Commentary

Understanding the cancer/tumor biology from 2D to 3D

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Cancer is now the second leading cause of mortality worldwide. It predicts that there are 595,690 cancer deaths and approximately 1.7 million new cases diagnosed in 2016 in the United States (1). Therefore, great efforts have been made in the cancer treatment. First of all, the cancer biology needs full understanding. Experiments with cell monolayer have allowed the interpretation of complicated biological phenomena (i.e., molecular biology, stem cell differentiation, and tissue morphogenesis) and are the most popular protocol. However, as early as 1972, the differences between cells cultured and grown on a flat surface and three-dimensional formats were explored (2). The cell-cell and cell-extracellular matrix (ECM) interactions are tremendously reduced in the 2D cell culture with the limited mass transport of molecules within the culture environment, leading to unnatural interactions with soluble factors, polarized integrin binding, and mechanotransduction. In 2D cell culture, there is a homogenous concentration of nutrients, growth factors, and cytokines interacting with the surrounding medium. Therefore, there is a large discrepancy in cytotoxicity chemo- and radio-therapy results, oncogenesis, and stem cell differentiation between *in vitro* 2D cell culture and three dimensional (3D) culture or animal models. In 2011, only 12 oncology drugs were approved by the Food and Drug Administration (FDA) despite almost 900 anti-cancer medicines and vaccines were in clinical trials or under review.

Solid tumors are poorly vascularized and result in the decreased oxygen tension towards the center. Oxygen tensions of less than 10 mmHg lead to the formation of hypoxic cells. A phenotype has poor clinical outcomes, tumor recurrence, and diminished sensitivity to chemo- and radio-therapy because oxygen plays a significant role in the production of free radical species for the ultimate DNA damage in radiation (3). Large cellular spheroids (>1 mm in diameter) have hypoxic center with necrotic cells surrounded by living cells, due to nutrient starvation and metabolite toxicity. The characteristic length that balances oxygen and nutrient diffusion and metabolism from capillary blood vessels is typically hundreds of microns. Morphology alone influences the subtle cellular processes such as global histone acetylation as well as proliferation, apoptosis, differentiation, and gene expression (4). To properly study the cell physiology, cells should be cultured in 3D micron-environments recapitulating the important mechanical and biochemical cues in the natural ECM, including 3D topography and mechanical forces (e.g., shear stresses as well as cytokine distribution gradients), while facilitating the hierarchical processes (5). 3D micron-environments can be designed to promote the cell viability, adhesion, differentiation, proliferation, and migration. Histological analysis shows nearly indistinguishable structure of cell spheroids and human tumors.

Several types of 3D micro-environment for cell culture have already been developed. Gels and sponges offer the largest and richest range of 3D structure. Micro-carriers are small spheres, typically less than 500 μm in diameter but with enormous surface area up to 500 cm^2/g, can culture a large number of cells in the small volume (6). Cellular spheroids, self-assembled clusters of cell colonies, are the most popular 3D models because of their simplicity, reproducibility, and similarity to physiological tissues and typically generated by single or co-culture techniques (e.g., hanging drop, rotating culture, and concave plate) (7,8). Embedding cells in the hydrogels is usually applied in bio-printing, but not suitable for establishing cell-dense due to the poor mass transfer and ECM construction. Utilizing inter-cellular polymeric linker as a more natural ECM environment, 3D multi-cellular aggregates could also be

formed *in situ* in microfluidic channels (9).

