

Three-Dimensional *In Vitro* Tumor Models for Cancer Research and Drug Evaluation

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Abstract: Cancer occurs when cells acquire genomic instability and inflammation, produce abnormal levels of epigenetic factors/proteins and tumor suppressors, reprogram the energy metabolism and evade immune destruction, leading to the disruption of cell cycle/normal growth. An early event in carcinogenesis is loss of polarity and detachment from the natural basement membrane, allowing cells to form distinct three-dimensional (3D) structures that interact with each other and with the surrounding microenvironment. Although valuable information has been accumulated from traditional *in vitro* studies in which cells are grown on flat and hard plastic surfaces (2D culture), this culture condition does not reflect the essential features of tumor tissues. Further, fundamental understanding of cancer metastasis cannot be obtained readily from 2D studies because they lack the complex and dynamic cell-cell communications and cell-matrix interactions that occur during cancer metastasis. These shortcomings, along with lack of spatial depth and cell connectivity, limit the applicability of 2D cultures to accurate testing of pharmacologically active compounds, free or sequestered in nanoparticles. To recapitulate features of native tumor microenvironments, various biomimetic 3D tumor models have been developed to incorporate cancer and stromal cells, relevant matrix components, and biochemical and biophysical cues, into one spatially and temporally integrated system. In this article, we review recent advances in creating 3D tumor models employing tissue engineering principles. We then evaluate the utilities of these novel models for the testing of anticancer drugs and their delivery systems. We highlight the profound differences in responses from 3D *in vitro* tumors and conventional monolayer cultures. Overall, strategic integration of biological principles and engineering approaches will both improve understanding of tumor progression and invasion and support discovery of more personalized first line treatments for cancer patients.

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1. Introduction

Cancer is the major cause of death worldwide, and one in every four deaths in the United States is due to cancer-related diseases (Siegel et al., 2012). While cells in normal tissue reside in defined locations and maintain steady numbers, cancer cells remove these constraints through mutations in oncogenes and tumor suppressor genes (Esmaeilsabzali et al., 2013; Joyce and Pollard, 2009). Consequently, cells in the tumor tissues can sustain proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis (Hanahan and Weinberg, 2011). During cancer progression and metastasis, malignant cells maintain their close interactions with surrounding cells and the stromal extracellular matrices (ECM) (Fig. 1) (DeINero et al., 2013; Hanahan and Weinberg, 2011; Infanger et al., 2013; Nyga et al., 2011; Seo et al., 2013). Numerous stromal cells, including endothelial cells of the blood and lymphatic circulation, stromal fibroblasts, and innate and adaptive infiltrating immune cells together comprise the complex tumor microenvironment (Hanahan and Weinberg, 2011; Joyce and Pollard, 2009; Koontongkaew, 2013). The stromal ECM is composed of complex assemblies of collagens, glycosaminoglycans and proteoglycans and the molecules that bind to them (Jain, 1999; 2012). Tumor cells interact with those stromal components dynamically through growth factor-mediated tumor-stromal cell crosstalk (Murata et al., 2011) and integrin-mediated tumor-ECM interactions (Desgrosellier and Cheresh, 2010). Moreover, these interactions evolve along with the progression of the disease (Tlsty and Coussens, 2006), where the stromal microenvironment can initially exert inhibitory effects on even aggressive malignant tumor cells (Bissell and Hines, 2011; Joyce and Pollard, 2009; Xu et al., 2012a). However, as the disease progresses, cancer cells exploit and modify their surroundings to facilitate the inappropriate growth, angiogenesis, invasion and ultimately metastasis in a secondary site (Chung et al., 2012; Joyce and Pollard, 2009; Psaila and Lyden, 2009). In general, tumor growth and progression requires intricate interactions between cancer cells and their surrounding microenvironment.

