Advanced Drug Delivery Reviews 69–70 (2014) 1–18

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Critical analysis of 3-D organoid in vitro cell culture models for high-throughput drug candidate toxicity assessments ☆

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ARTICLE INFO

Available online 5 March 2014

Keywords:
Toxicity
Drug development
Nanotoxicity
Reasons for failure
Cell culture models
HTS screens
Cellular microenvironment
3-D organoid culture

ABSTRACT

Drug failure due to toxicity indicators remains among the primary reasons for staggering drug attrition rates during clinical studies and post-marketing surveillance. Broader validation and use of next-generation 3-D improved cell culture models are expected to improve predictive power and effectiveness of drug toxicological predictions. However, after decades of promising research significant gaps remain in our collective ability to extract quality human toxicity information from in vitro data using 3-D cell and tissue models. Issues, challenges and future directions for the field to improve drug assay predictive power and reliability of 3-D models are reviewed.

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Abbreviations: 2-D, two dimensional; 3-D, three dimensional; HTS, high throughput; IVIVCs, in vivo–in vitro correlations; HA, hyaluronic acid; ECM, extracellular matrix; SV40, simian virus 40; HPV, human papillomavirus; CYP, cytochrome; MMP, matrix metalloproteinase; PCL, polycaprolactone; PS, polystyrene; PLGA, poly(lactide-co-glycolide); tPA, type plasminogen activator; bFGF, basic fibroblast growth factor; TGF-b, transforming growth factor; PN-II, protease nexin II.

☆ This review is part of the Advanced Drug Delivery Reviews theme issue on "Innovative tissue models for drug discovery and development".

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http://dx.doi.org/10.1016/j.addr.2014.02.008
0169-409X/

1. Introduction

A general lack of reliable drug candidate toxicity screening methods is correlated with the current unacceptable failure rates of new chemical and biological entities progressing from therapeutic discovery toward clinical use [1–3]. Unacceptable toxicity during pharmaceutical development is estimated to be the second leading cause of post-marketing drug withdrawal [4,5]. This is also a primary area without much noted improvement for the past two decades [6]. These exceptional failure rates combined with lack of reliable toxicity predictor assays highlight the need for new approaches required to better predict drug-related toxicity in pre-clinical development pipelines [4,7]. Unfortunately, current concepts for in vitro–in vivo correlations (IVIVCs) [8] do not often focus on drug toxicity mechanisms, as IVIVCs have traditionally emphasized drug dissolution and bioavailability equivalence; FDA's In vitro–In vivo *Correlation Guidance*, for example, states that the primary objective of developing and evaluating an IVIVC is to enable the drug dissolution test to serve as a surrogate for drug in vivo bioavailability studies to reduce the number of bioequivalence studies required both for drug approval as well as during scale-up and post-approval changes [9] In contrast, the United States Pharmacopoeia (USP) defines IVIVC as the establishment of a rational relationship between a biological property, or a parameter derived from a biological property produced by a dosage form, and a physicochemical property or characteristic of the same dosage form [10]. No IVIVC is yet defined specifically for correlating aspects of drug toxicity observed in vivo with accurate or predictive toxicity testing in vitro, despite the clinically correlated and costly drug failures due to toxicity.

Table 1
Comparisons of cellular differences between culture systems with different compositions and architectures.

Literature citation	Primary results and conclusions
[191]	Cells that co-exist in vivo outcompete and dominate co-cultures in 2-D. Examples: immortalized macrophages and fibroblasts; endothelial cells and muscle cells. *Conclusion*: 2-D co-culture is difficult for in vivo-relevant cellular interactions.
[192,193]	Cells in 2-D respond to toxins with limited amounts of cytokine profiles found in vivo. Restoration of cytokine signaling is observed in 3-D cultures. *Conclusion*: 3-D cell cultures better restore inflammatory pathways indicative of in vivo-like function.
[130]	Actin organization and integrin translocation were found to be different in 2-D vs. 3-D cultures. The effects of culture dimensionality were also observed on 2-D cultures that were mechanically flattened from 3-D cultures with native ECM. *Conclusion*: Native matrix does not retain appropriate conformation or topology in 2-D, resulting in altered cell function.
[194]	Hepatocytes quickly de-differentiate within days in 2-D culture. When hepatocytes are grown in co-cultures with fibroblasts on micro-patterned surfaces, their functionality is retained for several weeks. *Conclusion*: Heterogeneous cell–cell interactions and molecular signaling might be critical in primary cell homeostasis in vitro.
[195,196,15,197,198]	Kidney cell line MDCK produced spherical cysts in 3-D collagen I or laminin-rich gels and could undergo differentiation into branching tubules upon stimulation. No cyst formation was observed in 2-D. Similar differences were observed for bladder epithelial cells, endothelial cells, and mammary epithelial cells on laminin-rich ECM. *Conclusion*: Epithelial cells grown in 2-D and 3-D produce differential architecture and polarization.
[15]	Blood vessels showing incorporation into native vasculature networks in vivo were formed in 3-D polylactic/polyglycolic matrices. Muscle tissue 3-D constructs with endothelial vessels were formed in co-cultures of myoblasts, endothelial cells, and embryonic fibroblasts. 3-D culture alone could not procure stable vessels. Functional fibroblasts (stromal cells) were critical for vessel formation. *Conclusion*: Stromal signaling in 3-D and multiculture systems are critical for complex structure formation.
[199]	HepG2 liver cells formed more bile canaliculi and were more metabolically active (assessed by albumin secretion) in 3-D polystyrene (PS) culture than on 2-D surfaces. HepG2 cells were also less sensitive to toxic methotrexate in 3-D vs. 2-D. Cells in 3-D secreted more ECM than in 2-D. *Conclusion*: Cell–cell interactions and 3-D cultures are important in producing in vivo-relevant cell morphology and functionality.
[200]	Human liver cell line C3A had significantly increased drug metabolism by the two most important cytochrome p-450 (CYP) enzymes in human liver, CYP1A2 and CYP3A4, when cells were cultured in 3-D using alginate scaffolds vs. traditional culture. *Conclusion*: Phase I and phase II metabolism functions important to IVIV correlations depend on intracellular 3-D interactions.
[201,202,12,203]	Anti-cancer drug influences on migratory behavior of tumor cells are proposed to be regulated by communication with extracellular matrix. Human fibrosarcoma HT1080 cancer cell line expanded on plastic, 2-D plastic coated with collagen type I and fibronectin, and 3-D collagen I gels and treated with doxorubicin. Migration was significantly inhibited on plastic, while other conditions did not show antimigratory effects. Data from ECM-coated conditions match predictions from in vivo studies that cellular environment contributes to cancer survival after chemotherapy. Similar effects were shown for breast cancer cells that grew into gland-like acini on laminin, produced 'inside-out' polarized acini in collagen I, and disorganized masses without ECM. *Conclusion*: Cell–ECM matrix interactions, not 3-D architecture alone, are important in clinically relevant cancer progression studies in vitro.
[197]	Paracrine signaling between primary stromal cells and epithelial bladder cells in 3-D to produce adhesion and acini formation was studied. Interactions with cytoskeleton via cadherin-E and TGF-β2 mechanisms were implicated in adhesion mechanisms. *Conclusion*: Cell–ECM paracrine signaling via adhesion molecules govern 3-D cell architecture. Presence of stroma did not affect amounts but influenced co-localization of adhesion molecules with actin in 3-D.
[204,205]	Glioblastoma cells were assessed for N-cadherin expression in a 3-D magnetically levitated culture. Expression was detected in membrane, cytoplasm and cell junctions similar to protein expression in tumor xenografts. Conversely, 2-D cells had N-cadherin dispersed in the cytoplasm and nucleus but absent from the cell membrane. Similar differences in N-cadherin expression were observed with primary articular chondrocytes cultured in agarose 3-D gels. *Conclusion*: Expression of adhesion molecules in 3-D culture mimics distribution profiles observed in vivo, and is dissimilar to 2-D profiles.
[11,76]	Melanoma NA8 cells in 2-D vs. 3-D produce differential gene expression profiles. The 3-D profile resembled in vivo metastatic cancer. Gene profiles are regulated by cross-talk between ECM and intracellular signaling pathways via transmembrane (adhesion) proteins. *Conclusion*: Tissue-like 3-D organization is critical for in vivo-like gene expression. Gene expression is regulated by ECM-cell interactions.
[198,206]	Cell polarization characterizes normal tissues and is lost in tumor cells. Primary mammary epithelial cells organized into acini in laminin-rich 3-D matrices and were resistant to cancer induction due to physiologically 'correct' polarization profile. The PI3K pathway was shown to be important in maintaining tissue polarity and suppressing cell proliferation. Polarization in these cells was shown to be regulated through ECM interactions via integrin α6β4 that interacts with intermediate filaments, facilitates hemidesmosome assembly, and regulates tissue polarity. *Conclusion*: Physiologically relevant polarization profiles as well as cell organization into secondary structures are directed by ECM–cell interactions regulated via integrins.

To address this and other issues that might better assist and expedite more reliable drug development, alternative cell-based in vitro testing models containing features for more clinically relevant assays are increasingly emphasized in 21st-century toxicology. In particular, the three-dimensional (3-D) organoid tissue culture system has undergone rapid development as an alternative to traditional 2-D cell cultures on plastic. Many reports have determined significant differences in cellular morphology, behavior and molecular signaling between the classic cell monolayer approaches and analogous 3-D cultures (see Table 1). These on-going validations clearly demonstrate inequities between these 2-D and 3-D types of cell culture models. The potential of the 3-D organoid culture to contribute improved data to basic science and drug development research is thought to derive from the method's ability to better harness hard-wired cellular programming within higher order cellular tissue organization (i.e., embryogenesis and organogenesis), cancer propagation and metastasis, angiogenesis, inflammatory injury and toxicity pathways [11–15]. None of this is possible in cell mono- or co-cultures in 2-D monolayers on hard plastic supports.

