Screening out irrelevant cell-based models of disease

[Au: please provide all author first names, and list them in the format 'Peter Horvath' etc]

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Abstract

The common and persistent failures to translate promising preclinical drug candidates into clinical success highlight the limited effectiveness of disease models currently used in drug discovery. An apparent reluctance to explore and adopt alternative cell- and tissue-based model systems, coupled with a detachment from clinical practice during assay validation, contributes to ineffective translational research. To help address these issues and stimulate debate, here we propose a set of principles to facilitate the definition and development of disease-relevant assays, and we discuss new opportunities for exploiting the latest advances in cell-based assay technologies in drug discovery, including induced pluripotent stem cells, 3D co-culture and organ-on-a-chip systems, complemented by advances in single-cell imaging and gene editing technologies. Funding to support precompetitive, multidisciplinary collaboration to develop novel preclinical models and cell-based screening technologies could have a key role in improving their clinical relevance, and ultimately increase clinical success rates **[Au: edits to shorten OK?]**.

Introduction

Although there have been many notable drug development achievements in recent years, several disease areas, such as neurodegeneration and aggressive cancers, remain largely intractable **[Au:OK?]**. For example, substantial investment in the development of novel therapeutics for Alzheimer disease, Parkinson disease and motor neuron diseases has largely failed [1-3]. This failure can be attributed, in part, to our limited understanding of the targets that may prevent or repair neuronal damage, and to a lack of robust disease-relevant preclinical models [1, 4]. In the field of cancer, important progress has been made in the discovery of new drugs, including those based on target-directed precision medicine strategies [5]. However, for aggressive cancers, such as glioma, pancreatic, oesophageal and several lung cancers, many promising drug candidates developed from standard cell-line screens and *in vivo* xenograft models did not show clinical efficacy. The poor clinical translation can largely be attributed to the failure of these models to recapitulate key pathophysiological features of the human disease, including complex inter- and intra-tumour heterogeneity, poor drug penetration through tissue, host-stroma–tumour cell interactions, and the cancer stem cell niche, all of which may have profound effects on therapeutic response *in vivo*. In addition, given the periodic emergence of new and old infections [6], and the pressing challenges from antimicrobial resistance and pandemic threats such as Ebola and Zika, modern disease-relevant cell-based phenotypic assay methodologies may represent valuable assets for improving our knowledge on the dynamics of host–pathogen interactions in their natural environment [7] and the development of new therapies (Box 1) **[Au:OK?]**. Finally, a substantial proportion of

clinical trial failures for novel medicines overall are due to safety issues such as cardiotoxicity and hepatotoxicity [8], and serious toxicity issues are often discovered only *after* clinical development has been completed [9][10]. Thus, more predictive toxicology models would contribute significantly towards more successful clinical translation and improved patient care **[Au:OK?]**.

Despite advances in target- and cell-based screening technologies, the majority of drug discovery projects remain dependent on cell culture systems that were developed several decades ago incorporating immortalized cell lines, the use of which many consider to be questionable owing to their poor disease relevance. While many traditional models provide valuable tools for studying drug mechanism-of-action and have helped identify successful drug candidates in the past, it is our opinion that the widespread use of contemporary cell culture assay systems must be re-visited and that efforts should be directed toward development of new models, new assay formats and innovative screening technologies which better recapitulate *in vivo* physiology.

In this article, after briefly summarizing the limitations of traditional cell-based models of disease **[Au:OK?]**, we discuss how emerging developments in (patient-derived) *ex-vivo* cultures, induced pluripotent stem cell (iPSC) technology, 3D co-culture and organotypic systems, complemented by advances in single-cell imaging, microfluidics and gene editing technologies, are well-positioned to advance preclinical disease modelling and drug screening across challenging disease areas. We outline a set of principles for defining “disease-relevant assays” (Table 1), and highlight methodological and analytical gaps, fundamental challenges and new opportunities to exploit and combine more

advanced *in vitro* models with emerging technologies. We conclude by discussing the need to further evolve translational funding schemes and precompetitive research consortia to support the future development of new preclinical models and assay-screening technologies that provide more robust target validation and greater clinical predictivity.

Limitations of traditional disease models

Traditional cell culture methods typically rely on cancer cells or immortalized cells grown within artificial environments, on non-physiological substrates such as functionalized plastic and glass. While these methods have facilitated the discovery of many basic biological processes, they often fail to provide an adequate platform for drug discovery owing to their inadequate representation of key physiological characteristics. These problems can be broadly categorized into the following limitations.

Limitations due to cells. Most cell-based assay screens have traditionally been performed using transformed or immortalized cell lines. These have been cultured for many generations, resulting in a substantial drift in their genetic, epigenetic and physiological characteristics, which means they are not a good model of primary tissue cells [11, 12]. The gross genetic and epigenetic abnormalities, characterized by multiple genetic rearrangements and amplified gene copy numbers, associated with long term culture confound pharmacogenomic and functional genomic studies. Genetic adaptation resulting from long-term *in vitro* cultures also contributes to heterogeneity in cultures of the same cell line between passages, batches and laboratories.

Limitations due to culture conditions. The media most commonly used for cell culture are designed for fast cell growth, incorporating large concentrations of fetal serum and nutrients, which may promote dedifferentiation of primary cell types into more embryonic or foetal-like phenotypes [13]. With the development of primary cell and differentiated stem cell cultures, the usage of high glucose and growth factor media has been eschewed for defined culture media to promote cellular identity rather than rapid growth. Cells are often grown in standard incubators under high oxygen partial pressure (approximately 20%), which does not represent the steady state conditions of human organs and tissues (fluctuating between 1%; dermis, and 14 %; arterial blood) [14-17]. Such conditions poorly recapitulate the distinct microenvironments that define normal and diseased tissue phenotypes. This has a profound impact on the cell metabolism, reactive oxygen species (ROS) production, mitochondrial functions and ultimately on the differentiation and function of cells [18, 19]. Additionally, conventional tissue culture systems do not readily permit the formation of short-range gradients of nutrients, hormones and oxygen that are often experienced by cells depending on the distance to the nearest blood vessel. The liver is a well-known example of this, with gradients in the lobules between the central vein and the portal artery leading to zonation [20]. This can be mimicked with the adoption of microfluidic systems that deliver nutrients, dissolve gases and remove waste products [21].

Limitations due to lack of appropriate cell culture substrates and bioengineering tools. The 2D planar substrates on which cells are typically grown are stiff (gigapascal), which are unlike most substrates found in a human body (milli– to kilopascal,) with the exception of bone and cartilage [22]. Hence the plastic or glass used in cell culture may be

far from representing the normal *in vivo* mechanical environment [23, 24]. For example, in the case of liver culture, most differentiation protocols require the use of sandwich cultures, where the cells are grown between layers of either collagen or other extracellular matrix (ECM) proteins. The mechanical properties of these supporting matrices are generally not well characterised, despite the fact that such properties are known to have a significant impact on cellular function and differentiation in tissues [25, 26]. In these types of experiments where a minimal quantity of deposed hydrogel is employed, it is likely that the cells would encounter a stiff environment, which the liver would normally only encounter during fibrosis or cirrhosis. Thus, toxicology assays are typically carried out under pathological rather than healthy liver conditions. Microfluidic devices, which utilize mechanical actuation systems to recapitulate mechanical forces or generate the shear forces that tissues experience in living bodies are beginning to be used; however, they will require further development and refinement if they are to be used for more general screening applications [27].

A further challenge evident in tissue modelling within current *in vitro* assays is the absence of more physiologically relevant extracellular matrix (ECM). For example, the popular use of Matrigel and collagen type I as an ECM substrate in hepatocyte cultures does not represent the predominant ECM proteins found in the liver [28]. Many pathologies are associated with changes in ECM production that have significant impact on cell and tissue function. Thus, recapitulating both physiological and pathophysiological ECM composition and structure is an important consideration for *in vitro* cellular models.

