates is illustrated in Figure 1.

Conference sessions

The conference included keynote lectures, and presentations in the following seven sessions:

- Advanced Skin & Respiratory Models
- Novel Models for Studying Neurological Diseases

• In vitro Models for Animal Replacement
• In vitro Cancer Research
• Advanced Cell Culture; 3-dimensional (3D), Fluid Flow & Co-culture
• Diabetes and other age-related disorders
• Animal Free Research

We summarise all the papers presented in the sessions below. The video recordings of the live presentations in this conference are available on request from Kirkstall Ltd (York, UK).

Advanced Skin & Respiratory Models

Prof. Rosalind Hannen, Queen Mary University of London, UK, chaired the session which started with the keynote lecture by Dr. Samuel Constant, Epithelix, Switzerland. The lecture described the 3D human epithelial models, MucilAir™ and SmallAir™ system for studying severe acute respiratory syndrome cornonavirus 2 (SARS-CoV-2) pathogenesis and evaluating novel therapies against this virus [Pizzarno et al; Boda et al; Hopkins special issue]. These models have been successfully used for the development of antivirals against influenza, rhinoviruses, respiratory syncytial virus, amongst others. The current research strategies to stop SARS-CoV-2 infection were outlined by Dr. Constant who also highlighted how these reconstituted human airway epithelial models can be used to characterise viral infection kinetics, tissue-level tropism and transcriptional immune signatures induced by the SARS-CoV-2.

The session talks were:

- *Advancing in-vitro models of the alveolar epithelial barrier for their anatomical and physiological relevance for toxicology testing* (Martin Clift, Swansea University Medical School, Swansea, UK).
  Prof. Clift highlighted the increased attention given to the development of advanced *in vitro* technologies, specifically models of the alveolar epithelial barrier of the human lung [Clift 2020]. Most focus has been given to constructing systems based upon multiple cell types since these provide the ability to formulate models that exhibit anatomically correct structures [Miller 2017]. These systems have been used with great effect to study the potential adverse biological impact of numerous xenobiotics (*e.g.* chemicals, particles and nanomaterials), as well as for initial drug efficacy studies [Faber and McCullough 2018].

- *Modelling respiratory infections for developing novel anti-infectives* (Claus-Michael Lehr, Helmholtz Institute for Pharmaceutical Research, University of Saarland, Germany). Prof. Lehr described the cell culture models of the alveolar mucosa, an approach to implementing microfluidics, and growing bacterial biofilms on pulmonary cell cultures. Regardless of the recent pandemic caused by Corona virus, the problem of antimicrobial resistance continues to increase while the number of new antibiotics and even the number of companies engaging in those is decreasing. Besides the need for new targets and molecules for anti-infective compounds, for example, pathoblockers, there is also a need to deliver those across biological barriers preventing access to the target site. Relevant barriers in this context are the body's outer epithelia including the gut, the skin and the lung, but also the bacterial cell envelope as well as non-cellular barriers, such as mucus and bacterial biofilms. To study the cellular interactions of drugs and nanoparticle deposition in the deep lung, Prof. Lehr's group has pioneered human alveolar epithelial cell models [Elbert 1999; Kuehn, 2016]. More recently, their research has been inclined towards microfluidic devices and more complex co-cultures, allowing to mimic the air-blood-barrier in health and inflammatory state and chronic infections by biofilm-forming bacteria [Costa 2019; Artzy 2019; Montefusco 2020]. In vitro studies on such

systems suggest that novel self-assembling nanocarriers [Ho 2020], capable of co-delivering Tobramycin and modern PQSI may allow significant reduction in the dose of the antibiotic to eradicate the infection and reduce the risk of inducing antimicrobial resistance.

Novel Models for Studying Neurological Diseases

The session was chaired by Prof. Geoffrey Pilkington (University of Portsmouth, UK) and chaired by Prof. John Haycock (University of Sheffield, UK) who gave the keynote lecture titled 'Combining biomaterial scaffold and imaging technologies for advanced neurological models in-vitro'. Prof. Haycock described the use of biodegradable scaffolds to produce 2D and 3D in vitro/ex vivo models of peripheral nerve injury and regeneration to provide improved nerve regeneration guidance channel implants focussing largely on chick embryo dorsal root ganglia cultures under sophisticated microscopical imaging conditions.
Ultimately, human model systems would be developed informed by the studies in chick embryos. Human tissue from early post-mortem cadavers or human peripheral nerve iPSC-derived cultures might benefit these complex systems but each brings with it technical, practical, and ethical obstacles.
In this session, Dr. Prospero Civita and Dr. Catia Neto presented studies on the tumour microenvironment (TME) in glioblastoma (GB), a pivotal factor in supporting tumour progression and therapeutic resistance.