In vitro studies aimed at gaining molecular understanding of cancer progression or the identification of effective anti-cancer therapeutics rely on the availability of a versatile platform that closely recapitulates pathophysiological features of the native tumor tissue and its surrounding microenvironment. Conventional two dimensional (2D) platforms (Hutmacher et al., 2010) are well established and straightforward to use. However, the absence of the third dimension can obscure the experimental observations, generating misleading and contradictory results (Hutmacher, 2010; Hutmacher et al., 2010). Additionally, screening in 2D may miss promising lead compounds whose actions are suppressed when cells are adhered to plastic. Often, promising results obtained from 2D cannot be translated similarly into *in vivo* settings (Goodman et al., 2008). Whereas cells on 2D are exposed to a uniform environment with sufficient oxygen and nutrients, cells in solid tumors are exposed to gradients of critical chemical and biological signals (Mehta et al., 2012), which can exert both stimulatory and inhibitory effects on tumor progression (Mehta et al., 2012). Intriguingly, certain tumor cells from cancer patients are intrinsically resistant to a broad spectrum of chemotherapeutic drugs without any previous exposure to those cytotoxic agents (Sanchez et al., 2009; Zhu et al., 2005; Zhu et al., 2012). This intrinsic drug resistance has been attributed to the overexpression of the multidrug resistance (MDR) proteins by tumor cells (Sanchez et al., 2009; Wartenberg et al., 1998; Zhu et al., 2012). Characteristics of the tumor tissue, namely hypoxia (Milane et al., 2011; Zhu et al., 2012), low nutrient supply (Zhu et al., 2012) and low pH (Webb et al., 2011; Wei and Roepe, 1994; Xu et al., 2014), all have been suggested to upregulate the expression of MDR proteins through specific cellular signaling pathways. Although one can partially recreate a MDR-conducive environment in 2D cultures, the lack of a three dimensional (3D) architectural context precludes the recapitulation and the maintenance of MDR behaviors (Correia and Bissell, 2012; Faute et al., 2002). Finally, the lack of the complex 3D ECM network structures in monolayer cultures can affect drug testing results. While anti-cancer agents applied to a monolayer cell culture typically reach cells without physical barriers, the same therapeutics delivered *in vivo*

encounter an entirely different environment that significantly restricts the partition of the drugs throughout the entire tumor (Goodman et al., 2008). The 3D organization of the tumor mass, as well as the associated stroma, fundamentally alters the diffusion profile for drugs, both through the cell-cell contacts and cell-matrix interactions (Chauhan et al., 2011). Detailed descriptions on physicochemical properties of native tumor microenvironments including cell-cell and cell-matrix interactions, tissue structure and mechanics, as well as juxtacrine and soluble factor signaling, can be found in recent reviews by Fischbach and coworkers (DeINero et al., 2013; Infanger et al., 2013; Seo et al., 2013).

Realizing the limitations of monolayer cultures, and inspired by the complexity of the native tumor microenvironment, researchers have developed various 3D models that recapitulate certain features of solid tumor tissues, such as tumor morphology (Gurski et al., 2012), gradient distribution of chemical and biological factors (Fracasso and Colombatti, 2000), expression of pro-angiogenic and MDR proteins (Fischbach et al., 2009; Xu et al., 2014), dynamic and reciprocal interactions between tumor and its stroma (Xu et al., 2012a). Moreover, compared to 2D monolayer cultures, cells in 3D generally exhibit a reduced sensitivity to some chemotherapeutic agents (Fong et al., 2013). This review adds to the existing literature by summarizing recent advances in *ex vivo* assembly of pathologically-relevant 3D tumor models using custom-designed culture devices and biologically-derived or biomimetic matrices. Critical assessments are provided to highlight the applications of these models toward a mechanistic understanding of cancer biology and in therapeutic evaluations of anticancer drugs, both free and nanoencapsulated. Possible mechanisms for the altered drug sensitivity observed in 3D culture conditions as compared to the corresponding 2D systems also are discussed.

2. Device-assisted assembly of tumor models

Three-dimensional multicellular tumoroids can be grown using engineered devices that maximize cell-cell interactions and solute transport without the undesirable interference from

scaffolding materials. Outlined below are two types of devices employed in cancer research: tissue engineering bioreactors and microfluidic systems.

2.1. Tissue engineering bioreactors

The multicellular tumor spheroid (MCTS) model, developed by Sutherland and collaborators (Sutherland et al., 1970) in the 1970s, has become a classic model system for cancer research. The straightforward procedure and the reliable reproducibility have contributed to the success and wide-spread usage of this model (LaBarbera et al., 2012). Using traditional cell culture plates or spinner flasks, MCTS can be produced by various techniques, such as liquid overlay, hanging drop or suspension culture (Page et al., 2013). Depending on the cell types, their substrate consumption rates and cell packing densities, tumor spheroids as large as 600 μm can be generated (Kunz-Schughart et al., 1998) and under certain conditions, MCTS with a necrotic core and gradient distributions of critical metabolites and growth factors have been obtained (Grimes et al., 2014).

