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Preface

# Cell-based drug testing; this world is not flat☆

The actual costs for successfully transforming a lead drug candidate from new molecular entity (NME) to clinical product are debated to lie between $800 million and $1.2 billion with development timelines spanning 8–12 years. Yet, the costs of failure in this process are substantially greater: new therapeutics to treat key diseases are not yet available, any future progress based will only be more expensive using status quo strategies, newly approved drug products are withdrawn more rapidly and more frequently, increasing claims for substantial equivalence in new drug approvals, few "first-in-class" drugs, all question both therapeutic innovation and progress. Industry drug development R&D costs increase annually, with unimproved productivity as reflected in the reduced numbers of new drug approvals at double the cost from a decade ago [1]. Consistently high failure rates for NMEs in lead development, especially those in expensive late-stage clinical trials, indicate that currently employed pre-clinical models do not adequately provide critical information required for go/no-go decisions on NME selection or about possible adverse drug interactions, pharmacologies and patient variabilities required to de-risk the streamlining process [1–8]. And all of this despite record global resources and financial drivers seeking successes…

While diverse non-scientific factors (e.g., poor standardization, policy, attitudinal, financial, economic, regulatory) are strongly co-implicated in these failures [1], scientific and technological contributions that seek to inform and improve the drug development process have historically focused on two objectives:

- Providing more accurate knowledge to inform and guide drug discovery processes, and thereby improve NME success rates (i.e., through improved cell-based assays, improved analytical methods, applied genomics, computational structure-based drug models, data analysis algorithms, and combinatorial chemistry).
- Creating entirely new therapeutic approaches that by-pass traditional small molecule drug development bottlenecks and pitfalls (i.e., antibody, gene, and cell-based therapeutics).

Traditionally, far more resources and efforts have been devoted to the former strategy over the latter, although the future could change this balance. Efforts are focused on recognized deficiencies in the lack of predictive animal disease models, poor quality evidence for mechanism of action (MOA)-based drug efficacy and MOA-based toxicity, poor formulating properties and pharmacokinetics, and predictors for drug safety.

Nonetheless, it appears that academic and industrial processing of increasing amounts of new scientific information made available from new drug assessment technologies on drug leads might actually lengthen drug development timelines. Such experiments frequently add to the current processing effort and timeline without supplanting traditional development processes. They also add more information that must be parsed and analyzed, and might not necessarily directly inform better decision-making in NME selection. The idea that more knowledge necessarily aids and abets science but not necessarily the decision-making required to continue or kill pursuit of a NME is the problem with new knowledge provision. More data might not be better data, especially for critical development path decisions to continue or stop an NME study. New industrial drug development models now emphasize use of improved technology or information to help pharmaceutical candidates 'fail faster' in drug development processes, notably in preclinical models, using ADME methods and other in vitro prediction and in vivo validation tools.

The on-going inefficiencies in creating new therapies with higher reliability and lower costs are costly and humiliating. They also continue to erode the credibility of the pharmaceutical industry and academic researchers in advocating their traditional pipeline development strategies yielding only modest drug innovation successes. Drug development screening tools that reliably improve either predict translational success or the "fail faster process" add substantial value to the pharmaceutical pipeline through streamlining time and expense. Their continued utility in producing more reliable decision-making for drug developers would be immensely valuable. However, if drug developers do not embrace the extents or natures of various NME screening problems, then value added from new, improved NME screening methods will not be realized.

The persistence of traditional in vitro monolayer cell-line monocultures on rigid plastic supports as the basis for cell screening for NME toxicity and efficacy is troubling. It is long recognized that two-dimensional (2D) cell mono-cultures lack many of the requisite phenotypic details often expected for their utility in predictive drug assays. Mono-cultured cells are not tissues or organs and cannot be expected to produce similar structures or functions as tissues, especially as rigidly adhered monolayer cultures. Additionally, the human body comprises >200 normal human cell types, each with characteristic and specialized functions governed by unique gene expression patterns, cell–cell regulatory mechanisms and signaling networks, embedded in context-specific soft extracellular matrices and active transport networks. Essential cross-talk between these multiple cell types in tissues that respond to and are affected by drugs and their metabolites is certainly missing in even the most intricate co-culture systems. Some improvements have been directed at throughput, cell sourcing and phenotype validation, and assay prediction reliability for in vivo translation.

