Drug discovery through stem cell-based organoid models ☆

Adrian Ranga, Nikolche Gjorevski, Matthias P. Lutolf

Laboratory of Stem Cell Bioengineering, Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland

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1. Introduction

The development of new drugs is currently a long and costly process in large part due to the failure of promising drug candidates identified in initial in vitro screens to perform as intended in vivo. New approaches to drug screening are being developed which focus on providing more biomimetic platforms. This review surveys this new generation of drug screening technologies, and provides an overview of recent developments in organoid culture systems which could afford previously unmatched fidelity for testing bioactivity and toxicity. The challenges inherent in such approaches will also be discussed, with a view towards bridging the gap between proof-of-concept studies and a wider implementation within the drug development community.

Drug discovery today is at a crossroads: while increasingly large and varied compound libraries are synthesized and tested in primary screens, the promise of the identified lead compounds remains largely unrealized. Indeed, while tremendous investments in automation have enabled the costs and turnaround time for large to medium-scale primary screening to fall significantly [1], the gap between lead compound validation and success in the clinic is still wide, suggesting that a process still beset by significant limitations in efficiency.

To a significant degree, this inefficiency in taking lead compounds into the clinic may be due to the discrepancy between the simplified in vitro assays currently performed and the complexity of real in vivo pathologies. Indeed, while both drug safety and efficacy intrinsically linked to administration into a complex and heterogeneous three-dimensional (3D) physiological system, most primary drug screening campaigns are still carried out with cell lines grown on two-dimensional (2D) plastic, an entirely reductionist approach where important parts of the drug-biology interaction are lost. The outcome of this primary screening process is the identification of "hits", which satisfy very specific molecular targets or phenotypic requirements. A key problem is that these lead compounds are then validated and optimized in similarly over-simplified culture models. The process of ADMET evaluation (adsorption, distribution, metabolism, excretion, toxicology), while having undergone significant improvements in the last 15 years [2], could still be considered one of the main bottlenecks in the drug development process and could afford the greatest return on technological innovations [3].

As an important additional requirement, regulatory agencies require that identified pro-drugs be tested in two animal models before granting approval to proceed to any human clinical trials. This costly process of validation in animal models often fails due to physiological events linked to fundamental differences between human and animal model physiology. At this increasingly costly step, due to well-known differences in mechanisms of metabolism and toxicology between species, there remains a significant lack of fidelity between current testing procedures and human outcomes, particularly as related to appropriate evaluation of toxicity and drug dose.

These shortcomings have been clearly recognized within the pharmaceutical industry [4], yet few fundamental solutions have currently been implemented. The behavior of cells and their response to drugs continue to be studied in vitro mostly in 2D cell cultures that completely fail to mimic the complexity of the microenvironment. Not surprisingly, drug responsiveness in these settings is therefore often not predictive of the in vivo situation, which dramatically increases the costs of drug discovery.

At the same time, a vast amount of research has been carried out in academia to develop more relevant test-beds for screening and validation efforts (Fig. 1). In particular, there has been a push towards the development of multicellular spheroid models [5], notably in cancer modeling [6], as well as a number of miniaturized approaches culminating in organ-on-chip systems [7]. More recently, there has been a tremendous interest in developing increasingly complex multicellular constructs termed "organoids" [8–10] (Fig. 2). These morphogenetic models, often recapitulating developmental programs from embryology or harnessing adult stem cell-based regenerative processes, have allowed molecular and cell biologists to understand key signaling events required for the initiation and maintenance of multicellular organs. By recapitulating not only the form but also the rudiments of function of their in vivo counterparts, these constructs have the potential to move from laboratory proof-of-concepts to relevant tools in the drug discovery pipeline. Indeed, such organoids could finally provide a key missing link between compound screening and clinical trials, and could serve as models for testing drug efficacy in target organs, for toxicity in liver models or for bioavailability through intestinal system models. In particular, by using primary human cells, especially patient-derived cells with relevant pathologies in conjunction with cellular reprogramming strategies, these techniques could provide an invaluable link to disease-specific human drug screening models.