A vast and ever-increasing number of approaches are described for producing 3-D cell cultures: this review touches on aspects of several major development tracks. Despite their diversity, all organoid models share the common goal to capture complex biological interactions by re-creating physiologically relevant organ mechanical functions, cellular diversity and spatial architecture [16-18]. Recapitulating physical tissue characteristics appears to be key to reinstating clinically important biological biomarkers, quasi-normal cellular metabolic and communication pathways [5,16]. The physical similarity implies the critical physiological similarity between in vivo and 3-D cultures. This has been a central paradigm in obtaining tissue models that retain certain complex intra- and extra-cellular communication networks, cell signaling and differentiated cell types sharing requisite physiological responses with in vivo models. For example, both mechanical and chemical compositional properties of the extracellular matrix (ECM) were shown to be critical in controlling mammary epithelial cell phenotype within a 3-D culture [19]. Similarly, organ-on-chip designs demonstrate the importance of both flow and subtle mechanical tissue stimuli, including peristaltic movements associated with the gut or mechanically active 'breathing' alveolar–capillary interface representing the human lung, for creating both physiological and pathophysiological tissue equivalents [17,18]. This semblance closely aligns with in vivo data for intracellular forces induced by cell interactions with underlying substrates that are a key for cellular migration, spreading and tissue shape formation [20,21]. Similarly, enhancing in vivo-relevant cell–cell interactions through addition of either niche-specific cell populations or tissue architectures promotes developmental morphogenesis in vitro in an epithelial lung airway model. This occurs by inducing branching of 3-D tissue lung organoids [22], spontaneous formation of blood vessels that could be incorporated into existing in vivo blood circulation [15,23] and organization of epithelial kidney, thyroid and mammary cells into cyst-like formations with biologically-relevant polarization [24–26]. Separate work has also shown that retention of physiological similarity to tissues of origin significantly extends the differentiation potential and cellular functionality of cells in vitro [27]. This also improves the ability of 3-D culture organoid models to reproduce complex physiological responses and processes [28].

Significantly, despite abundant knowledge and evidence of successful implementation of 3-D culture in fundamental biological research, exploitation of these models for toxicity assessment has been limited. Notably, the approach fails to capture molecular and cellular aspects of tissue injury necessary for taking preclinical toxicology into animal-based safety testing and human clinical trials. Tissue toxicity incorporates diverse complex processes of cellular organ damage due to active and passive agent accumulation, their chemical alterations through interactions with cellular enzymes, particularly cytochrome (CYP) P450 enzymes, DNA damage, induction of inflammatory processes and cellular death [5]. Producing reliable in vitro–in vivo correlations for predicting drug toxicity would truly benefit the drug development process to avoid costly late-stage candidate development failures, adverse events, black box warnings and product recalls.

This review discusses the relevance of current 3-D culture techniques with respect to their capabilities to recapitulate complex spatial morphology and physiological features sufficient for reliable drug development assays. This review also highlights recent advances in 3-D organoid models and presents required characteristics important in making these models function effectively as in vivo-relevant, predictive high-throughput screening (HTS) platforms effective for toxicity assessments required for drug translation.

2. Underlying causes of failure in drug toxicity screening models in vitro

The first 3-D organoid model techniques were described in the 1960s [29] and have been used since that time in various forms to study a variety of normal and pathological processes [14,15,19,22,23,27–29]. The vast majority of work with these models, however, has been dedicated to establishing universal methods and support materials for creating tissue replicas rather than organ-specific models. There is also an alarming absence of information on 3-D culture comparisons, undermining the diversity of 3-D culture methods in their complexity, in vivo likeness and use of artificial support systems. Lack of model-centric approaches as well as minimal efforts directed toward inter-3-D organoid tissue equivalence comparisons and validation of their biological structure–function relationships has limited preclinical assay changes and adoptions by drug development organizations. Most published work exhibiting such potential fails to compare differences between traditional 2-D (monolayer) cell culture methods and newly developed approaches, with even a limited number of clinically relevant comparative indicators (see Table 1). Many current 3-D in vitro culture methods claim to recapitulate the physiological microenvironment and therefore be more suitable for drug testing than precedent monolayer cultures on plastic. However, no universal acceptance of any 3-D culture system is yet achieved due to lack of acceptable validation against primary tissue biopsies, organoids and standard culture methods. Additionally, lack of consistent production, automation facilitating for scale-up of cell production, to provide experimental flexibility, and a seamless transition from in vitro to in vivo applications remain important issues for this technology.

In moving 3-D models for drug toxicity assessment forward, it is essential to appreciate the specificity of each organ's unique physiological pathways, the need for models that respond with a wide variety of in vivo-relevant biomarkers, as well as possible physiochemical complications associated with mixing cells, media and materials into cell culture for organoid processing. These issues are addressed in greater detail in the following discussion and on more model-specific bases in Section 3.

2.1. The enigma of a universal 3-D culture matrix

Drug toxicity is a complex, multifaceted physiological event with specific and often distinctive features, affecting several or specific cell types perhaps unique to that tissue or organ system. Toxicity in vivo can be exerted by the drug itself, its metabolites, combination of drug and metabolites, and drug–drug or drug–protein conjugates. Such effects can be from both acute and/or chronic/cumulative drug exposures. However, all current assay strategies to reflect these complexes in vivo

scenarios rely on generic culture materials and methods designed to incorporate most any adhesion-dependent cell type in a pre-selected 3-D matrix culture system. Such approaches include immortalized and primary cell encapsulation in natural polymers (i.e., agarose [30], gelatin and collagen gels [31]) or synthetic materials (polycaprolactone (PCL) [32], polystyrene (PS) [33], poly(DL-lactide-co-glycolide) (PLGA) [34]). These polymer scaffolding approaches continue despite mounting data showing that under normal physiological conditions, extracellular matrix (ECM) derived from different tissues exhibits distinct, unique tissue-specific architecture, mechanical properties (i.e., elasticity, rigidity), biochemical composition and molecular complexity [35]. Additionally, these ECM properties are altered and modulated as a function of normal or pathological processes such as wound healing, tissue turn-over and regeneration, aging or tumor development [36].

A single universal "one-size-fits-all" culture method using a generic 3-D culture matrix and media that accommodates the current multi-well HTS assay configuration makes both economic and practical sense. Nonetheless, this generic goal has repeatedly proven to exhibit limited biological or predictive success for replicating tissue-like feature necessary for drug toxicity determinations. A more reliable approach should be model-centric by design and not method-centric for cost or convenience unless validated against toxicity markers [37]. Because preclinical in vivo screens tend to focus on a finite number of most common organ toxicity injuries, i.e., cardio-, nephro-, neuro-, gastrointestinal or pulmonary toxicities, improved culture models that correctly predict only these organ-specific pathologies are compelling even if they do not all use identical or generic methods. Organ-specific models may also be fiscally advantageous over the current wayward search for a single ideal 3-D culture solution to a diverse drug toxicity parameter set.

2.2. Incomplete 3-D model biological assessment

Given consistent, overwhelming data describing in vitro cell model limitations as biological mimics and resulting issues in drug development, the limited validation for 3-D model biological relevance in various contexts is indeed also remarkable. Literature emphasizes improving and characterizing a variety of biomaterials-based support matrices and culture conditions, but not necessarily validating the model's predictive value for specific purposes. The presumption that adding another material–spatial dimension to in vitro culture of isolated mammalian cell types (i.e., into 3-D matrices) intrinsically alters cell properties and better reflects "tissue" has been shown to be inaccurate by reports evaluating physiologically relevant, complex functions such as toxicity, cancer development and inflammatory processes [11–15]. Similarly, demonstrating cellular behavioral differences between 2-D and 3-D model data does not validate the in vivo significance of either format, but only the capacity to generate model-dependent results (see Table 1). Drug development requires new models to teach how different 3-D culture approaches provide clinically important indicators, with predictive power for preclinical translation.

2.3. Limited use of in vivo-relevant indicators

Toxicity measurements in vivo rely on statistically significant correlated changes in a wide variety of histological or secondary biomarkers. These data in turn are used to accurately inform mathematical approaches for pharmacokinetic, biodistribution and tissue injury predictions. Reliable organ-specific drug toxicity assessment, particularly analysis of in vivo-relevant toxicity biomarkers, is critical to successful drug candidate translation. Most in vitro comparisons, particularly from 2-D to 3-D models, are limited to evaluating drug candidate concentrations that produce 50% reduction in viable cell numbers (EC_{50} values). While standardized, the EC_{50} determination has no physiological validity until shown to mirror some organ toxicity marker. Without validated prediction of human toxicity profiles from in vitro laboratory data, this extrapolation remains tentative.

A more rational approach would rely on biomarkers of toxicity already verified and currently used in animal and human studies for assessment of toxicity in analogous cell culture models, rather than on arbitrary EC_{50} values. A growing volume of evidence indicates that toxicity in vivo is manifested by profound alterations in cell functions within respective organs without cellular death. These markers are *not accurately reflected in vitro* by cell EC_{50} or apoptosis studies. Examples of such cellular changes include shedding of intra- and extracellular enzymes, alterations in cell polarization and permeability, disruption of the cytoskeleton, induction of gene expression and post-translational modifications [38,39].

3-D cell culture offers real advantages to produce cell toxicity biomarkers previously shown to be only attained in vivo. For example, two different 3-D models of kidney toxicity have been able to attain important end-points of renal toxicity [28,40]. Similarly, models of embryotoxicity using established cell lines of primary embryonic cells allow for morphological and molecular method predictions of embryotoxicity in vitro using test methods validated by the European center for the Validation of alternative Methods (ecVaM) [41]. The key to generating physiologically relevant information is retention of the cellular environment and using cell sources preserving native functionalities. It is unlikely that any preclinical in vitro models respond to drug dosing with all known drug toxicity indicators: many simply cannot since some biomarkers are no longer expressed in certain cell lines used in vitro. Nonetheless, clinically important or even required biomarkers for each organ toxicity model should be produced to guide validation of new test models. Clear definition of such performance expectations in terms of biomarker validation would shift the focus from arbitrary advocacy of cell toxicity models often based on in vivo-irrelevant outputs to systems with high general physiological, pharmacological and toxicological extrapolation value. Similarly, transparency in expected model performance outcomes would greatly simplify inter-comparisons for scientific publishing and funding bodies, as well as level the playing field for further utility of these test beds.