Limitations due to lack of appropriate co-culture methods. Cell-culture screening assays traditionally use a single cell type, whereas cells *in vivo* are either in direct contact or communicate over a long range with many different cell types. As most biological processes and pathologies involve the interaction of multiple cell types, ideally, these should be incorporated into *in vitro* cellular assays whenever possible. For example, most toxicology assays use only hepatocytes, but while 80% of the liver volume consists of hepatocytes (60% of the cells), other important cell types within the liver include stellate cells, resident macrophages (Kupffer cells), sinusoidal endothelial cells and some non-parenchymal cells. Both stellate cells and Kupffer cells are known to be important for some compound toxicities and should therefore be incorporated into *in vitro* toxicology assays [29, 30]. Neurodegeneration, where both astrocytes and glial cells are responsible for protecting neurons but are also known to cause neural death, provides a compelling case for the use of mixed cell cultures of distinct cell types [31]. Further development of co-culture methods, which incorporate disease cells with relevant immune subcompartments, are also urgently needed to help better understand and address the role of the host immune system in the pathogenesis and therapeutic outcomes of many diseases [32]. These considerations are of particular importance for pathogen biology and infectious diseases, which operate at multiple cellular and tissue levels (Box 1).

Tackling the limitations. Addressing the translational gaps presented by current limitations in preclinical assays by employing new models, which better predict efficacy or toxicity observed in patients still presents a number of imposing challenges including how to: mimic the microenvironment and heterogeneity of normal and disease tissue; take into account the environmental and genetic/epigenetic factors governing disease aetiology

and therapeutic outcomes; understand the effect of drugs upon the whole physiological entity, e.g. across multiple cell types and organs of the human body; and interpret the role of the host immune system in the pathogenesis of a particular disease. While it is clear that no single preclinical model or screening assay will faithfully recapitulate the full complexity of human disease, we outline below and in Figure 1 the latest developments in cell-based models and assay technologies that begin to address the limitations of traditional and contemporary *in vitro* assays.

Developments to improve disease modelling [Au:OK?]

Primary and patient-derived cell models. The adaptation of patient-derived primary cell samples, as well as fresh human tissue samples, for *ex vivo* and *in vitro* translational research applications aims to overcome many of the disadvantages of using transformed cell lines for drug discovery [33]. They also offer a more clinically relevant model for testing novel gene and cell-based therapies. However, the lack of culture systems with the robustness, scalability and flexibility needed by companies has hampered the adoption of *in vitro* primary cell-based research tools, including patient-derived cell models, at the earliest stages of drug development.

In cancer, highly selective drugs targeted at genetically defined clinical subtypes are needed to support a more patient-centric approach to drug development [34, 35]. Potential drugs have been tested *in vitro* and *ex vivo* against well-characterized patient-derived primary cancer subtypes for various cancers, but often without a direct impact on treatment [36-38]. In leukemias, however, where the *ex vivo* material (e.g. suspension cells) is more readily available for drug testing than in solid tumours, patient-derived

samples have recently been utilized for potential drug repositioning [39] and combined with molecular profiling to identify clinically actionable AML-selective targeted drugs [40] **[Au:OK?]**. Although primary leukemic cells can be used without further expansion for *ex vivo* drug testing [33, 41], the drug responses may vary depending on the cell culture assay conditions. Importantly, several studies highlight the importance of the interaction of leukemic cells with the bone marrow stromal microenvironment, which can be partly mimicked by using co-cultures of leukemic cells with human bone marrow-derived mesenchymal stem cells [42, 43].

The extension of patient-centric primary *ex vivo* drug profiling to higher-throughput applications and primary cells derived from solid tumours or normal tissue presents several challenges [44]. The development of co-culture protocols [45] has enabled a relatively rapid production and scale-up of high amounts of conditionally reprogrammed cells from surgical and accessible biopsy specimens, both from healthy and tumorigenic tissues such as lung, breast, prostate, pancreas, colon, and kidney [44, 46]. *In vitro* cell culture conditions modify cells over time, and may even lead to loss of expression markers of the original sample and the enrichment of specific cell populations. It is therefore essential to ensure that these cells represent the original tissue and genomic background of the individuals from whom they were derived by extensive genotypic and single-cell phenotypic characterization. Living organoid biobanks for solid tumours can complement cell line- and xenograft-based drug studies by providing an improved model for complex tissue architecture. This was demonstrated in a recent proof-of-concept study, where the living organoids of 20 colorectal cancer patients, sharing identical gene expression profiles and genetics with the corresponding original tumours, were screened

against 83 compounds; findings revealed good reproducibility and correlation with individual oncogenic mutations [47].

Overcoming the challenges related to the expansion of primary patient-derived *ex vivo* cultures for higher-throughput screening across distinct patient cohorts will require access to numerous representative patient samples for simultaneous testing, scale-up of limited primary cell material, and effective integration of drug sensitivity phenotypic data with the molecular characterization and clinical data associated with each patient sample. Access to high-quality patient-derived primary samples requires close collaboration between researchers, clinics and biobanks to find representative samples combined with relevant clinical data, and to establish standardized sample handling procedures for sensitive live tissues and cells. The Finnish Hematology Registry Biobank (FHRB) provides an exemplar of an operative biobank, functioning as a valuable source of patient material for precision medicine approaches in leukemia [40, 48]. The exploitation of biobanks to support drug testing of *ex vivo* patient cells collected from across both large and smaller patient cohorts can help to prioritize and de-risk drug candidates for larger-scale clinical testing, to support patient stratified medicine strategies and to systematically identify novel drug-repositioning opportunities [48].

Further expansion of primary cells for high-throughput screening (HTS) of small-molecule or antibody libraries remains challenging, however, and will require development of new technology platforms, including miniaturized assay screening formats and defined culture conditions, that provide sufficient sample throughput and stability.

Until these challenges are met, HTS of compound libraries across transformed and immortalized cell line models, when integrated with molecular profiling, may still provide useful opportunities to advance drug mechanism-of-action studies, target identification and patient stratification hypotheses. A recent study used correlation-based analyses to associate the sensitivity of 481 compounds tested across 860 human cancer cell lines with the basal gene expression profile of each cell line to reveal new target mechanisms for several compounds [49]. Furthermore, application of multiparametric genetic or image-based phenotypic profiling assays in established cell lines, combined with multivariate statistics and machine learning methods, has been used to pattern match compound induced transcriptomic or phenotypic fingerprints with reference data sets to predict compound mechanism-of-action and postulate new disease indications [50-52]. Thus, while transformed cell line assays may poorly represent disease, integration with in-depth genomic and phenotypic profiling to understand mechanism-of-action and elucidate new targets may represent the best use of these well-characterized transformed or immortalized cell line culture resources.

Induced pluripotent stem cell technology. While primary human and patient-derived *ex vivo* models are considered to be of high value, the availability of the relevant tissue is a limiting factor for modelling many disease phenotypes. The ability to scale up and expand primary cell-derived cultures for HTS applications, whilst still maintaining the relevant genomic epigenetic and tissue architecture of the original tissue, also remains a challenge. These limitations have hampered drug discovery in several disease areas, most notably in neurodegeneration and psychiatric disorders.