Ultimately, the wider implementation of these bio-mimicking approaches within a still-conservative drug development community will require the level of reproducibility and consistency currently achieved with cell lines. Thus, such culture models will require 3D culture conditions which afford the needed flexibility to achieve precise control over the cellular microenvironment as well as a level of scalability. Furthermore, the applicability of such models will be greatly enhanced by adapting to existing infrastructure, notably automatic robotic platforms for experimental setup and assay readouts.
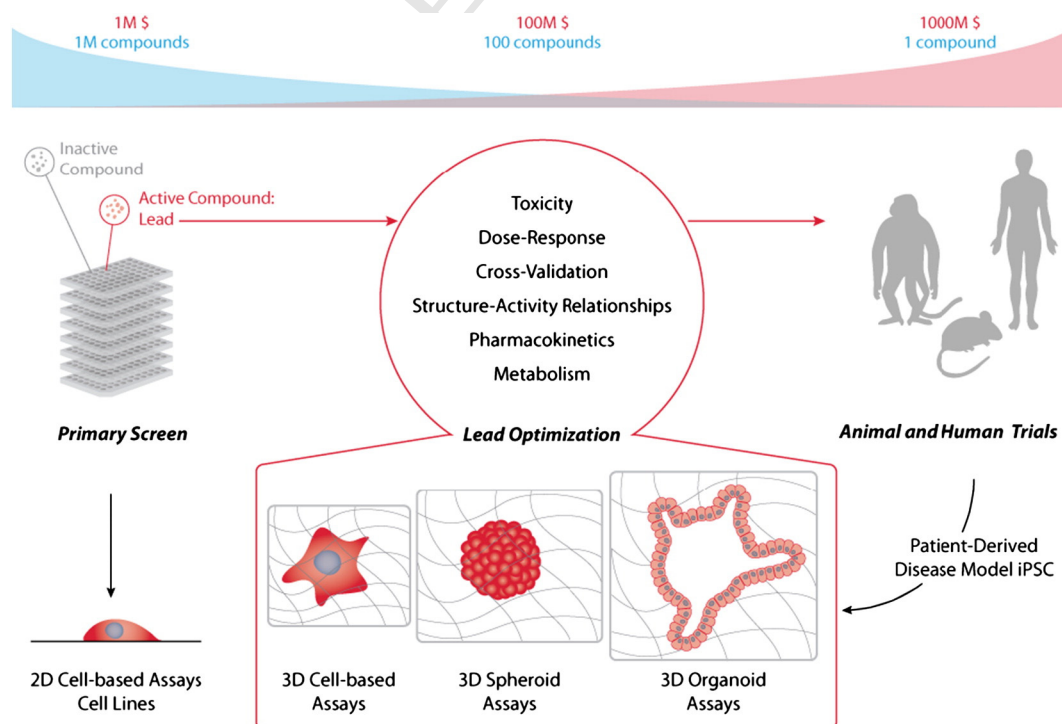
Fig. 1. 3D assays could bridge the gap between primary screening and animal and human trials. Drug discovery pipeline typically proceeds from multiple compounds tested at relatively low cost to few compounds in high-cost high-risk trials. The process of lead optimization and validation can benefit from increasingly representative in vitro technologies.
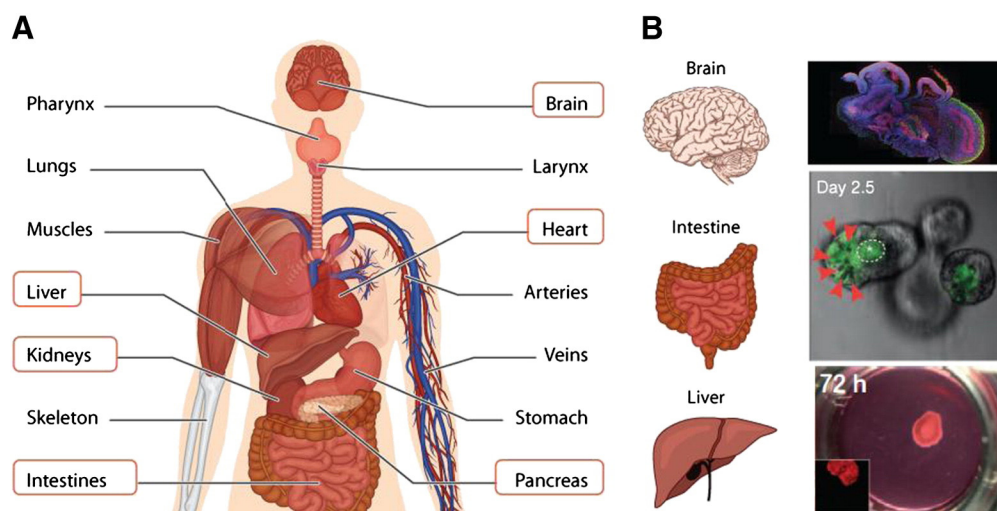
Fig. 2. Current organoid models. (A) An ever-increasing number of organs have in vitro organoid equivalents. Organoid-based assays present a novel and potentially high-value de-risking strategy, particularly when generated as iPS cell-derived disease models. (B) Among recently reported self-assembling cellular constructs, cerebral [73], intestinal [43] and hepatic [44] organoids are prominent examples of organoid cultures with potential applications in drug discovery.

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Thus the purpose of this review is first to provide a selected survey of existing state-of-the-art 3D models of in vitro drug evaluation, then to introduce some key recent developments in organoid systems, and finally, through a critical evaluation of the limitations of such systems, to propose some advances which could lead to the adoption of such models by making the case for a real functional value in helping de-risk this process.

2. From phenotype to organotype: high-throughput screening and the 3D paradigm

2.1. Phenotypic screens: an additional dimension

Phenotypic drug discovery has become increasingly popular in early stage drug discovery. Unlike target-based screens, in phenotypic screens, there is no a priori understanding of the molecular mechanism of action, and the effect of compounds on cell phenotype is observed directly. Such an approach has emphasized the importance of biologically-focused assays: between 1999 and 2008, in a period where target-based screens still predominated the drug discovery process, out of 75 first-in-class drugs with new molecular mechanism of action approved by the US Food and Drug Administration, the contribution of phenotypic screening to the discovery of first-in-class small-molecule drugs exceeded that of target-based approaches — with 28 and 17 of these drugs coming from the two approaches, respectively [11]. As cell-based assays continue to gain prominence and widespread adoption, it is clear that the new approaches focused on further enhancing biological relevance are necessary.

In the last few years, there has been a significant effort to develop 3D culture systems which better represent in vivo biology. Within this vast field, there have also been numerous approaches focused on high-throughput and miniaturized implementations of such technologies. Here, we will present such approaches, particularly focusing on most recent developments pertaining to implementation in high-throughput systems. Oncology has been one of the most important targets of drug discovery; in this field where the presentation of the pathology is often heterogeneous, and drug effectiveness, resistance and toxicity manifest itself in many ways. It is therefore in this field that a number of advances in the creation of more physiologically relevant approaches have been most prominent. Indeed, using a number of established cell lines, the cancer spheroid model as well as complementary assays for invasion, migration, and angiogenesis have been most readily explored.

2.2. Scaffold-free multicellular cancer tumor spheroids

Multicellular cancer tumor spheroids (MCTS) represent a well-established in vitro model for avascular tumor growth and this model has become a classic reference for 3D studies [5,12]. By forming an aggregate of cells which form cell–cell interactions, striking changes in morphology and gene expression are evidenced [13] (Fig. 3A) as a number of pathophysiological characteristics of an in vivo tumor are recreated, notably, oxygen gradients, glucose distribution, lactate accumulation, DNA strand breaks, ATP distribution and histomorphology/proliferation characteristics [14]. It is also particularly appropriate as numerous cancer cell lines, particularly those from the NCI-60 DTP human tumor cell line screen established by the NIH, have been shown to form spheroids without the addition of exogenous matrix materials [14].