2.4. Lack of long-term adverse effects assessment

Drug toxicity in vivo results from acute and/or chronic exposures of cells, organs or organ networks to a bioactive agent. However, current in vitro testing methods are commonly criticized for favoring acute rather than chronic exposure toxicity screening. Reasons for this are mostly technical: chronic exposure assessment requires models capable of maintaining viable cells in relevant differentiated states in vitro for as long as several months without contamination. In just a few weeks, primary cells de-differentiate in 2-D cultures, many 3-D culture matrices lose their mechanical integrity, and static 3-D models induce significant cellular death as a result of limited nutrient transport and perfusion. Therefore, many current approaches fall well short of ideal models for longer-term outcomes. Fortunately, use of bioreactors, 3-D culture approaches, materials with variable Young's moduli and recapitulation of native microenvironments, such as ECM, cell–cell interactions and tissue architectures, improve many of these limitations. For example, 3-D kidney organoid cultures that maintain native organ-specific cellular microenvironments sustain cellular differentiation in cultures for up to 6 weeks [27] and primary hepatocytes retained their biotransformation abilities for 60 days in a clinically used hollow-fiber bioartificial liver device [42]. Investment of time and resources into developing and validating new models amendable to chronic exposure toxicity evaluations would greatly improve the value of preclinical data derived from in vitro systems.

2.5. Lack of rigorous drug-construct and assay-construct interaction characterization

Every model is only as good as the information produced in characterizing, benchmarking and validating it against standards. Hence, understanding each model's limitations and benefits is a key to their proper utilization. However, with many 3-D culture constructs using natural or synthetic support materials (see Section 3.3), much more effort is placed in extensive characterization of the materials in these models, such as cell isolation purity and polymer network properties, than into assessment of cell phenotypes and drug and assay interactions with the 3-D construct. The majority of natural and synthetic polymer supports utilized for 3-D culture have chemistry (e.g., formal charge, composition), matrix porosity, density (swelling) and tortuosity that affect matrix diffusion and transport characteristics for solutes in media. Since most drugs tested as well as many assays used for toxicity assessment rely on diffusion of charged molecules (i.e., drug) in and out of the matrix with assumed homogeneous, unhindered access to cellular uptake, it is critical to consider the following points in such testing designs:

- *Drug interactions with the matrix.* Many drug candidates are weak acids or bases where the media pH and drug pKa must be compared to understand both solubility and possible interactions between drug and matrix (i.e., acid–base, hydrophobic, electrostatic, cation bridging). Such interactions alter drug transport and distributions with the matrix, resulting in variable drug exposure to encapsulated or suspended cells in the matrix.
- *Drug transport through the matrix.* Matrix porosity and possible tortuosity, and gel mesh size may affect drug transport properties, requiring some assessment of this impact on drug distribution within the culture system and possible local depletion in areas of high cell density and uptake. Local dosing and assumptions regarding local drug concentrations producing toxicity biomarkers are unknown without transport analysis.
- *Drug exposure time in assay.* The duration of drug exposure relative to the drug's diffusion properties within the matrix must be considered to ensure appropriate drug–cell exposure. Short-term experiments under static conditions in dense media, and/or with drugs with low intrinsic diffusivity or matrix interactions could result in poor drug distribution and heterogeneous exposure.
- *Matrix–assay interactions.* Each culture matrix has its own optical characteristics, dielectric, charge and residual chemical group distributions. These properties affect drug interactions with assays that rely on imaging, release of redox-active and charged molecules or exploit in situ chemical reactions (e.g., enzyme-catalyzed electronic or colorimetric readouts). Assay validation and background elimination must be considered as a part of sound experimental design with appropriate controls that demonstrate assay reliability and signal linearity for appropriate markers.
- *Medium effects.* Many drugs, as hydrophobic, low solubility agents partition into blood plasma components (i.e., lipoproteins, albumin, erythrocyte membranes) significantly (many at >99%), affecting their cellular availability, pharmacokinetics/dynamics and transport. Media that use serum for in vitro cell culture contain these proteins. Serum-free media do not. Hence, medium selection will affect drug solubility, partitioning, transport and cell processing in culture. Additionally, cells cultured in serum-free media can become serum-starved, producing increased pino- and phagocytic rates as they seek normal physiological nutrition from media. Relevance of serum-containing vs. serum-free media to desired cell toxicity profiles should be carefully assessed. Lastly, local hypoxia from poor oxygen transport or depletion in high metabolic zones within 3-D culture beds (i.e., high cell densities of proliferating cells) also results from poor medium exchange in 3-D matrices. Necrotic zones within these cultures as well as altered cell phenotypes from hypoxic stress result, altering the toxicity profiles and drug processing capabilities.

Erroneous results and conclusions about cell–drug interactions, processing and tolerance under faulty assumptions can unfortunately be a consequence of poorly controlled or misunderstood test conditions where culture system limitations and assay interference are not assessed. Each drug/matrix formulation/construct/assay design combination should be analyzed for transport and cell stability issues.

2.6. High-throughput and scale-up limitations

HTS is often proposed as an efficient strategy for large-scale target validation and toxicity assessments of new candidate therapeutics. Individual screening of libraries containing tens of thousands of potential drug candidates is not economically, ethically or practically feasible. Therefore, HTS methods are automated for efficiency, with handling protocols adapted to rapid automation. A primary drug development challenge is the innovation and improvement of screening models reliably scaled to HTS assessment while retaining the necessary biological complexity to yield valid answers. Yet, how these important assay answers – typically, cell viability, cell density, phenotyping, morphology, various cell metabolic functions, genotoxicity – are determined remains challenging in 3-D culture formats. HTS cell assay endpoints are frequently interrogated in situ using adaptations of cell characterization methods long-used for 2-D cultures on plastic. These are frequently optical methods, including in situ microscopies and imaging based on exogenous reagent additions. Image analysis for endpoints is confounded by optical issues and aberrations in 3-D: matrix curvature, cell and matrix density, light scattering, assay reagent interference and homogeneity, assay signal/noise, metric reliability and analytical sensitivity and specificity from in situ optical images [43–47]. Few 3-D methods to date are amenable to quantitative HTS analytical approaches, typically limited at the critical step of assay read-out or answer determinations in multi-well plates or droplet arrays using in situ optical imaging or spectrophotometric determinations. Methods often require termination of culture and disassembly of the components for conventional cell analysis. New analytical tools and 3-D formats that permit greater unhindered, in situ access to reliable assay answers and more sensitive metrics are required.

3. In vitro organoid 3-D cell culture models

Published approaches to model organ complexity in vitro can be loosely grouped into the following designs: 1) cells cultured on inserts or micro-carrier beads, 2) cells embedded in synthetic or natural polymers, and 3) cells organized into multicellular spheroid aggregates (see Fig. 1, Table 2). Models may use primary (i.e., low-passage animal- or human-derived) or immortalized (secondary) cell lines or a combination of the two. However, tissue and organs comprise several tightly coordinated but phenotypically distinct cellular species, often interacting via signaling pathways. Many models therefore use more than one cell type to attempt to recapitulate in vivo-like cell heterogeneity through co-culture [191] or organotypic culture. Genetically modified and pluripotent (e.g., stem and stem-like) cells will likely be increasingly important in future toxicity model development [48]. Each of these complex cell-based cultures has inherent limitations associated with cell handling and stability. Primary cells are generally thought to have greater capability to reflect complex repertoires of cell biochemical and physical behavior simply because they are used in their unadulterated form. This fidelity is adversely affected by repeated passaging; low passage numbers (often suggested to be less than 10) are recommended, although this is cell type-specific.

In terms of producing tissue injury and inflammatory processes associated with toxicity, primary cells have shown to be superior in their ability to reflect induction of cellular biomarkers of injury [28], metabolite and cytokine production, phenotype and intrinsic activation states [27,49]. However, primary cell utility is greatly complicated by arduous procedures needed for extraction and purification of

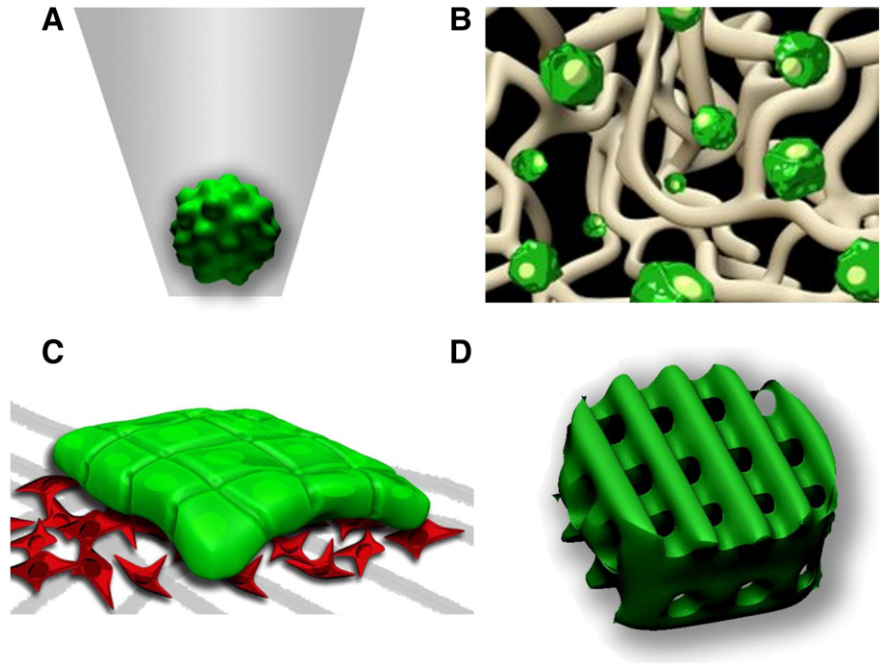


Fig. 1. General 3-D cell culture models used for screening and toxicity assessments: A) spheroid aggregate in a hanging drop; B) 3-D cells in a gel; C) skin model on a fiber mesh; D) polymer 3-D porous matrix for cell seeding.

homogeneous populations. Additionally differences in cellular post-isolation conditions vary as a result of technique. Primary phenotyping is tedious. One must also consider the expense of cell isolation and validation and ethical issues associated with the use of animals or human tissues as cell sources, as well as limited windows of phenotypic stability due to quick primary cell de-differentiation in vitro [50].