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Yet, despite repeated calls for improved cell assay reliability in drug screening, most workhorse efforts in both industry and academia remain focused on 2D cell culture drug screening methods. *What are the essential features of tissues, organs and dynamic metabolic systems required for accurate recapitulation* in vitro *to serve effectively in drug screening assays*? *Can certain minimalist technological* in vitro *cell-based approaches reliably predict* in vivo *human drug responses?* Only by more clearly distinguishing new cell-based methods that improve in vitro and preclinical NME screening reliability and translation to the human condition can their value in informing better NME streamlining be emphasized.

This theme issue of *Advanced Drug Delivery Reviews* provides extensive review and expert insights into many aspects of emerging three-dimensional (3D) cell-based and whole-organism methods for drug development. This theme issue is organized into sub-sections that group contributions along common theme lines and subtopics. Section 1 "3D cell culture technologies and their fundamental impact" opens with Astashkina and Grainger [9] overview of current challenges in current 2D cell culture models involved in drug toxicity screens. They argue, based on their research results, that accurate recapitulation of nephrotoxicity (as one example important to drug screening) requires co-cultures of kidney proximal tubule cells maintained within their native matrix context. Organoid 3D models satisfy this requirement by retaining native tissue structure and complexity. They then extensively review and critique the various 3D cell culture methods and systems reported, pointing out benefits and shortcomings. A primary conclusion is that despite the multitude of new 3D cell culture technologies, few are validated against actual clinical or in vivo responses. This sentiment is echoed consistently through the contributions to this theme issue. The second contribution by Ranga et al. [10] extends the organoid culture theme, surveying a new generation of drug screening technologies and recent developments in new stem cell-based organoid culture systems They propose that complex self-organized organoid proof-of-concept examples support their utility as miniaturized proto-organs that effectively bridge between primary high-throughput screening and costly animal and human trials. Safety and efficacy of lead compounds for treating a target pathology and also tumor response could be assessed not only in isolation but co-culture systems within an organoid of interest. For reproducibility and value, synthetic and tailored 3D matrices as well as meaningful, multiplexed readouts are important challenges. This first sub-section closes out with the contribution by Thoma et al. [11]. They assert that phenotypic heterogeneity of cancer cells, cells' biological context, their heterotypic crosstalk and cellular microenvironmental niches are key determinants in the multistep processes of tumor development. To screen tumor therapies, accurate tumor models are required. They discuss 3D cell culture technologies for growing tumor micro-spheroids as advanced tools for exploring molecular tumor growth determinants and facilitating drug discovery efforts. They survey several important technological aspects for on-line analysis and post-processing of 3D micro-spheroid models, emphasizing the needs for a high Z-resolution and image analysis depth commensurate with tumor micro-spheres. For single-cell resolution, optical microscopes must be capable of accessing cells close to the microsphere core at high resolution and at high image speeds.

Theme issue Section 2 "Complex 3D in vitro systems and disease models" first examines 3D matrix cultures systems seeking to duplicate important structural, mechanical and pathological aspects of breast tissue in a contribution by Weigelt et al. [12]. Profiling the genomic aberrations in breast cancer has revealed the diversity and complexity of the disease at the genetic level. The functional consequences of the specific mutations and copy number changes on signaling pathways in breast cancer require model culture systems that recapitulate the disease. Their review of models of normal mammary epithelial cells and cell transitions to breast cancer, including primary tumors and dormancy, highlights the challenges that need to be met for reliable generation of heterotypic breast cancer model systems amenable for high-throughput approaches. They conclude that improved understanding of the epistatic interactions between genetic aberrations, the structural and stromal microenvironment and drug response, 3D heterotypic cell culture models more representative of the different types of primary breast tumors and the metastatic setting are required. Håkanson et al. [13] then describe new in vitro models of physiologically relevant microfabricated cell-culture systems intended for drug development. Their two stated primary motivations for use of miniaturized systems include 1) the benefit of miniaturization to facilitate control of microenvironmental cues, enabling more predictive outcomes; and 2) reduced reagent consumption that enables use of scarce patient-derived samples for personalized approaches. The many novel in vitro models for capture, manipulation, analysis and drug response evaluation of cancer cells has exploited novel microfluidic devices with better control of culture environments, cell capture and positioning, and new methods for assay read-out. However, validation against clinical data to assert improved predictivity is still largely missing. They assert that technologies for automatic handling and read-out methods are also needed.