- *Modelling glioblastoma microenvironment using h-iPSC derived-cerebral organoid incorporating iPSC derived- microglia and patient-derived Glioblastoma Multiforma (GBM) cells: a sophisticated tool for disease modelling and pre-clinical drug screening* (Prospero Civita, University of Portsmouth, and Cardiff University, UK). Dr. Civita presented a 3D hyaluronic acid-gelatine based hydrogel model (HyStem™-HP) with GB cells and astrocytes to understand the impact of non-neoplastic cell and EMC component on GB progression and therapeutic resistance. The study showed that non-neoplastic astrocytes in both 2D and 3D in vitro co-culture models form Tunnelling Nano Tubules (TNT) connections with GB cells and transfer non-neoplastic mitochondria via a TNT connection and that this mechanism may be related to GB drug response as well as proliferation and migration [Leite 2020; Civita 2019]. To understand the impact of each cellular component within the brain upon GB progression, Dr. Civita and group used the iPSC technology to generate human cerebral organoids [Linkous 2019], " mini-brain", which include patient-derived glioma cells and iPSC-microglia cells to generate an in vitro microenvironment similar to the human brain in order to study GB as human disease and provide a valuable in vitro model for high-throughput therapeutic screening. To enhance the utility of the model for therapeutic testing and drug delivery the tumour micro-environment would, optimally, include the cellular components of the blood brain barrier and potentially blood flow as well as be carried out under appropriate cerebral oxygen conditions.

- *Understanding the glioblastoma immuno-environment: The impact of Caveolin-1 in microglia phenotype* (Catia Neto, Cardiff University, UK). Glioblastoma multiforme (GBM) is a highly lethal brain tumour. Microglia, the brain immune cells are highly abundant in GBM, and are responsible for creating an inflammatory microenvironment that promotes tumour progression [Quail 2017]. Caveolin-1 (Cav1) is involved in multiple signal transduction events and cytoskeletal dynamics, interacting with multiple pathways. Cav1 in GBM was identified as an independent prognostic marker [Quail 2017], and in immune cells seems to be involved in pro-inflammation [Martin 2009]. Dr. Neto concluded that induced pluripotent stem cell (iPSC)-derived microglia can respond to pro-inflammatory and anti-inflammatory stimuli. In microglia, Cav1 might be involved with inflammation. Suppression of Cav1 in iPSC-derived microglia drives ~~to~~

tumour invasion. Use of human iPSC-derived microglia along with GBM cells provides a sophisticated model for evaluating the role of the membrane protein Cav-1 in immune response regulation and invasion in GBM. The use of high passage, homogeneous GBM cells, however, restricts the utility of the system. The use of freshly segregated GBM cells from human brain tumours or human GBM organoids along with the iPSC-derived microglia sub-populations may provide a better reflection of the in situ tumour microenvironment.

Geoff Pilkington, on behalf of Karen Pilkington, University of Portsmouth, presented an interesting project on the evaluation of in-vitro brain tumour studies.

- *Systematic Approach to review In-Vitro Methods in Brain Tumour Research (SATORI-BTR): Developing preliminary guidance for evaluation of In-Vitro Studies in Brain Tumour Research'*. The project used a systematic process to identify, assess and agree criteria for assessing in-vitro brain tumour research. Forty-eight draft criteria were reviewed using a Delphi (expert consensus) process across two rounds by a panel of senior researchers from nine countries. This process has resulted in preliminary guidance for appraisal of in-vitro brain tumour studies [Bracher 2020]. Further development and dissemination of the guidance is planned. Some of the gaps in this field are: lack of widely accepted criteria for assessing the quality and human relevance of in-vitro studies, making implicit knowledge and expertise used by those in the field when assessing in-vitro research explicit, and gaining consensus from senior researchers located across different institutions and countries [NC3Rs 2020]. This project attempted to addresses these gaps by agreeing a set of provisional criteria for assessing quality and human relevance of in vitro brain tumour research with the experts. These provisional criteria could be used by those designing or reporting such research. The criteria are generic and could be adopted or adapted by other fields of in-vitro cancer research [Hartung 2019].

In Vitro Cancer Research

The session, also chaired by Prof. Geoff Pilkington, included a keynote lecture by Dr. Victoria Marsh Durban (Cellesce, UK). In her talk titled 'Making 3D possible: large scale organoid production using bioprocess design', Dr. Marsh Durban described the barriers to the adoption of organoid technology in the pharmaceutical industry, namely, the high skill and labour requirements for organoid culture, and the inability to produce large batches of organoids with the homogeneity required for screening applications. She illustrated the organoid expansion process at Cellesce, and the comparison of bioprocess-expanded organoids to those expanded by manual process. The talk also covered the proof-of-principle studies for colorectal cancer organoids in drug screening.

Session talks:

- *Air liquid interface (ALI) multi-layered co-cultures mimic biochemical mechanisms of the cancer cell-fibroblast cross-talk involved in* Non-Small-Cell Lung Cancer (*NSCLC) Multi-Drug Resistance in patients* (Dania Movia, Trinity College Dublin, Ireland). Lung cancer is the leading cause of cancer-related deaths worldwide. Patient prognosis is extremely poor for advanced (NSCLC), its most common form. Inhaled therapeutics are under development. However, at present, reliable preclinical models to support the development of such drugs do not exist [Movia 2020]. Dr. Movia described a 3D multi-layered cell culture of human NSCLC cells grown at the ALI, established by the research group, as the first in vitro tool for screening the efficacy

of orally inhaled drug products for NSCLC treatment [Movia 2018; Movia 2019]. The study demonstrated the establishment of a cancer cell-fibroblast crosstalk, capable of modulating Multi-Drug Resistance (MDR) and feedback activation signalling processes in vitro as per in vivo conditions.