For screening purposes, spheroid-forming assays have been developed in a most rigorously standardized manner within liquid media cultures. A key consideration in such studies has been the control over spheroid size, with the intention to establish a spheroid-based screen with clear pathophysiological gradients but without central necroses at the onset of treatment [15]. Indeed, a spheroid size of 400 μm was found to be ideal to recreate hypoxic conditions at the core, as well as proliferative gradients, which have significant impact on radio and drug-resistance as well as indirect effects of hypoxia-driven gene expression. Monitoring of spheroid growth kinetics to determine growth delay and regrowth upon drug administration consists of the primary analytical endpoint, and is performed by standard phase-contrast imaging. The acid phosphatase assay (APH) has also been established to monitor cell integrity and viability, with IC50 values estimated from dose–response curves determined through such an APH cell viability assessment.

Such a basic approach has been enhanced with the use of specifically designed high-density plates which have been engineered to allow for high-throughput hanging drop culture systems. Indeed such systems have been optimized for droplet stability [16], have been shown to be robust in fluorescence- and colorimetric-based assays through Z-factor calculations [17], and have allowed for the determination of differential effects on growth arrest with drugs. For example, fluoracil (5-FU) was determined to be more effective as an anti-proliferative agent in 2D,
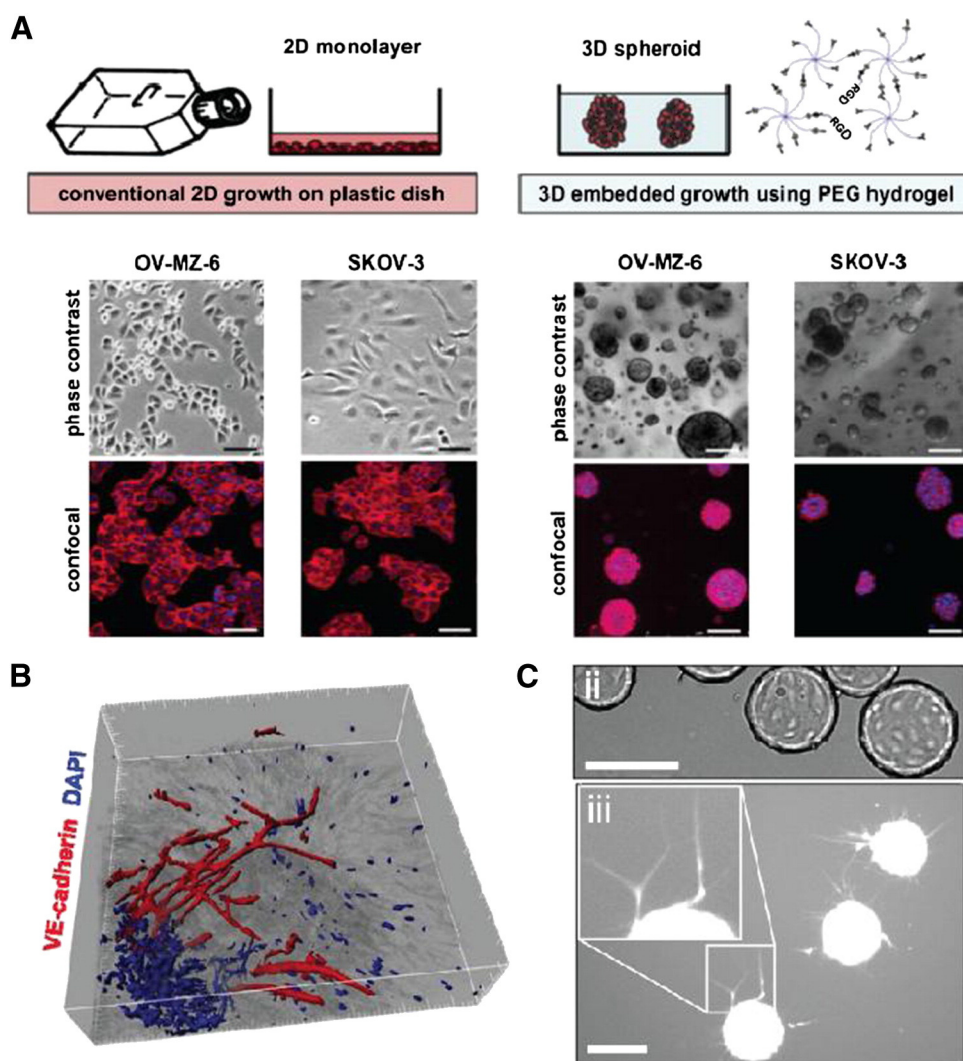
Fig. 3. State-of-the-art 3D assays allow for evaluation of multicellular tumor spheroid proliferation and quantification of angiogenesis and lymphangiogenesis sprouting. (A) Cancer cell line morphology and proliferation are clearly different between conventional 2D plastic culture and within synthetic PEG hydrogel (actin filaments stained with rhodamine phalloidin, nuclei with DAPI) [13]. Reproduced by permission of Elsevier. (B) Isosurface rendering of characteristic VE-cadherin immunofluorescence staining for VE-cadherin from ex vivo 3D (3D) assay of sprouting angiogenesis with arterial explants from human umbilical cords in Matrigel™ [74]. Reproduced by permission of American Society of Heamatology. (C) Lymphatic endothelial cell-coated beads showed cellular protrusions that sprouted into the collagen gel [30]. Effect of lymphangiogenesis inhibitors was quantified using identification of sprout number per bead by in-house developed software. Reproduced by permission of National Academy of Sciences of the United States of America.

whereas hypoxia activated drugs such as tirapazamine (TPZ) were seen to be more effective in 3D hanging drop cultures [18]. Hanging drop culture systems have also been recently used in an elegant high-throughput manner to show how co-culture models of cancer and stromal cells could unveil novel regulatory pathways [19].

2.3. Scaffold-based multicellular cancer tumor spheroids

The generation of large-scale liquid media spheroids has been the focus of technological advances in up-scaling; by creating "droplet micro-arrays" with the possibility of generating thousands of droplets with defined geometry and volume using superhydrophilic–superhydrophobic patterned surfaces [20], or enhancements in hanging drop spheroid manipulation through incorporation of magnetic-based technologies [21].

