3D Dynamic Cell Culture Systems

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The traditional two-dimensional (2D) cell culture methods have a long history of mimicking in vivo cell growth. 3D cell carriers have been gradually developed to provide a 3D matrix-like structure for cell attachment, proliferation, differentiation, and communication in static and dynamic culture conditions. 3D cell carriers in dynamic culture systems could primarily provide different mechanical stimulations which further mimic the real in vivo microenvironment.

Keywords: 3D cell culture ; mechanical stimulation on cell behavior ; bioreactor ; cell interactions ; drug testing ; tissue engineering ; regenerative medicine

1. Introduction

Since Harrison Ross first carried out in vitro cell culture using a sterile coverslip in 1906 [1], the era for cell culture began. Nowadays, the cell culture technique is one of the most common techniques in many fields of biomedical sciences, from basic research to large-scale industrial production of biological products. It offers an efficient approach to achieving different purposes without using animals.

To culture most of the cell types outside of a living body, artificial devices are usually required to allow the cells to adhere and grow. Glass devices such as coverslips were most commonly used in the first few decades of cell culture history [2]. Later, plasma-treated polystyrene was invented by the Falcon Plastics Company and showed excellent properties for cell adhesion and growth [3]. More recently, plasma-treated polystyrene has dominated the research consumer market with different configurations designed for various research purposes, such as flasks, dishes, and plates. These cell culture devices allow adherent cells to grow in a monolayer on a two-dimensional (2D) planar surface under static conditions. With the established techniques in cell seeding, subculture, cryopreservation, and harvesting, it is convenient to perform in vitro experiments, typically using a single cell type, in a 2D and static environment at a relatively low cost. Therefore, such 2D and static monoculture have become the major approach for cellular biology investigation and drug testing on the laboratory scale.

Other 2D culture devices, such as the transwell system [4], have been developed to satisfy the demand for studying cell interactions via co-culture of two types of cells and individual analysis of multiple cell types. The transwell system includes an insert with a microporous membrane and a traditional 2D cell culture plate. While one type of cell can be seeded on the microporous membrane, the other type can grow on the companion well, forming a co-culture system without direct contact between the two types of cells. This approach is widely used to study cell–cell interaction through paracrine or endocrine [4][5][6]. Alternatively, two types of cells can be seeded on two sides of the membrane, forming a direct cell-to-cell contact co-culture, which is suitable for investigating barrier-structured tissues, such as the intestinal barrier [7], the blood–brain barrier [8], the blood–placenta barrier [9], the alveolar–capillary barrier [10], and the vascular model containing endothelial and vascular smooth muscle cells [11]. In both non-contacting and contacting settings, two types of cells share the cell culture medium and the secretome with each other. Although the transwell system has been used extensively, certain limitations exist; for example, it does not allow the individual analysis of more than two cell types, it limits controlling the cell ratios in the setting, and it is relatively expensive.

Despite the convenience and the extensive use, these traditional 2D and static culture devices have been questioned recently regarding their in vivo relevance because in a living body, cells are grown in a three-dimensional (3D) and dynamic, rather than a 2D and static, environment. Studies suggest that cells cultured in a 2D [12][13][14][15] or static [16][17] environment may lose some cell-specific properties observed in vivo, including morphology, polarity, differentiation, and metabolic profile. Cells grown on a standard cell culture plate have a more flattened shape because of the static culture method and a single growth direction [18]. Cell structure changes can influence nuclear morphology, which may alter the transcription and translation of genes [19]. It is also shown that a single cell can only interact with the same type of cells surrounding it when grown in a culture plate, which may be detrimental to the differentiation process [20]. This can explain

why many new drugs show effectiveness in 2D and static cell culture systems in vitro but fail in further clinical trials [21]. A more realistic and vivid cell culture system is considered to benefit pharmaceutical development and toxicity tests [22]. Despite the lack of relevance to the in vivo condition, 2D and static cell cultures have other limitations for industrial applications. For example, in the large-scale production of cell protein or stem cells, the 2D culture has some limitations, such as surface-to-volume ratio and a lack of monitoring and control of critically metabolic parameters [23].