The next contribution from DesRochers et al. [14] focuses on recent advances in tissue engineered kidney disease models exhibiting a capacity to address traditional limitations of models of polycystic kidney disease, drug-induced nephrotoxicity, and the native glomerulus. They emphasize tissue-engineered models of autosomal dominant polycystic kidney disease (ADPKD) and drug-induced nephrotoxicity (DIN) because most progress has been associated with these models using tissue engineering strategies. They point out that despite progress from increasingly sophisticated, engineered systems, current efforts require extensive further development as these test systems must better recapitulate in vivo drug metabolism, uptake, and mechanism of action in order to stand as biologically accurate models. Merely establishing an LD50 to describe drug reaction in tissue engineered models in insufficient. Systems that properly metabolize drugs with active transport proteins to facilitate proper uptake are needed. Mechanisms of cell death must more accurately recapitulate that seen in vivo. Data generated in 3D models to date is not validated against that in an in vivo environment — a critical current deficiency. Full recapitulation of the human kidney with respect to structure and function will require compartmentalized fluid flow, reliable kidney cell lines capable of continued passaging and long-term cell growth, and maintenance of proper phenotype and function. They claim that development and characterization of improved material scaffold systems capable of promoting and retaining cell organization and function while maintaining complex kidney architectures are needed to move beyond structural randomness of current in vitro 3D cultures and cell monolayers in microfluidics. The contributions from Mathes et al. [15] moves to the technologically mature area of 3D tissue models of human skin, developed extensively for clinical transplantation of in vitro grown tissue substitutes and by the cosmetics industry as alternative test beds that replace animal models. Their review summarizes and discusses the diversity of different approaches to skin model development within the context of drug development. In this application, they are of particular interest for assessing systemically acting drugs applied topically to the skin, and topical drugs acting locally at the site of application (i.e., for skin therapies). While often used as stand-alone approaches for drug development processes, cross-over comparisons of various skin models are needed. Other challenges include standardized production procedures, process automation, establishment of sensitive analytical methods, and data correlation. Requirements for specific applications could demand more sophisticated skin structures or rapid scalability. Definition of appropriate and relevant performance standards for skin constructs, high reproducibility among experiments; correlation to in vivo data and systematic investigations of impacts from different drug application and formulation methods were identified as needs.

Giese and Marx [16] contribution examines methods for immunogenicity testing of biopharmaceuticals, nanomedicines and adjuvant systems where conventional 2D culture assays have little success to date.

They examine 3D approaches to emulate innate immunity in non-lymphoid organs and adaptive immune response in human professional lymphoid immune organs in vitro. The critical relationship between changing the architecture of professional lymphoid organs at rest and when activated by pathogens should be matched with models of immunity identified in vitro. Lymphoid tissue architectural models relevant to development of sustainable adaptive immune responses in vitro are summarized, despite most being at a very early stage of development. Recent efforts and achievements in 3D modeling of immune-competent human gut, lung and skin are reviewed as important facilitators for the future integration of the resident elements of innate immunity into these models in organotypic architectures in the near future. The few existing dynamic tissue culture platforms of professional lymphoid 3D organ equivalents they review provide a basis for further improvements of lymphoid tissue architecture, cellular composition and the antigen exposure process for development of a sustainable adaptive immune response. Nonetheless, the existing immune-competent non-lymphoid or professional lymphoid 3D in vitro systems they describe provide a translational alternative to recapitulate immunogenicity pathways in humans; neither do the human in silico immunity models. They conclude that human immunity in vitro can only be reached by improvements to the "human-on-a-chip" concept.