Culturing cancer cells in an aggregated state statically does not always lead to the rapid formation of complex 3D structures. In tissue engineering applications, bioreactors are designed to overcome mass transfer limitations associated with traditional static cultures (Wendt et al., 2009) and/or to introduce physiologically relevant biomechanical and biophysical stimulations to cultured cells (Tandon et al., 2013; Tong et al., 2013; Tong et al., 2014). By maintaining culture parameters with tissue-specific biological, chemical, or physical cues, these bioreactors (Martin et al., 2004) have facilitated the engineering of complex and functional tissue constructs (Wendt et al., 2009). In the context of cancer progression, invasion and metastasis, bioreactors have been utilized both to miniaturize the natural counterparts, to introduce relevant forces or to create a controlled environment to foster the assembly tumor-like tissues (Hutmacher et al., 2010).

To facilitate accelerated cell growth and to maintain unrestricted cell-cell interactions, a rotating wall vessel (RWV) bioreactor, originally designed to recapitulate certain conditions that occur in the microgravity environment of space, was adapted for the culture of cancer cells, with the goal of generating 3D tumor aggregate structures that simulate the native tumor tissue (Becker and Souza, 2013; Zhau et al., 1997). As shown in Fig. 2A, RWV is designed as a horizontally rotating vessel without internal mechanical agitator. Additionally, the vessel is completely filled with cell culture media; therefore there is no air-liquid interface in the device (Ingram et al., 1997). Within the RWV, microcarrier beads are utilized to provide the solid support for adherent cells to attach and aggregate. As the RWV rotates, culture fluid can reach a near laminar flow condition and the cellular aggregates cultured in the fluid are in a state of free fall, but never reach the bottom of the vessel because of the constant rotation of the device (Becker and Souza, 2013; Goodwin et al., 1993). As the tumor aggregates grow, multiple cell-covered microcarrier beads coalesce, undergo cellular bridging and as a result, form high-density, 3D aggregate structures. Of note, when microcarrier beads are included as an integral component of a RWV bioreactor, the resultant tumor construct will contain a non-degradable, bio-inert core that compromises the overall biological integrity of the engineered tumor. The removal of the solid core is not straightforward and can result in the dissociation of the cell aggregates (Skardal et al., 2010).

The RWV cell culture technique has enabled the production of large batches (10 to 500 mL) of 3D multicellular aggregates (Hammond and Hammond, 2001) in millimeter scale (Goodwin et al., 1992). Tumor agglomerates cultured in this type of bioreactor develop small areas of necrosis during continued growth (Becker and Souza, 2013). Therefore, these tumor constructs can be used to model bulky tumors with regions of low proliferative activity and increased drug resistance (Becker and Souza, 2013). Various tumor models, including hepatocellular carcinoma (Chang and Hughes-Fulford, 2009), neuroblastoma (Redden and Doolin, 2011), breast adenocarcinoma (Kaur et al., 2011), and melanoma (Marrero et al., 2009),

have been successfully engineered using RWV-based bioreactors. This type of bioreactor also has been utilized for the *ex vivo* culture of tissue explants. For example, bone marrow biopsies from multiple myeloma patients cultured in an RWV bioreactor exhibited viable myeloma cells inside the bone microenvironment and overall well preserved histo-architecture. This system then was applied successfully to evaluate the cytotoxic effects exerted by a proteasomal inhibitor not only on myeloma cells but also on angiogenic blood vessels (Ferrarini et al., 2013).

While the RWV system minimizes physical forces, other bioreactors are designed to model naturally occurring forces in the tumor microenvironment. Owing to tumor-associated angiogenesis and lymphangiogenesis, as well as to changes in the tumor stroma, tumor tissues exhibit elevated interstitial fluid flow (Munson et al., 2013; Shields et al., 2007) Using a model system that incorporates Matrigel™ with entrapped cancer cells and interstitial flow, established by a pressure head of 1 cm water at an average velocity of 0.2 $\mu\text{m/s}$, researchers examined effects of flow on tumor cell migration, with and without lymphatic endothelial cells. Overall, physiological levels of interstitial flow strongly enhance tumor cell polarization and migration. Moreover, tumor cells utilize interstitial flow to create and amplify autologous transcellular chemokine gradients and thus are chemotactic toward the draining lymphatic (Shieh and Swartz, 2011).

