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Biophysical microenvironment and 3D culture physiological relevance

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Force and substrate physical property (pliability) is one of three well established microenvironmental factors (MEFs) that may contribute to the formation of physiologically more relevant constructs (or not) for cell-based high-throughput screening (HTS) in preclinical drug discovery. In 3D cultures, studies of the physiological relevance dependence on material pliability are inconclusive, raising questions regarding the need to design platforms with materials whose pliability lies within the physiological range. To provide more insight into this question, we examine the factors that may underlie the studies inconclusiveness and suggest the elimination of redundant physical cues, where applicable, to better control other MEFs, make it easier to incorporate 3D cultures into state of the art HTS instrumentation, and reduce screening costs per compound.

Conventionally, 3D cell culture simply refers to providing a 3D spatial microenvironment for the cells to grow in. However, in our recent work, the meaning of three-dimensionality has been extended to providing the total microenvironment that supports the formation of microtissue that exhibit 'complex' physiological relevance (CPR) or better emulation of the *in vivo* microtissue functionality in a manner not possible in 2D cultures [1]. A good example of CPR outcome is the formation of bile canaliculi-like structures by HepG2 hepatocyte cells (Fig. 1) is 3D but not in 2D culture formats. The literature has provided guidance that leads to three main categories or microenvironment factors (MEFs) or 'three-dimensions' of: (i) chemical or biochemical composition, (ii) spatial (geometric 3D) and temporal dimensions, and (iii) force and substrate physical properties [1–3]. However, as pointed out by Lai *et al.* [4], because of the lack of a quantifiable entity or biomarkers of three dimensionality, the optimum composition of the microenvironment that is required for the cells to provide a physiologically relevant response has remained elusive. It might be that one microenvironmental factor is more important than the other to emulate *in vivo*-like functionality or if the cells are provided with some initial cues they might be able to create their own endogenous microenvironment rendering the other exogenous factors less important [4].

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Evidence in support of varying degrees of importance of MEFs comes from the success of the various commercially available 3D cell culture platforms that provide different MEFs that elicit similar functional or structural CPR from the cells. For instance, mammary epithelial cells (MCF-10A) grown in Matrigel (BD) have formed acini-like or hollow lumen, a structural element associated with glandular cells *in vivo* [5]. Similarly, a spheroid culture of MCF-7 cell line has also shown the presence differentiation features like lumen and budding formation [6]. Interestingly, both the growth platforms, although considered 3D, are very different in nature, with Matrigel providing all the three aforementioned microenvironmental cues to the cells while the spheroid culture just provides a 3D space for them to grow in. This begs the question as to the minimum level of exogenous MEFs that give rise to the *in vivo* conditions that emulate or produce a structurally and functionally analogous *in vitro* tissue model. It is reasonable to argue that the composition of the microenvironment is not standard, rather the optimum MEFs' combination that depends on the application (e.g. cell type, relevant CPR, among others). While in the field of regenerative medicine, a precise emulation might be necessary as the construct is meant for implantation *in vivo*, on the contrary, in the field of drug discovery, exogenous emulation of the microenvironment to the level found *in vivo* (e.g. for CPR expression) may not be necessary. If this is true, the redundancy in the MEFs of interest may be eliminated which may result in