While these techniques utilizing hanging drop or liquid suspension cultures provide an undeniable ease of use, such a free-growing construct certainly does not recapitulate the physical constrains experienced by in vitro tumors and may miss important mechanisms of extracellular interaction-mediated drug resistance. In order to address this issue, multiple platforms have been developed to allow cancer spheroids to be grown within simplified extracellular matrices (ECM). To cite only a few recent examples, methylcellulose has been used as a simple 3D culture system for pancreatic ductal carcinoma cells and compared to standard 2D culture conditions; in the 3D case cell metabolism was seen to shift towards glycolysis, and, notably, while most drugs tested were shown to be less effective in 3D, two were identified as having particularly significant effects in this model [22]. In another study a 3D soft agar matrix was adapted to high-throughput screening, and 1528 natural product compounds were screened against colorectal carcinoma colonies [23]. Notably, this study showed how by comparing tumor-only cells with a co-culture model incorporating colon epithelial cells, it was possible to distinguish tumor-specific agents from general cytotoxic ones. A number of these technologies are now commercially available and have been shown to be compatible with a number of downstream assays beyond imaging. For example, in alginate-based scaffolds while cytotoxicity was measured by AlamarBlue® assay and drug effectiveness was measured by imaging, additional readouts such as apoptosis were evaluated by immunohistochemistry and RT-PCR, where cellular uptake of drugs and nanoparticles could also be

evaluated [24]. Again, it was shown that IC50 values for a number of cancer drugs were significantly higher in a spheroid model as compared to 2D. Thus, as demonstrated in these selected examples, the multi-cellular tumor spheroid model, despite its simplicity as a first approximation of a 3D tumor microenvironment, has already shown value in a high-throughput drug discovery pipeline.

2.4. Migration, invasion and angiogenesis in 3D

While aberrant cellular proliferation may be the primary manifestation of solid tumor cancers, it is via remodeling and migration through the tissue microenvironment that malignant cells metastasize to spread to adjacent tissues or distant sites via lymphatic or angiogenic means. A better understanding of the role of the microenvironment in initiating and promoting these processes is critical if the prevention of metastasis is to be used as a target for oncologic therapeutics. With appropriate 3D culture models, it is possible to monitor not only bulk volume increases of spheroids (*i.e.* tumor proliferation), but also, given appropriate matrix conditions and chemotactic cues, the outgrowth of individual cells from such spheroids (*i.e.* cell migration). A number of approaches to assay migration and invasion exist, with varying degrees of physiological relevance and ease of implementation [25]. A prominent example includes filter-based transwell assays, which can be additionally coated with a layer of ECM components such as collagen or Matrigel™. More complex models incorporate a level of dimensionality, such as "sandwich assays" where a monolayer of cells is entrapped between two layers of ECM. Most relevantly, cellular spheroids such as the ones described above can be seeded onto a relevant matrix, or completely embedded within a 3D matrix [25]. Angiogenesis is one of the central hallmarks of cancer progression, and significant efforts have been carried out to determine ways to understand and inhibit this process in increasingly biorelevant model systems [26–28] (Fig. 3B). For example, a 3D vascular network assay showed considerable sensitivity to several angiogenic inhibitors, including kinase inhibitors and monoclonal antibodies and led the development of a 3D model of tumor-driven angiogenesis, in which angiogenic outgrowth was sustained by spheroids of prostate cancer cells in the absence of exogenous growth factors [29]. Another notable use of 3D-based spheroid assays was developed to screen for inhibitors of lymphangiogenesis [30], a process akin to angiogenesis and highly implicated in tumor progression (Fig. 3C). In this study, spheroids were formed by coating lymphatic endothelial cells around cytodextran microcarrier beads, and were then tested in a 3D high-throughput sprouting assay over the LOPAC collection of pharmacological compounds. By enhancing the power of such a screen by deploying auto-mated microscopy in conjunction with custom-developed advanced image processing software, novel regulators of lymphangiogenesis could be detected. In particular, a previously unknown link was established between statins and the inhibition of lymphangiogenesis, which has potential implications not only directly in the treatment of cancer but also for interactions with and management of cardiovascular disease.

2.5. 3D assays for toxicity evaluation

While cancer is a primary target for the deployment of advanced high-throughput screening strategies, and has been seen in examples above to benefit from a 3D approach, it is by no means the only area where spheroid-based assays have been used. Indeed the spheroid assay has found significant use in organ- and cell-type-specific toxicology studies, constituting another important step in the drug validation process. While dose response and toxicity must clearly be evaluated in the cell type of interest, systemic toxicity, and particularly liver toxicity, is also of primary concern. As such, toxicity studies are routinely performed in HepG2 cells, a hepatocarcinoma cell line which has been frequently used as a model system to study liver metabolism and cyto-toxicity. 3D spheroids of HepG2 and closely related HepaRG™ cells have been cultured in hanging drop suspension cultures and compared to 2D cultures. In one study, it has been shown that activity of CYP4A4, a member of the P-450 monooxygenases involved in the metabolism of a broad range of compounds from steroids to drugs and toxins, was higher in the 3D cultures compared to 2D [31]. Moreover, 3D cell cultures were more sensitive to a drug that is only toxic upon metabolic activation in the liver (aflatoxin B), suggesting that such an organotypic system better represents in vivo liver metabolism. Notably, within such an organotypic culture, the EC50 of acetaminophen was similar to in vivo toxicity, a phenomenon which could not be reproduced in 2D, demonstrating once again the importance of a 3D model for capturing in vivo response [32]. In a further advance, liver microtissues have also recently been constructed from primary human hepatocytes and liver-derived non-parenchymal cells [33]. Interestingly, significant species-specific differences in drug hepatotoxic response were found between rat and human microtissues [34], highlighting the importance of developing human cell-based 3D culture systems.

### 3. Organoids as in vitro organ models: promise and challenges

Despite the increasingly acknowledged value of in vitro 3D culture, of which some examples were reviewed in the previous section, animal models have remained as the immediate next test bed for promising new compounds after an initial primary 2D screen. However there is a significant gap between the still highly simplified models of spheroids and the systemic effects seen in an animal, with all the possible confounding effects which cannot be clearly deconvolved. Moreover, there have also been some significant data over the years indicating substantial differences between animal and human modes of drug response. For example, in a survey of a dozen pharmaceutical companies, with data from 150 compounds with 221 human toxicity events, data from rodents failed to predict 57% of incidents of human toxicity [35]. Furthermore, a recent study showed a complete lack of correlation in the genomic response to acute inflammatory stress between human subjects and murine models, suggesting that the use of such unrepresentative models may have accounted, thus far, for the failure of all compounds which have been put through clinical trials intended to block the inflammatory response in critically ill patients [36].

