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feature

# Is time an extra dimension in 3D cell culture?

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**Time or the temporal microenvironment is a parameter that is often overlooked in 3D cell culture. However, given that the 3D system is a dynamic entity, there exists bidirectional signaling between the cells and their microenvironment and, in time, cells can develop the capacity to modulate their environment. We make this case here by illustrating the relation between the temporal dimension and other microenvironmental parameters and demonstrate how the exogenously incorporated microenvironmental factors (MEFs) can be rendered less significant with time. Such knowledge can guide construct design to make 3D platforms architecturally simpler by eliminating redundancy. We further show that there is a need to establish the point at which the construct is complex enough such that its use yields responses that more closely emulate in vivo outcomes.**

## Introduction

With extensive research and recent developments in the field, 3D cell cultures are being defined as a platform that provides a comprehensive or an *in vivo*-like microenvironment for cells to grow in that elicits complex physiologically relevant (CPR) structural and/or functional outcomes from the microtissue formed in a manner not seen in traditional 2D monolayers. The microenvironment can be broadly classified into three MEFs: or 3Ds namely: (i) chemical or biochemical composition; (ii) spatial (geometric 3D) and temporal dimensions; and (iii) force and substrate physical properties [1–3]. Commercially available 3D platforms provide varying degrees of these exogenous MEFs. However, cells are a dynamic entity and there exists bidirectional signaling between the cells and their microenvironment. This means that it is not only that microenvironmental cues have a profound effect on the behavior of cells, but that the cells also have the capacity to modulate their environment, which in time might render the exogenously incorporated MEFs less significant.

Certain initial cues might be required to initiate microtissue formation, but after cell adaptation, time emerges as the master controller of all the other factors. To elaborate further, extracellular matrix (ECM) adhesion molecules (biochemical factors), such as collagen or laminin, can be incorporated in the platform design; however, in time, the cells develop a physiologically relevant endogenous ECM that might make the exogenous proteins unimportant. This is further supported by the fact that cells growing as spheroids in hanging drops that lack biochemical factors exhibit similar complex functional and structural characteristics. Similarly, cytokines that are essential for cell growth and functionality could be incorporated exogenously in the system as another biochemical cue to achieve more platform complexity; however, this might be redundant, because the cells can produce cytokines themselves in a time-dependent manner. This switch from the microenvironmental regulation of cells to cell modulation of the microenvironment might be the stepping-stone towards the generation of a fully functional and structurally similar autonomous 3D microtissue. However, when this transition occurs is unknown, which makes it important to analyze cell behavior in 3D in a time-dependent manner. This would provide a design principle that could lead to the development of a construct with a relatively simpler architecture that still elicits complex functionality from the cells growing within it.

As with other MEFs, the temporal factor is a relative entity and the optimum time depends on the application. For example, in the field of

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regenerative medicine, a higher degree of *in vivo* emulation might be necessary because the construct is meant for implantation, which 'intuitively' means a longer culture time. By contrast, for drug discovery applications, emulation of structural and functional characteristics to the level found *in vivo* might not be necessary. However, the nature of the relation between time and tissue complexity has not been clearly established and the field still lacks consensus as to when a 3D construct is *in vivo*-like enough to be used for the desired experimentation. Observation of cell behavior with respect to the temporal dimension might shed more light on this relation.

## Time as a modulator of the 3D microenvironment

As mentioned above, an 'outside-in' as well as an 'inside-out' signaling dynamic exists between the cells and the microenvironment. Changes in the microenvironment are recognized by cell surface receptors and subsequently manifest themselves through gene expression. However, the resultant expression has the ability to alter the microenvironment itself. As in embryonic development and tissue morphogenesis, complex cell adhesion and differentiation contribute to spheroid formation *in vitro*. The generation of a multicellular spheroid has been described as a three-step process by Lin and Chang [4]. First, ECM fibers with multiple RGD motifs for integrin binding provide the initial cues for rapid aggregation of dispersed cells. This is followed by a delay phase, where upregulation of cadherin occurs and, finally, homophilic cadherin–cadherin binding between cells confers strong intercellular cell adhesion, leading to the formation of a spheroid. Cadherins and ECM fibers are essential for spheroid formation and this requirement is observed in most cell types, although ECM fiber and cadherin dependence can vary from cell to cell. These events can be correlated with the growth kinetics of a spheroid (hepatocellular carcinoma; HepG2) as shown in Fig. 1a,c [5,6], where initially a larger-sized, loosely packed aggregate of cells undergoes compaction, followed by a uniform increase in size resulting from cell proliferation (Days 3–5; Fig. 1a,c).