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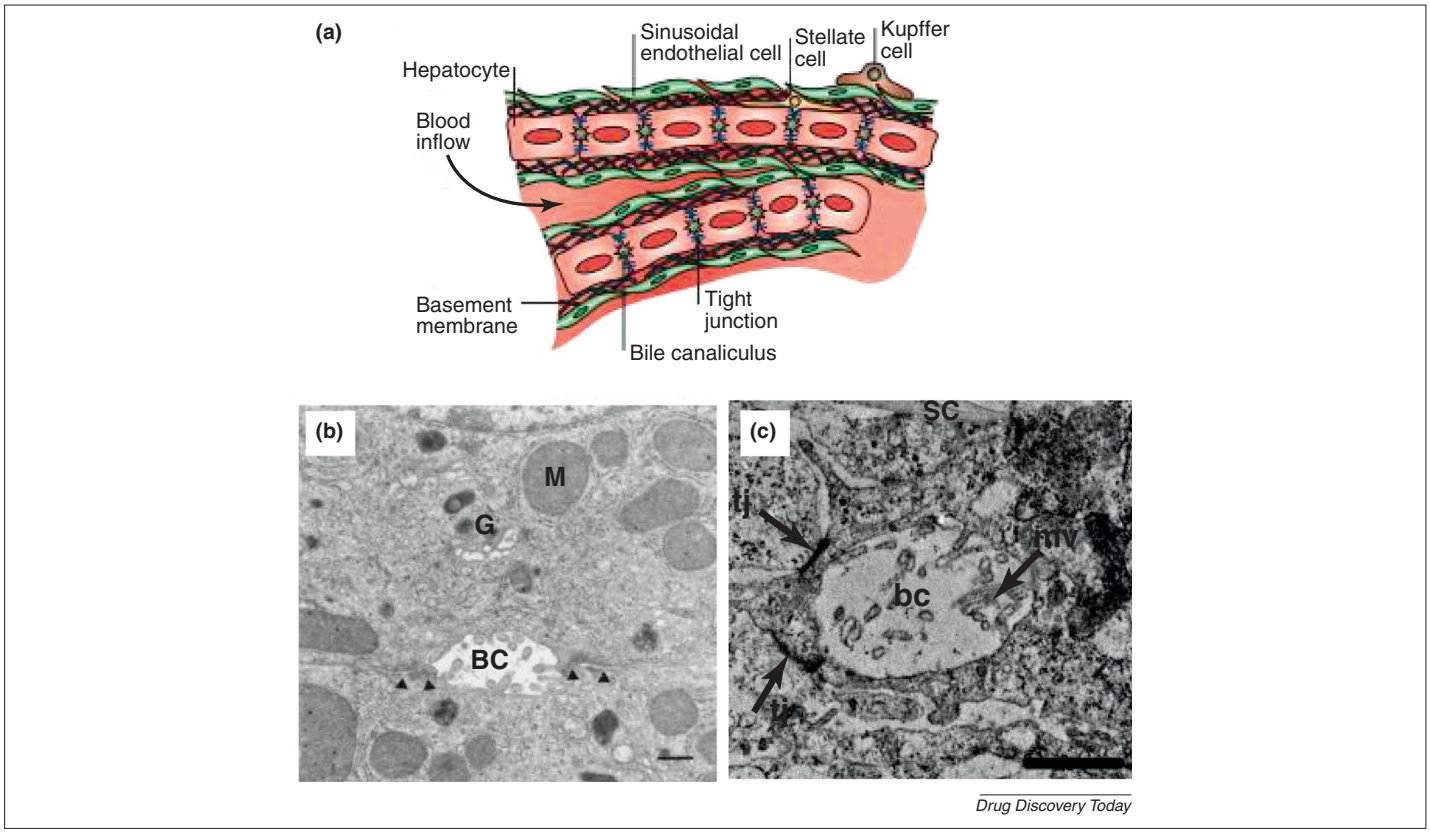

FIGURE 1

Formation of *in vivo*-like bile canaliculi structures by HepG2 hepatocytes that is only observed in 3D but not 2D culture formats. **(a)** Illustrative schematic of liver tissue *in vivo*. **(b)** Transmission electron micrographs showing pericanalicular region of two adjoining periportal hepatocytes of adult rat liver tissue – labeled structures are the bile canaliculus (BC), mitochondria (M), Golgi apparatus (G) and tight junctional regions (arrowheads). **(c)** Almost identical transmission electron micrograph of HepG2 cells cultured on 3D porous polystyrene scaffolds (sc) for 21 days [61], exhibiting tight junction (tj) complexes between adjacent cell; The void formed in-between cells in (c) closely resembles the *in vivo* bile canaliculus (bc) in (b) and is similarly lined with microvilli (mv). Bar = 500 nm.

reduction in cost and making it easier to configure for state of the art high-throughput screening (HTS) instrumentation. Herein we illustrate this 'conjecture' with a focus on substrate/scaffold pliability MEF, one of the three 'dimensions.'

