3D Matrices for Anti-Cancer Drug Testing and Development

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Until recently, basic research testing the efficacy of novel anti-cancer drugs was performed on cells grown on two-dimensional (2D) glass or plastic platforms. An emerging view finds that traditional 2D cell culture may not accurately mimic the three-dimensional (3D) environment in which cancer cells reside. Specifically, the unnatural 2D environment may provide inaccurate data regarding the predicted response of cancer cells to chemotherapeutics. For this reason, there is now considerable interest in developing 3D *in vitro* systems for testing the efficacy of anti-cancer drugs. Both biologically derived and synthetic matrices have been developed for culture of cancer cells. Some of the most recent work involves co-culture of cancer cells in 3D along with other cells normally found in the tumor microenvironment. This article details the reasons for using 3D matrices for anti-cancer drug development, describes the currently available state-of-the-art matrices, and speculates about future advances in the field of 3D cell culture that soon may bring these technologies to the clinical setting that emphasizes personalized medicine.

Why Use 3D Matrices for Drug Development?

To test the response of cancer cells to anti-cancer drugs the industry standard—until recently—was 2D systems or *in vivo* animal models. Both of these systems have major weaknesses. Drug sensitivity data gleaned from unnatural 2D systems often are misleading, while animal models are expensive, time consuming and present ethical dilemmas. A new type of model system is needed to provide a bridge between relatively easy to use, but sometimes inaccurate, 2D systems and more difficult, but physiologically relevant, *in vivo* systems. *In vitro* 3D systems could provide the bridge for this gap.[1]

The two most common forms of 3D cell culture systems are the prefabricated scaffold and the hydrogel. Scaffolds can be made from natural or synthetic materials and, through the use of various production techniques, can be engineered with diverse pore and fiber sizes to allow cells to migrate and grow within the network of the scaffold.[2,3] A hydrogel is a biologically compatible (biocompatible) polymer network with a high water content and physical properties that closely mimic the natural extracellular matrix (ECM). Like the prefabricated scaffold, hydrogels can be made of either natural or synthetic materials and can be produced with various pore sizes.[4] Cancer cells can be cultured either in or on a hydrogel.

Recent research has demonstrated consistently that cells cultured in 3D show different behavior and expression profiles compared to cells cultured on 2D. The behavior and expression profiles shown in 3D culture may better reflect cancer cells in their native, *in vivo*, environment.[5] In *vivo*, both the ECM and the mechanical properties of the 3D environment affect the behavior and gene expression of the cancer cells. *In vitro* 3D systems can provide similar ECM cues and mimic the mechanical environment of the *in vivo* cancer microenvironment.[6] Accordingly, several striking differences are observed when cells, previously cultured on 2D, are moved to 3D cultures.

- *Tumor cells in 3D adopt a different morphology than on 2D* (see Figure 1). While cancer cells on 2D adopt an unnatural spread morphology, cancer cells in 3D adopt a clustered, rounded morphology which is reminiscent of tumors *in vivo*.[7,8]

- *Tumor cells cultured in 3D grow more slowly when compared to the same cells cultured on 2D*.[9] The growth rate in 3D better reflects mathematical models of tumors *in vivo* than does the growth rate of cancer cells on 2D.[10]

- *Tumor cells also show increased glycolysis in 3D*[11] *and often display a different gene expression profile*. Differentially regulated genes include those responsible for angiogenesis, such as vascular endothelial growth factor (VEGF);[12,13] chemokines, such as interleukin-8 (IL-8);[14] and genes responsible for cell migration and invasion, including Rho GTPases[15] and focal adhesion kinase (FAK).[16]

- *Cancer cells cultured in 3D also show differences in anti-cancer drug sensitivities when compared to 2D culture*. Several research groups have shown that culturing cancer cells in 3D makes them resistant to some chemotherapeutics.[17,18] Others have shown that culture in 3D matrices either sensitizes or desensitizes cancer cells to anti-cancer drug treatment, depending on the cell and/or drug type.[7,19] These sensitivity differences seen in 3D culture may be representative of the way cancer cells *in vivo* respond to chemotherapeutic treatment.