Immortalized cell lines are generally modified by deliberate insertion of oncogenes to facilitate more rapid, facile proliferation and to enhance both phenotypic stability and longevity to overcome these issues in primary cells. Transduction of primary cells with genes from a DNA tumor virus, such as simian virus 40 (SV40), human papillomavirus (HPV), and Epstein–Barr virus [51,52] is common to produce commercial cell lines. Both SV40 and HPV viruses have been shown to be useful for immortalization of cells from different origins; Epstein–Barr virus is used to infect cells of lymphoblastoid origin [53]. SV40 and HPV E6/E7 oncogenes interfere with p53 and retinoblastoma proteins that regulate senescence induction and restrict proliferation, respectively [54–59]. Both oncogenes are thought to require a two-step immortalization process for cell line creation: 1. transduction of oncogene, which extends the proliferation potential of cells past the senescence point of normal tissues, but does not rescue cells from shortening telomeres, and 2. selection of cells with further randomly occurring mutations that alter telomere activity maintenance mechanism through extensive subculturing of transduced cells [51].

This profound modification of primary cells comes at a price; cells with significant alterations to their genotype often manifest other phenotypic changes in differentiation potential, protein expression and cellular functions. Toxicity-relevant examples of secondary cell line compromise and non-equivalence include loss of ligands, transporters, and cellular receptors involved in drug pharmacodynamics [60–62], partial activity of P450 drug metabolizing enzymes [63,64], limited correlation to in vivo-relevant cytokine profiles [27,49] and passaging-related drug paracellular transport artifacts [65–67]. These phenotypic dissimilarities with their tissues of origin and primary cells certainly have profound implications for the abilities of immortalized cell lines to reproduce clinically-relevant drug uptake, metabolic, genotoxic, therapeutic and tissue injury processes. Lastly and significantly, many secondary cell lines are over-passaged, poorly characterized and frequently highly contaminated or unverified [68–71]. Karyotyping (for interspecies comparisons) and DNA fingerprinting (within a single species source) can be used to verify cell genetic identity. Tumor cell lines are frequently found to exhibit anomalies not entirely representative of either tumors or normal cells [72,73]. Therefore, even in the simplest culture models, without valid phenotyping, cellular components alone can raise numerous questions about the model relevance.

3.1. Cells cultured on inserts and micro-carrier beads

3-D organoid cell cultures supported by beads or meshes represent a great diversity of culture models, ranging from fundamental epithelial cell models to skin drug distribution platforms to cancer migration and metastasis systems (Table 2). While 3-D in principle and form, these cell culture approaches share many similarities with traditional 2-D cultures in terms of techniques, assays assessment and use of bioreactors. Varying degrees of in vivo relevance are exploited for study — from basic science to toxicological evaluation. Skin replacement/epithelial models and micro-carrier cultures demonstrate this spectrum as examples. Epithelial models that mirror skin microenvironment and stratum corneum ultrastructure are one type of 3-D organotypic cell model (Fig. 1C, Table 2). Organotypic culture generally describes in vitro cell models that incorporate several types of cells to recapitulate physiological intracellular and cell–matrix interactions. Skin replacement models used in basic science, pharmacology and toxicology vary in their complexity but typically include a layer of primary/immortalized keratinocytes cultured on a monolayer of fibroblasts supported by wire grids or Transwell® inserts. Growing cells on a permeable support membrane, unlike traditional 2-D culture, allows cells to retain or develop apical–basal polarity in vitro [74]. Retaining epithelial cell polarization is important (see Table 1) for physiologically relevant molecular presentation of exogenous agents. Additionally, dynamic reciprocal communication with the surrounding ECM as well as reproduction of fundamental biological changes induced by pathological processes, such as toxicity or cancer growth, is also important to these models [75–78]. The simple laboratory-generated or commercially available skin cultures may better resemble the structure of epidermis rather than full skin. For example, EpiSkin® by L'Oreal is a construct

comprising keratinocytes over a collagen I matrix coated with a film of human type IV collagen [79]. SkinEthic® (SkinEthic Laboratories) is created by culturing human keratinocytes at the air–liquid interface on polycarbonate filters [80]. However, more complex living skin equivalents can contain both dermal and epidermal skin layers. Epidermal 3-D culture by CELLnTEC, EpiDerm FT™ by MatTek, Apligraf by Novartis, and EpiSkin® with fibroblasts cultured for 20 days, and skin reconstructs for induction of melanoma lesions are bi-layered skin-like tissue models that utilize collagen I and fibroblast mixtures as an underlying dermal substitute [81–85]. The current skin equivalent models do not come in the 96-well format. However, the relative simplicity of skin layer organization makes it HTS compatible.

These skin-equivalent models have been shown to have excellent in vivo profile correlation due to environmental exposures such as UV, corrosive and genotoxic substances, drugs, chemical warfare agents, ionizing radiation and nanoparticles [85–89]. Furthermore, skin reconstructs have been essential in evaluation of drug targets and investigation of signaling pathways associated with melanoma development and chemoprevention strategies [81,84] as well as in actual treatment of skin in burn victims and venous leg and diabetic foot ulcers (e.g., Apligraf, Dermagraft-TC, Integra Artificial Skin and Original BioBrane). The success reported with both preclinical and clinical use of these models has been attributed to the degree of resemblance between real skin and epithelial skin 3-D organoid models. Currently, skin replacement substitutes represent best examples of how recapitulation of cellular microenvironment results in products and predictable, validated in vitro models with substantial pharmaceutical and preclinical significance.

3-D models are now commonly highlighted for purported capabilities to more accurately reproduce physiologically relevant processes. However, the range of what is considered to be "3-D culture" varies widely within the scientific literature, depending on motivation and application. Strategies that exhibit most any perceived improvement over traditional 2-D cell culture techniques on plastic – for example, cell polarization, differentiation, formation of intracellular interactions or spheroidal growth morphologies – are commonly labeled as "3-D cultures" even though the demonstrated cell enhancements are not clearly attributed to either addition of a third spatial dimension or recapitulation of dynamic cellular interactions or new culture conditions. Although this market-style semantic labeling itself is not really the issue, the advocacy and use of these models as 'new' or 'innovative' without appropriate evaluations of specific, novel 3-D properties connecting in vitro conditions to in vivo physiology undermine their claims to in vivo relevance.

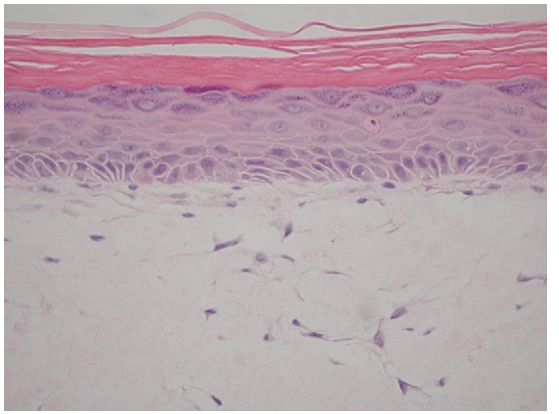
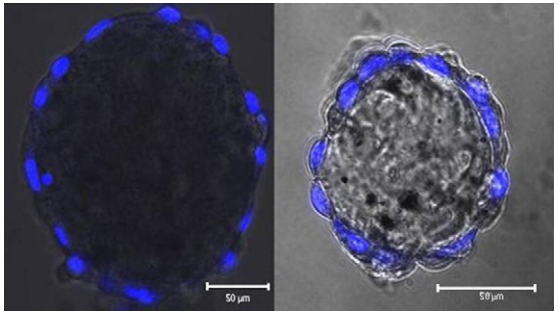
Micro-carrier cell support culture systems used with bioreactors are one example with four decades of development in cytotechnology. Techniques using microbead suspensions as cell adhesive and growth supports in stirred-tank bioreactors were originally developed to improve cell culture yield in early protein and vaccine fermentation processing. High surface-to-volume ratios of micro-carrier beads in combination with controlled reactor mixing conditions provided significant scale-up benefits and industrial value for cell harvests and recombinant product yields. This success prompted commercial development of several important cell micro-carrier culture systems such as dextran-based Cytodex (GE Healthcare) beads, polystyrene SoloHill Engineering and Synthemax® II (Corning) beads, glass beads (Sigma-Aldrich, Inc.) and gelatin beads (Cultispher). Micro-carrier cell culture approaches typically result in adherent monolayer cell attachment although some cell non-contact inhibited lines produce multiple cell layers. Furthermore, reduction of shear stresses during incubation due to improvements in bioreactor design or microgravity allowed bridging between cell-coated beads and formation of cell-bead aggregates encompassing as many as 15 beads [90,91]. This observation provided the inspiration for further development of tissue-like constructs that actually derive biological inspiration in their formation. Rotating Wall Vessel (RWV) bioreactors represent an exceptional result of utilization of this phenomenon for 3-D tissue-like relevance. The RWV is a microgravity device originally designed by NASA for scientific experimentation in deep space. However, under standard laboratory conditions, low-shear associated with this cell bioreactor allows for deliberate, controlled inter-cellular interactions based on native cell affinities. RWV-derived micro-carrier models have been used to mimic tissue structures as found in the lung, colon, bladder and liver. These were shown to produce architecture, cellular differentiation and polarization as well as functionalities associated with these microenvironments [92–99].