- *The development of a HepG2 3D spheroid model for the preclinical assessment of nano-biomaterials for cancer applications* (Melissa Tutty, Trinity Centre for Health Sciences, Ireland).
  There are large attrition rates and slow progress to the clinic associated with nanobiomaterials (NBMs). Current safety profiling methods fall short when assessing the risk of these materials, with only 60-70 % of hepatotoxins being detected from conventional screening methods [Duval 2017]. This is thought to be in part due to inappropriate and reductionist 2D in-vitro pre-clinical assessment methods and inter species variation in animal models [Kapałczyńska 2018]. This talk presented an alternative method for assessing NBMs accurately, using more in vivo like 3D cell culture, in the form of spheroids [Mandon 2019]. The work involved screening NBMs specifically, utilising a 3D model of the liver, and incorporating relevant cell types.

Advanced Cell Culture, 3-Dimensional (3D), Fluid Flow & Co-culture

Professors Elisa Budyn (Université Paris-Saclay, France) and John Haycock (University of Sheffield, UK) co-chaired this session. The keynote lecture by Dr. John Connelly (Queen Mary University of London, UK) was 'Engineering vascularised and immune-responsive human skin equivalents via indirect bioprinting'. Dr. Connelly noted that the diversity of chronic skin wounds which can be very painful and debilitating, presents several challenges in determining optimal individual treatment regimes. Dr. Connelly's research group utilised microfabricated wound healing models in combination with high content imaging and systems biology to develop systems-level understanding of keratinocyte migration and re-epithelialization [Costa 2014]. He also presented unpublished data on the development of bioinks for 3D human skin equivalents, methods for 3D bioprinting vascularised tissue models using sacrificial biomaterials, and the incorporation of immune cells into human skin equivalents.

The session talks were as follows:

- *Harnessing an innovative completely chemically defined, xeno-Free, albumin-free controllable cellular microenvironments for long-term culture of human mesenchymal stem cells (hMSCs) for production of ATMPs and cell-therapy applications* (Mohammed Essameldin Abdelgawad, Helwan University, Cairo, Egypt). Prof. Abdelgawad presented an innovative, chemically defined and controllable cellular microenvironment optimised for either hMSCs or hMSCs-extracellular vesicles (EVs) allowing long-term survival, expansion and maintenance of the phenotypic, proliferative, differentiation and paracrine-signals/secretome features of hMSCs. This approach is valuable as it paves the way for production, scaling up and diversification of pure hMSCs and/or their EVs for various precision and targeted therapy of urgent diseases like Covid-19.

- *Advanced in vitro management of three-dimensional cell cultures and explanted tissue (Sebastian Kress, University of Natural Resources and Life Sciences, Vienna, Austria).*
  The use of 3D cell cultures has gained importance in medicine and pharmaceutical research. However, commercially available systems are operated under conditions not yet optimised for 3D applications, for example, lack of availability of tailor-made,

minimally invasive monitoring systems with integrated sensors to monitor, optimize, and standardize culture conditions. In this talk, Dr. Kress presented a platform technology combining perfusion bioreactor systems with integrated biosensors to facilitate in vitro management of three-dimensional cell cultures and explanted tissue. This provides a potential solution for repetitive time-resolved non-destructive sampling, and monitoring of metabolism, secretome, and functional maturation of 3D cell- and tissue-based models (manuscript in preparation). This might enable the reduction of sample size and variation, bridging the gap between in vitro and in vivo studies.

- *Studying human bone formation in a Bone-on-Chip* (Elisa Budyn, Université Paris-Saclay, France). Prof. Budyn presented a model based on decellularized human bone with re-cellularisation with human bone cells to study bone geometry, cell morphology and differentiation, and bone assembly. A bone-on-chip composed of one or more decellularized bone pieces recellularised with either human primary adult MSCs or foetal osteoblast progenitors provided the relevant 3D environment for the cells to differentiate into stem cell derived osteocytes (SCDOs). These systems were cultured up to 26 months and mechanically stimulated in either 3-point bending or compression to mimic exercise. Synchrotron ATR FTIR and mechanical tests showed fetal cells produced highly mineralized and fatty tissue compared to adult cells. These systems displayed multi-level spatial reorganization of the cells, and the collagen fibres [Budyn 2018]. Hence, this bone-on-chip system makes it possible to simultaneously study the effect of mechanical stimulations and age on bone cell differentiation, their calcium mechanobiology and the quality of the bone they form over very long periods of time (at least over two years).

- *Miniaturised Microbioreactor for Controlled and Lower Cost Cell Culture* (Zulfiqur Ali, Teeside University, UK). Prof. Ali presented a microfluidic 24-element cell culture array for cell-based cytotoxicity assay and a miniaturised microbioreactor with integrated sensing and use with downstream analytics. Figure Ali.A shows that each culture element (2mm diameter) has two pairs of horizontal and vertical pneumatically actuated normally closed valves for control fluidic. The culture array is fabricated as a three-layer (fluidic, flexible PDMS membrane and actuation) structure by casting on an SU-8 mould [Pasirayi 2014]. A separate microbioreactor system was described using pressurised fluid driving and integrated pH, dissolved oxygen, and optical density measurements. The microbioreactor element comprising three layers (sensor, fluidic and headspace) that are joined by adhesive layers (Figure Ali B) and could be operated in batch, continuous and perfusion modes as well as allowing collection of supernatants for further downstream analytics [Parekh 2020]. The developed system can be used flexibly for a variety of applications including with synthetic biology, cell and gene therapy and organ-on-a-chip.

