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feature

Microtissue size and hypoxia in HTS with 3D cultures

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The three microenvironmental factors that characterize 3D cultures include: first, chemical and/or biochemical composition, second, spatial and temporal dimensions, and third, force and/or substrate physical properties. Although these factors have been studied individually, their interdependence and synergistic interactions have not been well appreciated. We make this case by illustrating how microtissue size (spatial) and hypoxia (chemical) can be used in the formation of physiologically more relevant constructs (or not) for cell-based high-throughput screening (HTS) in drug discovery. We further show how transcriptomic and/or proteomic results from heterogeneously sized microtissues and scaffold architectures that deliberately control hypoxia can misrepresent and represent in vivo conditions, respectively. We offer guidance, depending on HTS objectives, for rational 3D culture platform choice for better emulation of in vivo conditions.

Traditionally, the meaning of three-dimensionality in cell culture has been simply associated with providing a 3D spatial microenvironment. In our recent work, the meaning 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]. The literature has provided guidance that lead to three main categories or microenvironment factors (MEFs) or 'three-dimensions' of: first, chemical or biochemical composition, second, spatial (geometric 3D) and temporal dimensions, and third, force and substrate physical properties [1–4]. Although much attention has been paid to biochemical factors, such as integrin adhesion and presence of RDG peptides (integrin recognition site) in 3D cultures and biophysical cues, such as loading and unloading of collagen matrices, interactions among the factors have not been well studied.

The effect of the aforementioned microenvironmental factors is not exclusive, but they act synergistically to propel the cells towards a specific outcome. For instance, although regulating the size of the microtissue might seem trivial, it might indirectly have major implications on the functional response of the cells. If the tissue formed is too small, it might lack the physiologically relevant complexity and might not emulate the complex functionality present *in vivo*. Conversely, if the tissue is large (spatial factor), the oxygen diffusion (biochemical factor) limitation might lead to a necrotic core, reducing the viability and influencing the phenotypic outcome. It is known that oxygen can diffuse across 100–200 μm of tissue thickness and it is generally desirable to maintain the optimal aggregate size approximately 250 μm to prevent hypoxia [2]. However, this size should not be treated as a gold standard, as the optimal size might depend on the application. In the field of regenerative medicine, circumventing hypoxia to produce larger tissues with higher viability for implantation *in vivo* is relentlessly pursued, however, the field of drug discovery might benefit from incorporating hypoxia in platform design. After all, hypoxia is a physiologically relevant phenomenon and is important for many *in vivo* processes, such as development and tumor progression. Indeed hypoxia has been widely implicated as the initiator of angiogenesis in avascular tumors, vasculogenesis during embryonic development and regulator of terminal differentiation [5]. As such, it is important to regulate the size of the tissue in 3D

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TABLE 1

Microtissue size control in the commercially available 3D platforms

Company	Trade name	Type and material	Cell aggregate/ scaffold pore size
3DBiomatrix	Perfecta3D plates Perfecta3D scaffolds	Hanging drops Hydrogel	NA NA
InSphero	GravityPlus plates	Hanging drops	NA
BD	Matrigel	Laminin, Collagen	NA
Glycosan Biosystems	Extracel	Hyaluronic acid and Collagen	NA
GlobalCellSollutions/Hamilton	GEM	Magnetic alginate microcarrier	NA
Trevigen	Cultrex 3D Matrix	BME, Laminin, Collagen	NA
Sigma	HydroMatrix MaxGel	Synthetic Peptide Hydrogel Human ECM	NA NA
QGel	MT 3D Matrix	Hydrogel	NA
Kollodis BioSciences	MAPTrix HyGel	Chemically defined Hydrogel	NA
Synthecon Inc.	BIOFELT Biomerix 3D Scaffold	PGA, PLLA, PLGA, custom Polycarbonate polyurethane-urea	NR 100–250
Invitrogen	Geltrex AlgiMatrix	Laminin, Collagen Alginate	NA 40–300
ZellWerk	Sponceram	Ceramic	NA
amsbio	alvetex	Polystyrene	NR
3DM Inc.	PuraMatrix	Peptide	50–400
Corning	UltraWeb	Polyamide	300–500
3DBiotek	3D Insert PCL 3D Insert PS 3D Insert PLGA β-TCP Disc	Polycaprolactone Polystyrene Poly(DL-lactide-co-glycolide) β-Tricalcium phosphate	300; 500 200; 400 500 NR
MicroTissues Inc.	3D Petri Dish	Agarose	Multiple