In addition to evidence that cancer cells cultured in 3D may respond to drug treatment more like cancer cells *in vivo*, practical reasons exist for testing anti-cancer therapeutics in 3D systems. Many types of cancers, particularly the largely untreatable bone metastatic varieties, adhere very poorly to 2D plastic cell culture surfaces. This poor adherence makes many bioassays very difficult because washing steps cause the cells to detach from the surface and be lost.[7] Additionally, other non-solid cancer cell types, such as leukemia and other hematopoietic malignancies, often are non-adherent and, therefore, are grown in suspension culture. For these non-adherent cells types, 3D cell culture techniques may provide an alternative to suspension culture.[20] Finally, the effects of a drug on neighboring, non-cancerous cells can be tested using co-culture model systems, in which endothelial, stromal, and/or epithelial cells are introduced in the
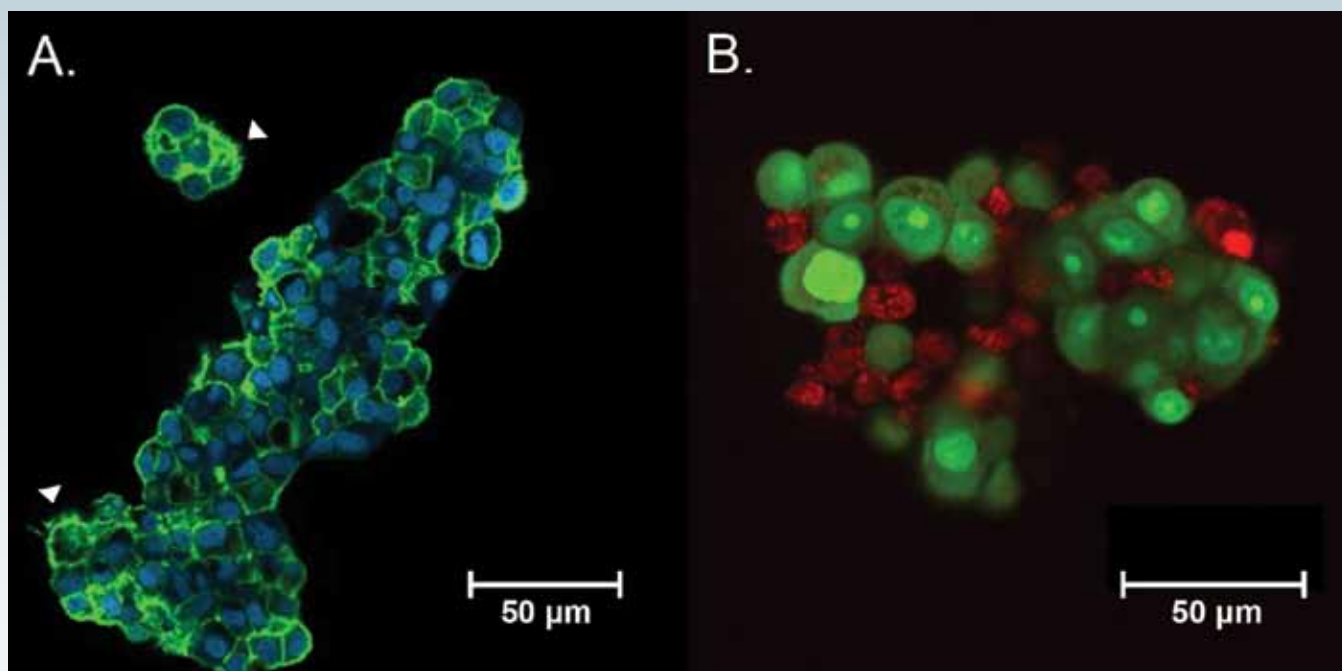
Figure 1. Prostate cancer cells cultured in 3D can be used to test anti-cancer drugs.

A. Confocal microscope images of cells cultured in a 3D hyaluronic acid hydrogel. Cells were stained for cytoskeletal structural actin (green) and nuclei (blue). White arrows indicate cancer cell invasion of the 3D matrix.

B. Confocal images of cancer cells cultured in a 3D hyaluronic acid hydrogel and treated with a chemotherapeutic drug (camptothecin). Cells were stained for dead (red) cells and live (green) cells to show cell killing.

same culture with cancer cells.[21] For more on the advantages and uses of 3D co-cultures, see discussion on page 23.