Perhaps this might be the transition point, where the cells become independent of the exogenous cues and develop into a '3D' autonomous unit capable of creating and modulating its own microenvironment. The microtissue displays consistent behavior in this period and continues in a quasi-steady state until the size reaches a critical limit and oxygen and nutrient (biochemical factors) diffusion limitations lead to the initiation of a hypoxic phase (Days 7–10 for this specific cell density; Fig. 1a). This is particularly important in constructs that cannot impose a physical constraint on the size of the microtissue, such as hydrogels and spheroids (type I and II; [7]). The modulation of microtissue behavior by hypoxia and its subsequent effect on the response to the administered treatment (e.g., chemotherapeutic drug) have been reviewed in detail by Asthana and Kisaalita [8]. As the microtissue undergoes angioadaptation, cell expression of cytokines [vascular endothelial growth factor 9 (VEGF) and interleukin 8 (IL-8)] is upregulated, changing the biochemical milieu (Fig. 1b; [9]). Any cytokines or angiogenic factors included exogenously in the construct design might be rendered redundant at this time point. Therefore, prior knowledge of the growth kinetics of the microtissue and subsequent exogenous incorporation of the essential cues only can make the platform architecture simpler and more cost effective. The same treatment administered at different time points during the microtissue growth cycle can elicit varied responses and their interpretation with respect to the physiological state of the spheroid would be more meaningful.

With increasing culture time (past day 5; Fig. 1c; [6]), cell aggregates mature and form solid spheroids coated with an ECM protein layer, appearing as a smooth surface with individual cells no longer recognizable. This shows that changes in the short-range biochemistry of the tissue microenvironment are also time dependent. Given that cells have the ability to develop a physiological ECM themselves, growing them on a 'cell-derived' ECM platform [10] would be an unnecessary labor-intensive and cost-ineffective step, at least for high-throughput sequencing (HTS) drug discovery applications. However, cells do require certain minimal initial adhesion cues to coalesce and form a compact structure. As stated above, long-chain ECM fibers with multiple RGD motifs for integrin binding can fulfill this requirement. It is already known that fibronectin (FN) is rich in the RGD sequence that acts as a primary ligand for integrin $\alpha5\beta1$ adhesion. Evidence for FN being the major component required for compact spheroid formation and activation of fibroblasts comes from a study by Salmenpera *et al.* [11]. In this study, cells lacking in FN expression ($FN^{-/-}$) or having a mutated ligation site for integrin $\alpha5\beta1$ (FNRGE/RGE) were found to be loosely adherent. Also, FNRGE/RGE cells showed reduced accumulation of endogenous FN compared with wild-type cells, but no such decrease in accumulation of FN was evident in 2D cultures. This suggests that the formation of compact spheroids and accumulation of FN was RGD dependent and is more dependent on RGD–$\alpha5\beta1$ integrin interaction in 3D than in monolayers. Moreover, in the same study, treatment with anti-$\alpha5$ and anti-$\beta1$ (but not anti-$\alpha$v) antibodies also led to loosely formed spheroids and lower accumulation of endogenous FN. Furthermore, integrin interaction with FN also promotes intermolecular association between the FN dimers, leading to the formation of fibrils, which further increases cell adhesion and cohesivity. Additional evidence comes from the fact that FN monomers failed to support aggregate formation [12] and this supports the concept that FN matrix assembly might contribute to aggregate cohesivity by creating a scaffold, or an organized 3D matrix, which functionally links the cells to each other. One might argue that cell adhesion might be biased towards RGD ligand by exogenous incorporation of FN. However, this is not the case, as evident from culturing cells on cell-derived matrices, where adhesion was found to be solely dependent on integrin $\alpha5\beta1$ [10]. Intuitively, it was predicted that the set of integrin involved in 3D matrix adhesions would be complex because of the presence of multiple molecules in the physiological matrix; however, this was not the case in this instance.

With the development of the endogenous ECM, the exogenous biophysical environment also becomes less significant because the ECM provides a physiological milieu for the cells to reside in. This is further substantiated by the fact that microtissues growing on platforms providing different pliability elicit similar CPR outcomes from the cells, as reviewed by Asthana and Kisaalita [7]. The reason for this observation can be traced back to the steps involved in microtissue formation, where, following the initial integrin–adhesion ligand binding, cadherin-dependent intercellular cohesive forces become more dominant than the cell–substrate interaction. Therefore, with increasing time, and as cohesion between the cells becomes stronger and the microtissue becomes more compact, the tissue might lose any dependence it might initially have had on the exogenous biophysical factors.