Abbreviations: NA, not applicable; NR, not reported.

culture to include or eliminate the effects of hypoxia depending on the physiological phenomenon of interest. To illustrate further, in anticancer drug discovery, test compounds might be scored 'hits' with early stage tumor progression models, however, these compounds might be incompetent if tested with a microtissue tumor model having a larger size, owing to the drug resistance associated with hypoxic tumors. To make the case for the importance of microtissue size, we present many 2D and/or 3D comparative transcriptomic and proteomic studies where the effect of the culture platform studied, might have been confounded by the heterogeneous microtissue sizes and thus the effect of hypoxia on gene expression was affected. Taken together, we stress the importance of inclusion of hypoxic conditions in constructs through size regulation for certain applications. Such a realization is important in rational design and/or choice of a 3D platform, where the need for strict control of microtissues is balanced against the need for flexibility to alter microtissue size to better emulate the *in vivo* conditions.

Spatial constraints in 3D platforms

Most of the 3D platforms that are commercially available (Table 1) can be broadly classified into three categories based on the spatial constraints that they impose. First, Hydrogel-forming (alginate, agarose, chitosan, fibrin, hyaluronan and collagen to name a few) that cannot exert control on the size of the microtissues giving rise to heterogeneous microtissues (Type I; Fig. 1a). In such constructs, the microtissues range from being just a cluster of few cells to larger tissues that are above the crucial size for oxygen diffusion and might provide an adulterated response as discussed in the previous section. The sizes of the tissues depend on the seeding density and the proliferation rate of the cells and because the scaffold material is pliable there is no physical constraint on the size of the aggregates formed. Recently, a gradient depressurization strategy has been used to control the porosity and pore diameter in chitosan hydrogels. However whether this technology is successful with hydrogels made of other substrates remains to be seen [6]. Scaffold-free spheroid cultures also fall into the same category because their size also depends on the aforementioned factors rather than physical restrictions. The sizes of the aggregates growing in rotary wall vessels (RWV) or continuous stirred-tank reactors (CSTR) can be regulated by physical factors, such as revolutions per minute (rpm) and fluid shear stress, however; this control is activated only when the spheroids grow above a crucial size. Moreover, the larger tissues are broken down into random smaller sizes giving rise to heterogeneously sized microtissues. Also, smaller tissues present in the culture, ranging from single cells to just below the threshold limit are not affected by this mechanism. Second, Synthetic microporous scaffolds made of stiff materials (e.g. polymers, such as polystyrene) that put a physical restraint on the size of the microtissues but the range of the pore size is large, again resulting in a variable sized population (Type II; Fig. 1b). However, the extent of variation is lower than Type I cases and the variability is defined as the range of pore sizes is known to the user. Third, Polymeric scaffolds that have a defined geometry and homogeneous pore sizes and provide a strict spatial control on