Cell–substrate interaction – biophysical constraints in 3D platforms

Cells exert stress on their matrix during morphogenesis, tissue remodeling, differentiation, and normal physiological activities. The rigidity of the matrix along with the number of receptor-mediated adhesions formed by the cells with the microenvironment influence the extent to which the matrix can be contracted by them [7]. This in turn generates intracellular tension which leads to the formation of stress fibers in the cells. If the matrix is rigid, it is more difficult for the cells to contract it, resulting in differential cell functions [2], wherein lies the importance of providing an optimal biophysical microenvironment to the cells *in vitro*. Most of the 3D platforms that are commercially available (Table 1) can be broadly classified into three categories based on their rigidity: (i) Hydrogel-forming (alginate, agarose, chitosan, fibrin, hyaluronan and collagen to name a few) that are pliable and provide a 'soft' environment for the cells to grow in (Type I); (ii) Synthetic microporous ('spongy') scaffolds or constructs fabricated by freeform technology are generally made of stiffer materials having high modulus of elasticity such as polystyrene, PLLA (Poly-L-Lactide Acid), PLGA (Poly(lactic-co-glycolic acid)) and PCL (Polycaprolactone) (Type II); (iii) Scaffold-free 3D formats such as spheroids or cellular aggregates, produced with hanging drop or the Rotating Wall Vessel (RWV) configurations that lack cell–exogenous material interaction (Type III). Advantages and disadvantages of the three platform types are discussed below.

Type I

The pliability of hydrogels can be altered within the physiological range (100–10,000 Pa) by either changing the concentration of the polymer ((Poly(ethylene glycol))PEG, agarose), the extent of cross-linking or the proportion of the (Extracellular matrix) ECM proteins (hyaluronan, collagen, fibronectin, laminin) incorporated in the substrate. Modifying the concentration of the ECM proteins (also biochemical cues) might also lead to a change in the number of adhesion ligand sites present for the cells to bind to. However, this can be managed by mixing a more compliant ECM component like Matrigel with a stiffer constituent (collagen I) in defined proportions that allow a relatively constant chemical–ligand concentration within a variable stiffness range. Furthermore, the rigidity of the hydrogels can also be increased by using the hydrogel in an 'attached' configuration, where the hydrogels are bound to the bottom of the culture dish and resist the forces that are exerted by the cells, rather than a floating raft or suspended mode. An increase in gel rigidity generally results in the