## Relation between time and CPR

For a 3D construct to be meaningful, it should produce responses that are physiologically relevant (structural and/or functional; CPR); that is, they are known *in vivo* but are absent in 2D formats. CPR outcomes for cells derived from the three tissue types of most interest in preclinical drug discovery (epithelial, neuronal, and cardiac)

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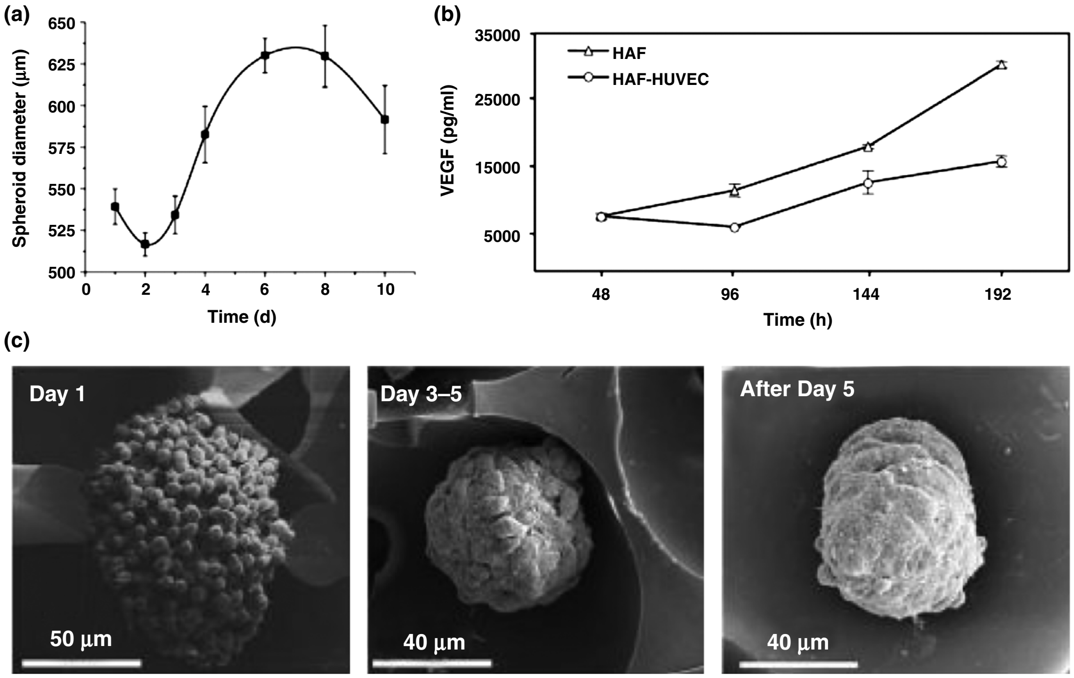

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**FIGURE 1**

Effect of time on the spatial **(a)**, biochemical **(b)**, and biophysical **(c)** microenvironmental factors. After initial compaction, the tissue grows in size until it is limited by oxygen diffusion (a). The production of cytokines also increases with time as the tissue emulates *in vivo* conditions (b). Individual cells aggregate, surface morphology gradually changes, and, with increasing culture time, the deposition of extracellular matrix (ECM) increases, and it becomes hard to distinguish individual cells in the spheroid (c). *Abbreviations*: HAF, human aortic fibroblasts; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor.

have been discussed in detail by Asthana and Kisaalita [7]. Intuitively, this complexity increases with time; however, a consensus regarding the time point at which the construct is complex enough for use has not been established. The temporal dynamics of CPR outcome in hepatic and muscle 3D tissue constructs is discussed below.

### *Hepatic cells*

Immortalized liver epithelial cell line (QSG-7701) cultured as spheroids in Matrigel showed bile canaliculi formation that led to the development of polarized acini with time (past day 5; Fig. 2a [13]). This phenomenon can be considered a marker of structural CPR. However, the question is whether the status of the construct before this time point can be considered '3D' or physiologically complex. Structural CPR is an end-point that is either observed at a particular time point or not and, given the lack of a non-invasive quantifiable marker that could correlate with or predict its expression, it is difficult to judge the state of the microtissue before this point. Moreover, once the microtissue expresses structural complexity, its behavior with time is not known. As stated above, once structural CPR is realized, the tissue might continue to exhibit it but other changes that might lead to alterations in complexity are not generally translated into observable phenomenon. A biomarker of three dimensionality, as discussed by Lai *et al.* [14] can translate CPR into a quantifiable entity and can also act as an indicator of complexity throughout the culture period and not only the end point. Knowledge of the point at which the microtissue is at its optimal performance would help decide when to best use the construct in experimentation and that would make the response from cells more meaningful. Interestingly, the secretion of albumin, which is considered a functional CPR outcome for cells of hepatic origin, has been shown to decrease with culture time in various 3D platforms [Fig. 2b, nanofibrous poly-L-lactide (PLLA) [15]; Fig. 2c, Matrigel [5]]. If albumin secretion is correlated with *in vivo* emulation, then a decrease in the albumin content with time can be interpreted as an indicator of decreasing complexity. As the data from structural and functional studies conducted on 3D platforms point to the contradicting temporal–complexity dependence (Table 1), a consensus for establishing an optimal time point for platform use in drug discovery studies is necessary.