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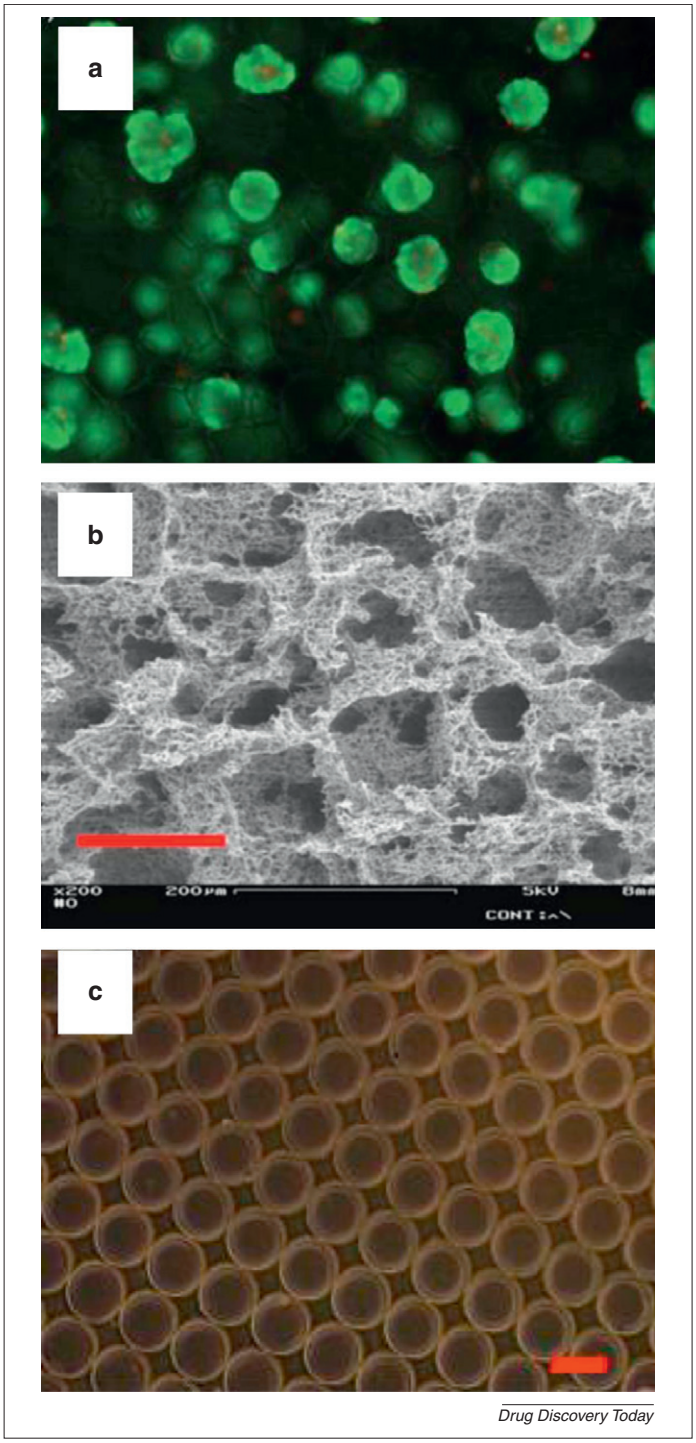

FIGURE 1

Three categories of 3D platforms. **(a)** Hydrogel (AlgiMatrix) with heterogeneous (C3A human hepatocytes) microtissues stained with live/dead dye kit. Dead cells (stained red) are visible at the center of large aggregates [51]. **(b)** Polystyrene scaffold that imposes a size constraint but offers a wide range of pore sizes. The scaffold was fabricated following procedures described in [52]. **(c)** SU-8 (photoresist material) microwell scaffold that provides a uniform pore size. The scaffold was fabricated following procedures described in [53]. Scale bars = 100 μm.

the size of the microtissues (Type III; Fig. 1c). If seeded at the optimal cell density, such scaffolds produce a population of equally sized aggregates; however they might lack flexibility and can be application specific. For instance, a scaffold having a defined pore size of 200 μm might not be suited for studying or developing a drug against a late stage cancer, where the tumor is hypoxic and adapting for angiogenesis. Conversely, while developing tissue models for drug testing by differentiating stem cells, a larger pore size (500 μm) might produce microtissues with hypoxic cores and this might influence the differentiation capacity of the cells, giving rise to a heterotypic model. The impact of hypoxia in both these scenarios is discussed below. Owing to such variability, the response generated due to a specific treatment by cells growing on different 3D platforms might be different and too difficult to compare. One must consider that variability in tissue size might manifest itself in the form of hypoxia or other unknown forms and perturb gene expression leading to an adulterated response to the administered treatment.

Relationship between microtissue size and gene expression

As discussed above, if the size of the microtissue grows beyond the threshold for oxygen diffusion, the cells in the core of the aggregate become hypoxic. Hypoxia can manifest itself in the form of gene expression perturbation because it regulates the expression of a wide variety of genes associated with oxygen transport and iron metabolism, glycolysis and glucose uptake, angiogenesis, extracellular matrix (ECM) and coagulation systems, drug resistance, pH regulation, transcription and growth factors and cytokines (Table 3) [7,8]. The relationship between microtissue size and gene expression was established by Kelm *et al.* [9] where it was shown that larger myocardial spheroids (230 ± 11 μm in diameter) produced high levels of vascular endothelial growth factor (VEGF; a marker of hypoxia) while it was absent in smaller spheroids (130 ± 11 μm in diameter) although both the sizes showed CPR (synchronized beating frequencies). This relationship is further substantiated by a transcriptomic study [10] showing that neural progenitor (NP) cells growing as neurospheres (larger size) had a higher number of upregulated genes than those growing in 3D microporous scaffolds (controlled smaller size) when compared with 2D cultures. It was suggested that this might be owing to hypoxia associated with the larger size of neurospheres as was evident by the upregulation of macrophage inflammatory protein-2 (MIP-2) gene, which is induced by hypoxic conditions [11].