- *A dynamic perfusion based blood-brain barrier model for cytotoxicity testing and drug permeation* (Basma Elbakary, Aston University, UK).
  The blood-brain barrier (BBB) serves to protect and regulate the central nervous system (CNS) microenvironment. The development of an in vitro mimic of the BBB requires recapitulating the correct phenotype of the in vivo BBB, particularly for drug permeation studies. However, the majority of widely used BBB models demonstrate low transendothelial electrical resistance (TEER) and poor BBB phenotype. The application of shear stress is known to enhance tight junction formation and hence improve the barrier function [Johnson 2011; Cuculo 2011]. We utilised a high TEER primary porcine brain microvascular endothelial cell (PBMEC) culture to assess the impact of shear stress on barrier formation using the Kirkstall Quasi Vivo® 600

(QV600, Kirkstall Ltd. UK) multi-chamber perfusion system. The application of shear stress resulted in a reorientation and enhancement of tight junction formation on both coverslip and semi-permeable inserts, in addition to enhancing and maintaining TEER for longer, when compared to static conditions. The functional consequences were demonstrated with the reduction in flux of mitoxantrone across PBMEC monolayers. The QV600 perfusion system may service as a viable tool to enhance and maintain the high TEER PBMEC system for use in in-vitro BBB models [Elbakary 2020].

- *Automated Three-Dimensional Cell-Based Assays in Animal Free Nanofibrillar Cellulose Hydrogels* (Jonathan Sheard, UPM, Helsinki, Finland).
  Animal-derived hydrogels often used for 3D assays have known challenges, e.g. batch variation and presence of undefined components. GrowDex® hydrogels (UPM, Finland) made from plant-derived nanofibrillar cellulose (NFC), are fully defined and reproducible, ready to use, and biocompatible for cell culture resembling the extracellular matrix (ECM) whilst allowing free diffusion of small molecules such as nutrients, drugs and oxygen. Shear thinning properties, temperature stability, and tunable stiffness allows for their use in a multitude of applications, including automated 3D cell-based assays. GrowDex® has been shown to be an effective support matrix for many cell types including primary tumour cells [Rinner 2017], stem cells [Lou 2014], liver cells [Malinen 2014], pancreatic islets [Chen 2019], and cancer cell lines [Barnawi 2019], allowing them to form spheroids or organoids. The screening of these 3D cultures against chemotherapeutic drug libraries revealed differences in drug responses between 2D and 3D culture conditions. Primary hepatocytes and liver cell lines were shown to form 3D spheroids with polarized structures and could be cultured for extended periods with stable metabolic activity, thus enabling drug hepatotoxicity tests in repeated dose assays. Multicellular structures can be recovered for downstream analysis without impact to cell viability, phenotype, or function.

- *Status of Organ on a Chip technology and progress towards replacement of Animals* (John Malcolm Wilkinson, Technology for Industry, UK).
  Organ on a Chip (OOAC) technology (now also known as microphysiological systems, MPS) has made great progress in recent years but has failed to make significant inroads into replacement of animal testing. Dr. Wilkinson reviewed the major commercial and academic efforts to develop robust, physiologically relevant and, more importantly cost-effective assays and tests that can be used in the study of disease or for screening new drug candidates. Microfluidic MPSs have been under development for over 15 years and have been very much a technology driven field until now but there is a shift as more attention is being paid to the market requirements and needs of customers. The companies involved are mostly start-ups and they are still refining their business models. Some are selling product, but most are following grants or research contracts. There have been many academic research papers, but they have not been significantly adopted by the pharmaceutical industry. Recent workshops [Marx 2016] have identified several of the challenges and hurdles that are being faced. These include technical, economic, and slow regulatory acceptance as well as communication gaps between potential users and developers. This paper set out clear functional and performance standards as well as economic criteria that will need to be met, both at the general level and for some specific applications (Figure Wilkinson).

Diabetes and other age-related disorders

Dr. Victoria Kearns, University of Liverpool UK, chaired this session. Prof. Lorna Harries, University of Exeter, UK, in her keynote lecture titled 'Cellular stress and beta cell identity changes', talked about a humanised cell culture system for EndoC-βH1 beta cells [Jeffrey 2017].

Session talks were as follows:

- *A Multicellular Scaffold Based Pancreatic Ductal Adenocarcinoma Model – Towards Animal Free Research* (Eirini Velliou, University of Surrey, UK, now University College London, London, UK).
  Dr. Velliou presented a novel 3D scaffold base hybrid, multicellular pancreatic tumour model consisting of pancreatic cancer cells, stellate, and endothelial cells [Totti 2018; Gupta 2019, 2020]. This scaffold-assisted pancreatic cancer model recapitulates the tumour niche due to tuneable mechanical and biochemical properties and structural integrity along with enabling cell-cell, cell-Extracellular Matrix (ECM) interactions. The model remains viable with physiological features for up to 2 months, enabling the conduction of fractionated treatment and long-term post-treatment observations in vitro.