TABLE 1

Elastic moduli for commercially available 3D platforms

Company	Trade name	Type and material	Elastic moduli (kPa)	Ref.
3DBiomatrix	Perfecta3D plates Perfecta3D scaffolds	Hanging drops Hydrogel	NA NR	
InSphero	GravityPlus plates	Hanging drops	NA	
BD	Matrigel	Laminin, collagen	0.45	[62]
Glycosan Biosystems	Extracel	Hyaluronic acid and collagen	0.011–3.5	[63]
GlobalCellSollutions/Hamilton	GEM	Magnetic alginate microcarrier	0.7% – 0.203 ± 0.013 1.5% – 1.3 ± 0.129 3.0% – 3.01 ± 0.084	[64]
Trevigen	Cultrex 3D Matrix	BME, laminin, collagen	0.45	[62]
Sigma	HydroMatrix MaxGel	Synthetic peptide hydrogel Human ECM	1.59–14.7 120–380	[65] [66]
QGel	MT 3D Matrix	PEG hydrogel	0.448–5.408 (0–2% PEGDA)	[67]
Kollodis BioSciences	MAPTrix HyGel	Chemically defined hydrogel	NR	
Synthecon Inc.	BIOFELT Biomerix 3D Scaffold	PGA, PLLA, PLGA, custom Polycarbonate polyurethane-urea	NR NR	
Invitrogen	Geltrex AlgiMatrix	Laminin, collagen Alginate	0.45 0.7% – 0.203 ± 0.013 1.5% – 1.3 ± 0.129 3.0% – 3.01 ± 0.084	[62] [64]
ZellWerk	Sponceram	Ceramic	NR	
amsbio	alvetex	Polystyrene	77	[11]
3DM Inc.	PuraMatrix	Peptide	1.59–14.7	[65]
Corning	UltraWeb	Polyamide	0.725	[68]
3DBiotek	3D Insert PCL 3D Insert PS 3D Insert PLGA β-TCP Disc	Polycaprolactone Polystyrene Poly(DL-lactide-co-glycolide) β-Tricalcium phosphate	500 3,680 (fibrous) 3,000 (porous) 24.6 ± 0.95 to 78.6 ± 2.36 ($\times 10^6$)	[69] [70] [71] [72]
MicroTissues Inc.	3D Petri Dish	Agarose	3.2% – 294 3.9% – 496 6.7% – 626	[73]

Abbreviations: BME: basement membrane extract; NA: not applicable; NR: not reported.

enhancement of proliferation and inhibition of differentiation because of elevation in the phosphorylation of focal adhesion kinase and the formation of focal adhesions, as shown by Paszek *et al.* [8]. Even though such platforms provide an *in vivo*-like pliable environment for the cells to grow in, they lack a defined geometry and fail to impose any physical constraints on the size of the aggregates. As such, the microtissues formed range from being just a cluster of few cells to larger tissues that are above the crucial size for oxygen diffusion and this might generate an adulterated outcome in response to drug exposure [9].

Type II

Because the pliability of these scaffolds is above the physiological range, it is usually assumed that they fail to provide the optimum biophysical cues for the cells, yet they have been successful as shown by their commercial adoption (Table 1). However, it should be noted that it is not just the material that affects the pliability but also the form in which it is presented. For example, polystyrene in its bulk state as used in tissue culture plates has a very high elastic modulus (2–4 GPa; [10]) but when used to fabricate salt leached microporous scaffolds, it exhibits a considerably lower modulus (77 kPa; [11]). Also, ECM proteins can be coated on such scaffolds to provide adhesion sites for the cells to attach. The protein coating might provide a more compliant surrounding for the cells, however it has been argued that the coating is very thin and it is a known fact that cells can sense and adapt in response to the topography independent of the adsorbed proteins [12]. The major advantages of type II scaffolds are that they have a defined geometry and controllable pore sizes that provide a strict spatial control on the dimension of the microtissues [9] and are better suited for incorporation in HTS state of the art instrumentation.

Type III

Scaffold-free 3D culture production is either achieved in static conditions such as gravity-enforced hanging drops or dynamic conditions, such as RWV (Rotating Wall Vessel), Stirred Tank Reactors (CSTR), spinner vessels, and microfluidic chambers. In the dynamic configuration, shear stress generated because of the fluid flow constitutes the primary driving biophysical factor in absence of stress created because of the cells pulling onto to the material as in the previous cases. Microtissues generated by the above mentioned techniques have been used as *in vivo* surrogates far and wide [13,14], however, these systems are generally labor intensive and are difficult to adapt in state-of-the-art HTS instrumentation.