A major breakthrough in the ability to develop tissue specific cell-based disease models, including patient-derived cell assays at scale, has been achieved through the development of iPSC technology [53]. New opportunities presented by iPSC technology in disease modelling and translational research have recently been reviewed in depth [54, 55]. We therefore focus our discussion below on advantages and some limitations specifically related to cell-based assay development and screening, and selected example applications for neurodegenerative diseases, cardiotoxicity testing and metabolic diseases **[Au:OK?]**.

iPSCs have several advantages as a platform for drug screening. They represent normal primary cells with a mostly stable genotype compared with transformed cell lines and they possess an intrinsic capacity for self-renewal, facilitating their propagation and expansion for drug screening. iPSC can also be reprogrammed into many different tissue specific cell types and can be derived from any patient in unlimited quantities. Importantly, iPSC are amenable to detailed genetic characterization and to new gene editing technology, therefore presenting an excellent opportunity for directly linking phenotype to genotypes. These properties facilitate pharmacogenomics studies and the development of matched pairs of genetically defined disease phenotypes and isogenic controls for screening.

iPSCs also have several limitations. Firstly, the persistence of residual epigenetic memory, from the somatic cells from which the iPSC cells were derived, may adversely influence or confound phenotypic response to testing candidate therapies and in drug screening [56, 57]. Secondly, iPSC disease models have tended to focus on rare

monogenic hereditary forms of disease rather than more common spontaneous forms that are characterized by complex genetic traits involving multiple unknown genetic and epigenetic factors. New advances in multi-gene editing and synthetic biology approaches in patient-derived iPSC models may begin to address these challenges [58-60]. Thirdly the rapid differentiation protocols and embryonic nature of iPSCs and their derivatives may not be optimal for modelling late-onset disorders associated with aging. Long differentiation protocols have been applied to help develop mature differentiated cell types and exogenous stressors have been used to induce aging-like features of iPSC-derived models of Parkinson disease [61] **[Au:OK?]**. However, these approaches only partly address this issue of cellular aging. Finally, a practical limitation of iPSC models is the long differentiation protocols required. Despite these limitations, iPSC-derived **[Au:OK?]** cultures of cardiomyocytes [62-66] neurons [66-68], intestinal [69] and lung [70] tissue have been developed as heart, cerebral, intestinal and pulmonary disease models, and have been used in drug screening [62, 63, 65-67, 71].

For example, the development of automated phenotypic screening assays incorporating differentiated iPSCs that address specific neurodegenerative diseases has recently begun to yield new potential therapeutic targets and lead compounds. Neurons made from iPSCs, derived from a patient with Rett syndrome, exhibiting reduced spine density and smaller cell bodies, were employed in a drug screen that identified two molecules, IGF1 **[Au:OK?]** and gentamycin, able to rescue synaptic defects [72, 73]. In a recent study of a large cohort of healthy controls and ALS patients, fibroblasts were reprogrammed into pluripotency. The cells were subsequently configured into a high-content chemical screen, resulting in the identification of several FDA-approved small molecule modulators,

demonstrating the feasibility of patient-derived iPSC-based disease modelling for drug repurposing and screening [74]. In another study, α-synuclein-defective cortical neurons were generated using iPSC from patients at risk of developing Parkinson's disease. By identifying pathogenic phenotypic endpoints suitable for cell-based screening assays, the studies identified new potential therapeutic targets, such as the ubiquitin ligase NEDD which rescues the α-synuclein toxicity associated with Parkinson's patient-derived neurons [75]. Another recent small-molecule chemical screen using human iPSC-derived **[Au:OK?]** dopaminergic neurons in a rapid 96-well screening format identified several potential neuroprotective candidates for Parkinson's disease [76].

Cardiotoxicity testing has traditionally been focused on *in vitro* electrophysiology assays to assess the risk of arrhythmia, yet a major limitation has been the dependence on use of cell lines engineered to express single ion channels [77, 78]. These reductionist approaches are poor predictors of the risk of arrhythmias since cardiac action potential *in vivo* involves the cooperation of multiple ion channels. Stem-cell technology can be used to provide an unlimited supply of cardiomyocytes that more faithfully reproduce human cardiac electrophysiology and that can be used for *in vitro* HTS approaches, which are beginning to revolutionize the field [79]. However, current HTS approaches are still limited by the maturation state of stem-cell-derived cells, which do not recapitulate completely the contractile function of adult cardiomyocytes [80]. *In vitro* engineered 2D [81] and 3D [82] cardiac tissue models have now been developed as low-or medium-throughput screening platforms using stem-cell-derived cardiomyocytes with improved maturation status and hold great promise for the future study of cardiotoxicity and myocardial dysfunction. Screening platforms have also been developed from neonatal rat

primary cardiac myocytes [83, 84]. While highly efficient, some functional discrepancies to human models exist. Medium and high-throughput screening technologies for recording cardiomyocyte function currently span from the classic radio-ligand binding assays [77] to automated patch clamp [85, 86] and microelectrode arrays (MEA) [78, 83, 86]. Optical-based high-throughput assays have been developed for monitoring voltage or ion sensitive dyes using kinetic plate readers and high content imaging platforms [78, 87-89]. Stem-cell-derived cardiomyocytes in combination with image-based high-content screening technology have proven to be very effective for analyzing structural cardiotoxicity associated with anti-cancer therapies [90]. Drug-induced heart failure can also arise from impaired cardiac function characterized by changes in cardiomyocyte contractility, which can be studied using real-time cell analysis (RTCA), an impedance-based high throughput technology [91]. However, monitoring cardiomyocyte contractility is rarely assessed in preclinical toxicology studies and remains a particularly challenging task due to the high speed of cell beating and the complexity of the process, which requires advanced phenotypic approaches. Several new methods have been developed to quantify cardiomyocyte contractility based on digital holographic microscopy [92], muscular tissue films (MTF) [93] or dynamic monolayer force microscopy (DMFM) [94]. With the exception of label-free DHM, which is relatively inexpensive, the complexity and cost of many of the other techniques currently limit their application to HTS.

Culture of patient-derived fibroblasts have been used extensively to characterize and/or to find new treatments for inherited metabolic diseases, such as genetic enzyme deficiencies and lipid storage diseases (e.g, Niemann-Pick disease type C [95]). The advantage of fibroblasts is that they are easy to collect, store and expand for several passages and, in

general, they do not require complex culture conditions. Importantly, the fibroblasts can be reprogrammed to iPSCs and further differentiated into the most appropriate cell types relevant to specific metabolic disorders. In type 1 diabetes, this approach has been utilized for *in vitro* production of functional stem cell-derived β-cells from fibroblasts of diabetic patients [96], which can be used to search for new diabetes targets and molecules that promote pancreatic β-cell proliferation and function or suppress β-cell apoptosis [97] **[Au:OK?]**. Similar approaches exploiting iPSC technology are being applied to other diseases such as muscular dystrophies [98], cardiovascular disease [99] and aldehyde dehydrogenase 2 deficiency [100]. Importantly, many of these iPSC models can be readily expanded for HTS.

Overall, it is too early to accurately measure the impact of iPSC technology. However, differentiated iPSC assays, combined with more informative functional screening technology, provide improved models of normal and diseased tissue compared with traditional assays, leading many investigators to anticipate they will ultimately improve clinical success rates.

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3D cell culture models. Culturing cells in 3D environments can favour the formation of multicellular tissues with the appropriate cell–cell and cell–extracellular matrix

interactions and architecture that are critical drivers of tissue differentiation and function. The use of 3D cellular models for *in vitro* disease modelling and screening is especially useful where aberrant tissue organisation is associated with disease pathology and progression; for example in neurodegenerative disorders, fibrosis, solid cancers and cystopathies.

Many options for 3D *in vitro* and *ex vivo* models are emerging that utilize both natural and synthetic biomaterials, each with advantages and limitations (Box 2 and Figure 2). New 3D assay formats have been developed specifically for medium- to high–throughput screening, including commercially available options, such as microtissue products (InSphero 3D InSight), nanoculture spheroid plates (SCIVAX), micropattern plates (Cytoo), aligned (NanoAligned) or randomly oriented (NanoECM) polymer nanofiber plates and low-density 3D cell suspension media (HappyCell), which provide robust 3D cell culture architecture in 96- and 384-well formats. Examples of advanced multicellular 3D spheroid screening assays, designed to address specific clinical scenarios, include the application of a co-culture model composed of normal human dermal fibroblasts (NHDF) growing together with RFP labelled breast cancer cells for high-throughput phenotypic screening of radiation resistant tumour cells [115]. This assay was quantified by real time high-content imaging in a format that is suitable for scale-up to HTS of drug combinations that sensitize cells to radiotherapy, chemotherapy or both **[Au:OK?]** [115]. Further high-content image-based 3D spheroid assays have been applied to small-molecule screens investigating compounds that specifically target dormant tumour cells within the inner core of tumour spheroids or compounds which prevent either fibroblast or immune invasion into tumour spheroids [116-118]. Such 3D spheroid assays formats have

also been used as tissue surrogates to study immune infiltration into specific tissue types and represent a rapid and cost-effective alternative to animal models for studying host-immune response [116, 117].