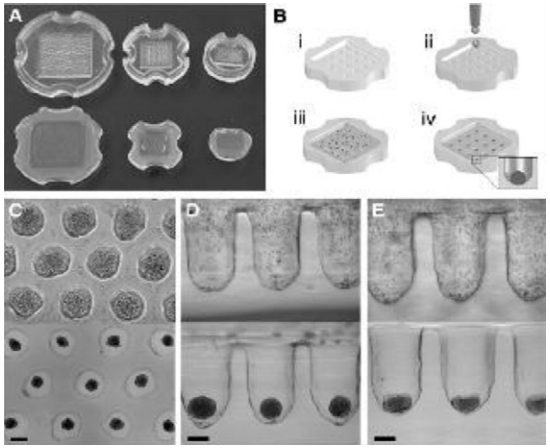
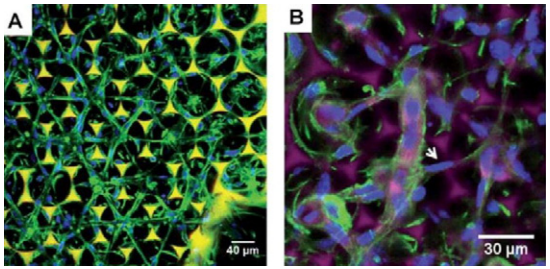
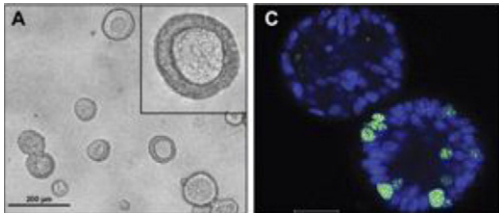
Unlike the validated RWV 3-D organoid model, other micro-carrier cell culture systems (e.g., Global Eukaryotic Microcarrier (GEM™, Global Cell Solutions)) have not yet made adequate verification of their value to tissue physiology, in essence of adapting traditional 2-D cell culture techniques to micro-carrier cultures even though branded as "3-D" cell culture (Table 2). Specifically, alginate-coated core magnetic GEM ™ beads not only have all the features and advantages of other micro-carriers such as large surface area, capability to be coated with different adhesive cell matrix proteins and HTS compatibility, but also can be easily dissolved for cell dissociation and collection due to their magnetic properties. However, while offering a curved support surface for cellular attachment and proliferation, these carriers offer little to actively control, induce or recapitulate specific cellular tissue or organoid microenvironments beyond traditional cell culture approaches. Similar to plastic 2-D surfaces, these carriers typically produce adherent cell monolayers [100] that exhibit intercellular associations and coating-dependent cell–ECM interactions (www.hamiltoncompany.com). Such capabilities contrast those from RWV-derived micro-carrier approaches. This is an important example of how very similar techniques yield distinct cell-based models with very different cell spatial organization, function, control and in vivo relevance. Specific selection and rationale for best use of each strategy should be evaluated. Extension of basic scientific principles that leverage the value of 3-D strategy beyond current marketing established technology with new terminology is more effective to contributions that advance pre-clinical cell assessment.

3.2. Spheroid models

Spheroid 3-D culture (i.e., microtissues) models represent a precedent organoid technique with substantial history (Fig. 1A, Table 2). Peer reviewed publications dating to 1952 report on spontaneous formation of cell aggregates following co-cultivation of myogenic and chondrogenic cells from early chick embryo [29]. The aggregates showed pronounced polarity with chondrogenic cells comprising the core and myogenic cells forming the outer coronal layers of the multicellular structures. Nine years later, the same group improved their original method by substituting stationary culture conditions with a newer rotational technique shown to facilitate more effective and consistent cell aggregate formation [101]. The stationary and rotational techniques as well as combination of the two – the liquid overlay method [31,102] – have been quickly adapted by other research groups and shown to work for both normal [103,104] and cancer cell lines in creating spheroids [105], giving birth to the original in vitro field of tissue culture multicellular spheroids. Current methods of 3-D spheroid generation expand these original approaches to include spontaneous aggregate formation from single cells or cell multilayers, methylcellulose conditioning and hanging drop arrays [106] as well as rocking and rotary cell cultures. The single cell spheroid formation technique is probably the simplest method, requiring only use of ultra-low adhesion surfaces with serum-free medium to create suspended cell structures [104]. Multilayer approaches generally describe use of adherent cells that spontaneously form spheroids and detach under 1) the influence of medium conditioning (for example use of insulin, L-glutamine, epidermal growth factor and linoleic acid for albumin spheroid medium with hepatocytes [107]), 2) use of adhesion-prevention surface coatings like agarose and agar, or 3) co-culturing conditions (i.e., methods involving overlaying mesenchymal stem cells with primary cells [108]).

Table 2
Summary of 3-D culture systems with advantaged and limitations.

Model type	Technological principal	Advantages	Limitations	Images
Skin model (3-D model grown on support)	Multi-layered skin-like tissue construct of primary/immortalized keratinocytes cultured on a monolayer of fibroblasts supported by wire grids or Transwell® inserts	• Resembles skin organization with appropriate intracellular organization • Retains cell apical polarization • Shown to have excellent in vivo profile correlation due to environmental exposures such as UV, corrosive and genotoxic substances, drugs, chemical warfare agents, ionizing radiation and nanoparticles • Used in clinical practice	• Different models may have significant differences in organization complexity • Relatively expensive • No 96-well (HTS platform) commercially available models	MatTek Epiderm 
Cells grown on beads	Single layer of cells are grown on beads that are cultivated in specialized bioreactors (GEM™)	• Great for scale-up and HTS platforms • GEM™ platform allows for easy cell detachment, collection and cell–ECM interactions • Sourcing, production and manufacturing reproducibility	• Basically a 2-D culture with significantly greater surface area • No 3-D cell-relevant interactions • No cell–native EMC interactions	GEM™ image with EAhy 926 cells [100] 

Spheroid model	Individual cell aggregates or microtissues are formed using various cell culture methods	• Intracellular interactions in 3-D • Easy for co-culture • Shown to allow cells to retain xenophobic metabolism and metastatic and cell proliferation potential • Great for HTS platforms	• No tissue-like organization • Variation in size and density • Possibility of necrotic core development	Microtissue preparation using molds [207] 
Synthetic 3-D matrix culture	Cells or cell aggregates are seeded on precast synthetic matrices	• Chemically endowed with specific motifs known to engage cells and ECM proteins • Tailored mechanically to match cellular requirements • Molded into complex geometric shapes or miniaturized components to suit diverse needs • Sourcing, production and manufacturing reproducibility • Sterilization convenience • Adaptation to HTS capabilities	• Unpredictable cell–polymer interactions • No tissue-like organization • Possibility of necrotic core • Lack of ECM complexity	Matrix-embedded endothelial cells (MEECs). From [208] 
Cell-derived and single protein/ polysaccharide 3-D matrix culture	Cells or cell aggregates are resuspended in the matrix that gels around them	• Chemically/naturally endowed with specific motifs known to engage cells and ECM proteins • Molded into complex geometric shapes or miniaturized components to suit diverse needs • Adaptation to HTS capabilities	• No tissue-like organization • Lack of ECM complexity (for single protein matrices) • Possibility of necrotic core • High interbatch variability (for cell derived matrices) • Possibility of contamination (for cell -derived matrices)	Metastatic 344SQ cells form polarized epithelial spheres in 3D Matrigel culture. Modified from [209] 

Similarly, methylcellulose additives to cell culture media in combination with non-adherent cell round-bottom coated surfaces were shown to be an alternative approach for creating multicellular spheroids. This method was validated for co-cultures of endothelial cells and smooth muscle cells with different mammalian origins [109].

Hanging drop cultures (Fig. 1A) collectively refer to technologies that cultivate spheroids by placing medium drops of cell suspensions on a surface incubated in vitro upside down, i.e., with the drops suspended from a contacting surface. The methods typically can be performed without any specialized equipment, but are laborious due to the limited droplet volume and need for frequent medium exchanges [110]. The process is greatly simplified and improved in reproducibility and HTS potential if combined with modified plate and fluidic handling, as commercialized in the GravityPlus™ technology (InSphero, www.insphero.com) or PERFECTA3D™ hanging drop plates (3D Biomatrix, http://3dbiomatrix.com). The InSphero technique utilizes plates with a unique vertical inlet channel for medium and cell deposition that relies on capillary and cohesion forces to stabilize hanging drops in culture. Spheroids typically form within 4 days, however, long-term incubation protocols are possible by facilitating medium exchange from the top via an interfaced fluidic inlet. The 3D Biomatrix platform utilizes similar technology with a modified inlet design accommodating both 96- and 384-well plate formats as droplet protective enclosures. The universal plate design enables culture of single cells and organotypic cultures of both immortalized and primary origins. Furthermore, the capability to accommodate direct microscopic evaluation and compatibility with standard 96-well plate laboratory methods make this method a viable option for HTS 3-D organoid analysis.

Both rocked and rotary cell culture methods, unlike previously described static culture techniques, are non-stationary culture systems, typically using spinner vessels with magnetic bars [111], rotating labware [112,113], rocking tissue culture plates [114] and commercially available bioreactors [115]. Non-stationary culture methods are found to produce larger, more compact spheroids than stationary methods due to enhanced collision dynamics during incubation [114]. As an alternative approach, in vitro spheroid formation could be facilitated using several different synthetic or natural matrices with bioengineered cell surface patterning approaches. In this case, spheroid formation is induced through spheroid formation and embedding into cell non-adhesive polymer micro-compartments. Micro-fabrication techniques allow for control of spheroid location, shape and size [116].