Several transcriptomic and proteomic studies that have compared gene expression variations between a variety of cells grown in 2D and 3D formats are listed in Table 2. These differential gene expression events have generally been solely attributed to the transition from 2D to a 3D platform, but hypoxia associated with larger tissue size might also be responsible for these gene perturbations as several of them might be

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TABLE 2

Hypoxia controlled genes/proteins found upregulated in 3D/2D comparative studies.

Cell line	3D Scaffold	Size (μm)	Genes[a]	Refs
Fibroblasts	Collagen matrix	ND	***IL-6***	[41]
IMR-90	Collagen–GAG matrix	80–100	HIG2, IL-8, CXCL2, VEGF, FTH1, FTL	[42]
MG-63, SaOS-2	Si-HPMC polymer hydrogel	ND	IL-6	[43]
NA8	Spheroids on pHEMA plates	ND	***IL-8***, CXCL2, Angiopoetin like4, CA-9, LOX, ADM, HIG2, BNip3, IGFBP3, Jun, ITGA2	[44]
R1	Cytomatrix RW-spinner culture	$\leqslant$150	Jun, IGF-2	[45]
L1236	RADA-oligopeptide matrix	ND	CCL5, TNF	[46]
PDAC	pECM	ND	***IL-6, IL-8***	[47]
OSCC3, U87 MDA-MB231	PLG, RGD alginate, Matrigel	ND	***IL-8, VEGF***	[48]
HFSF, CRL-2088 **HES** **HAL** **MRC-5**	Spheroids on Agarose plates	ND	***COX-2, CCL3,CCL5, CXCL8*** ***CXCL8*** ***CXCL8*** ***CXCL8***	[49]
BMSC	Spheroids on Agarose plates	400	CXCL12	[50]

[a]Bold italic indicates protein results, other results are only transcriptomic.

Abbreviations: IL, interleukin; CXCL2, Macrophage inflammatory protein 2; FTH1, Ferritin Heavy subunit; FTL, Ferritin Light subunit; HIG2, hypoxia-inducible gene 2 protein; ND, Not Defined.

missing in 3D cultures of smaller sizes [9,10]. The genes listed in Table 2 were found to be upregulated in 3D cultures when compared to 2D but they were also induced by hypoxia (Table 3) suggesting that gene expression due to hypoxic conditions might have been involved in these studies. Thus, to assess the genes whose expression is significantly altered specifically owing to 3D culture conditions, the size of the microtissue should be controlled; else the genes influenced by hypoxia might augment the total number of differentially expressed genes and mask the ones that are really of interest.

Physiological relevance of hypoxia – need for inclusion in construct design

Hypoxia can be a physiologically relevant phenomenon because it is a major characteristic of 3D microenvironments both *in vivo* and *in vitro*. Oxygen concentration in 3D tissues depends on the balance between oxygen delivery and consumption. *In vivo*, this balance is tightly regulated by evenly distributed capillary networks but *in vitro* homotypic 3D microtissues lack vasculature and therefore develop a hypoxic core as their size increases. This might lead to cells, producing chemical signals (cytokines) and programming themselves for developmental, adaptive or neoplastic angiogenesis depending upon their type (stem, committed or malignant, respectively). This is similar to the response generated by *in vivo* hypoxic tissues where balanced signaling cascades lead to vascular remodeling and angioadaptation until the tissue oxygen concentration is back within its normal range [12]. The central connection between physiological hypoxia and the cellular response is mediated by hypoxia inducible transcription factors (HIFs). Under hypoxic conditions, HIF-1α and HIF-1β are translocated to the nucleus [13] where they dimerize and bind to target gene motifs called hypoxia responsive elements (HREs) to alter gene expression [14]. *In vivo*, hypoxia is generally associated with the tumor microenvironment where it is responsible for angiogenesis, drug resistance and increased metastatic potential of the malignant cells and also with development where it regulates vasculogenesis, stem cell renewal and terminal differentiation. As such, hypoxic conditions need to be included in the 3D scaffold design, where applicable, to better mimic these conditions *in vitro*. This can be done in a physiologically relevant manner by strictly regulating the size of the microtissue.