The last contribution in this sub-topic section comes from LaBarge et al. [17] who discuss how combinatorial microenvironment microarrays and other biomimetic microenvironments reveal emergent properties of cells in specific micro-environmental contexts. These platforms are asserted to measure phenotypic changes within those contexts. They describe computational tools that can unify the microenvironment-imposed functional cell phenotypes with underlying "constellations of proteins and genes". They propose a new merger of these two disparate technologies to enable more accurate pre-clinical drug discovery by integrating new bioengineering approaches with multi-parametric measurement methods for cellular phenotypes. The authors detail combinatorial MEArrays comprising hundreds of ECM components that define specific microenvironmental features as important to determining cell contextual-dependent drug effects. The ultimate aim is to compare responses in the same cell types across numerous defined microenvironmental conditions. Nonetheless, significant gaps exist between combinatorial array approaches and accurate recapitulation of a tissue microenvironment. New computational approaches that build a modular framework for complex queries of genomic data, cellular profiling, and chemical structures are proposed to facilitate exploration of relative contributions of genomes and microenvironments in drug responses and other emergent phenotypes of high order tissue-level organization.

Theme issue Section 3 "Organotypic tissue models and multi-organ systems" first introduces the long-standing challenges associated with liver-drug interactions. Ebrahimkhani et al. [18] focus on liver as the central metabolic and immunologic nexus in the human body. Significantly, it is also the direct or indirect target of most molecular therapeutics in metabolic processing. Diverse therapeutic and technological motivations drive current efforts to capture liver physiology and pathophysiology in vitro. These include prediction of metabolism and toxicity of small molecule drugs, understanding off-target effects of therapeutics, and as disease models for drug development. The authors describe bioreactor-based models to address the challenges in drug discovery and development, emphasizing design challenges in maintaining long-term liver-specific function and how emerging technologies in biomaterials and microdevices are converging to provide new experimental models. While bioreactors for liver cell and tissue culture are in use for several decades, applications specific to drug development are still nascent. As the liver is a highly complex organ containing many cell types, in many instances in vitro models containing only a few cell types are insufficient to capture this complexity and accurately predict drug-tissue interactions or model disease processes. Traditionally, complex, three-dimensional and multi-cellular engineered tissue models that can be maintained for long time spans in the lab and produce reliable, readily obtained metrics in response to various stimuli are elusive. Evidence suggests that by co-culturing multiple cell types found in liver, in vitro models can be generated that are more physiologically relevant. Commercial bioreactors directed at liver are just now becoming available, using microfluidic and microfabrication technologies. The authors conclude that the emerging frontier is the influence of the gut microbiome on liver function with respect to drug uptake, metabolism, and efficacy. Models employing multiple communicating tissue types will yield fascinating results with new implications for in vitro models.

The next contribution from Esch et al. [19] describes development and use of multi-organ microdevices to improve the drug development process. Multi-organ microdevices exhibit potential to aid new therapeutic strategies by providing a platform for testing in the context of human metabolism (as opposed to animal models). Further, when operated with human biopsy samples, the devices could be a gateway for the development of individualized medicine. These devices are designed to mimic physiologic relationships of organs and their interactions via soluble metabolites, capturing inter-organ effects in vitro. The low cost of the devices permits testing a large number of drugs and drug combinations with human tissues instead of animal tissues. This bears the potential advantage of providing a higher degree of accuracy when predicting toxic side effects for humans. The authors show the devices' capabilities to simulate first-pass metabolism, the conversion of anti-cancer prodrugs and their subsequent effects on tumor tissue, the synergistic actions of two MDR modulators, and modulation of bioavailability and toxicity via barrier tissues and tissues that absorb and store chemical compounds. The contributions describe how devices are used to test hypotheses about mechanistic models of drug toxicity. Current DARPA- and NIH-funded efforts in the US will likely result in several platforms with authentic ability to mimic metabolism using devices with primary or stem cell sources.