An ideal in vitro analysis system would therefore comprise of human cells, in a construct complex enough to demonstrate physiologic-like composition, morphology and heterogeneity and, ideally, the rudiments of functionality, yet simple enough that it could still be readily assayed in vitro. Such a construct, capturing some of the complexity of a human organ in a dish, has been termed "organoid" [37] (Fig. 2). The idea of creating in vitro organoids is not a new phenomenon: leveraging cells' intrinsic ability to self-assemble into organized structures has been envisaged at least since the early generation of teratocarcinomas in 1954 [38]. Indeed, when embryonal carcinoma cells were transplanted into a host mouse, malignant tumors were found to develop and could form tissues of all germ layers, and surprisingly, could even in rare cases develop into complete organs. This became even more relevant with the advent of embryonic stem cells (ESC), which, under the same condition, *i.e.* reimplantation into a host animal, could generate equally histologically and morphologically complex structures. These early studies clearly demonstrated the potential of stem cells grown in vitro to recreate complex and organized structures, albeit when placed in the context of a complex host microenvironment. As well, these studies early on demonstrated the need for extrinsic microenvironmental regulation for growth and development. In the context of more relevant physiological studies in vitro, clearly there was an interest and a need to understand how such processes could be regulated, in order to then attempt to recreate these processes in vitro.

While the field of tissue engineering has made significant attempts to recreate in vitro organs in the last 30 years, these approaches have generally focused on scaffold-based cell seeding techniques, and arguably

have found limited success in recreating the complex and heterogeneous cellular organization found in vivo. More recently, new approaches based on developmental biology have focused on recreating morphogenesis underpinned by a more sophisticated molecular understanding, with the intent of harnessing the differentiation potential intrinsic in stem cells to allow for self-organization. Given the right cues, a number of increasingly complex structures have been recreated in vitro (Fig. 2A), which for the first time may allow for "function-in-a-dish". While their potential uses as replacement organs in regenerative medicine is the clearest and ultimate objective, a more likely and tractable shorter-term goal is to make use of such constructs in the context of drug discovery.
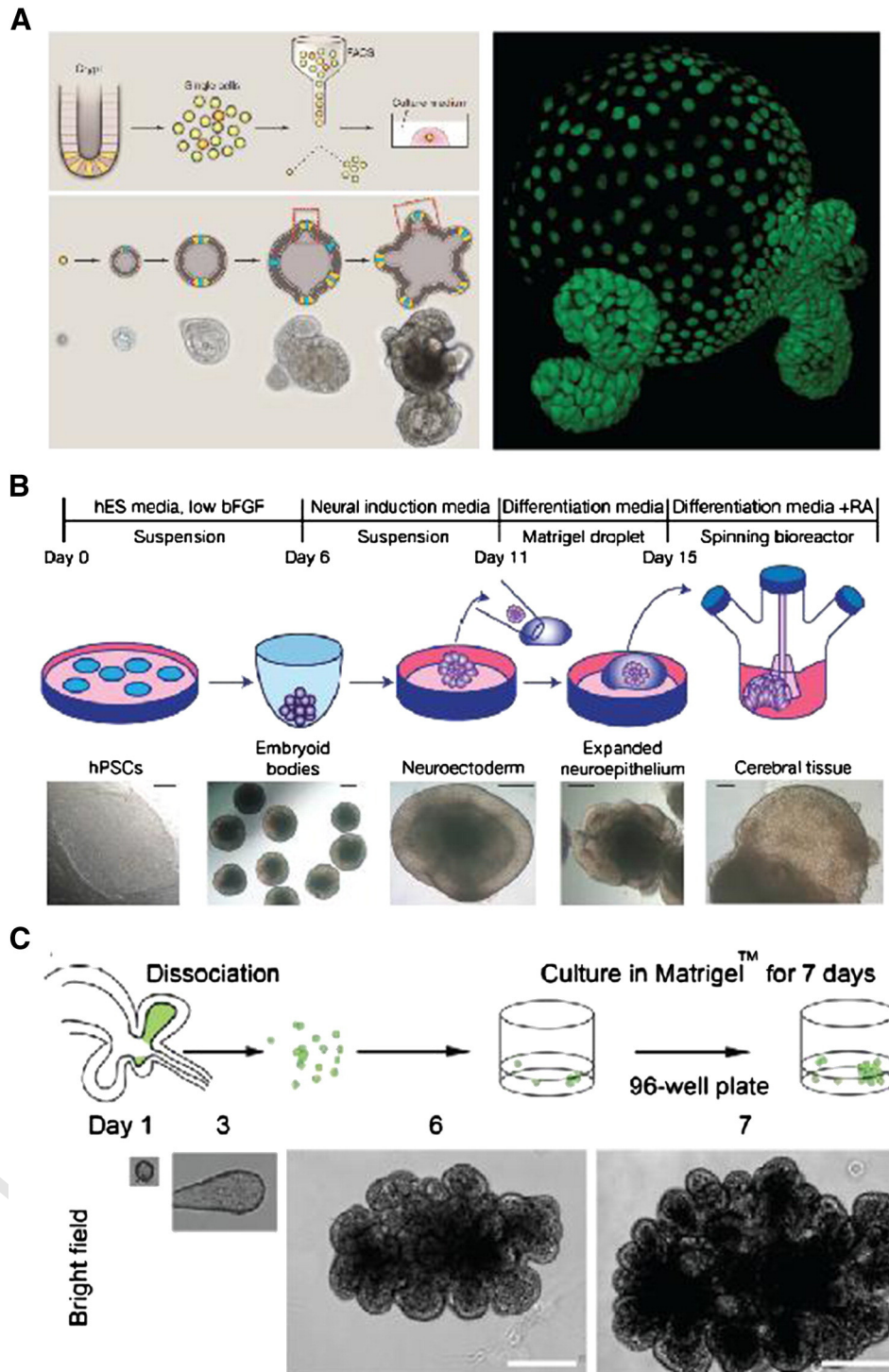
Fig. 4. Organoid culture systems require Matrigel as a 3D matrix for structural support and instructive signaling. (A) Development of an intestinal organoid from a single sorted Lgr5-GFP cell and architecture of a mini-gut organoid expressing histone 2B-GFP, with five crypts budding from a large central body containing the main lumen [8,75]. Reproduced by permission of The American Association for the Advancement of Science and Elsevier. (B) Schematic of cerebral organoid multi-step culture system including aggregation of ESCs in embryoid bodies, suspension in neural induction medium to form symmetric neuroectoderm, followed by expansion of neuroepithelium in Matrigel™ and final tissue maturation in spinning bioreactor [73]. Reproduced by permission of Nature Publishing Group. (C) Morphogenesis of pancreatic organoids from mouse pancreatic buds composed mainly of bipotent pancreas progenitors by seeding in growth factor-depleted Matrigel™ [53]. Branched organoid after 7 days contain ~10,000 cells. Reproduced by permission of The Company of Biologists Limited.