Despite a number of successful studies demonstrating practical implementation of 3D assays to small-, medium- and high-throughput screening assays [115, 119], adoption of 3D tissue culture into routine screening has been sluggish, in part owing to a number of remaining technical issues **[Au:OK?]**. First, although animal-derived basement membrane extract (BME) hydrogels often support the growth of difficult-to-culture cells such as primary cells, their physical and chemical properties are fixed, their composition is undefined and there is inevitable batch-to-batch variation associated with these natural products, which is considered a major hindrance to obtaining reproducible results [120]. To promote more cost-effective and reproducible 3D cell culture screening platforms, synthetic biomaterials have been developed (Box 2 and Figure 2). However, the lack of organic ECM proteins and appropriate extracellular environmental signalling cues mediated by ECM protein binding to cell-surface receptors limits the physiological relevance of synthetic 3D biomaterial substrates. Even peptide-derived gels have yet to recapitulate sufficient functionality for the development of 3D tissues from most cells. Those cells that do grow in inert hydrogels, scaffolds or in hanging drop/low attachment plates may do so through the secretion of endogenous extracellular matrix proteins or due to oncogenic mutations that confer anchorage independence. Adoption of hybrid matrices combining synthetic and organic biomaterial has gained recent popularity for drug testing in cancer cell models [121], tissue-engineering matrices [122-125], and development of more complex innovative immunocompetent 3D culture models comprising of dendritic

cells co-cultured with fibroblasts and keratinocytes.[116]. Indeed, addition of cells responsible for producing the extracellular matrix *in vivo* (e.g. stromal fibroblasts and stellate cells in the case of the liver) into 3D co-cultures systems represents an alternative approach to incorporating more physiological ECM constituents into synthetic 3D scaffolds.

Further practical limitations of 3D cell-culture models include: the high cost of biomaterials; higher viscosity and temperature-sensitive gelation hindering automated handling of gels in the liquid state; sample processing (for example, antibody staining and sample washing) for high-content analysis; and the challenge of defining optimal cell ratios, culture conditions and ECM constituents for 3D co-culture models. However, integration of factorial design strategies and evolutionarily inspired genetic algorithms, together with advances in cell culture automation and phenotypic analysis, are well placed to advance complex assay optimization [126-128]. Perhaps the most challenging aspect of high-content screening of 3D cultures, however, is image capture and analysis, which requires new advanced microscopy and image-informatics solutions. Nevertheless, emerging microfluidic and high-resolution 3D imaging technologies such as light sheet fluorescence microscopy (LSFM) and selective plane illumination microscopy (SPIM) hold great promise for advancing 3D culture-based assays, although they are not yet adapted to a screening setting [129]. Such technologies are discussed in more depth later in the article **[Au:OK?]**.

The poor penetration and perfusion of drugs into 3D *in vitro* models can present limitations for drug testing and screening but also new opportunities to mimic fibrotic and

poorly vascularized tissues associated with several diseases, where poor drug perfusion contributes to poor clinical efficacy [130, 131]. This aspect of pathophysiological drug resistance is not recapitulated in 2D cell culture models and may only be partly addressed in 3D multicellular spheroid models. This more complex aspect of disease pathophysiology can be recapitulated in some *in vivo* models, for example genetically engineered mouse models of pancreatic cancer [130]. However, *in vivo* models are not practical, cost-effective or sufficiently rapid for screening larger numbers of candidate drugs. 3D organotypic *in vitro* co-culture **[Au:OK?]** assays (for example, composed of stromal fibroblasts and cancer cells) are faster and recapitulate the fibrosis and poor drug penetration observed in genetically engineered mouse models of pancreatic cancer and in the human disease [132]. Such assays can predict the poor clinical response of solid tumours to small-molecule kinase inhibitors such as dasatinib, and they are suitable for identifying new drugs and drug combination strategies that combat poor tissue perfusion [133, 134]. Development of such predictive preclinical assays into higher-throughput and reproducible screening formats is imperative.

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[Au: section on principles of disease-relevant models moved later to help the flow]

Organ-on-a-chip and microfluidic technologies. New approaches that can offer a satisfactory level of biological complexity and clinical relevance while taking into account the issues of throughput, scale and cost are in great demand. One such approach is ‘organ-on-a-chip’ technology. These devices are essentially miniaturized microfluidic perfusion systems that permit long term *in vitro* growth and the propagation of primary,

stem cells and tissues in a format that is both economically and ethically viable with the potential to scale up for high-throughput discovery campaigns. Although still early in their development, several organ-on-a-chip assay formats have been evaluated; a liver-tumour-bone marrow-on-a-chip [156] and a liver-skin-intestine-kidney-on-a-chip are two such examples [157]. The advantage of these systems is that they offer a means of modelling the complex tissue microenvironment and the communication between distinct tissues *in vivo*. These systems are reported to produce levels of tissue and organ functionality not possible with conventional 2D or 3D culture systems, e.g., kidney tubular epithelial cells and, as previously discussed, hepatocytes [21].

Another advantage of these microfluidic systems is the ability to recapitulate the hemodynamic forces generated by blood flow, which are important in governing normal homeostatic function of the endothelial cell layer lining the inner lumen of the vascular wall and the sub-endothelial vascular smooth muscle cells. The development of microfluidic perfusion chambers that recapitulate the pulsatile nature of blood flow and regions of high and low shear stress known to regulate endothelia and smooth cell function have helped in more effectively modelling normal homeostatic vascular function and the pathophysiology associated with cardiovascular disease [158, 159] **[Au:OK?]**. The latest advances in microfuidic designs such as the multi-organ tissue flow (MOTiF) chip to enable more precise supply of nutrients and discharge of catabolic metabolites under controlled shear stress contribute to the increased utility and physiological relevance of microfluidic cardiovascular models [160].

Other examples of recent advances in microfluidic devices for advanced cell culture include the lung-on-a-chip, a microfluidic system that mimics the critical physical and biological features typically found at the alveolar-capillary interface of the human lung. This system can be used to mimic complex pathophysiological responses to stimuli such as those elicited when bacteria and inflammatory cytokines are introduced into the alveolar space [27]. Increasing efforts are being placed to produce cardiac tissue on microfluidic devices using cardiomyocyte models as beating heart-on-chip platforms which can be used to measure contractility and electrophysiology to test cardiac pharmaceuticals as well as to assess potential cardiotoxic effects during drug discovery [81] [82].

The development of microfluidic devices that include temporal and spatial measurements on single cells are further enhanced by methods that manipulate cell movement and collection. Hydrodynamic cell trapping systems have recently overcome the throughput limitations of previous methods for manipulating cells, such as acoustic tweezers or fluorescence-activated cell sorting, by enabling rapid, robust and high throughput handling of single cells [161]. Microfluidic manipulation of single cells has many applications, including elucidating mechanisms of stem cell renewal and differentiation [162]. Miniaturized methods for manipulating and analysing small populations or single cell phenotypes are complemented by recent advances in ultrasensitive methods for proteomic, and genomic analysis [163, 164]. As an example, Salehi-Reyhani et al. reported the development of a microfluidic antibody capture chip, integrated with TIRF detection and robust cell lysis to monitor p53 levels within single cells [165]. Progress in whole-genome and whole-transcriptome amplification combined with next-generation

sequencing platforms, have facilitated the advance of single-cell genomics. An integrated microfluidic device, which couples single cell capture, enzymatic reaction and quantitative mRNA detection within a single platform, has recently been developed [166]. This platform distinguished stochastic variation in gene expression between two distinct cell populations at the single-cell level, which would otherwise be masked when analysed at the population level [166].