### *Skeletal muscle cells*

Even though physiologically relevant and more predictive *in vitro* models have already been developed for liver, lung, and cardiac tissues [16], a functional model of human skeletal muscle had not been described until recently [17]. Skeletal muscle tissue is of particular importance because it is affected by a variety of dystrophic, metabolic, and neuromuscular disorders that still lack therapy. It is also impaired in diseases including diabetes and obesity, and has been shown to be involved in processes such as inflammation, cancer, and aging [18]. The recall of cerivastatin (a HMG-CoA reductase inhibitor), a cholesterol-lowering drug that was well tolerated in mice but caused rhabdomyolysis in humans [19,20], further exemplified the need for a more predictive preclinical model of the human skeletal muscle.

The effect of culture time on the structure and function of cells is not limited to the hepatic tissue, because it has also been seen in the aforementioned 3D construct of skeletal muscle cells. This study by Madden *et al.* [17] used primary myogenic cells from donor biopsies, cultured at a high density on a hydrogel (Matrigel and fibrinogen; medium containing thrombin)-based platform under dynamic conditions to generate a 3D 'myobundle' for *in vitro* studies of muscle physiology and drug development and screening. The myobundles were able to emulate key structural and functional

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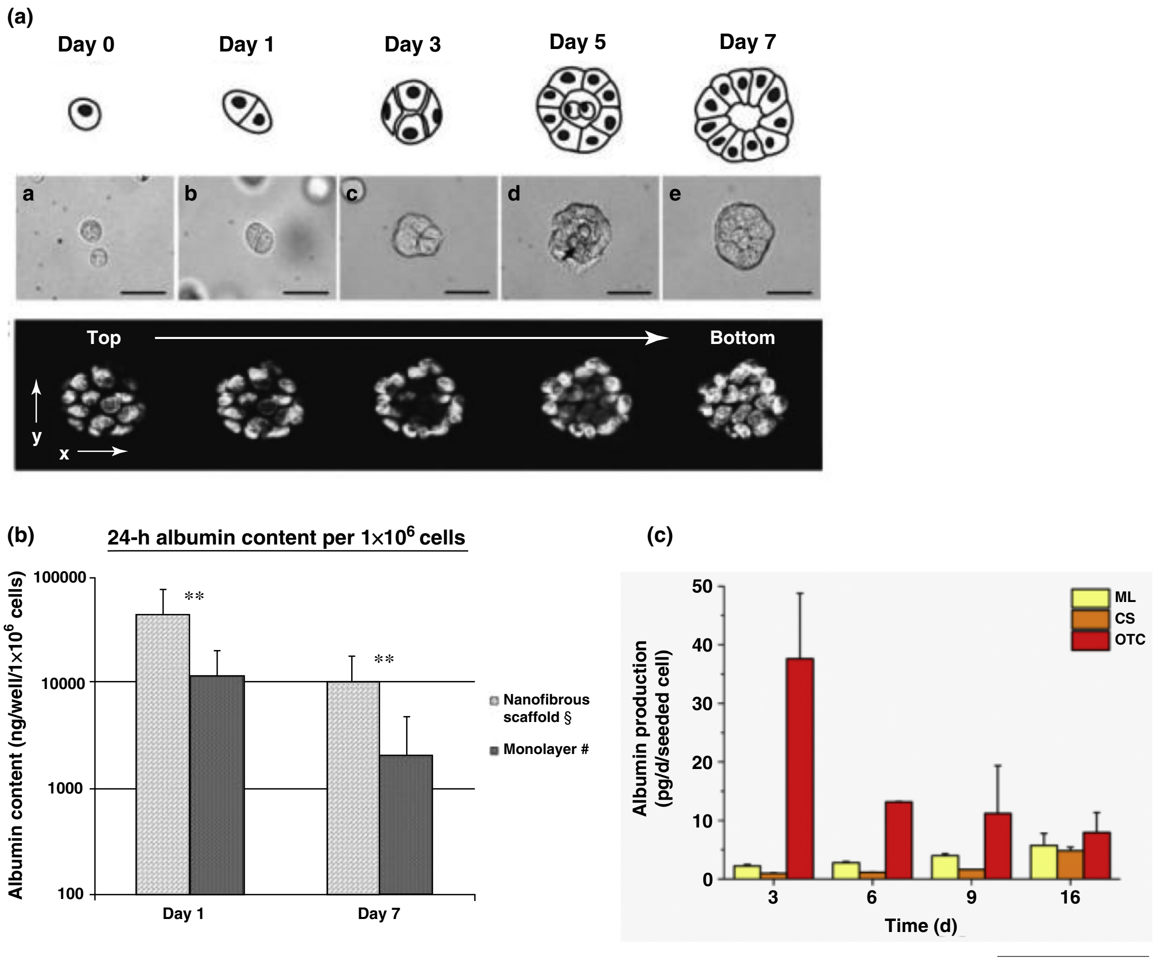