While spheroid-based approaches may be sufficient for testing drug efficacy in ablating tumor growth, mitigating migration and modeling to some extent angiogenesis, such approaches do not allow the equally important assessment of cytotoxic effects of drugs on a multitude of organs, nor the investigation of issues such as bioavailability when crossing the intestinal lining, liver metabolism, or blood–brain barrier effects. As key examples, we will focus here on recent developments in creating brain, liver and intestinal organoids (Fig. 2B), which could help in assessing these whole-organ effects.

4. State-of-the-art organoid culture systems: Matrigel™ as critical component

4.1. Intestinal organoids

In recent years, the organoid system that has gained the most attention is the mini-gut construct, or intestinal organoid (Fig. 4A). In landmark studies by the Clevers group [39–41], it was first shown that the transmembrane protein Lgr5 marks stem cells in the intestinal crypt, and that such cells exclusively contribute to the rapid self-renewal of the intestinal epithelium. Based on this in vivo knowledge, experiments were then carried out to demonstrate how an epithelial cell fragment isolated from the mouse intestinal crypt, when placed in an appropriate ex-vivo 3D culture system, could generate a 3D construct with some of the key characteristics of the intestine, including the establishment of a crypt-villi architecture as well as a lumenized interior [42]. Clearly, the stem cell niche concept plays a significant role in this system: factors such as R-Spondin, EGF, and Noggin are essential for the maintenance of the organoids in culture, and Matrigel™, the matrix used as 3D support, provides a set of structural and biochemical cues. Notably, it has been found that even a single Lgr5 cell could be sufficient to regrow an entire organoid, but this process occurred at very low efficiency (circa 5%) [42]. Significantly, it has been shown that organoid-forming efficiency was greatly enhanced when Paneth cell-Lgr5 cell doublets were used, instead of single Lgr5 cells, suggesting that factors secreted by the Paneth support cell are crucial for regulating the intestinal niche [43]. Such intestinal organoids have also been derived from human ES cells [44], thereby greatly enhancing the applicability of such a system. In turn, it is now possible to envisage that such intestinal organoids could begin to be used to detect drug–intestine interactions, and, more specifically, to investigate bioavailability and aspects of drug pharmacokinetics. Indeed, intestinal organoids could certainly be imagined as complement or alternative to the commonly used Caco-2 monolayer transwell assay, which is now the norm as an in vitro model of human small intestinal mucosa's ability to absorb orally administered drugs [45].

4.2. Cerebral organoids

The developing human brain acquires its complexity through a myriad of developmental steps, with various cell types and regions acquiring their fate in a tightly regulated and sequential manner. In vitro, it has been possible to establish 2D cell culture protocols to generate bulk populations of neuronal subtypes from ESCs for screening purposes in procedures which have become fairly standardized and even in some cases deployed in primary drug screening assays [46]. With the adoption of 3D culture techniques, it has been possible in recent years to generate increasingly complex neural subsystems which, to some extent, preserve their highly restricted in vivo spatial arrangement. Indeed, the formation of an optic cup from ESCs, including a multilayered neural retina containing rods and cones, was shown to occur using a multi-step protocol involving the creation of aggregates in a floating culture in serum-free and growth-factor-reduced medium, named SFEBq culture, or serum-free culture of embryoid body-like aggregates with quick aggregation [47]. The extraordinary self-organization seen in the optic cup construct involves nonetheless, both in mouse and human systems [48], the embedding of aggregates in Matrigel™. Indeed, while it may be claimed that the process is entirely driven by spontaneous self-organization orchestrated by local cellular interactions, it is quite possible that there are instructive matrix-derived cues at critical points in the process, which may perhaps even be the initiating impetus for key symmetry-breaking events within the homogeneous aggregates. More recently, a similar SFEBq-based protocol has been utilized to develop a human ESC-derived 3D organoid, termed cerebral organoids, where extensive patterning of brain regions can be seen, including characteristic cerebral cortex zones with mature cortical neuron subtypes [49] (Fig. 4B). In an elegant demonstration of how such an approach could be used for disease modeling, such a cerebral organoid was modeled from induced pluripotent stem (iPS) cells derived from a patient presenting with microcephaly, a disorder which has yet to be suitably reproduced in a mouse model. Such organoids were less developed than their normal counterparts, with an analysis of the constructs revealing a potential mechanism for disease progression rooted in defective, premature neuronal differentiation. Proof-of-principle studies such as this one confirm the promise that patient-derived iPS cells can serve to better understand disease and to identify potential molecular targets from a function perspective. In particular, cerebral organoids could also be further developed to study the blood–brain barrier and help overcome the difficulties in delivering pharmacological agents into specific areas of the brain. Such an approach, which would require at least some measure of vascularization, could potentially be achieved within a co-culture system, and could potentially identify novel molecular paths to entry or specific regions of the brain more sensitive to drug delivery.