- *MicroRNAs (miRNAs) responsive to the diabetic microenvironment in the human beta cell line EndoC-βH1 may target genes involved in cell function and survival* (Nicola Jeffery, University of Exeter, UK).
  MicroRNAs are small non-coding RNA species that comprise a key component of cellular stress responses. Altered expression of miRNAs has been reported in the islets of human donors with type 2 diabetes, but the effects of different aspects of the diabetic microenvironment, and the responses of beta cell genes at the level of gene ontology pathways have largely gone unexplored. Dr. Jeffery's group set out to identify the miRNAs dysregulated by different aspects of the diabetic microenvironment and to classify their targets into functional pathways. Dr. Jeffery detailed a fully humanised beta cell line model system that showed an improved glucose sensitivity exhibited by changes in the expression of protein, genes, and miRNA. Furthermore, global miRNA screen and validation showed altered expression of miRNAs associated with glucose function, cell function and survival.

Animal Free Research

Dr. Alpesh Patel, Animal Free Research, UK, was the chair of this special session. Dr. Carla Owen, Animal Free Research, UK gave the keynote lecture 'A systemic approach to transforming biomedical research and regulation'.

The session talks were as follows:

- *Identification of Bisphenol A-Regulated (BPA) Genes in ER+ Breast Cancer Using Animal Free Approaches* (Kerri Palmer, University of Aberdeen, UK).
  The exact mechanisms of BPA in the development of breast cancer are not fully understood. The bioinformatic analysis presented in this talk identified BPA-regulated differentially expressed genes between non-malignant breast tissue and ER+ breast cancer. This analysis also identified specific pathways regulated by these genes and identified adipose tissue as potentially having a more prominent role in breast cancer development. The endocrine disrupting chemical BPA is found in the environment at low levels and can leach from products such as plastic bottles leading to unintentional exposures [Grumetto *et al.,* 2008; Le *et al.,* 2009; Cooper *et al.,* 2011]. These exposures may influence breast cancer development, but exact mechanisms are incompletely understood [Diamanti-Kandarakis *et al.,* 2009; Macon & Fenton, 2013;

Gore *et al.,* 2015]. The bioinformatic analysis presented in this talk identified BPA-regulated differentially expressed genes between non-malignant breast tissue and ER+ breast cancer. This analysis also identified specific pathways regulated by these genes that may present future targets for breast cancer prevention. Furthermore, adipose tissue was identified as potentially having a more prominent role in breast cancer development and progression; a view that is also being increasingly shared by others [Wu *et al.,* 2019; Marino *et al.,* 2020].

- *Raising an aptamer against the peptide hormone Kisspeptin, for potential diagnostic use in Alzheimer's disease and Ovarian Cancer* (Sheree Smith, Leeds Beckett University, UK).
  Smith described the synthesis of a non-animal-derived affinity reagent (ssDNA aptamer) against the peptide hormone kisspeptin. This aptamer could be used for research and diagnostic purposes in Alzheimer's disease and ovarian cancer. This paper also highlighted the ease of aptamer generation and its applicability to many other fields of research. Whilst aptamers have limitations such as susceptibility to degradation and cross-reactivity [Lakhin, et. al., 2013], increasing the number of positive selection rounds can counteract the issues with cross-reactivity. Post-selection chemical modifications also exist to strengthen binding affinities and resist degradation [Odeh, et al. 2019]. Overall, aptamers have huge potential and some are already commercially available.

- *Endogenous Mas-Related G-Protein-Coupled Receptor X1 Activates and Sensitises TRPA1 in a Human Model of Peripheral Nerves* (Hayley McMillan, Queens University, Belfast). This study aimed to investigate the role for Mas-related G-protein coupled receptor X1 (MrgprX1) in pain signalling via its interaction with the pain receptor, Transient Receptor Potential Ankyrin 1 (TRPA1) using an in vitro model of human nociceptors derived from human dental pulp stem cells. Peripheral nerve equivalents (PNEs) were generated using a fibronectin differential adhesion protocol, established previously (Clarke 2017). MrgprX1 and TRPA1 protein expression was investigated using immunocytochemistry. MrgprX1 receptor signalling and the mechanisms through which it couples to TRPA1 were studied by Fura-2-based Ca^{2+} imaging. Immunocytochemistry confirmed endogenous protein expression of both MrgprX1 and TRPA1 in PNEs. Ca^{2+} imaging results showed that MrgprX1 couples to the $G\alpha_{q/11}$ pathway and activates TRPA1. In addition, MrgprX1 sensitises TRPA1 to agonist stimulation via Protein Kinase C (PKC). Hence, the study concluded that MrgprX1 both activates and sensitises TRPA1 in a model of human peripheral nerves, suggesting an important role for this receptor in the modulation of pain.

In Vitro Models for Animal Replacement

This session was chaired by Dr. Virginia Pensabene, University of Leeds, UK. In the keynote lecture 'Medical devices biocompatibility testing in vitro - Are we there yet?', Dr. Helena Kandarova, Slovak Academy of Sciences, Slovakia, addressed the status of the use of alternative methods by medical device industry and implementation of the validated in vitro assays into the ISO standard 10993 for biocompatibility testing. She described the development, validation and implementation of an in vitro test based on in vitro 3D reconstructed human skin models for intra-cutaneous testing of extracts from medical devices. The new ISO standard 10993-23, describing this method, has been published in January 2021. Dr. Kandarova noted that it took more than 10 years to develop, validate and implement this method into the ISO standards. She also highlighted that to accelerate the regulatory acceptance of the novel approaches, it is necessary to include the regulators from the beginning of the validation process. The team participating on this international

multicentric validation project has been awarded two Society of Toxicology (SOT) prizes and recognised by LUSH in their 2020 contest – as the “commended project” in the category ‘Lobbying for alternatives’.