Bioreactors also have been designed to enable the investigation of cancer cell interactions with stromal cells (Krishnan et al., 2011; Yates et al., 2007). For example, a compartmentalized bioreactor was utilized for the growth of osteoblastic tissue (OT) and the co-culture of OT with metastatic breast cancer cells (Dhurjati et al., 2008). The bioreactor contains two compartments separated by a dialysis membrane (6-8 KDa cutoff) (Dhurjati et al., 2006). One of the compartments was used for cell culture and the other one as a medium reservoir. The purpose of the dialysis membrane is to retain all the growth factors and cytokines (with molecular weights larger than the dialysis membrane cutoff) that are secreted into the cell growth compartment, because it is known that the spatially and temporally ordered sequence of

those secreted growth stimuli is closely aligned with specific stages in both the osteoblast development and the metastatic cancer colonization. The bioreactor-based co-culture system allows for the mechanistic study of the early stages of metastatic colonization of breast cancer cells to the bone tissue. In this work, sequential stages of the interaction between invasive cancer cells and the OT, including cancer cell adhesion, penetration and colony formation, reflected some of the features involved in breast cancer bone metastasis as observed in clinic (Dhurjati et al., 2008).

2.2. Microfluidic devices

Microfluidic technology was introduced as a tool for biological analysis in the early 1990s (Hong and Quake, 2003). It processes or manipulates small (10^{-9} to 10^{-18} liters) amounts of fluids, using microchannels with dimensions of 1 to 1000 micrometers (Zhang and Nagrath, 2013). In such regime, fluid flow within the microfluidic devices is strictly laminar, therefore, the concentrations of molecules can be well controlled both in space and time (Whitesides, 2006). Microfluidic technologies offer a number of useful capabilities for analysis including the ability to use very small quantities of sample and reagent and to perform experiments with short processing times, high resolution and sensitivity (Whitesides, 2006). Recently, microfluidics also has been used to simulate the physiological cues in cellular environment by precise spatial and temporal control over gradients of soluble biological factors and cell-cell contacts (El-Ali et al., 2006). Components for downstream cellular analysis (e.g. imaging or molecular characterization) also can be connected with the microfluidic cell culture device to form an integrated system (Zhang and Nagrath, 2013). These attractive features make microfluidics a powerful tool with which to study tumor progression, invasion and angiogenesis (Zhang and Nagrath, 2013).

For instance, a two-layer microfluidic system was developed for the culture of 3D multi-cell type spheroids of fluorescently labeled metastatic prostate cancer cells (PC-3 cell line),

osteoblasts and endothelial cells (Fig. 2B) (Hsiao et al., 2009). The system enables the formation of uniformly-sized spheroids, and also ensures the uniform distribution of PC-3 cells as well as the other two co-cultured cell types across all spheroids within the device. The engineered 3D microfluidic tumor model mimics the bone microenvironment in which the metastatic prostate cancer cells reside. This microfluidic device maintained high cell viability and permitted prostate cancer cells to grow at a physiologically relevant rate (Hsiao et al., 2009). These 3D microscale tumor tissue constructs have been used both for drug testing and for gaining in-depth understanding of cancer biology (Liu et al., 2010; Wu et al., 2008).

In another example, a microfluidic system was developed for the co-culture of tumor and endothelial cells under varying flow shear stress conditions (Buchanan et al., 2014). The system enables the study of the shear force effect on endothelial organization and paracrine signaling during tumor angiogenesis. Specifically, in this study, a central microchannel (711 μm) embedded within a collagen I-based hydrogel matrix serves as a neovessel through which tumor-relevant hydrodynamic stresses are introduced. Green fluorescent protein (GFP)-labeled breast cancer cells (MDA-MB-231) were seeded in the bulk of the collagen hydrogel, which surrounded endothelial cells lining the luminal surface of the central microchannel. The authors demonstrated that endothelial cells assemble a confluent endothelium on the microchannel lumen, maintaining the channel integrity under physiological flow shear stresses. The cancer cells co-cultured with endothelial cells under low flow conditions significantly enhanced the expression of pro-angiogenic factors (Buchanan et al., 2014).

In the native tumor microenvironment, an intricate network of chemokines and growth factors facilitates the crosstalk between cancer cells and stromal cells. Such intimate communications are conducive to cancer progression, through the recruitment of the immune system and new blood vessels (Mantovani et al., 2010; Roussos et al., 2011). Both tumor cells and stromal cells exhibit directional migration towards a chemokine source during disease progression and tumor metastasis (Mantovani et al., 2010; Roussos et al., 2011). To investigate

cancer migration and invasion, researchers have relied on microfluidic devices to generate stable and quantifiable concentration gradients of chemokines. For example, U87MG brain cancer cells seeded on channels in a microfluidic device were found to invade a type I collagen-based matrix in response to a gradient of epidermal growth factor (EGF). Specifically, cancer cells were guided towards higher EGF level, and the directional bias relied on both the gradient magnitude and EGF concentration (Zervantonakis et al. , 2010) In addition to providing a versatile and powerful tool for cancer research, microfluidic technology also has been utilized for the isolation of circulating tumor cells (Nagrath et al., 2007), molecular diagnosis (Pekin et al., 2011) and high-throughput screening of anti-cancer drugs (Kim et al., 2012). The readers are referred to a recent review article for in-depth discussions of these applications (Zhang and Nagrath, 2013).