Hypoxia and the tumor microenvironment

Neoplastic angiogenesis is an essential process in tumor progression and the initiation of metastasis [15]. The phenomenon of angiogenesis comprises a series of linked and sequential steps that finally leads to the development of a neovascular blood supply to the tumorous tissue [16]. Angiogenic growth factors secreted by infiltrating immune cells, adjacent stroma and tumor cells themselves bind to specific receptors on endothelial cells which leads to endothelial cell proliferation, migration and invasion, eventually culminating in capillary formation.

Angiogenesis regulation by hypoxia is an important homeostatic mechanism that links vascular oxygen supply to metabolic demand. Lately, molecular characterization of angiogenic pathways, establishment of HIFs as their key transcriptional regulators and the identification of hydoxylases that regulate HIF corresponding to the oxygen availability have provided novel insights into this process [17]. Hypoxia in the tumor microenvironment is of specific importance in drug discovery and development because optimal oxygenation is a primary requisite for many chemotherapeutic drugs, such as alkylating agents (melphalan), antibiotics (bleomycin) and podophyllotoxins (etoposide) to act at their maximum efficiency [7]. Most alkylating agents act by transferring alkyl groups to DNA during cell division, following which the DNA strand breaks or cross-linking of the two strands occurs, preventing subsequent DNA synthesis [18,19]. Hypoxic conditions can lead to resistance against these drugs directly by increased production of nucleophilic substances, such as glutathione, which might compete with the target DNA for alkylation, subsequently reducing the drug efficacy [20]. Another class of anticancer agents acts at specific phases of the cell cycle. As hypoxia causes the cell cycle to slow down or lead to pre-S-phase arrest in extreme conditions [21], it can also indirectly affect the apoptotic potential of these agents. Also, hypoxia leads to changes in the genome that can confer growth advantage to cells with p53 mutations [22] or deficient in DNA mismatch repair [23], while inhibiting

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TABLE 3

Genes induced by hypoxia

Biological function	Gene (abbreviation)	Refs
O_2 transport and iron metabolism	Erythropoietin (Epo)	[7]
	Ferritin (FTH1, FTL)	[7]
	Heme oxygenase-1	[7]
	Transferrin	[7]
	Transferrin receptor (Tfr)	[54]
	Ceruloplasmin	[55]
Angiogenesis	Vascular endothelial growth factor (VEGF)	[7,56]
	VEGF receptor-1	[7]
	Cyclooxygenase (COX)-2	[7]
	Leptin (LEP)	[57]
	Endothelin-1, -2	[7]
	Fibroblast growth factor (FGF)-3	[7]
	Angiopoietin-4 (Ang-4)	[58]
	Nitric oxide synthase (NOS)	[7]
	Placental growth factor (PlGF)	[7]
	Transforming growth factor (TGF)-α	[59]
	TGF-β1	[7]
	TGF-β3	[7]
Matrix metabolism and coagulation	Metalloproteinases	[7]
	Matrix metalloproteinase (MMP)-13	[7]
	Plasminogen activator inhibitor-1	[7]
	Urokinase receptor	[7]
	Collagen prolyl hydroxylase	[60]
	α-Integrin	[7]
Glycolysis and glucose metabolism	Adenylate kinase-3	[8]
	Aldolase-A,C (ALDA,C)	[8]
	Carbonic anhydrase-9 (CA-9)	[61]
	Enolase-1 (ENO1)	[8]
	Glucose transporter-1,3 (GLUT1,3)	[7,62]
	Glyceraldehyde phosphate dehydrogenase (GAPDH)	[8]
	Hexokinase 1,2 (HK1,2)	[63]
	Lactate dehydrogenase-A (LDHA)	[8]
	Pyruvate kinase M (PKM)	[8]
	Phosphofructokinase L (PFKL)	[8]
	Phosphoglycerate kinase 1 (PGK1)	[8]
	6-phosphofructo-2-kinase/gructose-2,6-bisphosphate-3 (PFKFB3)	[64]
Transcription factors	Hypoxia-inducible factor (HIF)-1α	[7]
	HIF-2α	[7]
	Activator protein (AP-1)	[7]
	Jun	[7]
	Nuclear factor-κB (NF-κB)	[7]
	Insulin-like growth factor (IGF) binding protein-1,-2, -3	[7]
	Cyclic AMP responsive-element-binding protein (CREB)	[7]
Drug resistance	Multi-drug resistance (MDR1)	[7]
Apoptosis	Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNip3)	[65]
	Nip3-like protein X (NIX)	[66]
Growth factors and/or cytokines	Insulin-like growth factor-2 (IGF-2)	[7]
	Platelet-derived growth factor (PDGF)	[7]
	Adrenomedullin (ADM)	[8]
	Interleukin-6 (IL-6)	[67]
	Interleukin-8 (IL-8; CXCL8)	[67,68]
	Tumor Necrosis Factor α (TNFα)	[67]
	Macrophage inflammatory protein 1-α (CCL3; MIP-1α)	[69]
	Macrophage inflammatory protein 2 (MIP-2; CXCL2)	[11]
	Stromal cell-derived factor 1 (SDF-1; CXCL12)	[70]
	Chemokine (C–C motif) ligand (CCL5; RANTES)	[71]