Prominent examples of commercially available spheroid-yielding surface patterned technologies are Micro-Space Cell Culture plates by the Japanese company, Kuraray (www.kuraray.co.jp), AggreWell™ plates by Stemcell™ Technologies (www.stemcell.com) or Microtissues® molds (www.microtissues.com). The Micro-Space plate comes in a 24-well format where each well is further subdivided into regular 200 μm long × 200 μm wide × 100 μm deep square micro-sized compartments [117,118]. Each of the squares represents an individual niche for spheroid formation. The plate has been shown to yield 3-D organoid cultures in 5 days and is amenable to primary and immortalized cells of normal or cancerous origins [117,118]. The Micro-Space Cell Culture plate conforms to the regular 24-well plate footprint and has similar transparency properties as conventional tissue culture plastics, conforming to all standard laboratory cell culture assessment equipment. AggreWell™ plates are specifically designed for stem cell expansion, which includes formation of detached cell aggregates or embryonic bodies (EBs). Use of the AggreWell™ plates allows creation of EBs with equivalent size and shape, improving the reproducibility of stem cell differentiation processes. This uniformity is accomplished through distributing the entire cell suspension for the plate among the microwells by centrifuging prior to start of the experiment. The AggreWell™ plates come with 400 μm and

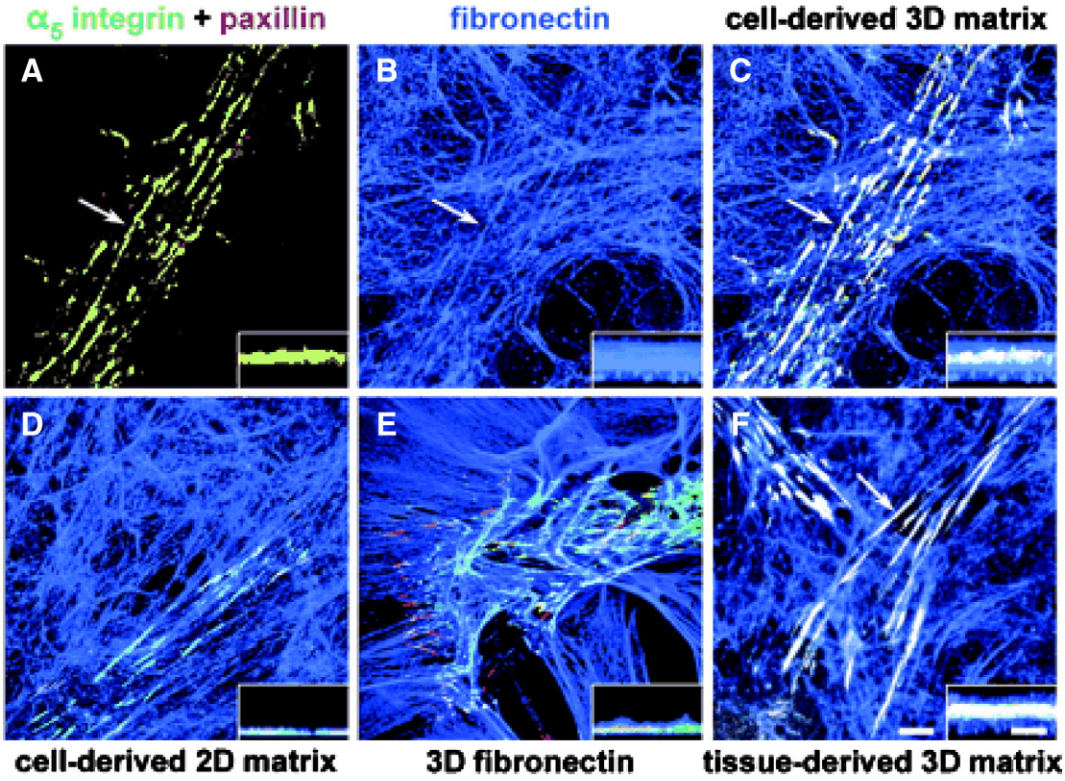


Fig. 2. Effects of tissue architecture and composition on extracellular matrix adhesion formation (from [130] used with permission). Cells grown as monolayers on top of cell-derived flattened ECM, inside 3-D cell-derived ECM, or within single protein 3-D matrix or tissue-derived 3-D gels exhibit distinct morphologies, integrin localizations and compositions of cell focal adhesions. Human fibroblasts cultured for 24 h in the (A–C) 3-D cell-derived matrix; (D) mechanically flattened 2-D cell-derived matrix; (E) fibronectin 3-D matrix; (F) 3-D tissue derived-matrix. Arrows point at 3-D matrix adhesions: focal adhesions (red, or purple due to merging red and blue) and fibrillar adhesions (turquoise, merged green and blue). The yellow stating structures in (A) are the merged labels of α5 in green and paxillin in red. Inlets are rotated projections showing the thickness of each matrix. Scale bars, 5 mm.

800 μm pyramid-shaped microwell widths and are compatible with standard size 24-well tissue culture plates. According to manufacturer, EB formation can be expected with 24 h, similarly to the traditional cell scraping method. Likewise, Microtissues® molds can be used to cast 3D Petri Dish® agarose patterns for 12- and 24-well tissue culture plates. The mold comes in a variety of shapes and sizes, but similarly to the Micro-Space Cell Culture and AggreWell™ plates, each creates small niches facilitating spheroid aggregation based on natural cellular affinities for each other in confined growth compartments. Spheroids can form in the cast as soon as within 24 h and can accommodate a variety of cell types. The success of these plates has been attributed to the uniformity of the compartments that leads to consistent and reproducible spheroid formation as well as HTS compatibility. Regulation of size is considered to be one of the key limitations of this system; uncontrollable expansion of the spheroids beyond 100–150 μm size [114] leads to poor diffusion of oxygen and nutrients, accumulation of toxic metabolites and tissue necrosis at the center of the 3-D organoids. This is an important lesson in 3-D cell cluster size scaling parameters that define culture medium transport limitations.

Overall, the relative ease and reproducibility of spheroid culture techniques make them the most popular and commonly employed 3-D organoid model used to create and represent a wide variety of normal and pathological tissue surrogates. However, each model's strength derives from hard-wired innate processes associated with collective cellular functions prompted by intercellular tissue interactions. Specific examples of promoting essential intra-cellular processes include xenobiotic metabolism of liver spheroids and cancer metastasis targeting studies [114,115,117,118]. Liver spheroids (e.g., hepatospheres) have been among the greatest successes of 3-D organoid culture systems in terms of toxicity predictions in vitro. The liver, the primary organ on drug detoxification, has been a substantial focus of research using both cell monolayers and 3-D cultures. Primary hepatocytes rapidly lose key metabolic functions under 2-D conditions. However, several studies found that hepatosphere cultures exhibited stable expression of enzymes associated with xenobiotic phase I/II and lipid metabolism, albumin synthesis, blood clotting and urea cycle [114,115,117,118]. These data have been validated for both static dynamic and spheroid cultures created using Micro-Space Cell Culture plates [114,115,117,118]. Gene expression data were verified by direct exposure to cytochromes P450 (CYP)1A1, CYP1A2 and CYP3A-specific drugs in rocked cultures [114], CYP3A, CYP2C9 and UGT1A1 substrates in the Micro-Space Cell Culture plates [117] and induction of clinically relevant biomarkers such as gamma-glutamine transferase and lactate dehydrogenase release [112].

Results suggest that 3-D organoid hepatic spheroid models are capable of liver-specific metabolism-essential or liver toxicity assessment. Similarly, different studies using 3-D spheroid models of melanoma showed that cells from different stages of this disease exhibited similar metastatic and cell proliferation potential in vivo and in vitro. Specifically, tumor spheroids made from cell lines derived from early stages of melanoma progression had poor invasion characteristics whereas cells from a metastatic stage quickly and effectively migrated throughout the entire gel, similar to invasiveness of these cells when injected into nude mice [119–121]. This behavior directly contrasts the cell line attachment and migration behavior seen on 2-D surfaces. Furthermore, recent studies using 3-D melanoma organoid models determined that matrix metalloproteinases (MMPs), a common target for drug migration in cancer, are a critical actor in cancer cell proliferation and melanoma metastasis [122]. Taken together, these cell migration experiments show that 3-D spheroid melanoma organoids could potentially contribute reliable information to cancer target identification and drug validation.

Example 3-D organoid spheroids have proven to be a versatile, relatively simple and easily augmentable model system for preclinical drug development and toxicity testing. The model allows for relatively fast (i.e., 4–48 h for dynamic and 1–5 days for static cultures) generation of tissue aggregates that can be further embedded in 3-D matrix, cultured in bioreactors or assessed in HTS manners. Moreover, recent use of the model has been gaining popularity due to robustness and relative ease of culturing conditions as well as wide accessibility of commercially available products. Companies like Kuraray, InSphero, 3D Biomatrix and Microtissues provide researchers with protocols, matrices, specialized plates and perfusion bioreactors. The convenience of the "out-of-the-box" models, however, comes also with their limitations. Depending on the experimental protocol, spheroid models vary in size and cell density, amounts of isolated single cells and time required for spheroid formation — all directly affecting pharmacokinetic and pharmacodynamic assessments [123]. Spheroids develop necrotic regions within their cores after reaching a mm-size scale, so practices often limit their growth, resulting in only limited cell–cell interconnections completely lacking the normal development of intra-spheroid connections [124,125]. Spheroid size and numbers therefore always appear to be correlated with the initial seeded number of cells. Hence, large deviations in sizes and numbers limit their controlled parameterization and reproducibility in culture. Furthermore, once formed, aggregates vary in degree of cell polarization [116] and functional enzyme activity levels [123]. Finally, these models generally lack in vivo-relevant cell–ECM interactions and typically last in culture for weeks, which is not adequate for long-term toxicology assessments. Nevertheless, spheroid models represent significant, insightful and established examples of how known, developed technologies can now be further harnessed to mirror intracellular interactions that enhance the breadth and value of in vitro cell–cell and cell–substance data. This includes new, improved information relevant to drug metabolism and toxicity.