Combining microfluidics with image-based or label-free methods for quantifying cell phenotypes at the single-cell level enables miniaturized phenotypic analysis of rare sub-populations and primary cells without the need for bulk expansion *in vitro*. Developments have included the use of optically encoded droplet-microfluidics to enable HTS of compound libraries across single cells [167]. Collaboration between industry and tissue engineering academic groups should encourage the further development and adoption of these technologies by a wider community, bringing microfluidic devices, artificial extracellular matrices of tuneable stiffness and mixed cell culture models to a greater number of laboratories.

Advanced microscopy and image analysis tools. An advantage of automated microscopic imaging over other HTS platforms is its provision of information on functional data points together with associated spatial information in x, y and z dimensions. This allows cell-based screening assay formats to progress towards more complex, heterogeneous co-culture and 3D models. Novel *ex vivo* cell models such as those previously described from solid tumour patients as well as whole-organism models used in drug testing, are often a source of heterogeneous cell types and present challenges for assay quantification. The

heterogeneity, which characterizes these models and the original tissues that they represent, may drift as a consequence of cell culture conditions, and should be taken into the account during assay development and analysis. The widely used whole-well measurements, based on standard luminescence, fluorescence, or other similar assays, do not reveal the heterogeneity in response to culture condition or drug exposure. Such assays also do not recognize or quantify sub-populations of cells carrying specific markers (e.g. an amplification of a cancer biomarker to inform patient stratification), which can be scored by high-content imaging, and other single-cell technologies.

Microscopy technologies have progressed remarkably over the past few years. Advances in optics, robotics and computational techniques, as well as an expanding repertoire of contrast markers, including functional live-cell reporters, are contributing to the widespread adoption of image-based screening platforms that provide highly dynamic and quantitative fluorescence readouts in cell-based assay systems [168-170]. Non-invasive label-free imaging techniques have recently emerged fulfilling the requirements of minimal cell manipulation for cell-based assays in a high-content screening context. Among these label free techniques, digital holographic microscopy (DHM) provides quantitative information that is automated for end-point and time-lapse imaging using 96- and 384-well plates [171-173]. Similarly, label-free optical techniques, such as phase contrast or differential interference contrast (DIC) can be digitally reconstructed and quantified [174]. Light sheet fluorescence microscopy (LSFM) holds great promise for the analysis of large numbers of samples, in 3D high resolution and with fast recording speed, and minimal photo-induced cell damage. LSFM has gained increasing popularity in research areas such as neurosciences, plant and developmental biology, toxicology and

drug discovery, although not yet adapted to an automated screening setting [129, 175, 176]. Currently, the majority of 3D image analysis software is applied to single images or in a semi-automated low-throughput manner using predominantly custom solutions because no community-wide accepted tools exist [177, 178]. Image-based multi-parametric phenotypic profiling, including morphology, topology, and texture parameters such as wavelet and image moments, have begun to address the challenges of automated segmentation and mathematical descriptor extraction for 3D cell profiling [178, 179].

Optimizing bioinformatics solutions and phenotypic analysis towards systems pharmacology. Developments in microscopic imaging provide a strong example of the impact of new technologies on functional biology and cellular pharmacology studies. Rapid and transformative advances in other technology areas, including proteomic, lipidomic, transcriptomic, epigenetic and mass spectrometry imaging technologies, all support a move away from reductionist approaches in drug discovery to a more holistic "systems pharmacology" approach. Systems pharmacology describes a broader view of drug activity whereby targets are considered as part of integrated biological networks and where phenotypic response is linked with genotype and epigenetic considerations, supporting more in-depth understating of drug mechanism-of-action and potential personalized healthcare strategies [180]. The CANScript technology was recently developed to combine *ex vivo* phenotypic responses of heterogeneous patient-derived tumour tissues with next-generation proteomics and genomic data, to predict clinical outcomes [181]. Following integration of experimental *ex vivo* data with genomic, proteomic and clinical data, machine learning was utilized to predict the clinical outcomes of chemotherapy in head and neck squamous cell carcinoma and colorectal cancer patients

[181]. Further development of such “integrative” bioinformatics tools, combining clinical expression or mutation status of specific targets with cellular networks, chemical tools and preclinical activity, is exemplified by the CanSAR knowledge base, which enables evaluation of target biology, drug mechanism-of-action and patient stratification hypothesis within the context of pathway networks and integrated biological systems [182, 183].

While such bespoke bioinformatics solutions demonstrate promise, a major challenge to exploiting new functional genomic, proteomic and phenomic technologies is how to integrate large orthogonal datasets in a robust manner to accurately inform the drug discovery process and predict viable clinical development strategies [184]. New advances in network biology and graph theory offer approaches for such integration but require further development and validation [185]. A common weakness of current bioinformatics databases incorporating functional preclinical data is a lack of any standardization of phenotypic assay operation, data reporting and evaluation of the relevance of the phenotypic assay. Indeed, inconsistency is apparent between two recently reported large-scale pharmacogenomic studies in cancer cell lines **[Au:OK?]** [186-188]. This could be due to the lack of standardization in experimental assay design, execution and data analysis. However, high correlation for both drug testing and the genomic data could be achieved if common standards for experimental assays and bioinformatics methods are used [189]. These examples further highlight the need for new, better-defined and more robust screening assays through the establishment of strict standard operating and bioinformatics data analytics procedures, allowing statistical comparisons and validations across laboratories [190]. Further investment in preclinical assay development,

standardization of compound profiling, screening formats and quality control over phenotypic analysis will be required to fully exploit the potential of new integrative drug discovery bioinformatics tools and the systems pharmacology approach.

Principles of disease-relevant assays

Although important progress has been made in developing more physiologically relevant and predictive cell-based assays, as described above **[Au:OK?]**, many key aspects of these technologies must be improved to promote adoption, with the ultimate aim to reduce clinical attrition rates. Key considerations include:

- Regular DNA fingerprinting and karyotyping of cell cultures, to confirm their origin and genomic integrity, hence facilitating accurate disease modelling and robust pharmacogenomic analysis and biomarker discovery. Additionally, deep genotyping and phenotyping of the patients and the healthy volunteers from which primary cell and iPSC models are derived will help us to understand phenomena such as cell heterogeneity and drug resistance and how *in vitro* culture conditions influence representation of disease. These efforts could be supported, for example, by the generation/utilization of biobanks of well-annotated patient derived cells, ensuring improved characterisation of genomic attributes of these disease models. Such approaches should also help us to understand the contribution of clonal variation within models by monitoring, quantifying and compartmentalizing cell heterogeneity, leading to a better understanding of the underlying causes of attrition resulting from patient heterogeneity and variation in efficacy and toxicity across diverse human populations. In-depth molecular characterization of patient samples and derived cell models at genetic and post-translational pathway levels would

support reverse engineering of assays that recapitulate clinical drug resistance mechanisms. Such detailed characterization of cell models may also partly address the lack of reproducibility across laboratories, which may be due to genomic variation.

- Identification of reproducible protocols for the generation and differentiation of consistent somatic cell phenotypes to facilitate the expansion of primary cell and iPSC models for candidate drug profiling and HTS. Such developments will enable evaluation of new target biology and drug mechanism of action across multiple diseases, patient cohorts and healthy donor samples. It will also help identify issues arising from poor experimental reproducibility and therefore better understand pharmacogenomic effects that maybe inherent in these systems. Additionally, it should be ensured that the cell models used in such studies represent the appropriate maturity (e.g. embryonic or adult characteristics) for the expected age of the patient, therefore providing a more accurate disease model.

- Ensuring that the environmental (macro and micro) conditions of *in vitro* models are appropriately tailored to represent tissue types, disease pathophysiology and disease aetiology. This will involve the accurate modelling of the nutrient and metabolite concentrations, pH and dissolved gases. Special attention should also be focused on the mechanical conditions in which cells and tissues are propagated. These include ECM constituents specifically those that contribute to the stiffness of growth substrates, the interaction of cellular adhesion molecules, mechanical deformation and shear forces. Finally, many culture systems will require the controlled introduction of defined and relevant disease-causing environmental and physiological perturbagens to develop more

accurate models of disease progression **[Au:OK?]**.