Theme issue Section 4 "Impact of 3D cell culture on drug discovery and development" begins with a contribution from McGivern and Ebert [20] who discuss commonly used cell lines for drug screening purposes and how pluripotent stem cells (PSCs) have or could fit into currently used approaches for drug discovery and development. Challenges moving forward include reducing cost, standardizing screening methods by reducing cell differentiation variability and enriching specified cell types, improving maturation of cells towards more adult phenotypes, and producing important validation data for toxicity, safety, and disease phenotypes against known compound standards. The huge financial burden of growing, maintaining, and differentiating PSCs compared to other rodent or human cell lines can be overcome by maintaining PSCs as aggregate cultures. This allows scaling up of differentiation while reducing maintenance time. Roth and Singer [21] then provide an overview of the different approaches undertaken to produce pre-clinical safety assessments, particularly organ toxicity. Development of more in vitro models relevant to human conditions, including models involving multiple cell types in 3D cultures, is of great interest to improve organ toxicity predictions. For example, a liver model capable of recapitulating formation of reactive metabolites, hepatocyte nonparenchymal cell interactions, and liver damage after prolonged incubation, is essential. Models incorporating liver Kupffer cells, stellate and endothelial cells adjacent to hepatocytes are important. Furthermore, a cardiac system that retains ion channel interactions beyond hERG, including structural damage-associated functional impairments is targeted using combinations of electrophysiological measurements and multi cell-type cardiac cultures. As both stashkina and Grainger [9] and DesRochers et al. [14] explain (vida supra), the kidney remains an in vitro toxicology challenge. Its structural complexity may only be duplicated by meticulously defined scaffold-based methods and semi-permeable membranes to model exchange of ions and drug transport. Such platform-based approaches provide a basic setting adaptable to different cellular and tissue models. Such platforms may be used not only to grow different organoids in isolation but may even be interconnected to recapitulate liver-generated metabolites

affecting heart or kidney (see Esch et al. [19] and Ebrahimkhani et al. [18] vida supra).

Berg et al. [22] then provide a contribution that reviews use of primary human cells and their co-cultures in drug discovery and describe key characteristics of co-culture models for inflammation biology (BioMAP systems), neo-vascularization and tumor microenvironments. They describe technical trends to enable and impact the development of physiologically relevant co-culture assays based on their previously described phenotypic assay platform that employs a suite of primary human cell-based assays, BioMAP systems. Each assay system contains primary cells or co-cultures in stimulatory conditions that reflect various aspects of tissue and disease biology of different tissue types: the vasculature, skin, lung, immune and inflammatory tissues. A key aspect of these systems is the use of combinations of stimulatory factors and cell types to mimic the in vivo pathophysiological state of the affected tissues. Because of the networked architecture of signal transduction systems within cells, these assays are claimed to produce cell responses qualitatively and quantitatively different than typical monoculture assays, and more representative of physiologic settings and disease states where multiple factors and cell types are present.

Seo and DelNero [23] focus on engineering approaches to drug delivery as a function of tumor-associated changes of vasculature and extracellular matrix (ECM). The authors provide a current biological understanding of these components and discuss their impact on transport processes. Genomic and epigenomic instability in these pathologies produces diverse clonal populations within a single tumor that cross-communicate among the different cell (pheno)types. This property and signaling is lost in uniformity of conventional monocultured cell lines. Current microfluidic, tissue engineering, and materials science strategies to recapitulate vascular and ECM characteristics of tumors are then reviewed. Finally, the challenges and future directions of the field that may ultimately improve anti-cancer therapies are described. Integrated cancer biology and engineering approaches are asserted to create realistic tumor models to advance current anti-cancer therapies. Both qualitative and quantitative parameters currently complicating drug transport in tumor-associated blood vessels and stroma may be elucidated with this approach. Future integration with organ-on-a-chip devices may not only help recapitulate systemic aspects of the disease, but also better define the spatiotemporal relationships and patient-inherent complexity complicating drug transport in the clinic.

Theme issue Section 5 "Microbial and animal models" features a contribution by Vyawahare et al. [24] addressing drug development issues involving microbial drug resistance. Bacterial resistance to antibiotics, or tumor resistance to chemotherapy depends on the tissue niche and the surrounding heterogeneous tissue microenvironment. As in vitro drug testing is performed in homogeneous environments, these assays do not duplicate the condition. Recent advancements in microfluidics and micro-fabrication now provide opportunities to develop in vitro culture models that mimic the complex in vivo tissue environment in this context. The authors first discuss the design principles underlying such models. They demonstrate two types of microfluidic devices that combine stressor gradients, cell motility, large populations of competing/cooperative cells and time-varying drug exposures. Using adaptive pressures that produce in vivo drug resistance, the authors show that drug resistance in vitro can occur at much greater rates in niches than in well-stirred environments. The contribution closes with their description of future directions for in vitro microbial culture models and how these can be extended to cultures of eukaryotic cells.