Three-dimensional matrices fall into three broad categories: 1) biologically derived matrices, 2) synthetically derived matrices, and 3) biologically inspired synthetic matrices or hybrids.

Biologically Derived Matrices

The most commonly used 3D matrices are derived from the ECM of biological sources. In addition to providing the mechanical cues that affect cell behavior, gene expression, and drug sensitivity, constructing 3D matrices from ECM molecules has the advantage of the 3D matrix itself initiating signal cascades in the tumor cells. Because ECM molecules themselves can function as bio-active molecules known as ligands for a number of cell surface receptors and integrins, a matrix composed of ECM molecules can activate downstream target proteins through its interaction with receptors and integrins.[22]

The most commonly used biologically derived matrix is BD Matrigel™, a mouse-tumor-derived basement membrane that is liquid at cold temperatures and solidifies at 37°C. BD Matrigel™ contains all of the common ECM molecules found in basement membrane (i.e., laminin, collagen IV, perlecan, and nidogen/entactin) and has the advantages of being relatively easy to use and commercially available.[23] Because it mimics an *in vivo* basement membrane, Matrigel™ is best used for cancer cells that still resemble those residing in epithelial tissues.[22] The ECM components of Matrigel™ activate various signaling pathways in cancer cells that control angiogenesis,[24,25] cancer cell motility,[26] and drug sensitivity.[27]

To model stromally derived or bone metastatic cancers, networks constructed from collagen I or gelatin are popular. Collagen I is a common ECM molecule found in stromal compartments and bone. Gelatin is simply a denatured form of collagen I. Collagen I and gelatin can be isolated from various biological sources including bovine skin, rat tail, and human placenta. Both materials also are available from multiple commercial sources.[28] Collagen I and gelatin can form gels[29] or can be electrospun into membranes,[30,31] either of

which can support 3D cell growth. Additionally, collagen I's interaction with integrins affects gene expression.[32] Target genes include those that alter production of matrix metalloproteinases (MMPs), enzymes that degrade ECM components allowing for cancer invasion,[33] cell sensitivity to anticancer drugs,[34] cell proliferation, and migration.[35,36]

Hyaluronic acid (hyaluronan or HA) is an increasingly popular biologically derived matrix.[7,17,37] HA is a ubiquitous ECM component, making 3D gels of this type suitable for nearly any cancer cell type. Most commercially available HA is of bacterial origin, causing it to be of very pure, homogeneous quality and devoid of eukaryotic growth factors that can complicate experimental results. HA also is easier to chemically modify than many other ECM molecules, allowing for more extensive engineering options in the crosslinking chemistry (i.e., the way in which the molecules are connected).[38,39] HA signals through its receptors, cluster designation 44 (CD44) and receptor for hyaluronan-mediated motility (RHAMM) to activate a variety of pathways including those affecting cell motility and drug sensitivity.[37,40,41]

The biggest advantage of biologically derived matrices is that they are more biologically relevant than their synthetic counterparts. As described above, the natural ECM molecules activate various signaling pathways that affect cell behavior and drug sensitivity. Therefore, the behavior of cancer cells in biologically derived 3D matrices may reflect better the *in vivo* cell behavior than does that of cells grown in synthetic 3D matrices. The disadvantages of biologically derived matrices stem from the natural heterogeneity of biological systems. Biologically derived materials can vary in composition or contain vertebrate growth factors, both of which can lead to irreproducible or misguiding results.[38]

Synthetic Matrices

Although less commonly used in 3D cell culture than biologically derived matrices, synthetic matrices have gained popularity because of their chemical purity and flexible engineering options. 3D culture of cancer cells can be viewed as a special case of tissue engineering in which malignant tumors, instead of healthy tissues, are engineered *in vitro*. Consequently, synthetic matrices that are commonly used as tissue engineering scaffolds can be translated readily to the 3D culture of cancer cells.