- Developing multiscale tissue assays to mimic organism level physiology to accurately model drug metabolism, co-morbidities and systemic paracrine effects.

- Incorporating clinically equivalent dosing strategies, which mimic known or predict expected *in vivo* pharmacokinetic properties of various therapeutic modalities.

- Derivation of iPSCs from multiple patients and tissue types, containing multiple diverse genomic alterations, in order to reflect broader patient populations and more common disorders **[Au:OK?]** .

- Comparison of the drug–response data between patients and patient-derived cell models to evaluate the predictive value of the model.

- Integration of molecular genetic, epigenetic and proteomic data sets with robust phenotypic measurements in both clinical and preclinical settings with computational bioinformatics, supporting multiparametric validation of drug targets and assay relevance through a less-reductionist “systems pharmacology” approach.

- Adoption of a human physiology assay checklist, based on unbiased evaluation of which assay conditions accurately recapitulate the human tissue physiology or pathophysiology under study, and the limitations that each assay will inevitably have with regards to recapitulating the full complexity of human disease.

• Definition of the relevant assay endpoint. In diseases where the underlying causes are multifactorial it will in many cases be challenging to isolate the relevant endpoint(s) from *in vitro* cell and tissue-based assay systems. For example, in neurodegenerative diseases that are associated with plaque formation, it is currently a matter of debate as to whether the current readouts (the inclusions) represent a mechanism of protection or if they are a pathological endpoint leading to irreversible damage. Similarly, in type 1 diabetes, it is unclear whether assays measuring the insulin production/release or degradation of granules should be utilized to guide new therapies focused towards stimulating the production and release of insulin or whether the degradation of old insulin secretion granules should be prioritised in order to allow newly synthesised granules to fuse with the plasma membrane. Thus as previously discussed by others [143] the selection of relevant endpoints, which translate into clinical phenotypes or biomarkers of disease outcome may be most beneficial.

With the considerations above in mind, in Table 1**,** we provide our proposal for a set of "defining principles of disease-relevant assays" and justify their potential impact upon drug discovery. While many principles are generic and can be applied across disease models, certain principles have disease specific considerations, which require more in-depth investigation and modelling as indicated in Table 1. We anticipate that no single model or assay will be perfect and many of them will fail to recapitulate the full complexity of the human disease conditions, which they intend to represent. However, we believe that careful consideration of the limitations of each assay and the defining principles outlined in Table 1, will lead to more critical evaluation of the predictive value

of every cell based assay, and to more cautious and appropriate interpretations of the results derived from these assays.

The defining principles are also intended to encourage further discussion and debate of preclinical assay screening standards and their clinical relevance; to help drive forward the field of preclinical assay development towards improved clinical success rates. We anticipate that broad communication of such principles across diverse scientific disciplines will both stimulate and guide further investment in innovative solutions and new enabling technologies which address the challenges. In Figure 3, we highlight how integration of the technologies discussed above could contribute in early-stage drug discovery **[Au:OK?]** .

Assay validation and backward translation

Validating new preclinical models for physiological and disease relevance is not trivial, and arguably the best validation is evidence of prediction of clinical efficacy or toxicity. The majority of new cellular assay technologies described in this article have not yet reached a level of maturity that would permit any clear conclusions regarding their predictive value. Currently, the adoption of new technologies often depends on an organic rise in utilisation and acceptance. However, rather than simply waiting for a sufficiently large body of evidence to be generated by pioneering drug discovery efforts, a concerted effort to compare the predictive power of new cell-based models for outcome in patients is needed. Such efforts would ideally involve the testing of collections of known molecules that have demonstrable clinical efficacies and toxicities to support backward translation to determine the predictive value of *in vitro* models. Backward translation

studies may also encompass reverse engineering of *in vitro* model systems to predict past clinical trial failures through recapitulating known mechanisms of drug resistance or relapse observed in the clinic.

Recent examples of such work have been focused on evolving hepatotoxicity assays utilizing, primary hepatocytes, stem-cell derived hepatocytes and hepatocyte cell lines cultures grown on a range of defined organic or synthetic matrix substrates. These systems were then tested against a panel of drugs to predict agents that result in drug-induced liver injury in humans [135-137]. Similar approaches using stem-cell-derived cardiomyocytes to predict cardiotoxicity are also under development, as previously discussed.

Examples of backward translation studies to predict clinical efficacy of therapeutic agents are rare, which is probably a reflection of the lack of research funding available to support backward translation studies and assay model development. However, to demonstrate the predictive and translational potential of new preclinical models described in this review we highlight two case studies: the Hedgehog signalling inhibitor vismodegib and an αvβ6 blocking antibody (264RAD). Both of these examples exploit the unique aspects of the 3D organotypic pancreatic cancer model previously described, which is composed of a co-culture of established pancreatic cancer cells cultured on dense collagen matrix contracted by fibroblasts [132, 138]. This model recapitulates *in vivo* pharmacology issues relating to poor drug penetration and inadequate clinical efficacy [133, 134].

In the first example, studies that used both the *in vitro* organotypic cell culture model and comparative *in vivo* transgenic models showed that cyclopamine, which targets Hedgehog signalling in the stromal cell compartment, leads to increased penetration and hence activity of the anticancer drugs dasatinib and gemcitabine in pancreatic cancer **[Au:OK?]** [130, 134]. Clinical trials of the approved Hedgehog signalling inhibitor vismodegib have been instigated in pancreatic cancer and recent publication of early results indicate an improved efficacy response [139] **[Au:OK?]** . In the second example, pioneering studies demonstrating that targeting αvβ6 suppressed cancer cell invasion in 3D organotypic assay formats [140, 141] have contributed to the progression of the novel αvβ6 blocking antibody therapy (264RAD) into a phase 1 trial in pancreatic cancer: (http://bci.qmul.ac.uk/news/general-news/item/264rad-antibody-bci-medimmune-cruk-trials).

Finally, a recent article published by Scannell and Bosley provides further justification for increased investment in backward translation studies [142]. This work, we, which describe a quantitative decision-theoretic model of declining R&D productivity, illustrates how small improvements in the positive predictive value (PPV) of preclinical assays can outperform the documented advances in assay automation and throughput with regard to improving R&D productivity as defined by the successful translation of drug discovery to positive clinical outcomes [142]. Thus, a clearly defined pathway to assay validation and a subsequent development of a repository of assays with known positive and negative predictive values (PPV and NPV) would be of significant value. As far as we are aware there is no unifying systematic approach to measuring and recording the clinical impact of established assays or new assay technologies and approaches. We suggest that a routine

evaluation by measurement of PPV or NPV of all assays used in drug discovery would provide a valuable resource to the translational research community.

The need for funding and consortia

Academic-industrial partnerships have existed in some form since the birth of the pharmaceutical industry over 100 years ago, but have increasingly appreciated and pursued more recently in a wide range of contexts, from identifying and validating novel therapeutic targets through to addressing drug development challenges that are too broad for any one organization to tackle effectively alone, often through consortia **[Au:OK?]**. There remains, however, a gap in the development of such consortia in the area of preclinical model development, and here we put forward three proposals to address this **[Au:OK?]** .

First, we propose that direct funding for the development of preclinical models and assay-screening technologies with improved clinical relevance is an aspect of the drug development process that can be most effectively addressed by academic-industrial partnerships. Although target-based drug discovery is directly supported through academic-industrial partnerships such as Bayer's Grants4Targets [195]; GlaxoSmithKline discovery partnerships with academia (DPAc scheme) [196] and the IMI European Lead Factory consortium [197], the general strategy by drug discovery groups within both industry and academia, at present, is to "tap into" academic groups that have developed more relevant assays for their own research and apply them to existing drug discovery programs. Such academic assay systems and models have typically not been developed for the purpose of screening or guiding clinical decision making and thus remain to be

validated for robustness, reproducibility and clinical predictivity [198]. We therefore feel that translational funding schemes and pre-competitive research consortia must be significantly expanded and re-balanced from hypothesis-driven, target-directed research to support the development of new preclinical models and assay-screening technologies that can provide improved prediction of clinical outcomes.