4.3. Liver organoids

Indeed, such a co-culture system has been employed in an approach focused on generating a liver organoid. A liver bud exhibiting similar markers to its in vivo counterpart was generated after aggregation of three cell types at very high cell densities (human umbilical vein endothelial cells (HUVEC), human mesenchymal stem cells (MSC) and iPS cell-derived hepatic cells) and embedding in Matrigel™ [50]. As in the neural and intestinal systems, self-organization occurred within the organoids; here a notable advance involved the additional development of nascent endothelial networks, which, helped by MSCs thought to function as a source of pericytes promoting vessel stability [51], allowed the limb bud to integrate into the host vasculature when implanted into ectopic extrahepatic sites in a mouse. Within two months in vivo these organoids matured and resembled adult liver histologically, and had developed bile canaliculi (though not bile ducts). Importantly, multiple transplanted liver organoids were able to rescue mice from subacute gangyclovir-induced liver failure. As with other newly developed in vitro organoid systems, a fully mature miniaturized organ with a complete set of functional features has not yet been achieved, but it can be speculated that a better understanding of complex signaling pathways involved in establishing morphogenesis, co-culture approaches and longer maturation times could lead to the types of function seen here in this case after in vivo implantation. Still, even immature human-cell based organoids such as the one depicted here, may be more responsive and predictive of acute liver injury which is difficult to detect in the course of the current drug discovery pipeline.

The strikingly complex organoid model systems described here represent only a selection from the rapidly expanding organoid literature. Indeed, there have also been in the last year reports of such diverse organoids as the pituitary gland [52], inner ear [49], pancreas [53,54] (Fig. 4C) and hair follicle [55]. For all these systems, whether derived from single cells or from pre-aggregated pluripotent stem cells, the overarching feature has been a significant level of self-organization over time mediated by a 3D matrix.

5. ECMs and 3D screening: towards synthetics and scalable approaches

5.1. Engineering better hydrogel systems

A number of challenges present themselves in the pursuit of an effective translation of these organoid culture systems from an academic laboratory proof-of-concept to the kind of robust and reliable assay required for a drug discovery program. The first of these challenges is the need for a reproducible, well-defined and scalable 3D gel system. Indeed, all the organoid cultures presented in the previous section made use of the commercially available Matrigel™ system, which is an extract from Engelbreth–Holm–Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins. Its main component is Laminin-1, an abundant extracellular component found in basement membrane, while other components of Matrigel™ include a mixture of collagen IV (30%) and entactin (6%) as well as heparin sulfate proteoglycans and a variety of growth factors in varying proportions, including transforming growth factor beta (TGFβ), epidermal growth factor (EGF), fibroblast growth factor (FGF), tissue plasminogen activator, as well as residual matrix metalloproteinases (MMPs) and growth factors occurring naturally from in the tumor [56]. It appears clear that the organogenic bioactivity of this matrix therefore derives both not only from its three-dimensionality but also from the complex mixture of signaling cues. Yet, Matrigel™ remains a natural extract with relatively imprecise composition and unquantifiable batch-to-batch variability, rendering it unsuitable for reproducible and large-scale assays. Furthermore, Matrigel™ presents a number of practical limitations in handling and processing: it requires careful manipulation and must be maintained at constant cold temperature throughout cell-encapsulation processes, which is incompatible with current implementations of large-scale robotics, which have generally been adapted to handle cell culture reagents within a different temperature range. Matrigel™ also tends to have a widely variable degradation profile, which, depending on handling procedures as well as cell-mediated responses, can partially degrade in an uncontrolled manner within the time span of the assay.

Thus, to begin to consider the implementation of organoids as suitable in vitro drug discovery tools, a better-defined matrix which would minimize or preferably completely eliminate animal-derived components as well as be easy to handle and reproducibly degraded is necessary. While other purified natural materials such as alginate and collagen I have also been shown to support cell encapsulation, it is doubtful whether such materials could allow for the complex morphogenesis seen in Matrigel™, due to their relatively poor abilities to be functionalized with additional required signaling cues and lack of independent modulation of structural and chemical properties.

Currently, synthetic and highly tuneable approaches to materials engineering can provide hydrogels with the versatility and consistency which would be required for large-scale compound screening (reviewed in [12,57]). Such artificial ECMs can allow the experimenter to determine in an independent and highly reproducible manner both the physical and biochemical properties of such matrices. For example, poly(ethylene glycol) (PEG)-based hydrogel approaches allow us to create biophysically active materials. These materials allow for modulation of mechanical properties as well as cell-mediated degradation in response to matrix metalloprotease (MMP) secretion [13, 58,59]. For example, in a model of epithelial ovarian cancer, it was shown by exploiting the design flexibility of the hydrogel characteristics, that proliferation in 3D was dependent on cell-integrin engagement and on the ability of cells to proteolytically remodel their immediate extracellular microenvironment while maintain hydrogel stability in long-term culture [13]. Additionally, it is possible to chemically tether instructive ECM-derived signals directly onto the PEG backbone, thereby modulating the biochemical microenvironment. Indeed, by engineering protein or peptide constructs compatible with the chosen cross-linking system [60–62], it is possible to build a material toolbox whose elements can potentially be used in a set of combinatorial rearrangements [63].

5.2. High-throughput approaches for microenvironment optimization

While Matrigel™ presents multiple and potentially interacting cues, defined synthetic matrices would need to be precisely tuned to achieve the required optimal properties. In such a reductionist and defined approach, the faithful recreation of organoid microenvironments would have to go beyond the "one-size-fits-all" approach. A Matrigel™-like organ-specific synthetic analog would certainly require an as yet unknown combination of biophysical properties and biochemical signaling cues, and it would be necessary to establish a screening paradigm to systematically identify unique microenvironments which would optimally support robust and reproducible organoid development.