New cell culture systems that allow cells to grow in a 3D environment have been developed to overcome these shortcomings in recent years. In addition, based on the 3D culture model, a dynamic cell culture system has been designed to provide a more reliable cell growth environment in vitro. Firstly, a dynamic cell culture system can offer a continuous dynamic environment which is able to mimic cell growth conditions in vivo to promote cell proliferation and differentiation [24]. Besides, the dynamic culture method can help researchers to study the effects of physical stimulation during cell growth [25]. The third beneficial effect of dynamic culture is that different research purposes can be satisfied by adjusting the frequency, range, and period of the dynamic model [24]. The current 3D culture systems can be mainly categorized as (a) static 3D culture systems such as an extracellular matrix (ECM)-mimicking scaffold, which provides an ECM-like 3D environment for cell growth; (b) microcarrier and bioreactor-based systems, which provides a high surface-to-volume ratio suitable for large-scale production in a 3D and dynamic environment; and (c) organ-on-a-chip, a system combining various settings of cell culture platforms and microfluidics devices, which can provide 3D and dynamic environments and allow for multicellular co-culture and individual cell type analysis. With the development of such new cell culture devices, the application expands from culturing cells in vitro to serving as a platform to simulate the in vivo cellular interplay of specific organs/tissues.

2. Effects of 3D and Dynamic Culture Environment on Cell Behaviors

In a traditional 2D cell culture system, the lack of a suitable 3D background environment and structural framing will influence cell behavior. For instance, normal epithelial cells always lose their differentiation ability and perform like cancer cells when they grow as 2D monolayer cells. Cells growing in a 3D environment show different behaviors in various aspects: morphology [26], proliferation [27], function [28], etc. In addition to the 3D environment, cells in vivo live in a dynamic environment that encounters continuous mechanical stress derived from the blood flow, interstitial flow, and body movement. Currently, regardless of the 2D or 3D environment, most cell culture approaches can only enable the cell to grow in a static environment without mechanical stimulation. Cells growing in static environments lack mechanical stimulation, which is an indispensable factor in modulating cell behaviors along with chemical stimulation. Cells in vivo can experience multiple mechanical force types, such as tension, pressure, and shear force, which significantly change cell behaviors. For example, myocardial cells grow under periodic tension from heartbeats [29]. Hemocytes, as a part of blood, flow across different blood vessels and are under shear force all their lifetime [30]. The transitional epithelium of the bladder receives pressure from the storage of urine [31]. Therefore, it is necessary to understand the different behaviors of cells growing in 2D and 3D environments and between cells under static and dynamic conditions.

2.1. Cell Behaviors in 3D Culture Environment

2.1.1. Cell Proliferation and Differentiation in 3D Culture

The influence of 2D and 3D cultures on cell proliferation has been extensively studied. In nearly all the cell lines, tumour cells showed a higher proliferation rate in 2D monolayer cell culture than in 3D culture [20]. Research proved that the expression of polarization and differentiation associated of tumour marks integrins (β1 and β4) is higher in tumour cells from 3D culture, which suggesting the proliferation, adhesion, and viability of tumour cells are impaired [32][33][34][35][36]. Similarly, it was reported that endometrial cancer cells growing in a 2D environment had less specific function and organization than in a 3D culture. In another study, a 3D culture system using amphiphilic polydepsipeptides (HYDROX) was found to promote the differentiation of induced pluripotent stem cells (iPSCs) into the hepatic cell. Meanwhile, *CYP3A4*, an important metabolic enzyme-gene, which mostly expressed in human liver, was upregulated in primary human hepatocytes cultured with HYDROX, and these cells showed higher activity compared with those cultured in the 2D system [37]. Cells culture methods also determined the expression of genes associated with cytoskeletal protein, contractility, and matrix remodelling [24]. It was reported that cells growing in a 2D environment showed higher expression of ECM proteins than in a 3D environment [38].

2.1.2. Cell Apoptosis in Cancer Drug Test in 3D Culture

Research showed that, when exposed to anticarcinogen drugs, apoptosis is more likely to occur when breast cancer cells grow in a 2D monolayer culture rather than when they form a 3D cell aggregate [39]. That was mainly because, in 2D culture, the absorption of drugs was not dependent on the gradient of cells, as dead cells would disperse into the medium,

and living cells would always come into contact with the anticarcinogen [40]. Conversely, when cancer cells aggregated into a spheroid, the interior partitions could not fully contact drugs like surface cells, which suggested that inner cells would not be sensitive to the drugs and steadily divide into new tumour cells [41]. In addition, 3D cancer cell spheroids can produce cancer cell-derived ECM [42][43][44].