Secondly, we propose that biobanking should be encouraged and developed **[Au:OK?]** . The initiating step in moving towards more clinically relevant primary human assays to support experimental medicine and personalized treatment paradigms is a standardized flow of high-quality samples from the clinic to the research bench. Such samples require relevant and anonymized clinical information, complete pathology reports and timelines from surgery for optimal integration with molecular profile data. Currently, the sample flow is often initiated through disease-specific research projects where ethics boards provide permission for patients sampling. However, in the long term, biobanking operations with a single consenting procedure provide a much more viable solution. The development of drug testing pipelines for human cell-based or *ex vivo* samples must at least aim to achieve diagnostic grade assays with quality standards in place, to enable relative ease of routine procedures and to be scalable in terms of maximizing both sample amounts and screening capacity. Such translational efforts are often nation-centric with the limitations of national laws, which unfortunately confound larger cohort studies and contribute to duplication of efforts with limited standardization.

Third, we propose that international collaboration and consortia to derive primary human cell biobanks with relevant annotation and standard operating procedures will promote a

step-change in the derivation and use of primary human cell assays to support drug discovery, experimental medicine, drug repositioning and personalized healthcare studies. Quality control, standardized procedures, standardized data analysis and validation of assay results across reference laboratories will be critical to the efficient development and acceptance of such assays, by driving improved data reproducibility and greater general confidence in promising early translational research results. Collaboration between dedicated assay development and assay screening groups together with clinicians, biostatisticians, computer scientists and IT experts adequately supported from industrial, academic, government and charitable funding sources are necessary to address the challenges (Figure 4).

Conclusion

The emergence of new cell-based assay technologies will be important in enhancing conventional target-directed drug discovery (TDD) by supporting more robust target identification and validation and secondary screening assay cascades. Such advances are also well placed to support a new era of phenotypic drug discovery (PDD) (Box 3). However, to reach their full potential, there must be an acknowledgement of the value of functional biology and physiological-based assay systems in drug discovery and the need to further develop more predictive *in vitro* models, and new assay screening approaches towards greater clinical relevance. We propose that consideration of physiological relevance and the defining principles of disease-relevant assays proposed in this article should be the starting point of any cell-based assay development. Adhering to these principles may lead to increased research costs, but in our view, the benefit justifies the increased investment and effort. As a translational research community, we should drive

the field towards greater disease relevance in drug development and call for funding bodies to support more advanced cell assay development and robust hypothesis-generating translational research as standalone grants. The current focus on reductionist hypothesis-driven research combined with the lack of robust disease-relevant preclinical models to evaluate targets, novel drug candidates and to predict clinical outcomes is clearly hindering advancement. Our hope is that the principles laid out in this article should function as a signpost for greater investment in the development and uptake of advanced preclinical disease modelling technologies and infrastructures. It is our view that such investment will complement the existing investments in academic and industrial drug discovery and substantially increase the likelihood of success for drug discovery and experimental medicine projects.

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Box 1 | Development of improved models of infectious diseases [Au:OK?]

Pathogen biology and infectious disease operate on multiple cellular and tissue scales, wherein the biology of the infectious microbe and its target cell depend upon the contextual interplay of pathogen development stages with distinct host tissues, organs, and the immune response. One important example of this complexity is the lifecycle of *Plasmodium falciparum*, the parasite that causes malaria in humans. There are several possible strategies to screen for lead compounds using phenotypic approaches, including screening at the human liver-, blood cell- or the insect- stage of the parasite life cycle. The

use of cell-based phenotypic assays is offering promise in the identification of novel therapeutic classes that target multiple stages of the parasite's development [101].

However, the nature of the targeted cells and the virulence of an infectious agent are intimately linked, and can sometimes only be appropriately recapitulated by very specific host-pathogen combinations. Along these lines, a series of studies to identify new chemical entities active against *Leishmania* subspecies, the causal agents of human leishmaniasis, clearly demonstrated that developing an assay targeting the insect promastigote stage of the parasite does not yield viable lead compounds [102-104]. Instead, a primary macrophage assay supporting infection competent (replicative) parasites requires a much longer and more sophisticated methodology, reducing screening throughput, but greatly enhancing the quality of results and identification of viable lead compounds [102-104].

A major concern in the field of infectious disease modelling is the use of perpetually cultured laboratory strains of infectious disease organisms despite their poor resemblance to "real-world" pathogens. Bacterial growth conditions *in vitro* differ fundamentally from the conditions found in natural ecosystems, including infection sites. Indeed, as a result of the high degree of plasticity of their genome, bacterial strains adapt quickly to the optimized laboratory mono-culture conditions and, therefore, rapidly lose key pathophysiological characteristics [105]. In the late 19^{th} century, Louis Pasteur had already recognized that laboratory adaptation of bacteria is associated with attenuation of virulence towards the host species and this idea was exploited by his colleagues Calmette and Guérin, leading to the development of the BCG vaccine in 1921 [106].

It is now well established that bacteria do not exist in isolation but rather live as communities, behaving collectively to adapt to new host environments and modes of growth [107]. Quorum-sensing (QS), *i.e.* cell-to-cell communication by production and release of autoinducers in the environment, plays a key role in *Pseudomonas aeruginosa* pathogenicity. The large variation of expression of QS-genes observed upon culture in different environments emphasizes the pertinence of clearly identifying and mimicking the actual habitat that bacteria encounter *in vivo* when developing an assay to design new therapies [108]. Along these lines, most bacterial chronic infectious diseases are associated with the formation of polymicrobial biofilms. Biofilms protect the bacteria from the host innate- and adaptive- immune response and represent an ideal setting for horizontal gene transfer (HGT), thus creating new virulent strains and resistance by way of creating a communal distributed "supra-genome" [109].

A way to develop more physiological infectious disease models is to mimic as closely as possible the *in vivo* ecosystems that the pathogens might encounter in an infected host, in order to recreates the microenvironment require for the virulence and eventually to identify drugs that might boost the host defence mechanisms **[Au:OK?]** . Many pathogens that infect humans **[Au:OK?]** have been studied in model animals such as *D. melanogaster, C. elegans, zebrafish* or mice; unfortunately, not all of them are easily amenable to higher-throughput drug screening, or properly model the human response to pathogen infection. Nonetheless, in an elegant study, Kim and co-workers describe how host-cell autophagy activated by antibiotics is required for effective anti-mycobacterial drug response through conserved mechanisms between fly and mammal [110]. While small model organisms hold many promises for infectious diseases drug discovery, they

still have many drawbacks such as their temperature, discrepancies between the anaerobic nature of the human intestine and non-conserved host targets across species. These shortcomings might be overcome by using synthetic micro-tissues such as the human gut-on-a-chip [111] or small airway-on-a-chip microfluidic devices [112].

Many pathogens require high biosafety level laboratory confinement (BSL3 and above) presenting several challenges to developing assays under these conditions. The continued development of automated liquid handling and automated microscopy platforms, which enable remote control, including image acquisition and analysis help support assay development and screening under high level biosafety restrictions. The lack of effective cure, or preventive measures against, emerging antibiotic-resistant bacteria and re-emerging viral pandemics (*e.g.* Ebola, Marburg and Zika) calls for renewed efforts to innovate technologies and methodologies for drug screening in the infectious disease area. Comparative phenotypic screening across microbial resistant sub-species combined with relevant host systems may guide drug discovery toward novel therapeutic classes targeting infectious disease resistance mechanisms and new host-oriented therapies [113, 114].

Box 3 | Phenotypic drug discovery versus target-directed drug discovery

Target-directed drug discovery (TDD) has been the predominant strategy of the pharmaceutical and biotechnology industry for the past 25 years. It is characterized by the identification and optimization of compounds that modulate a pre-nominated target implicated in disease progression, often using high-throughput screening to identify initial

hits. Phenotypic drug discovery (PDD) has been defined as the generation of hit or lead molecules without any prior knowledge of the target. Such compounds are typically identified and developed through empirical testing in physiological *in vivo* models or from cell-based phenotypic screening assays.