Poly(D,L-lactic-*co*-glycolic acid) (PLGA or PLG), polylactide (PLA), and poly(ε-caprolactone) (PCL) are biodegradable polymers that have been approved by the Food and Drug Administration (FDA) for biomedical applications, a testament to the biocompatibility of these polymers.[42] Polyvinyl alcohol (PVA) is another biocompatible synthetic polymer that is popular for the production of synthetic scaffolds.[43] Various composites of these materials have been used in tissue engineering applications, including

engineering tumors for drug selection applications.[5,43]

Polyethylene glycol (PEG) hydrogels are one of the most popular synthetic matrices used in tissue engineering. PEG is a type of polyether that is available commercially in a variety of molecular weights. Its advantages include being easy to chemically modify, highly biocompatible, and relatively inexpensive.[44] Unlike pre-formed scaffolds that require a separate step to load cancer cells into the matrix, many PEG-based hydrogels are engineered to allow for cell encapsulation, simplifying the experimental procedures.[45] Various cell types including kidney cells[46] and bone forming cells (osteoblasts)[47] have been successfully grown in PEG hydrogels.

The advantages of synthetically produced matrices include that they are chemically pure, contain no uncharacterized growth factors or other contaminants, and are relatively easy to engineer chemically. The multitude of engineering options provide elegant crosslinking and modification options that allow the scaffolds to be used in a variety of ways. The main disadvantage of synthetic matrices is that they are not as biologically relevant as biologically derived 3D matrices, and do not contain the signaling molecules and sequences which ECM naturally provides.

Biologically Inspired Synthetic (Hybrid) Matrices

This type of 3D matrix combines the biologically derived and synthetic matrix technologies to provide a scaffold that is chemically pure and easily engineered, and also contains the biological cues to allow for cell adhesion and changes in gene expression. While synthetic 3D matrices can provide the mechanical cues to change gene expression, the matrix itself cannot bind cell receptors which initiate signaling cascades and promote cell adhesion. For these reasons, biologically inspired peptides, classically arginine-glycine-aspartate (RGD) peptides, often are linked to the synthetic matrices to promote cell adhesion, migration, and spreading. Newly developed, biologically inspired synthetic sequences have been used for tissue engineering and 3D cancer cell culture as well.

Peptide scaffolds are synthetically prepared peptides produced in biologically inspired sequences. These scaffolds classically were made of peptide sequences that allowed the scaffold to self-assemble under physiologically relevant conditions, permitting cell encapsulation in the scaffold.[48] Popular examples include the self-assembling peptide hydrogel, PuraMatrix™,[49] peptide amphiphiles,[50] and β-hairpin peptides.[51] More recently, there has been considerable interest in incorporating biologically active sequences into the self-assembling peptides, allowing cell adhesion and other biological processes.[52] Cancer cells, among other cell types, have been cultured successfully in peptide scaffolds, and one study has used this type of sys-

*is that they **are more** than their synthetic counterparts.*

tem to test the efficacy of anti-cancer cells in 3D.[34] As one of the newest 3D matrix options, peptide scaffolds likely will show many new developments in the upcoming years.

Hybrid hydrogel materials consisting of synthetic polymers and bioactive peptide segments have gained recent popularity as tissue-engineering matrices.[53,54] These types of hydrogels have the flexible engineering options of synthetic matrices, but also provide biologically relevant signals to the embedded cancer cells due to the bioactive peptide component. For example, hydrogel matrices based on PEG have been engineered to contain adhesive signals and enzyme-sensitive crosslinkers, the functional groups on the PEG that allow for crosslinking. These types of hydrogels allow for cell adhesion, migration, and spreading.[45,55]

Hybrid matrices have the advantage of being chemically pure and easy to engineer while still providing biological signals to the encapsulated cells. While hybrid matrices allow for some integration of biological signaling peptides, these matrices still are lacking when compared to biologically derived scaffolds. Additionally, peptide-based scaffolds cannot be produced in large quantities and can be mechanically weak and prone to dissociation, making them more likely to come apart.

3D Co-cultures

In trying to provide the most physiologically natural environment for cancer cells in which to study drug sensitivity, all aspects of the tumor's surroundings need to be considered. While ECM cues and the mechanical signals from 3D culture provide part of this environment, one major component is still missing. In the patient, a tumor would be surrounded by other cell types, and the ways in which the tumor cells interact with the surrounding normal cells affect the cancer's aggressiveness and response to anti-cancer drugs. For example, stromal cells can induce chemoresistance[56] and encourage metastasis.[57] Additionally, endothelial cells provide the blood supply to the tumor that allow it to grow, but also are responsible for carrying therapeutics to the cancer.[58] To best model the natural environment of a tumor, the surrounding cells should be included as well.