Cellular complex physiological relevance and drug discovery outcomes

Moving to second generation 3D cell culture platforms is being driven by the brief that the responses generated by the cells growing in a 3D format are not just 'different,' but are physiologically more relevant, when compared to cells cultured on traditional 2D surfaces. It is necessary to conclusively show that these responses produced in 3D formats are emulations of those that are seen *in vivo*. To be meaningful, these physiologically relevant outcomes (structural or functional) that are also known *in vivo* should be absent in 2D formats and such outcomes (CPRs) should be established for cells derived from the four major tissue types (epithelial, muscle, connective, and nerve). These outcomes can serve as a standard for determining how close a 3D culture is to its native tissue or which out of a given number of 3D platforms is better suited for a given application. Below, we explore some of the well established or provisional CPRs for cells derived from the three tissue types of most interest in preclinical drug discovery (epithelial, neuronal and cardiac).

CPR in liver tissue-derived cells

Because liver is the primary organ involved in the metabolism of xenobiotics, 3D constructs of hepatocytes are often used as a tool to screen for drug toxicity and test compounds that may affect liver cell function. As such, because of the vast amount of literature available, both structural and functional CPR responses for liver cells can be established with ease. One of the basic structural phenomena that distinguish liver cells in the native tissue from those cultured in 2D formats is polarity. While those in their natural environment possess structural and functional polarity [15,16], the ones that are isolated and cultured on most flat non-porous surfaces do not [17,18]. In their native conditions, hepatocytes maintain a cuboidal shape, with two to three basal surfaces facing the sinusoid. The lateral domain between adjacent cells is divided by a polygonal network of microvilli-lined bile canaliculi (Fig. 1) which is formed by membranes contributed from contiguous cells and comprises the apical domain of cells. Furthermore, position specific processes are carried out in each domain. For instance, proteins involved in the shuttling metabolites from the blood capillaries are centralized to the basal surface, while those involved in bile acid transport are confined to the apical domain. In monolayer, the development of such bile canalicular networks is sparse, heterogeneous and transient [16,19] further differentiating it from native tissue and substantiating this structural element as a valid CPR outcome. In terms of function, hepatocytes in their native environment display high levels of liver specific activities like high cytochrome P450 activity [20], transferrin secretion [21], albumin production [21,22], tyrosine aminotransferase induction [23] and ureagenesis [24] than their monolayer counterparts. Cells growing in a 2D format entirely lack or have very low levels of many Cytochrome P450 enzymes (CYPs) and transporters found in hepatocytes *in vivo* [25,26]. The CYPs are a family of phase 1 metabolizing enzymes that consist of 50 isoforms, six of which metabolize 90% of drugs [27]. The primary isoforms in human liver include CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, making them a very important family of enzymes not only for screening purposes but also in validating the CPR of 3D liver cell cultures.

CPR in nerve tissue-derived cells

The literature on cells of neuronal origin has not provided a consensus toward establishing a neuronal microtissue CPR, however, several phenomena with high CPR outcome potential are more worthy of further exploration. For example, intracellular calcium oscillations are an innate characteristic of neural cells *in vivo* and have a pivotal role in synaptic signal transmission. Although calcium oscillations have been observed in both 2D and 3D nerve cell cultures, we submit that there should be differences in the nature of oscillations between the two cultures. The frequencies of these oscillations found in 3D cultures are considerably lower (e.g. 3.42/600 s, brain slices [28]; 8/600 s, NP cells derived from neurospheres [29]) than those found in 2D cultures (e.g. 60/600 s [30], 60/600 s [31]) and is closer to many *in vivo* experiences (e.g. 3.6/600 s [32]). Interestingly, the differences in voltage-gated calcium channel (VGCC) function in 2D and 3D cultures might partly be the underlying cause, because these channels are central to the movement of calcium into and out of cells [33]. It has been shown that intracellular calcium transients are significantly lower in 3D as compared with 2D cultures and this is more representative of the *in vivo* situation [11,13]. Also, L-type VGCC agonists and antagonists have previously been shown to enhance and abolish calcium oscillations, respectively, in nerve cells [34–37], substantiating involvement of VGCC in the differential frequency of calcium oscillations found in different culture formats. These findings suggest potential for calcium transients and/or oscillations to serve as nerve cell CPR outcome.