Session talks were as follows:

- *Comparing an in vitro Organ-on-Chip model of Non-Alcoholic Steatohepatitis (NASH) to murine models and liver tissue from patients* (Tomasz Kostrzewski, CN Bio Innovations Ltd, Cambridge, UK). The presented studies demonstrated a liver Microphysiological System (MPS) to culture primary human liver cells for multiple weeks and to induce a disease state using physiologically relevant stimuli. The phenotype of the model was interrogated in detail and shown to correlate with patient samples at the transcriptional and proteomic level [Kostrzewski 2017, 2020; Vacca 2020]. To demonstrate its utility in drug development, the MPS was treated with the bile acid analogue Obeticholic acid which caused dose responsive reductions in a wide range of disease endpoints. NASH is a disease that involves multiple tissues and peripheral immune cells and therefore, the model could be further enhanced with the addition of other cell types or by being interlinked with other tissue models (e.g., adipose, gut, pancreas) (Figure Kostrzewski).

- *Current regulatory guidelines and technological development of 3D models: closing the gap* (Janette E Turner, Safer Medicines Trust, Kingsbridge, UK).
  The scientific progress of 3D models over the last 10-15 years has resulted in estimates that a reduction in total research and development costs for drug development of up to 26% is expected through organ-on-chip (OOC) or microphysiological system (MPS) technologies. Dr. Turner examined how these New Approach Methodologies (NAMs), with specific reference to 3D models, are increasingly being recognized as viable models in some aspects of human drug discovery but due to legacy regulations which mandate animal use in preclinical testing and an undeveloped process of validating these models, they are not being fully adopted across countries or regulatory agencies. The need for harmonisation and standardisation in this field was discussed. The development and adoption of these models in non-regulatory settings was also highlighted to show how their use may help provide a better understanding of disease and help tailor precision medicine treatments.

- *The potential of inflamed human Alveolar Epithelial Lentivirus immortalized (hAELVi) cells as an in vitro test system* (Julia Katharina Metz, PharmBioTec Research and Development GmbH, Germany; Department of Pharmacy, Saarland University, Saarbrucken, Germany).
  Dr. Metz described a stepwise in vitro approach to investigate lung inflammation. Development of an in vitro test strategy for orally inhaled drug products, the adverse outcome (AO) represented by the reduction of the epithelial barrier during an inflammation was addressed. The hAELVi cells [Kuehn 2016] were inflamed by stimulation with the proinflammatory cytokines tumour necrosis factor-alpha (TNF-α, 25 ng/mL) and interferon-gamma (INF-γ, 30 ng/mL) for 48 hours. The apparent permeability coefficient (P_{APP}) of the tracer molecule sodium fluorescein (NaFlu) rapidly increased when falling below a TEER of 245 Ωcm². This value was calculated by a piece-wise linear fitting of the transepithelial electrical conductivity (TEEC), the reciprocal of the TEER (Figure Metz) [Metz 2021]. The approach included the combination of a human cell line hAELVi and a standardised protocol to induce inflammation, thus potentially enabling a better comparison with human data and improving reproducibility. By calculating a specific threshold value (critical

Transepithelial electrical resistance, TEER value), a categorisation of the inflammation level could be possible.

Changing the world: Good science is not enough to persuade researchers to replace animal testing – a roundtable discussion

At the end of the conference, a roundtable session was run with special focus on animal-free research. This highly interactive session, moderated by Dr. Wilkinson, Technology for Industry Ltd., UK, started off with five short talks (5 minute each) delivered by pre-selected panel members. These talks aimed to make introductory positional statements to steer the discussion towards thought-provoking open-ended questions, followed by 35 minutes of dialogue among the participants of the session. All the delegates of the conference were invited to participate in the roundtable discussion.

The panel talks were:

- *The need for Economic Justificatio*n (John Malcolm Wilkinson, Technology for Industry Ltd., UK).

- *The role of scientific skills in bringing about the change* (Bhumika Singh, Kirkstall Ltd., UK).

- *The End Game – a strategy to end animal modelling for the benefit of patients* (Alex Irving, Andre Menache, Patients Campaigning for Cures, UK & EBVS® European Veterinary Specialist in Animal Welfare Science, Ethics and Law).

- *Need for Regulatory Change* (Janette E Turner, Safer Medicines Trust, Kingsbridge, UK).

- *Catalysing the world's transition to human-specific medical research and testing* (Jarrod Bailey, Centre for Contemporary Sciences, USA).