2.1.3. Cell Motion and Migration in 3D Culture

The migration of cells appears differently between 2D and 3D cultures, since cells may have more complex interactions when they form a 3D aggregate [38]. In 3D cell aggregate, inner cells could hardly migrate towards the outside mainly because their migration is hindered by surrounding cells [45]. This phenomenon is critical in investigating cancer metastasis and other disorders. For instance, culturing in basement membrane extract, fibroblasts migrate much faster in the 3D environment (about 1.3 times than in a 2D environment). Additionally, more migration-associated signal cascades could be found in 3D culture than that in 2D culture. It was reported that β1-integrin could send several interaction signals to epidermal growth factor receptors in 3D cell culture, a phenomenon missing in the 2D environment [46].

2.2. The Effects of Mechanical Force on Cell Behavior/Function

2.2.1. Stretching

Stretching is a common biological phenomenon defined as cells being put into a lengthening position caused by muscular movement or external forces. The stretching here refers to not only muscular but also skin and neuro-guided movement. Cells from these tissues will be stimulated during the stretching process. Cell stretching plays essential roles in both cell proliferation and apoptosis, which depends on the magnitude, frequency, and duration of mechanical extension. For example, in the ulna-loading study, low-strength mechanical stimulation (4000 μ-strain) could relieve osteocyte apoptosis-resulted bone resorption. In contrast, high mechanical stress (8000 μ-strain) caused osteocyte apoptosis and micro-damage of bone tissues [47]. In the clinics, continuous stretching force with different magnitudes and duration has been widely used in orthodontic treatment. This kind of stretching could activate signaling pathways such as p38 MAPK, JNK, and ERK in the human periodontal ligament (PDL), promoting the expression and activity of alkaline phosphatase (ALP), which is an early stage marker of osteogenic differentiation [48]. In regular breath, the stretching of the diaphragm muscular can induce the expansion of alveolar cells during the breath [49]. In burn cases, patients usually wear pressure suits in case of the formation of scar tissues. That is because the stretching of the skin can form a relatively anoxic environment in the burned part and inhibit fibroblasts from producing collagen [50]. In addition, the pull of the muscle causes the excitation of sensory nerve terminals in the muscle spindle, resulting in the motor neuron exciting the impulse to transmit sequentially through the spinal nerve anterior root and spinal nerve to the muscle, causing an opposite directional stretching of the power, termed “myotatic reflex” [51].

2.2.2. Compression

Compression here is defined as cells being squeezed, normally by external forces, to achieve a smaller size or a flatter shape. Compressive force plays a crucial part in the bone remodeling process. Compressive force may initiate osteoclastogenesis during orthodontic tooth movement (OTM) [52]. Consequently, an excessive compressive force would lead to finger-like bone fracture [53]. Besides, under compressive force, TNF-α expression would be induced in periodontal ligament fibroblasts during OTM, directly related to the elevated RANKL expression and consequently resulting in induced osteoclastogenesis [54]. Moreover, in MC3T3-E1 cells (a pre-osteoblast cell line), the osteogenic differentiation could be impaired by compressive force through the ClC-3 chloride pathway and the expression of EphB4 and ephrinB2 [26][55].

2.2.3. Contraction/Relaxation

The contraction and relaxation are the biological forces generated by the intestinal tract which can be helpful to the digestion and absorption of the nutrients. It was reported that the contraction and relaxation of drosophila midgut could promote the transportation of calcium ions and differentiation of intestinal stem cells. Similarly, the human intestine exhibits the same movement to affect the differentiation of stem cells [56].

2.2.4. Shear Stress

Shear stress is the biological force generated by the blood flow on the endothelium, the inner layer of the blood vessel. In vivo, the heterogeneous phenotypes of arterial endothelium cause changes in blood flow patterns. Pulsatile or steady laminar flow could stimulate the endothelial cell (EC) to secrete functional factors and suppress coagulation, supporting EC survival [57]. On the other hand, ECs chronically experience arrhythmic changes in haemodynamic forces and exhibit different a behavior by enhancing cellular turnover (proliferation and apoptosis) and facilitating the adhesion of monocytes onto endothelium [58]. This change suggests a higher risk of function loss and atherosclerotic plaque formation [59]. In an in

vitro study, the flowing culture medium was generated to mimic the hemodynamic shear stress in blood to stimulate ECs. The shear stress could be transformed into biological signals through integrin, which would be received by phosphoinositide 3-kinase (PI3-kinase) to activate the downstream signaling pathways in ECs [60].

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