CPR in muscle tissue-derived cells

Myocardial microtissues are of particular importance as they find application in HTS Q-T elongation assays, required for screening every drug in development [38]. Some recent examples of drugs that have been withdrawn from various markets because of Q-T interval prolongation effects include Droperidol (Inapsine; Akorn; 2001), Dofetilide (Tikosyn; Pfizer; 2004) and Thiordazine (Mellari; Novartis; 2005). As such, it is essential to establish a consensus for the CPR of cardiac microtissue to provide an *in vivo*-like tissue model for drug development and screening. A couple of phenomena that are characteristic of the native cardiac tissue *in vivo*, found in 3D cultures but are lacking in traditional monolayers include beat frequency and contraction force. In a study by Kelm *et al.* [39], neonatal rat cardiomyocytes (NRC) showed rhythmic contractions at a beat frequency of 60 beats per minute (bpm) when they were grown as spheroids in a hanging drop culture. Similar beat frequencies (43 ± 21 bpm) have been seen when NRC were transplanted *in vivo* in adult rat and formed a microtissue [40] which is considerably lower as compared with 2D culture (83.4 ± 4.5 bpm [41], 85.6 ± 9.3 [42]). This is consistent with the fact that cells in monolayers exhibit exaggerated responses, specifically VGCC function which is responsible for cardiac cell contraction and has been discussed earlier. However, there are a few studies that have suggested otherwise, such as Zimmerman *et al.* [43] who reported the beat frequency of NRC growing as Engineered Heart Tissue (EHT) of 180 bpm. It should be noted that hearts from 2-day-old rats have a beat rate of 135–155 bpm [44].

CPR in HTS 3D platform validation

The two examples of liver and muscle cell culture used above, to illustrate the CPR concept, have traditionally been associated with low throughput later phases of discovery applications like toxicology. A relevant question is how relevant the examples are in early HTS phases of discovery. In response to this question, we submit that as long as the cells can express a target of interest, their 3D assay can be used for both HTS and low throughput discovery applications. For example, there is no reason why HepG2 (human hepatocellular carcinoma) cannot be used in both toxicology (low throughput) and chemotherapeutics HTS. Therefore, CPR should not be thought about only in the context of low throughput applications. As a matter of fact, 3D culture platforms are slowly finding their way into HTS laboratories. For example, studies of HTS assay robustness (in terms of Z' factor) with 3D cultures in high well-density plates (e.g. 96–384 wells) are beginning to appear [45,46].

A drug exposure might generate a different, and in some cases a 'better,' response, such as differentiation toward a particular lineage, from cells growing on a 3D format as compared with those in a monolayer. However, in the absence of a validated CPR outcome, there would be no way of telling if the outcome is predictive of the *in vivo* response. For example, Tung *et al.* [47] showed that two cytotoxic drugs had different effects on cells growing as spheroids in hanging drops and those in 2D. Had it been shown that the 3D spheroids produced in this particular platform emulated the native tissue and were validated using a particular CPR outcome, then the effect of the drugs on the spheroids would have been considered more predictive of its *in vivo* effect. Therefore, it is more desirable that the existing and the upcoming novel 3D culture platforms are validated with respect to a CPR outcome known in the native tissue and then the results of a drug exposure would be a more physiologically relevant outcome that should lead to a better success rate in the drug development process.

Relationship between CPR and biophysical factors – are they independent?