Summary of the roundtable discussion

Many core in vitro assays are still reliant on animal (non-human) cell types. Substituting the existing animal tests with more in vitro tests may be risky as relevance to humans and predictivity may be lost. New Approach Methodologies (NAMs), with specific reference to 3D models, are increasingly being recognised as viable models in some aspects of human drug discovery. Microphysiological systems (MPS) may be employed in many areas of drug discovery and perhaps hold the greatest potential in delivering personalised treatments and patient screening of therapies. But, due to legacy regulations which mandate animal use in preclinical testing and an undeveloped process of validating these models, these are not being fully adopted across countries or regulatory agencies. Regulatory acceptance may be accelerated by generating data comprising of comparison of animal in vitro and in vivo outcomes with the corresponding human in vitro and in vivo outcomes. The need for harmonisation and standardisation in this field was discussed. The development and adoption of these models in non-regulatory settings was also highlighted to show how their use may help provide a better understanding of disease and help tailor precision medicine treatments.

So, we noted, that although advances have been made in the scientific research to adhere to the principles of the 3Rs, the extent of engagement required at many fronts in the current society to implement the outcome is inadequate. Much more collaboration during the

development of MPS (or any other novel platform) is needed so all stakeholder perspectives and requirements are captured early on.
There was general consensus amongst delegates that the way forward to 'changing' the world into adopting human relevant methodologies is by establishing collaborations with all the stakeholders, educating them, training current and new students, and setting up of new organisations dedicated to promoting the scientific advantages of such approaches.

The following are the key comments and ideas that emerged from the discussion:

- *Imparting confidence in all parties is key*. W*e need all stakeholders together. These stakeholders are companies requiring registration of drug or chemicals, contract research organisations (CROs), regulatory bodies, non-governmental organisations, in vivo scientists and technicians, consultants, and innovators*.

- *There has been a communication gap between regulators, end users and developers, except in genetic toxicology and safety pharmacology. This has been recognised and activity to bring all stakeholders is taking place (c.f. US EPA). Regulators should clarify i) their needs ii) establish what data is required to address that need iii) accept performance criteria and iv) provide a clear pathway for the evaluation of new methods if adoption is to succeed.*

- *The sharing of success stories from end users/developers would help educate regulators, entrepreneurs and investors.*"The replacement of mice for Botox testing is a success story.''

- *Set up of organisations.* One recent example is the Center for Contemporary Sciences (CCS - contemporarysciences.org), USA, launched in 2020 to help catalyse a paradigm shift in research to human-specific investigation. Dedicated solely to this vision, the organisation is developing educational, academic, collaborative and policy programmes to achieve it. Other older organisations including the UK based NC3Rs (https://www.nc3rs.org.uk/) launched in 2004, and the North American 3Rs Collaborative (NA3RsC, https://na3rsc.org/) have been propagating the importance of the 3Rs by promoting training, funding, and collaborative opportunities.

- *Educating and training of the stakeholders, students, and innovators. Showcasing scientific breakthroughs, publishing our own reviews, conducting proactive workshops to solve research issues using human-specific methods would help increase the pipeline of early career scientists.*

- *Working with educational establishments, ensuring students are informed about NAMs, preferably via course content. "I only had a brief introduction the 3Rs during pharmacology undergrad but was fortunate to be funded by NC3RS for my PhD. They have some really interesting educational resources online which would definitely benefit pre-PhD level students to consider.''*

- *There is a need for smart data interpretation and stratification of assays depending on the complexity and the context of the research question being investigated.*

Discussion

The ACTC conference included seven theme-based sessions including keynotes lectures, session talks and roundtable panel discussion as part of the two-day programme. The

overall aim of this event was to bring the scientific community up to date with the latest research and technological advances in the cell and tissue culture field primarily the in vitro modelling for drug discovery and disease mechanisms. Due to the imperative need to find more predictive human-relevant systems and valid alternatives to invasive animal experimentation, there has been increased attention given to the development of advanced in vitro technologies. For example, models of the alveolar epithelial barrier of the human lung [Clift 2020; Movia and Prina-Mello, 2020; Kopanska 2019]. It is now proven that the extension of the models beyond single cells to multiple cell types would allow them to exhibit anatomically correct structures, thus increasing the accuracy, predictive power of the models [Miller 2017]. These systems have been used with great effect to study the potential adverse biological impact of numerous xenobiotics (*e.g.,* chemicals, particles, and nanomaterials), and for initial drug efficacy studies [Faber and McCullough 2018]. Despite the clear advantages of multi-cellular in vitro model compared to monoculture systems due to allowance of important cell-to-cell interplay [Movia and Prina-Mello, 2020], the static nature of multi-cellular in vitro systems is unable to provide the physiological, dynamic environment of the in vivo scenario. Therefore, focussing upon re-creating the dynamic physiological environment together with multi-cellular models is imperative to progress this area of alternative technologies. Approaching the advancement of multi-cellular models *via* improving their physiological characteristics, in essence making them dynamic in nature, similar to in vivo, will enable increased sensitivity and make in-roads towards in vitro replacement strategies towards animal experimentation. Theoretically, it is all possible, but it is not yet achieved. The ability to make versatile models and understand the intrinsic role of the microenvironment requires the creation of clinically relevant systems that embrace the complexity of the tissue under investigation, for example, the human brain. Despite considerable research, modelling brain tumours is still challenging due to the difficulties in reproducibility and the inherent complexity of both the neoplasm and the organ in which it grows. Current pre-clinical glioblastoma (GB) models, although progressive, fail to address the critical issue of the interaction between tumour cells and normal host tissue. Recently, Civita's group developed all human GB models where both non-neoplastic microglial and astrocytic cells were shown to grow with GB cells within a 3D extracellular matrix (ECM) which confers resistance to several drugs with different modes of action. New and highly sophisticated in vitro 3D approaches, stemming from either patient-derived tumour cells or pluripotent stem cells could provide in vitro models to study diffuse gliomas or metastatic brain cancer. However, these models still have limitations due to the absence of stromal component or functionalized vasculature (the blood-brain-barrier). These models also often lack reproducibility. Incorporation of co-or multiple- stromal cell types to the system along with human serum supplementation and appropriate oxygen concentrations could significantly improve the model and be used to test a wide range of nano-delivery vehicles and agents. In another example, the in vitro model of lung presented by Dr. Movia offers refinement of early-phase clinical testing which may allow for a better selection of drugs with disease-specific activity and, subsequently, more likely to succeed in Phase 3 trials [Paul 2010; Movia 2020]. This model will could be further improved by inclusion of the complexity of the lung tumour microenvironment (TME). The TME is a complex structure comprising cancer cells, extracellular matrix (ECM) and stromal cells. Physical interactions are formed between the ECM and cancer (NSCLC) cells, regulating tumour growth/invasion, and abrogating the effects of drug intervention. Optimisation of controlled cellular microenvironment is very tedious, expensive and time consuming. An approach to build robust biomechanical and biophysical control of the TME in the model of the pancreatic duct adenocarcinoma presented by Dr. Velliou, University of Surrey, would enable accuracy in the disease modelling and treatment screening. Next, with regards to age-related and chronic disorders, the ability of human cell model to maintain their quality to reproduce