A number of approaches have been proposed to begin to assess the effect of large-scale combinatorial biomaterial libraries on cell behavior. The repurposing of DNA microarray printers to produce what have been termed "cellular microarrays" has been a particularly popular technique. In one implementation, a combination of synthetic polymers with different material properties including wettability, surface topography, surface chemistry and elastic modulus were arrayed onto glass slides and were assessed for their ability to maintain human ESC self-renewal [64]. In another example, ECM proteins and soluble factors were assessed in combination to determine optimal conditions for primary hepatocyte maintenance and early hepatic differentiation of ESCs [65]. These platforms focused on directly functionalizing microscope slides have relied on cell adhesion to provide a cellular readout. Other approaches have focused on the creation of structured microwell arrays which served to entrap cells and could for example track individual cell fate in a more precise manner via time-lapse microscopy, and platforms where the simultaneous physical and biochemical properties of the matrix (i.e. hydrogel substrate stiffness and surface protein
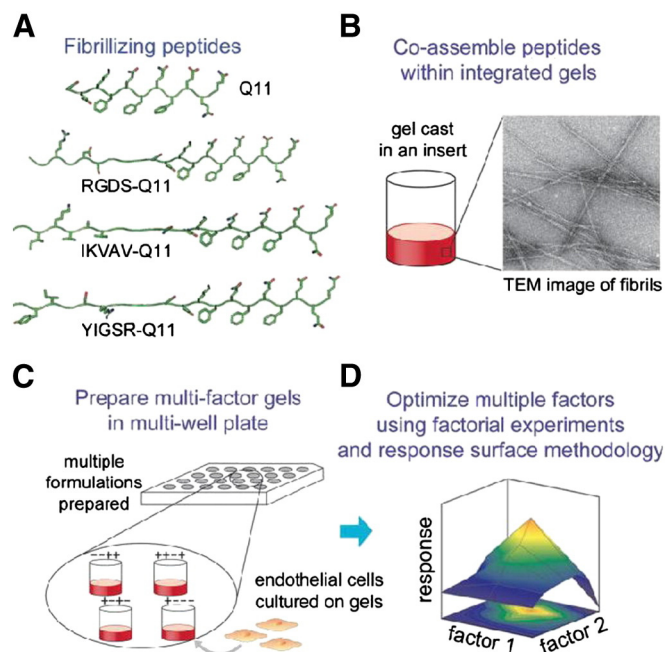
Fig. 5. Modular 3D scaffolds enable multifactorial experimentation [68]. Different ligand-bearing peptides were synthesized (A) and co-assembled into fibrillar hydrogels (B). HUVECs were cultured in combination of gels with different combinations of peptides (C). Full factorial experiments and response surface methodology were used to explore the direct and interactive effects of various immobilized ligands on cell growth, and optimum formulations that maximized proliferation were identified (D).
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functionalization) that could be modulated have enabled truly multifactorial explorations of extrinsic microenvironmental control [66].

5.3. Challenges in implementing engineered organoids

While much can be learned from the combinatorial technologies seen above, a major limitation has remained that only adherent cells or cellular aggregates such as neurospheres [67] in liquid media could be assayed in such systems. Despite technical difficulties which have hampered the use of high-throughput combinatorial studies in 3D, the deployment of rational approaches based on design of experiment (DOE) methodologies has proved to be instructive in ways to assess a combinatorial space for 3D cellular response. In one notable example, multiple peptide ligands were incorporated into engineered self-assembling peptide hydrogels. An iterative process consisting of single-factor experiments for setting initial bounds followed by factorial experiments for identifying main effects and interactions between ligands served to identify previously unknown antagonistic interaction between the laminin-derived peptide mediating HUVEC cell attachment and growth. In a final step, response surface methodology experiments were carried out to identify optimal formulations of these ligands, which led to endothelial cell growth equivalent to that on native full-length fibronectin [68] (Fig. 5).

Ultimately, once such synthetic matrices would be defined for an organoid of interest, it is possible that such artificial extracellular microenvironments could be widely deployed, either in standardized formats using liquid handling robots, or via miniaturized arrays such as the ones described for toxicity testing [69]. With organoid morphology and function being significantly more complex than spheroids or single cells, one important challenge will be in the systematic assessment of function. More advanced readouts would have to be developed to address these issues; while confocal microscopy currently provides the standard imaging tool for assessing cellular function within such constructs, it is certainly limited in throughput. As such, newer techniques currently being developed for whole animal imaging allowing for rapid and highly accurate scanning of large areas, such as light sheet fluorescence microscopy or high-resolution optical coherence tomography could be deployed in this context. Light-sheet microscopy, where only the fluorophores in the light sheet's plane contribute to the image, is particularly useful in reducing out-of-focus blur from three-dimensional samples [70]. Additionally, a technique known as biodynamic imaging, which uses short-coherence dynamic light scattering to evaluate intracellular motions [71], has recently been specifically applied to study the multicellular cancer spheroid model. However, the key for such promising new imaging technologies to gain wider application will be to modify them in appropriate ways in order to conform to the standardized formats and throughput required in the drug discovery process. Furthermore, image analysis tools focused on simple cellular morphologies would have to be adapted and customized to yield relevant and quantitative data. Beyond imaging, a number of multiplexed techniques could also be used to interrogate for functional outputs and to maximize sample value. For example, Luminex/XMAP bead-based assays can be used to detect hundreds of proteins or genes of interest within a single sample, while gene expression can be assessed by such technologies as the Fluidigm qPCR gene expression profiling system [72] as well with as a battery of increasingly inexpensive sequencing technologies. Today, some pharmaceutical companies are actively.

6. Conclusions and outlook

Numerous studies have now shown the advantages of 3D cell culture, in particular in the context of the multi-cellular tumor spheroid model, with notably different drug responses compared to 2D contexts which in some cases compare favorably to the in vivo observations. Such 3D cultures have also been used to demonstrate more physiological responses in other contexts such as migration, invasion, angiogenesis and lymphangiogenesis, as well as in toxicology. We have proposed in this review that complex self-organized organoids, which have recently come to the fore as striking proof-of-concept examples of in vitro developmental biology, could be appropriate test platforms for future drug discovery efforts. Indeed, such miniaturized proto-organs could be used as a significant validation bridge between primary high-throughput screening and costly animal and human trials. Safety and efficacy of lead compounds could be tested directly on in vitro organs for the target pathology. It could also be imagined that in the context of oncology, cells and tumor response could be assessed not only in isolation but in a more realistic co-culture system within an organ of interest. Ultimately, such organoid systems could be systemically linked in order to begin to build a "human in a dish" as a technology with unprecedented fidelity to human disease and drug response. Today, the promise of such transformative advances is largely limited by issues of historical importance in the drug discovery process: reproducibility, standardization, validation and quality control. For these far-reaching objectives to be accomplished and for such technologies to move from the university laboratory to a broader use in the commercial drug discovery process, we have emphasized the need to focus on implementations based on synthetic and tailored 3D matrices amenable to medium to large-scale automation, as well as meaningful multiplexed readouts.

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