Cases where the biophysical MEFs do not seem to influence the CPR outcome have raised the question as to how much importance they command or to what extent they need to be exogenously included in construct design for HTS. Summarized in Table 2 are studies where liver cells were grown in type I, II and III scaffolds that provided vastly different biophysical cues with no difference in cellular function outcomes. Encapsulation of primary human hepatocytes (HHY41) in alginate beads (pliable; type I) has been shown to promote the growth of these cells leading to the formation of 3D aggregates and upregulation of liver specific functions [48]. Cells grown in this particular 3D format have prominent structural CPR features, such as junctional complexes and microvilli-lined network of canaliculi along with improved secretion of liver specific proteins, cytochrome P450 function, and urea synthesis. As the pliability of alginate beads is comparable to that of native hepatic tissue (bovine liver: 0.62 ± 0.24 kPa and 0.94 ± 0.65 kPa by ultrasound and Instron Young's modulus, respectively [49]) and their resultant biophysical MEF can be considered optimal. Considering this as a standard, a porous polystyrene surface (rigid; 77 kPa) that has an elastic modulus considerably above the physiological range can be considered

TABLE 2

Liver cell studies with different platforms, but similar CPR outcomes

Cell line and type	Scaffold type and material	Structural CPR	Functional CPR	Ref.
Rat small hepatocytes (SHs)	Stacked layers on microporous membranes	Bile canaliculi with luminal microvilli, tight junctions, desmosomes	Albumin secretion, tyrosine aminotransferase expression	[74]
HHY41	Alginate beads	canaliculi with network of microvilli; desmosomes and junctional complexes	Albumin secretion, fibrinogen, α-1-antitrypsin production, cytochrome P450 1A1 activity, urea synthesis	[48]
HepG2 and HHY41	Alginate beads	Desmosomes, junctional complexes and canaliculi lined with microvilli	Albumin secretion, CYP1A1, CYP1A2 cytochrome p450 activity	[75]
Lig-8 cell line (adult rat liver progenitor)	Peptide hydrogel (Puramatrix)		Albumin secretion, CYP1A1, CYP1A2, and CYP2E1 cytochrome p450 activity	[76]
Primary rat hepatocytes, HepG2	Chitosan-collagen coated PET mesh scaffold	microvilli	Albumin secretion	[77]
Primary rat hepatocytes	Nanofibrous/porous PLLA scaffolds	Tight junctions, bile canaliculi, gap junctions	Glycogen storage, HNF-4 positive, albumin secretion	[54]
HepG2	Porous PS scaffold	Tight junctions, channels with microvilli	Albumin secretion	[50,51]
Primary rat hepatocytes	PMMA or PC polymer scaffold	Junctional complexes, luminal microvilli	Albumin secretion, cytochrome P450 activity, tyrosine aminotransferase induction	[78]
Fetal porcine hepatocytes	PLLA scaffolds		Albumin secretion, cytochrome P450 1A1/2 capacity, ammonia removal, urea synthesis	[79]
Primary rat astrocytes	Spheroids	Bile canaliculi, microvilli		[57]

Abbreviations: PET: Poly(ethylene terephthalate); PC: Propylene carbonate; PMMA: Poly(methyl methacrylate); PS: Polystyrene.

sub-optimal and the liver cells grown on it are expected to produce an aberrant or at least a significantly different outcome. However, studies by Bokhari *et al.* [50,51] have shown that HepG2 cells grown on porous polystyrene scaffolds (type II) exhibit CPRs similar to those shown by microtissues grown on softer materials like higher viability, structural integrity and formation of bile canaliculi, enhanced liver function and drug response comparable to *in vivo* activity.