3.3. 3-D matrices

An alternative approach to 3-D organoid cultures uses pre-cast matrices or gels crosslinked in situ (shown in Figs. 1B and D, Table 2). Such matrix-centered approaches are based on creating support networks that encourage cellular attachment, proliferation and tissue-relevant intercellular and cell–ECM microenvironment interactions. The rationale for these models lies with the increasing body of evidence that successful cell–ECM engagement is key to prompting proper complex 3-D cell networks with signaling and interactions shown necessary for normal tissue maintenance in vivo. Many cell network interactions are stabilized by tensional mechanical forces generated between extracellular ECM and internal cell cytoskeletal components via membrane receptor binding. These connections are continuously co-regulated in vivo using cytokines, hormones and growth factors [5,126–129]. Forces transmitted via ECM adhesion molecules in the matrix and cellular membrane receptors, cell–cell contacts, and cell–soluble cues depend on expression patterns, conformations and activation states of diverse, dynamic arrays of involved protein complexes [5]. Given this complexity, it is not surprising that none of the extracellular environment characteristics, such as 3-D matrix architecture and mechanics, and (bio)chemical composition alone are sufficient for recapitulating physiological interactions. Specifically, cells cultured in cell-derived and expressed ECM display different migration and attachment characteristics than cells cultured in purified single ECM protein gels comprising collagen I, laminin and purified fibronectin [130]. Similarly, cells grown as monolayers on surfaces of natural flattened ECM, within 3-D natural ECM or within single protein 3-D gels exhibit distinct morphologies, integrin localizations and compositions of cell focal adhesions (seen in Fig. 2) [130]. Furthermore, single protein ECM 3-D gels do not appear to reliably replicate key physiological processes such as cell infiltration and scar formation [131].

Beyond matrix (bio)chemical composition, matrix mechanics and stiffness are known to be responsible for certain changes in migratory behavior and in controlling small GTPase Rho-dependent pathways [132,133]. The biological significance of 3-D matrix properties has been reviewed at length recently [5]. Compliant gels seem to promote

amoeboid Rho-independent phenotypes [132]. However, more rigid supports encourage mesenchymal phenotypes and more natural Rho-dependent migration [133]. Taken together, these data reflect the mechano-chemical complexity of cell metabolism, matrix-signal processing and cellular interactions at the cell niche-level and effects on establishing and sustaining tissue physiology.

The central importance of ECM in cell–cell and cell–matrix engagement has actively inspired the creation of a wide variety of substrate-based 3-D culture systems seeking to duplicate these interactions in vitro. In essence, these model designs can be divided into two categories, based on either synthetic or natural polymer scaffolding. Methods for development, synthesis and fabrication of these substrates are a major focus of the tissue engineering field. Approaches are extensively reviewed to contrast traditional cell monolayer culture methods for tissue mimicry [11,14,126,130,134–143]. Confluence of tissue engineering and regenerative medicine goals with those of 3-D in vitro bioreactor and HTS culture strategies provides substantial synergies in advancing many 3-D tissue cultivation concepts. Since many aspects of 3-D organoid matrix models have been recently and previously reviewed [5,11,14,126,130,134–143], only some common examples of scaffolds investigated for preclinical research are described here.

3.3.1. Synthetic culture matrices

Synthetic cell-support scaffolds are among the most popular 3-D organoid matrices in both tissue engineering and in vitro 3-D cell culture (Fig. 1D, Table 2). Significant advantages of these systems are their abilities to be 1) chemically endowed with specific motifs known to engage cells and ECM proteins, 2) tailored mechanically to match cellular requirements, and 3) molded into complex geometric shapes or miniaturized components to suit diverse needs, 4) sourcing, production and manufacturing reproducibility, 5) sterilization convenience, and 6) adaptation to HTS capabilities. Furthermore, market availability of high quality synthetic polymers and calcium-based granulated ceramics ensures a wide selection of possible matrix choices. Commercially available 3-D scaffold systems reflect this diversity. For example, 3D Biotek (www.3dbiotek.com) offers pre-cast inserts comprising PCL, PS, PLGA and PS/PCL. Similarly, Reinnervate (www.reinnervate.com) developed the Alvetex® PS matrix culture system. These scaffolds offer excellent matrix pore interconnectedness, large pores (36–40 μm for Alvetex® and 150–500 μm for 3D Biotek products), lack of animal-derived additives and availability in a wide range of formats including 96-well plates facilitating routine HTS cell culture approaches. However, the synthetic nature of these matrices does not fully capture all qualities of natural ECM–cell localized niches and adds the unpredictable possibility of unnatural cell–polymer interactions. Cells seeded into artificial matrices will attempt to adhere and then secrete and modify their own ECM, remodeling their microenvironment as much as they can to accommodate their needs. However, continuous and dynamic remodeling of their surroundings is thought to keep cells in activated states and might adversely affect their phenotypes and resulting culture data [144]. Moreover, cellular ECM secretion is cell-, time- and perhaps also matrix-dependent, mandating some minimal understanding of reproducible ECM deposited for each model using 3-D matrices. Importantly, numerous tissue engineering reports show how these synthetic materials are modified with ligands, ECM components, or other chemistry, or mechanical inputs or substrate morphological influences to endow them with more reliable, natural cell signaling. Rational design that elicits natural tissue (as opposed to cellular) responses is currently guesswork, based on trial and error mimicry of natural scaffolding properties.

3.3.2. Cell-derived ECM matrices

Cell-derived or natural ECM matrix is a highly diverse set of glycoproteins, proteoglycans and polysaccharides excreted in soluble form and then crosslinked in situ using natural affinity interactions (e.g., heparin–collagen–fibronectin, or bFGF–heparin binding sites) as well as enzymatic processing. The resulting ECM is a hydrogel film directly contacting the cell external membrane receptors that deposits onto the external matrix as a cell-adhesive coating can also be harvested as a gel and then be used to encapsulate cells in vitro (Fig. 1B, Table 2). Natural ECM can be isolated from cell cultures or purchased from vendors and, therefore, is HTS compatible. Cell-specific ECM has been previously derived from human cell line tumors (Humatrix [145]), human fibroblasts (MaxGel™ Human ECM, Sigma-Aldrich [146]), mouse fibroblasts [130], Engelbreth–Holm–Swarm mouse sarcoma cells (Matrigel®, BD Biosciences, and Mouse ECM, Sigma-Aldrich) and embryonic tissues [130]. The main advantage of cell- and tissue-derived ECM is the retention of its intrinsic complexity that may include basement membrane molecules like laminin, type IV collagen, chondroitin and heparan sulfate proteoglycans, or combinations of basement and non-basement membrane molecules, including type I and type III collagens, as well as significant amounts of thrombospondin-1 angiogenic inhibitors, tissue-type plasminogen activator (tPA), MMP-2 and MMP-9 bound proteinases, basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-b, protease nexin II (PN-II) and a1-antitrypsin bound proteinase inhibitors [145].

This mixed heterogeneity translates into unparalleled advantages for promoting 3-D cell differentiation, proliferation and morphology, as well as the ability to form organoid hollow structures. For example, utilization of basement membrane ECM prompted the establishment of the first nerve regeneration models through use of Schwann cells that are capable of robust myelination under 3-D culture conditions [147]. Similarly, Matrigel® was used to create epithelial acinar-like models from breast epithelial cells, salivary gland cells, and pancreatic and prostate cells that demonstrated distinct tissue-specific architectures [148,149] and capillary-like structures that inspired 3-D screening models for angiogenesis studies [150]. A more recent and significant use of basement membrane ECM is its use in stem cell cultures as a substitute for fibroblast feeder layers (often as secondary cell line monolayers including 3T3 cells). Unlike single natural protein-derived fibronectin, collagen I and IV gels or other polymer matrices, Matrigel® and ECM-derived substrates are capable of supporting undifferentiated stem cells with normal karyotype, pluripotency markers and telomerase activity for up to 30 passages [151,152]. These observations clearly support the importance of natural ECM compositional and structural heterogeneity and in vivo-like organization in promoting development of complex physiologically relevant cellular models in culture. However, use of cell- and tissue-derived ECM should consider both the nature and composition of the matrix. Even though ECM replacement products like Matrigel® and Mouse ECM are complex mixtures of matricellular proteins, proteoglycans and growth factors, they more frequently represent biased molecular compositions associated with the basement membrane [142,145]. Furthermore, these products are commonly of non-human nature and are tumor cell-derived, resulting in distinct dissimilarities with normal ECM [153,154]. Given that tumor microenvironment has been shown sufficient to promote desmoplastic differentiation of normal cells, utilization of tumor-derived ECM should be approached with caution for models that represent normal morphology [36]. Additionally, all natural ECM replacements are subject to batch-to-batch variability and viral contamination issues. These concerns must be considered and addressed appropriately when using naturally sourced ECM for normal tissue replacement models.

3.3.3. Single protein/polysaccharide cell culture matrices

Single constituent matrices including hyaluronic acid, alginate and collagen gels (Fig. 1B, Table 2) represent some of the most frequently used 3-D ECM replacements in laboratory conditions due to their simplicity of use, ability to be augmented for HTS and bioreactor platforms, affordability and extensive list of commercial providers. Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan and primary ECM component found in varying amounts in the kidney, epithelial, neural and connective tissues throughout the body [155]. Unmodified HA does not polymerize in vitro, is water soluble

and therefore is chemically altered to create crosslinked 3-D matrices. Examples of commercially available HA-based gel matrix materials include Glycosan Biosystems (www.glycosan.com) and SentrX Animal Care (www.sentrxanimalcare.com), both using acrylate-modified HA to crosslink with thiol-containing additives in the presence of cell culture milieu, cells, proteins and matrix proteins [27,140,141]. Alginate is a marine polysaccharide extracted from brown algae. Alginate gels on its own in situ in aqueous media when combined with divalent cations (e.g., Ca^{2+}) through interchain polymer electrostatic binding, yielding 3-D networks [156]. Alginates have a significant history for cell encapsulation use and are recognized for batch–batch and compositional heterogeneity that can affect cell–matrix behaviors [157,158]. For 3-D ECM applications alginate gels are acquired as AlgiMatrix® (Invitrogen) and NovaMatrix®-3D (www.novamatrix.biz).