relevant features of human disease over long time periods will be key to furthering our understanding of chronic diseases such as diabetes. The ability of these models to reproduce and predict the response to treatments will allow them to contribute to new therapies. In addition to novel experimental approaches described above, computational systems biology tools will allow for additional avenues for exploration at multiple biological scales. This will enable more accurate comparison between in vitro and in vivo and more detailed integration of multi-parameter datasets and further improvement of the experimental design. Large scale studies which employee these models along with detailed pathological analysis, for example combination of histomorphology, molecular, genetic, and metabolic characterization, will be required to develop a full, scalable, sensitive, and reproducible in vitro model that could be robustly employed in the evaluation of compound efficacy and safety.

As discussed earlier, in vitro studies could inform optimal clinical drug use and improve understanding of tumour or disease pathobiology [Barcher 2020]. However, lack of general accepted criteria and guidance for in vitro studies has led to poor quality and reproducibility of studies for pre-clinical drug testing and delivery. The systematic approach presented by Prof. Karen Pilkington, using strategies, utilized mainly for clinical trials, has provided a sound base for discussion among in vitro experts although suffering from the low response to the initial questionnaire [Hartung 2019]. Results of the study provided a list of criteria for best practice in in vitro research and testing which are now being used by a panel of experts to produce documentation of criteria and guidelines which will standardise practice in a range of in vitro techniques, thus enhancing quality and reproducibility of such work.

So, what more do we need to do to achieve full, scalable, reproducible, sensitive, predictive, and most importantly, human-relevant in vitro models?

- Focus on characterising the models and comparing to the human organ being modelled with respect to structure and function. Design large scale studies with detailed all-round pathological analyses (for example combination of histomorphology, molecular, genetic, and metabolic parameters)
- Move away from animal-derived products used in conventional cell culturing techniques, as these can induce phenotypic changes in the human cells grown in vitro, influencing their responses to drugs and decreasing the confidence level of preclinical models.
- Help accelerate the uptake and use of models by major public sector and industrial stakeholders, as well as acceptance by legislative and regulatory bodies.

Conclusions

As in previous years, the ACTC conference succeeded in providing a systematic platform for researchers to present and discuss their scientific approaches and findings with a scientifically diverse community. This report detailed the proceedings of the latest conference that was held virtually over two days 30th (September and 1st October 2020). The virtual platform augmented the geographical reach of the event and the benefit was evident by the enrichment of the scientific participation from the UK, Europe, and wider geographies including North America, and Africa. The content of the sessions helped learn the state-of-the-art, understand the current challenges and how the new methodologies could address these challenges in the current landscape of the cell and tissue culture field. To conclude, replicating tissue microenvironment, multi-cellularity, utilisation of patient-derived cell types where appropriate and integration of dynamic flow systems are the future directions for advanced research in the in vitro modelling field. Microphysiological systems do hold the

potential to significantly increase the success rate of candidate therapies in clinical development. One common and the most important message that emerged from the discussions in the event was that the engagement of the stakeholders in the implementation of the new technologies at all levels needs to be strengthened. They are not sufficiently aware of the existence and capabilities of human-specific methods. Funding of, and confidence in, human methods are not as sufficient as the evidence demands. All need attention and appropriate action - greater funding, training and awareness, collaboration, investigation opportunities, and confidence in human-relevant approaches.

Conflict of Interest

The authors declare that the report was prepared in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Bhumika Singh is an employee of Kirkstall Ltd., UK, a manufacturer of advanced tissue culture fluidic devices.

Author Contribution

BS drafted the manuscript. All authors contributed to the content of the manuscript.

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