apoptosis, which also contributes to their therapeutic resistance.

Screening of such drug compounds on the aforementioned Type I and II 3D platforms, that produce microtissues of variable sizes, can lead to skewed responses because the compounds might be selectively effective against a certain population of cells. For such compounds, Type III platforms seem to be the more obvious choice, but the size cut-off that is meant for this particular application must be carefully chosen. Also, the difference between hypoxic cancer cells and normal cells provides a novel target upon which cytotoxic drugs can be designed. These drug designing strategies include: targeting the HIF-1

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transcription factor (thioredoxin-1 inhibitors – pleurotin, PX-12; [24]), hypoxia-selective gene therapy, pro-drugs activated by hypoxia (AQ4N, NLCQ-1) and the use of recombinant obligate anaerobic bacteria (non-pathogenic clostridia) [25]. As such, these novel strategies require a consistent population of hypoxic microtissues to be tested upon. One might argue that culturing cells under reduced oxygen pressure or adding a hypoxia-mimetic agent, such as desferrioxamine mesylate (DFX; iron chelator) to the media can produce hypoxic growth conditions but this might not recreate the natural oxygen and nutrient gradients that are associated with the tumor microenvironment and a 3D culture of larger hypoxic microtissues might be a better emulation of the *in vivo* situation, rendering the platforms that do not provide control on size or produce microtissues below the required size, less desirable for such applications. Recently, a report published by Rohwer and Cramer [26] listed several studies where hypoxic conditions and HIFs have been directly implicated in chemotherapeutic resistance leading to inefficacy of drugs along with the resistance phenotypes and the molecular mechanisms underlying the resistance. Had the potency of these drugs been validated on 3D microtissues of a particular size that incorporated a hypoxic core, it is plausible that they might have been rejected during the initial phase of discovery and this would have saved considerable resources.