Collagen is the most abundant protein in the body and a central component of tissue ECM [159]. To date, 29 different collagen types have been identified. However, collagen I accounts for about 90% of all collagen body volume [160]. While an insoluble, crosslinked fibrillar network in tissues, for 3-D culture purposes, collagen can be purchased as a pure soluble protein solution or mixed tissue extract with high market visibility from most major biomedical providers. Despite the variety of compositions and forms, all collagen 3-D culture materials intended for long-term cell maintenance require either chemical, physical or enzymatic crosslinking methods. This processing necessarily changes protein structure and may introduce other chemicals that adversely affect cell health [160]. Additionally, collagen and its related derivative, gelatin, have been found highly contaminated with endotoxin, a powerful activator of many cell pathways and possible lethal toxin at sufficient levels [161]. Such contamination would provide substantial confounding influences and false guidance to drug development and cell toxicity responses.

Utilization and manufacturing methodologies for these protein and polysaccharide matrices have been the subject of many excellent reviews and are beyond the scope of this work [14,78,134,141,162–164]. Some critical focus is needed on their capability to mimic in vivo ECM for creating 3-D cell aggregates. Specifically, it is important to recognize that both hyaluronic acid and collagen matrices do not represent the complete complexity of connective tissue matrices, and polysaccharide gels like alginate are completely foreign cellular substrates. Moreover, none of these provide the extensive repertoire of adhesion molecules and cellular ligands required to fully represent the physiologically relevant cell–ECM interface. This chemical and perhaps physical oversimplification has been shown to affect cell signaling and cell behavior in terms of movement, proliferation, attachment and phenotype regulation [130,165,166]. Furthermore, direct comparison of common matrices such as collagen I, fibrin and cell-derived matrices showed significant differences in human fibroblast morphology, migration directionality and cytoskeletal organization [167]. Similarly, cell-derived ECM, collagen I, laminin and fibronectin gels promoted different integrin localization (e.g., paxillin, α_5 integrin, fibronectin) and composition of focal adhesions (Fig. 2) [130]. These basic studies indicate significant differences in cell behavior and cell–ECM interactions in single component culture matrices vs. in vivo counterparts that likely translate into limited abilities of these materials on their own to reliably establish cellular models that replicate complex toxicity-related behavior. Importantly, how these natural materials should or could be further modified with other matrix components to better endow them with more reliable, natural cell signaling lacks rational design criteria beyond chemical and physical mimicry approaches to natural ECM.

4. Conclusions

In vitro 3-D organoid models exhibit potential to become highly predictive cell-based tools for preclinical drug toxicity assessments. The convergence of fundamental cell–ECM interaction analysis with new methods and biomarkers in toxicology using 3-D cell models reflects some success in mimicking select cellular microenvironments. However, this is only part of the validation required for reliable drug testing in these matrices. Most culture models are based on matrix materials and aggregation strategies known for decades from other fields. Critical aspects of tissue-like behaviors required for drug bioactivity assays are most often presumed however, without actual verification or drug- or organ-specific validation. Examples of organ-specific models that respond reliably to exogenous toxin exposures with clinically relevant biomarkers are very limited to date. The vast majority of new 3-D culture models demonstrate arbitrary and partial recoveries of toxicologically important mechanisms (e.g., certain CYP enzymes in 3-D organoid liver spheroids [114,156] or induction of nephrotoxicity genes and protein biomarkers in kidney cells grown in Matrigel® [40]). Most of these models simply show differences in drug EC_{50} values compared to traditional 2-D cell culture, and only for a few days of culture. Improvements are sought by introducing new matrices/methods to aggregate cells in 3-D, or optimizing method–cell combinations. This focus currently limits the performance of these systems, produces troublesome inconsistencies and restricts their wider adoption and screening use in drug development. The presumption that culture model function follows form is precarious and remains to be proven, as shown by tissue engineering examples. The fact that this is an important improvement to drug screening efficiency and reliability remains to be shown.

Improved cellular models for producing complex pharmacological processes, such as toxicity require better understanding of minimal biological complexities required in cell–matrix interactions to duplicate essential enabling pathways for the desired biology and toxicology. Toxicity results from compounds should involve intersecting cellular processes in drug metabolism and pharmacology. These critical pathways should exhibit actively and passively transported drug accumulations, cell metabolic and genetic damage, de-differentiation and apoptosis, CYP toxic metabolites, stress responses and reactive oxygen species production, as well as drug biotransformation via cellular and extracellular enzymes, protein binding and interactions with other cells (e.g., immune system) [5]. These are the common drug screening parameters for clinical toxicologists. Initiation and propagation of these and other tissue damage processes such as inflammatory pathways, cell and ECM changes, typically require on-going bidirectional communication between cells with their surrounding microenvironment. While complete recapitulation of tissue form and function in vitro is unlikely in the short-term, and even unnecessary to many well-defined, verified drug assays, few studies have actually assessed the minimal cellular features required in cultures to faithfully obtain a single reliable biomarker result and correlate it to drug toxicity in vivo. As a consequence, many current formats rely on measurements of cell apoptosis instead of in vivo-relevant graded biomarkers of toxicity as end-points.

Two challenges emerge from this approach. Firstly, apoptosis and cell necrosis are damage events occurring at far higher rates than many clinically assessed biomarkers or relevant clinical exposures. Secondly, no current pharmacological approximations exist to extrapolate physiological animal or human drug doses from such in vitro-derived EC_{50} values. Hence, improved in vivo–in vitro correlations of clinical value from HTS studies should target clinically relevant indicators of toxicity as benchmarks. Furthermore, organ toxicities in vivo are typically characterized by multiple soluble proteins associated with inflammation and cell-specific injury, changes in cell-specific gene expression, ex situ histology, and enzyme and mitochondrial functions. Therefore, cellular drug toxicity models should be designed around specific, minimal model performance requirements for reliable dose-dependent biomarker expressions in vitro and reliably duplicate a variety of important clinical tissue changes upon drug exposure, either acutely in short-term culture or chronically in more demanding systems.

Several 3-D culture approaches and commercially available platforms are currently HTS scalable as required to benefit drug

development. It is now opportune for microenvironment requirements that maintain physiologically important cell metabolic processes to be integrated into these systems beyond the simpler historical gel matrix extrapolations. This includes better defined culture media for soluble cellular cues (growth factors, cytokines, essential metabolites), molecular definition of scaffolds and matrices to represent natural ECM properties, improved culture designs with enhanced transport and mechanical properties (i.e., static and dynamic reactors with fluid shear and elastic modulus variability), and analytical methods for biomarker assay with increased HTS versatility, sensitivity and reliability. Both drug- and toxicity goal-dependent objectives – that is, acute versus chronic markers, escalated dosing versus IC_{50} predictability – linked both to cell phenotypic reliability and to biomarkers that need to be validated for toxicity in each culture case.

The prevailing presumption that seeded cell monocultures spontaneously attach in 3-D matrices to proliferate into tissue-like networks denies much current physiological support for the known multiple cell–cell interactions across many specific cell types in defined geometries. This is a likely contributor that fails to effectively delineate and distinguish critical tissue and organ responses, specifically multiplexed toxicities and pathologies, from cell responses to drugs. Current work in pancreatic cancer drug development manifests these difficulties. This work focuses on pancreatic ductal adenocarcinoma (PDA), a major contributor to cancer-related deaths (ranked 4th in the United States with only 4–6 month median survival [168,169]). Despite many advances in surgical techniques and targeted large molecular therapies [170–172], neither new approaches nor traditional chemotherapy/radiotherapy/small molecule regimens [170] improve patient survival. Clinical resistance to treatment is not reproduced in any in vitro culture. Specifically, cellular models including spheroids from cancer cell lines grown in agarose matrices [30], 3-D pancreatic cell lines in Matrigel® [173] and PuraMATRIX (BD Biosciences) [174], collagen gel spheroids in the RWV [175], other spheroid models [176], explant systems [177] and subcutaneous tumor cell line ectopic transplant models [178,179] all proved to be sensitive and responsive to conventional treatments, unlike PDA.

PDA's clinical resistance to therapy is attributed to increased interstitial fluid pressure generated by tumor-specific HA-rich ECM [180]. Genetically engineered mouse models demonstrate that altering the tumor microenvironment through administered HAse enzyme (PEGPH20) dosing greatly reduces IFP to improve drug permeability into tumor cells. This endogenous PDA matrix comprises a complex mixture of fibrous collagen, proteoglycans and glycosaminoglycans [181,182]. This matrix is not present in simplified 3-D organoid models lacking stromal cells which are created to study PDA disease. Culture models that provide additional ECM components such as collagen or protein-rich basement membrane surrogates failed to effectively simulate 'cancer organ' conditions. Dense PDA-characteristic ECM secreted by activated stromal fibroblasts, or myofibroblasts, is not present in cell culture models due to co-culture complications.

Recent evaluation of tissue equivalents in 3-D kidney cultures or liver-on-a chip models further shows the importance of physiological mimicry. Retention of intercellular and cell–ECM interactions as well as original proximal tubule tissue architecture elicits in vitro responses to nephrotoxic drugs with a wide range of clinically relevant biomarkers, an improvement over other 3-D kidney models with limited in vivo-like microenvironments [30,149,173]. These results, however, required entire harvested proximal tubule fragments isolated from fresh kidneys in their original tissue form, complete with multiple cell types, host tissue organization and endogenous matrix intact. Similarly, several recent organ-on-a-chip [17,18,36] models seek to move beyond technical limitations imposed on previous microfluidized culture systems. For example, recent liver-on-chip innovations feature the dynamic liver-skin model [183] that optimizes fluid tissue velocities; the cord-like liver equivalent [37,184,185] that enhances liver cell polarization, albumin production and higher phase I and II enzymatic activities, and the sinusoid-like equivalent [37,186] that produces near-physiological tissue densities and functional organ sub-division to emphasize the inherent relationship between organ architecture and clinically-relevant toxicity data. Examples where native tissue complexity is fully appreciated and either duplicated or retained in culture closely reflect older published results obtained from organ slices and whole organ perfused models that exhibit in vivo-like behavior in hormonal stimulation and agent toxicity [187–190]. Further improvements to existing models with HTS capabilities should therefore focus on selecting those requisite tissue characteristics for best resemblance to the native tissue markers desired.

Acknowledgments

The authors thank P. Hogrebe (University of Utah) for technical illustrations.

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