The field of 3D cell culture is particularly amenable to the co-culture of cancer cells with other associated cell types. Advances in 3D cell culture techniques have made growing diverse cell types together much easier. Sometimes the cell culture systems can consist of more than one type of 3D matrix to encourage growth of the different cells. Researchers working with 3D cell culture often are interested in making the tumor microenvironment as close as possible to the *in vivo* environment.[21] Diverse types of cancers have been co-cultured with normal cells, such as stromal fibroblasts,[59] bone cells,[60] endothelial cells,[60,61] neurons,[62] and macrophages.[63]

Future Directions and Applications

To date, papers describing 3D cell culture systems for testing cancer cell drug sensitivity primarily have presented proof-of-principle type data on use of the systems for evaluation of chemotherapeutics. The general content of these papers includes a description of the 3D system and its advantages compared to previously described systems, evidence that anti-cancer drug sensitivity can be tested in the system using previously evaluated therapeutics, and sometimes a brief speculation on why drug sensitivity data may differ between 2D and 3D systems.[7,19,36] The use of 3D cell culture systems for evaluating novel anti-cancer drugs has yet to become mainstream. However, with the scientific community's increasing interest in 3D systems and a number of available 3D matrices validated for drug sensitivity studies, papers describing the efficacy of novel chemotherapeutics likely will be including data derived from 3D cell culture systems along with 2D and animal study results.

While 3D matrices are readily available to most scientific researchers and could be incorporated easily into basic science publications on novel therapeutics, the potential uses for 3D drug sensitivity studies extend far beyond small-scale experiments in research laboratories. Pharmaceutical companies could benefit from using carefully designed 3D matrices to screen novel therapeutics for anti-cancer activity.[64,65] Drug efficacy data of this sort would be more physiologically relevant than data produced from 2D monolayer culture. Additionally, 3D cell culture is inexpensive compared to animal trials.[66] Poorly adherent cell lines routinely are disqualified from high-throughput drug activity screenings used by pharmaceutical companies because of their inability to withstand mechanized washing steps. 3D cell culture encapsulates poorly adherent cells, effectively immobilizing them in such a way that they can be used in mechanized bioassays. Additionally, methods have been developed to culture liver cells (hepatocytes) in 3D matrices to test the liver toxicity of novel therapeutics. Because hepatocytes quickly stop producing drug metabolizing enzymes when cultured on 2D monolayer, culturing these cells in 3D to retain these functions is highly desirable.[67,68]

Another potential use of 3D drug sensitivity trials is in the quickly growing field of personalized medicine. The treatment and prevention of cancer has benefitted greatly from the advent of personalized medicine, with genetic testing of susceptibility genes, such as breast cancer (*BRCA*) 1 and 2 mutations, and detection of the expression of proteins that may make therapies more effective, such as human epidermal growth factor receptor 2 (HER2) expression. Such specialized targeting of certain cancers accounts for the efficacy of trastuzumab (Herceptin®).[69] Using 3D matrices, one could envision a future in which tumor biopsies could be transferred to the laboratory, cultured, and then treated

To successfully translate
that of a community cancer center,
testing core model

with various anti-cancer drugs. By testing the efficacy of these drugs on the patient's own cancer cells, physicians could predict which of the available therapies a patient's tumor would most likely respond to and treat accordingly.

For 3D systems to be used in either pharmaceutical or clinical settings, several improvements to the presently available matrices will have to occur. Production of the matrices must be scaled-up in a fashion in which the matrix can be reproducibly engineered at a relatively inexpensive cost. Additionally, protocols for using the matrices should be simple and clear enough for laboratory technicians to routinely and successfully culture cancer cells in the 3D matrices. To successfully translate 3D cell culture to the clinical setting, such as that of a community cancer center, the Myriad Genetics commercial testing core model could be mimicked. This group accepts samples from the clinical community to test for genetic mutations that increase risk of several different types of cancer. A similar model could be employed in which well-trained workers produce the materials and culture patient biopsies shipped from various hospitals in the 3D matrices, then relay drug sensitivity information back to physicians. With the right research direction and the interest of clinicians, 3D drug selection for cancer patients can become a reality in the future. 📖

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