Hypoxia and stem cell niches

The availability of reliable cell types is the primary concern in the successful development of therapeutics against cellular targets in the drug discovery process. These cells are typically obtained from primary tissue, immortalized tumor cells or genetically transformed cell lines. Compared with primary and immortalized cells, stem cells are more advantageous as they are genetically normal and can be maintained in culture for a longer time, increasing their applicability in the screening process. However, to fully exploit their potential, successful and consistent protocols need to be developed for their renewal and maintenance and also differentiation into committed lineages. Recently, many studies have shown that 3D cultures consistently outperform 2D monolayer cultures in terms of promoting stem cell growth, differentiation and development of complex physiologically relevant structures and functionality. When compared with 2D, embryonic stem cells (ESC) grown in 3D cultures, differentiated into hepatocytes that had closer resemblance to their *in vivo* counterparts in terms of morphology, gene expression and biological behavior [27,28]. Similar results have been obtained with 3D cultures of stem cells differentiated into cells of neural [29–31], epithelial [29], endothelial [29,30], chondrogenic [30,32], and hematopoietic [33] lineages. Moreover, 3D cultures have also been shown to sustain long-term self-renewal of human ESCs, maintaining them in undifferentiated state, preserving their normal karyotype, and conserving their differentiation capacity as indicated by embryoid body formation [34]. Considering an increasing shift from the usage of traditional cultures towards 3D platforms for maintaining or differentiating stem cells to generate complex models of particular tissue types for drug testing, the hypoxic conditions associated with the 3D microenvironment should be taken into account as oxygen tension has been known to influence stem cell quiescence, proliferation and differentiation both *in vivo* and *in vitro* [35]. It has been shown that hematopoietic stem cells (HSCs) are more likely to reside in the low oxygen areas of the marrow, away from blood vessels and hypoxia appears to regulate hematopoiesis in the bone marrow by influencing the survival, metabolism and cell cycle of HSC and also provides protection against oxidative stress [36]. Also, the differentiation capability of bone marrow-derived mesenchymal stem cells (MSCs) is decreased in hypoxic culture conditions along with an increase in Oct-4 expression and telomerase activity, further substantiating the notion that low oxygen tension promotes an undifferentiated state of these stem cells [37]. Similarly, hypoxia also promotes survival, proliferation and maintenance of an undifferentiated state in neural crest stem cells and NSCs [38]. Hypoxic (physiologically normoxic) culture conditions also seem to maintain full pluripotency and enhance embryoid body formation of human ESCs [39] whereas normal environmental oxygen levels lead to a significant decrease in the expression of stem cell markers, SOX2, Nanog and Oct-4 [40]. Furthermore, silencing of HIF-2α and HIF3-α, but not of HIF-1α, also led to a substantial reduction in the expression of the aforementioned pluripotency markers [40] further implicating a role of hypoxia in the maintenance of pluripotency and stemness. Recently, HIF-2α has been shown to bind to the *Oct-4* promoter and induce its expression and transcriptional activity [5], thus hypoxia can contribute to generation of induced pluripotent stem cells (iPSC). As hypoxia has such an important role in determining stem cell fate, it is essential to regulate the size of the microtissue in 3D to either maintain the differentiation capacity of the cells or induce differentiation towards a particular lineage. Therefore a platform that does not maintain a homogenous population of equally sized microtissues might have a mixed heterotypic population of cells, some differentiated while others maintaining their differentiation capability, giving rise to differences in drug response. A mixed population is a physiologically relevant condition; however each microtissue should have the same distribution of each type of cells to generate a valid drug response. If the size of the aggregates is different, the smaller ones might have a higher proportion of differentiated cells whereas the larger ones might be replete with stem cells as their cores become hypoxic and maintain the stemness of the cells.

Concluding remarks

The tale of the spatial and the biochemical microenvironmental factors in 3D cell culture is that they are not mutually independent, because an increase in size leads to a depletion of oxygen and creates hypoxic growth conditions. Most of the commercially available 3D growth platforms either do not regulate the size of the microtissue or restrict it to prevent hypoxia. However, hypoxia is a physiologically relevant phenomenon encountered in avascular tumors and stem cell niches and affects the gene expression profiles of the cells. As such, it should be a key factor in rational design or selection of 3D HTS platforms for preclinical drug discovery. For example, to test the cytotoxic potential of chemotherapeutic agents, it might be more desirable to use hypoxic rather than smaller normoxic microtissues that might be more susceptible to their apoptotic actions and produce false positives. Similarly, for generation of tissue models from stem cells for drug screening, a size cutoff should be chosen according to the state of cells (renewal or differentiation) required for the application. Finally, in future transcriptomic and/or proteomic comparison studies, a marker for hypoxic conditions, such as lysyl oxidase (LOX) should be included or a fluorescent hypoxyprobe, such as Oxylite (Oxford Optonics; http://www.oxford-optronix.com/) should be used to determine whether the cells are hypoxic. Also, a subset of genes (Table 3) dedicated to hypoxia can be established and its hierarchy or the rank assigned to it by clustering programs might suggest the degree to which hypoxia might be responsible for results from differential gene expression studies.

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