One might argue that the type of cells (e.g. primary hepatocytes versus hepatocellular carcinoma) and substrate rigidity might be a factor in screening results as cancerous cells are known to have a higher elastic modulus than the non-malignant phenotype and can easily adapt to a more rigid environment or substrate (e.g. standard polystyrene plates). However, the modulus of HepG2 cells (1.1, 1.6 and 1.4 kPa cultured on Collagen I-, Laminin-, and Matrigel-coated substrates, respectively [52], 2 kPa by micropipette aspiration [53]) is not considerably higher than that of native tissue (bovine liver – 0.62 ± 0.24 kPa and 0.94 ± 0.65 kPa by ultrasound and Instron YM, respectively [48]), while the modulus of polystyrene is well beyond the adaptability of the cell, thus ruling out the difference in cell phenotype and respective optimal growth environment elasticity requirement as major factors. This is further substantiated by the fact that primary hepatocytes grown in nanofibrous PLLA scaffolds have also exhibited comparable morphological and functional CPR outcomes [54]. Interestingly, the level of albumin produced by cells cultured on this scaffold for a day was found to be 70 $\mu g/10^6$ cells (10 $\mu g/day/10^6$ cells in 2D) which is considerably higher than that found in alignate beads (55 $\mu g/day/10^6$ cells in 3D, 10 $\mu g/day/10^6$ cells in 2D [48]) and closer to that *in vivo* (140 $\mu g/day/10^6$ cells in adult normal rat liver [55]). Furthermore, to totally eliminate the effects of substrate pliability and cell–material interactions, spheroid systems that lack any physical scaffolding for cells (type III) can be considered. Such systems, whether employed in static (hanging drops) or dynamic state (RWV [56] or spinner flasks [57]) have yielded CPR outcomes similar to the previous cases. Hence, it can be inferred from these studies that neither the pliability (soft, rigid or scaffold-free) nor the structure (microporous or nanofibrous) of the substrate has a major effect on the CRP phenomena exhibited by the liver cells grown therein. A similar analysis is needed if this is also the case for cells derived from other tissue types.

A possible explanation for the above lack of pliability effect comes from the 'cell-on-cell' hypothesis [58], where cell-to-cell contacts appear to have a more pivotal role as neighboring cells provide a soft 'stroma' for surrounding cells and produce responses similar to those seen when cells are grown in softer gels. For example, prominent actomyosin striation can be seen in myotubes when they are cultured on top of a layer of muscle cells. The lower layer of myotubes that adheres more strongly to the rigid substrate shows formation of ample stress fibers, however, myotubes in the upper layers differentiate to a more physiological, striated state with an elastic modulus in a range similar to that of gels suited for differentiation and also the native muscle tissue [58]. Similarly, a basal layer of astrocytes grown on glass provides a pliable environment optimal for branching of neurons, which is similar to gels having brain-like pliability [59]. Also, when endothelial cells seeded at a high density become confluent, they have similar morphologies on both pliable and rigid substrates [60], which differ from cells in a monolayer that are attached only to an underlying rigid surface that cause actin cytoskeletal remodeling and spreading. A similar phenomenon might be responsible for the above discussed liver cell CPR outcomes from cells grown on rigid porous scaffolds (type II/high muduli) where only the outermost layer of the microtissue comes in contact and adapts to the pliability of the substrate while the cells in the core grow in the softer environment provided by peripheral cells. However, whether it is only the peripheral layer that is affected by substrate rigidity or a radial gradient in elastic modulus exists within the microtissue is not yet known and requires further investigation.

Concluding remarks

With the field of 3D cell culture moving into its second generation products, the significance of translational research is increasing. Up until now, much thought and effort has been put towards engineering an optimal 3D microenvironment that can emulate the characteristics of the native milieu. However, adapting state-of-the-art cell-based HTS platforms to accommodate 3D cultures requires a balance between simplistic architecture, control over microenvironmental parameters, structural and functional integrity of microtissues and cost effectiveness. Based on the current state of knowledge, it is fair to suggest that in some cases, biophysical factors might not be necessary for obtaining CPR outcomes *in vitro*, which are predictive of treatment/drug efficacy *in vivo* as the microtissues might be creating their own physical domain (endogenous ECM) rendering the exogenously incorporated factors less important. Elimination of such redundant physical cues might lead to a better control over important parameters like aggregate size and hypoxia, easier adaptability to automated handling and a reduction in high costs that are currently associated with 3D cell culture platforms. However, more translational research spanning different platforms, constituting a vast array of biophysical factors such as pliability (soft, rigid or scaffold free) and structure (microporous or nanofibrous) is required to establish a design philosophy consensus.

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