Phenotypic drug screening in whole organisms is usually more physiologically relevant and less artificial than in vitro cell-based approaches. In the next contribution, Schmitt et al. [25] provide a unique review of the rapid progress made in adapting novel genome editing tools (i.e., zinc-finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs), and CRISPR/Cas) to Xenopus embryos in arrayed cultures. Advantages of Xenopus embryos as in vivo models to study human inherited diseases and their utility for drug discovery screening are discussed. As a tetrapod, Xenopus complements zebrafish as an indispensable non-murine animal model for the study of human disease pathologies and the discovery of novel therapeutic agents for inherited diseases. When coupled with high-throughput whole organism models such as zebrafish and Xenopus, mutant lines of human-inherited diseases are more precisely engineered for in vivo phenotypic drug discovery screening. One disadvantage of drug screening using in vivo models include the loss of potential lead compounds due to poor drug absorption. Other issues include poor relevance of some animal models to human diseases, and species-specific differences in drug metabolism that contribute to drug candidate failures in late-stage drug development. The authors show how novel genome editing technologies now enable the development of more accurate animal models of human-inherited diseases. Furthermore, increased emphasis on Xenopus as an evolutionary less distant relative to humans than zebrafish will reduce likelihood of failure of drug candidates once they reach preclinical testing.

Another alternative whole organism drug screening model is described by O'Reilly et al. [26]. *Caenorhabditis elegans* has proven to be a useful model organism for investigating molecular and cellular aspects of numerous human diseases. More recently, this organism is frequently used as a tool for drug discovery. Although earlier *C. elegans*-based drug assays were labor-intensive and low throughput, recent advances in high-throughput liquid workflows, imaging platforms and data analysis software now make *C. elegans* a viable option for automated high-throughput drug screens. The authors outline the evolution of *C. elegans*-based drug screening, and discuss the inherent challenges of using *C. elegans*. They highlight recent technological advances demonstrating value for future *C. elegans* drug screens. Numerous proof-of-principle studies have already demonstrated the versatility of *C. elegans* in compound screening, drug target identification and in deciphering drug mechanisms of action. Furthermore, increasing numbers of companies utilize *C. elegans* models in various drug discovery stages; drugs are currently undergoing clinical trials. Progress to date indicates that *C. elegans* will become an increasingly important tool in the drug discovery process.

Theme issue Section 6 "3D cell culture: new opportunities for regenerative medicine" closes the volume with a single contribution by Emmert et al. [27]. They provide an overview of the current state-of-the-art for using in vitro regenerative strategies to address cardiac pathologies. Numerous strategies are under investigation for evaluating regenerative medicine strategies on the failing myocardium, including classical cell-therapy concepts, three-dimensional culture techniques and tissue-engineering approaches. These therapeutic approaches and diverse modes of action are proposed to be classified as a form of drug delivery where natural regenerating component provide the endogenous biochemical signals to restore tissue structure and function. The authors describe how transplanted cells and tissue provide both precursors that can differentiate into functioning myocardial cells and also paracrine effects that recruit new cells, mediate inflammatory response, remodel the extracellular matrix (ECM) and prevent deleterious downstream effects of fibrotic scar tissue formation. Transplanted therapeutic cells and tissues that regenerate new tissue and restore function are considered promising and highly effective means of drug delivery to the failing myocardium. In vitro cell culture models that enable this capability are in development.

Overall, this is a comprehensive set of expert reviews featuring extensive, up-to-date descriptions of the current challenges in moving cell-based drug screening and toxicity testing beyond 2D cell monocultures on plastic to relevant test beds that seek to capture the in vivo complexity and dynamics. Whether more, new information provided by these new living models will improve drug development successes remains to be seen. Model validation against the in vivo situation appears to be the consistent call. Nonetheless, as NME output is essentially linear while costs of producing NMEs increase exponentially, this increasingly desperate situation requires scientific and technological

attention. This issue suggests that the drug development world is not flat with regards to cell-based testing. Increasing both the dimensionality and biological sophistication of cell constructs provides new opportunities to inform and guide NME pipelines as well as new information on fundamental disease pathology and tissue physiology. I encourage you to read this issue to facilitate broader understanding of the state of this field, its creative successes, innovations and formidable challenges.

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