An initial retrospective analysis of all drugs approved by the US Food and Drug Administration (FDA) between 1999-2008 indicated that of first-in-class small molecule medicines approved, 37% (28 drugs) were initially identified by a PDD approach, relative to 23% (17 drugs) identified by TDD [199]. Follow-up retrospective studies in cancer [200] and across disease areas [201], produced different results, in part owing to differences in the definition of phenotypic screening and in part owing to differences in the time period studied, with TDD showing substantial success in cancer in more recent years **[Au:OK?]**. In cancer, 31 novel small-molecule **[Au:OK?]** drugs approved by the FDA between 1999-2013 were discovered by TDD (including 21 kinase inhibitors), whereas 17 novel small-molecule **[Au:OK?]** drugs approved during the same period were discovered by PDD (including a single kinase inhibitor, trametinib targeting MEK) [200]. Retrospective analysis by Eder and colleagues [201] across all disease areas, using a refined selection criteria, identified 45 novel small molecules approved during 1999-2013, discovered through TDD. At the same time, 33 approved small molecules were discovered by non-target directed methods, including 8 through unbiased phenotypic screening of large chemical libraries and 25 through chemocentric approaches, representing a combination of rational drug design and phenotypic screening/validation. These studies indicate that PDD and TDD provide complementary approaches. Further

investment in more predictive cell-based assay systems could advance both PDD and TDD strategies, ultimately resulting in improved clinical success rates.

Box 2 | 2D versus 3D cell biology

Cellular growth in 2D versus 3D *in vitro* culture models differ in critical environmental factors. First, the mechanical factors differ; cells grown in 2D cultures are subject to stiffer (i.e. less compliant mechanical conditions) than those grown in 3D cultures, which resemble better mechanical forces exerted to cells *in vivo*. Second, the biochemical environment differs; access to nutrients, oxygen, ions, gradients and drugs is a critical issue in 3D cultures, which lack vasculature, physiological gradients, and proper medium exchange found in tissues. Third, the environmental context differs, as physiological cell-cell and cell-extracellular matrix interactions are severely compromised in most 2D cultures. These factors can influence intracellular signal transduction pathways leading to differential gene expression patterns, with important implications in the polarization and differentiation status of cells [202-204]. Accordingly, screening run in parallel in 2D and 3D assays have led to different results [203].

As reviewed in [205-214], there are multiple static and microfluidic systems that facilitate the development of new 3D *in vitro* models of disease (Figure 2). A wide range of biomaterial scaffolds for improving physiological relevance of *in vitro* assays are increasingly being adopted using different natural and synthetic materials in 3D or 2D cell culture models, and for bioprinted organotypic tissue and organs [207, 212, 215]. Scaffold-based 3D cultures can be generated by seeding cells on an acellular 3D matrix or by dispersing cells in a liquid matrix followed by solidification or polymerization.

The most common scaffolds used fall into two broad categories. The first is biologically derived materials or natural hydrogels that commonly include, but are not limited to, collagen, fibrin, hyaluronic acid, Matrigel, and derivatives of natural materials such as chitosan, alginate and silk fibres [216]. The second category is synthetically derived matrices, includeing polyvinyl alcohol (PVA), polylactide-co-glycolide (PLG), polycaprolactone (PLA) Polyethylene glycol (PEG) hydrogels, which offer more flexibility for tuning chemical composition and mechanical properties since they can be selected or tuned to be hydrolysable or biodegradable [216, 217].

To enrich their potential as “bioactive” materials, those scaffolds are generally supplemented with ECM proteins [124], active peptide sequences [218, 219] or nucleic acid aptamers [220]. Magnetic nanoparticles such as magnetite (Fe3O4) [221] are used to create magnetic hydrogels, which allow for greater control of the swelling and collapsing properties of the hydrogels using an external magnetic field [222]. These 3D systems reflect better the *in vivo* scenario, allowing for example, epithelial morphogenetic processes, including formation of tubules and cysts and modelling epithelial acini (reminiscent of those found in lung alveolae, mammary and salivary glands and in pancreatic and kidney cysts), which in many instances have been reported to be functional [27, 223, 224]. Thus, different 3D assays are considered to bridge the gap between 2D cultured cells and *in vivo* models.

Figure 1 | Novel assay technologies and their integration. Advances in patient-derived primary cell models; iPSC technology; 3D ex-vivo and multicellular models, microfluidic devices; CRISPR-cas9 gene-editing; automated imaging and image analysis platforms; molecular cell profiling technologies, including advanced proteomic and genomic methodology such as next-generation sequencing and bioinformatics, individually and together present new opportunities for incorporating more relevant physiological models into drug discovery.

Figure 2 | Evolution of more physiologically relevant cell-culture assay systems. In contrast to traditional two-dimensional (2D) cultures of cells as monolayers on flat surfaces, three dimensional (3D) assays allow cells to grow forming more complex 3D structures, which reflect better the physiological architecture of tissues and organs *in vivo*. Several new technology developments and culture methods have enabled the design of more consistent and informative 2D and 3D cell culture assays, which can be tailored to address specific biological and clinical questions (Box 2). Specific developments illustrated here include; A. Synthetic nanofibre, hydrogel and polymer scaffolds for 3D Culture http://www.elmarco.com/gallery/nanofibers/ [219, 225-228]; B. Spheroid and microtissue assays [229]; C. 3D organic matrix assays [230]; D. Multicellular organotypic assays [132, 138]; E. *Ex vivo* tissue assays [231] as an example human ovarian cancer cells cultured on fresh human omentum tissue is shown (Carragher unpublished). F. Microfuidic and organ on-a-chip devices incorporating 3D cell culture substrates, defined mechanical stimuli and controlled perfusion with nutrient media [232-235]. Comparative models of increasing complexity and physiological relevance, while not always suitable for primary screening may also serve well positioned further down the cascade as

secondary assays or target validation tools to provide increased confidence in the translational potential of novel lead compounds and new target hypothesis.

Figure 3 | New cell-based assay technology contributions to early-stage drug discovery pipeline. We outline where the application of new emerging cell-based assay technologies can enhance the quality and clinical relevance of data derived from cell based models. Adoption of more robust, informative and relevant assays early within the drug discovery process will support more informed decision making on which opportunities to progress into preclinical development. Frontloading attrition earlier (Fast to fail) in the drug discovery process will reduce the substantial costs associated with late stage attrition due to poor efficacy and toxicity and contribute to the development of improved treatments for the most challenging diseases.

Figure 4 | Precompetitive consortia facilitating predictive assay development, experimental and personalized medicine strategies. The development of screening assays and preclinical models, which better predict clinical outcomes is a significant challenge, which has not received the necessary investment from translational funding bodies and industry. We present a model for the pre-competitive development of more predictive and reproducible cell-based assays though academic and industrial consortia. Facilitated by the access to human cell and tissue biobanks, we propose a network of core cell based screening laboratories, proficient in assay quality control and automated cell based screening technology, to develop assays to common standards. New assays will be qualified for disease relevance in partnership with disease area experts and clinicians performing retrospective studies with approved drugs and failed clinical candidates,

where possible, to determine the positive and negative predictive value of each assay. Assay protocols and assay results relating to identification of new targets, lead compounds and repositioning opportunities will be validated for reproducibility by benchmarking assay performance and confirming results between distinct core laboratories within assay development networks. We believe that academic, industrial and government funding of such pre-competitive activity will provide open source access to high quality assays and results to be exploited by the academic and healthcare communities and the biopharmaceutical industry. These activities will drive significant improvements in the quality of the academic literature publishing new target hypothesis and new drug combination and repositioning opportunities. Such pre-competitive activity will also improve the quality of assays used by academic and industrial groups for screening internal chemical and biologic libraries. Follow up studies on promising leads or drug candidates could be performed on larger cohorts of patient derived cell models through the consortia and/or access to the academic network of core laboratories.

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