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**FIGURE 2**

Time dependency of tissue formation and albumin production in hepatic 3D cultures. Increases in the structural complexity and development of a polarized acinus by QSG-7701 immortalized liver epithelial cells cultured as spheroids in Matrigel **(a)**; decrease in albumin section per unit of hepatic cells over time, grown on nanofibrous poly-L-lactide (PLLA) scaffold coated with collagen **(b)**, and in Matrigel (OTC; **c**). *Abbreviations*: CS, collagen sandwich; ML, monolayer; OTC, organotypic culture.

**TABLE 1**

**Time profile for the expression of liver-specific structure and function in 3D platforms**[a]

| Cell line and type | Scaffold type and material | Canaliculi | Albumin secretion | | Refs |
|---|---|---|---|---|---|
| | | | Peak time; conc ($\mu g/10^6$ cells/d) | Time of secretion (d) | |
| **Rat small hepatocytes (SHs)** | Stacked layers on microporous membrane | 3–14 | NR[1] | 2–14 | [21] |
| **HHY41** | Alginate beads | NR | 8; 60 | NR | [22] |
| **HepG2 and HHY41** | Alginate beads | NR | 8; 43.83 ± 2.5 | NR | [23] |
| **Lig-8 cell line (adult rat liver progenitor)** | Peptide hydrogel (PuraMatrix) | NA | NR | NR | [24] |
| **HepG2** | Porous PS scaffold | 21 | 7; NR | 1–21 | [25,26] |
| | Hanging drops | NR | 3; 38 | 3–16 | [5] |
| **Primary rat hepatocytes** | Nanofibrous/porous PLLA scaffold | 1–7 | 1; 47.21 ± 33.2 | 1–7 | [15] |
| **Primary rat hepatocytes, HepG2** | PMMA or PC polymer scaffold | NR | 6; 29 | 3–14 | [27] |
| | Spheroids | 3 | NA | NA | [28] |
| | Chitosan-collagen coated PET mesh scaffold | NA | 2; NR | 2–6 | [29] |

[a] NA, not applicable; NR, not reported; PC, polycarbonate; PET, polyethylene terephthalate; PMMA, poly(methyl methacrylate); PS, polystyrene.

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phenomena (CPR), which are observed in the native tissue, and their expression was found to be dependent on time. The myobundles matured with time, which was apparent from the progressive increase in myofiber diameter (13.5 ± 1.5 Mm and 21.8 ± 2.8 Mm at 1 and 4 weeks of culture). The maturation also led to an increase in the expression of muscle-specific proteins [myosin heavy chain (MYH), sarcomeric alpha-actinin (SAA), and muscle creatine kinase (MCK)], whereas the length and number of myofibers remained relatively constant with time. Furthermore, the functionality (contractile force) of the construct was also shown to increase with time. The amplitude of spontaneous and as well as electrically stimulated contractions (both twitch and tetanus forces) generated by the myobundles increased over 4 weeks in culture. Moreover, the myobundle platform was shown to phenocopy the clinical response to cerivastatin. The cells displayed a high sensitivity towards the drug and showed progressive weakness and lipid accumulation at a high drug concentration. The mechanism of action was also observed to be similar to that seen *in vivo*.

## Concluding remarks

In a previous 3D ‘Cartesian’ model of the three microenvironmental factors- Spatiotemporal, biophysical and biochemical - time and space were combined as one of the axes. In light of the foregoing discussion, a more representative model is one in which space and time are separated and each of the other variables is represented as a function of time. Taken together, time is a ‘dimension’ in its own right and we submit that the data presented above support the fact that time is the fourth dimension in 3D culture.

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