Whitesides and co-workers developed a new 3D culture system, cells-in-gels-in-paper, to study the cell responses to molecular gradients and evaluate the metabolic response of lung cancer cells to radio-therapy (10,11). Chromatography paper-based scaffolds need fewer steps in the fabrication than the paraffin mesh sheet and use a hydrophobic barrier to prevent the lateral oxygen diffusion. Because paper is thin (a few hundred microns), mechanically robust, and has a large void space (up to 80%) simultaneously, the cells in the paper-supported hydrogels are not limited by the mass transport of nutrients and oxygen or the loss of metabolic by-product. Cellular by-products (e.g., carbon dioxide, lactate, and cytokines) overlap in such setups. Cells at the stack top exchange with the surrounding culture medium as a normoxic environment similar to those near the blood vessel in a tumor. In contrast, cells at the stack bottom cannot contact the culture medium directly, recapitulating the growth-arrested, apoptotic, or necrotic core of the tumor. The paper-based cell culture system has many advantages in comparison to the monolayer and cell spheroids: multi-layered 3D constructs in the thickness of millimeters similar to solid tumors could be produced easily by stacking multiple layers with most of cell-based assays and high-throughput screens; the diffusion-dominated culture environment allows to study the effects of the concentrations of nutrients, waste products, and drugs on cells simultaneously; each single layer may have different types of cells and hydrogels; all the layers can be sectioned easily and isolated precisely without perturbation on the viable cells and histology procedure for quantitative analysis of metabolic activity optically or through the use of enzymatic assays; the effects of proliferation and migration could be decoupled. Decreasing the levels of oxygen can reduce the proliferation on cancer cells, and consequently, their metabolic sensitivity to radiotherapy. Insensitivity of the cells at the stack bottom is due to the decreased proliferation, which is consistent with the observed response in solid tumors that only cells close to blood vessels have a good response. Meanwhile, its disadvantages include: multiple measurements over time is impossible as the gradients formed in the cells cultured are destroyed; destacking the layers minimizes the influence of diffusion; cells cultured in these "loosely packed layers" have no gradient expression of VEGF and IGFPB3.

3D cell culture is more relevant to cancer/tumor than the counterpart of 2D one so that its development needs multidisciplinary approaches and expertise (e.g., materials science, cell biology, bioreactor, clinical applications, and regulatory practice). Although tremendous advances have been made in the past decades, immediate clinical and commercial expectations are unrealistic. Many complex biological responses (e.g., receptor and transcriptional expression, cell migration and apoptosis) differ significantly from the original organ or tissue. Bioreactors have been integrated with 3D culture production and engineered constructs for the precise and reproducible control over many environmental conditions, such as the temperature, pH, flow rate, oxygen, nutrient supply, and waste metabolite removal. A future design is the reproducible and automated production of tissues with all environmental conditions monitored and controlled simultaneously (12). Autologous cells are favoured for clinical implantation in order to avoid the immune rejection. However, they are not viable or capable of proliferation *in vitro* and not always available. Primary and stem cells, including inducible pluripotent stem (iPS) or embryonic stem (ES) cells, will become the focus of 3D cell culture. A common problem of primary cells is the unavailability or inability of producing sufficient numbers. iPS and ES cells are at their early stages in research, and great effort is required to derive from a neonate-like state to the more mature phenotype. Therefore, the use of progenitor and multipotent stem cells holds great promise (13). The inclusion of other cells (e.g., fibroblasts, endothelium, stromal cells, and epithelium) will provide a better and more realistic carcinoma model. Co-culturing and patterning multiple types of cells in 3D now provides good oncology models, particularly in the fundamental studies of cellular migration and metastasis, heterotypic cell-cell signalling and interactions (7). Quantitatively monitoring cellular responses deep into samples is highly required (14). A popular method for determining the viable cells quantitatively is to measure the rates of turnover of metabolic probes, but it is difficult in the thick 3D cultures because reagent diffusion competes with reagent turnover. In addition, it is impossible to quantify the cell proliferation of the 3D cultures noninvasively (15). Integrating the micro-engineered cellular phenotypes with sensors that can analyse their structure and function by optical, chemical, electrical, or mechanical approach is challenging.

3D cultures have greatly improved cell-based screening at an earlier stage of the drug discovery and revolutionized our understanding of cellular behaviour (16). Moreover, they can reduce the ethically-controversial animal experiment. Replacement of animal models in drug discovery and toxicity experiment is part of the agenda for the humane

handling of laboratory animals. However, adoption of 3D cultures is slow owing to the problems of consistency, scale, and cost (17). Microfabrication techniques are well-suited to create structures with defined shapes and positions on the micrometer scale (13). More efforts are needed to develop more complicated and realistic 3D cultures for biological and pharmaceutic research and applications. "Organs-on-chips" that both support tissue differentiation and recapitulate the tissue-tissue interfaces, spatiotemporal chemical gradients, and mechanical microenvironments allow the investigation of human physiology in an organ-specific context as novel models.

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Footnote

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