3. Matrix-assisted assembly of 3D tumor models

The intricate molecular network of tumor-associated stromal ECM is an important component of the tumor microenvironment, and plays crucial roles in cancer progression and invasion (Dutta and Dutta, 2009; Weaver et al., 1997). Studies have shown that the blockage of ECM-integrin interactions led to death of highly metastatic breast cancer cells, thereby restoring morphologically normal breast structures (Wang et al., 2002). In general, association of cancer cells with the ECM produce survival or death signals at cellular levels to influence the efficacy of chemotherapeutic agents (Fracasso and Colombatti, 2000). Because tumor-associated stromal cells exerts either positive or negative effects on tumor growth and propagation, the absence of the stromal components in MCTS models limits the utility of such models in cancer research (Joyce and Pollard, 2009). Advanced technologies developed in the field of tissue engineering have enabled cancer researchers to create matrix-derived 3D tumor models that more closely recapitulate pathophysiological features of native tumor tissues (Hutmacher et al., 2010). These 3D systems have improved our understanding of the dynamic and reciprocal interactions

between the solid tumor and their surrounding microenvironment, including the stromal ECM molecules, immune systems, stromal cells, as well as growth factors and cytokines (LaBarbera et al., 2012). In this section, we summarize various types of matrices (Table 1) that have been utilized for the assembly of 3D tumor models.

3.1. Naturally derived matrices

Three-dimensional tumor models have been created using fibroblast-derived matrices (Lee et al., 2011). This matrix, produced predominately by cancer associated fibroblasts, resembles the mesenchymal microenvironment typically associated with advanced carcinomas *in vivo* (Serebriiskii et al., 2008). Compared to 2D plastic culture, cancer cells cultured in the fibroblast-derived matrices exhibited distinctly different cell morphology, aggregation pattern, proliferation profile and invasive potential (Serebriiskii et al., 2008). Disadvantages of this type of cell-derived matrices include lengthy sample preparation and batch-to-batch composition variations. In addition, the membrane-like structures have limited thickness and cannot be physically manipulated without compromising their structural integrity. Finally, fibroblast associated matrices do not fully represent the composition and structure of the tumor microenvironment (Nyga et al., 2011).

Separately, basement membrane extracts have been widely utilized in cancer research (Hutmacher et al., 2010; Poincloux et al., 2011). The utility of reconstituted basement membranes from mouse tumor as cell culture substrate was first demonstrated in the mid-1980s (Kleinman et al., 1986). The product, known as Matrigel™ or Cultrex® (Fridman et al., 2012; Sasser et al., 2007) consisting of collagen type IV, perlecan/HSPG2 and laminin, has been considered for a long time as the material of choice for 3D cancer cell culture. For example, Price et al (Price et al., 2012) demonstrated the influence of Matrigel™ culture on the expression of microRNA (miRNAs), non-encoding RNAs thought to be involved in cancer cell adhesion, proliferation and invasion. These authors discovered that tissue-like miRNA

expression levels and patterns were found when colon cancer cells were cultured in Matrigel™, but not on 2D plastic (Price et al., 2012). Although critical microenvironmental cues are restored in Matrigel™, this type of matrix often contains residual heparan sulfate growth factors, lacks human motifs, and contains undefined substances, and suffers from considerable batch-to-batch variation (Zaman, 2013). These shortcomings make it difficult to compare and analyze results from various research groups (Hutmacher et al., 2010). The identification of specific matrix components and soluble factors that contribute to the observed cell behaviors also is challenging.

Collagen type I, a native ECM component produced by cells in the stromal environment, influences the growth and metastasis of resident cancer cells (Moss et al., 2009). Collagen can be collected from various biological sources including bovine skin, rat tail and human placenta (Elsdale and Bard, 1972; Pacak et al., 2014). Collagen-based hydrogels have been widely used for the construction of 3D tumor models (Chen et al., 2012; Ingber, 2008; Szot et al., 2011; Yip and Cho, 2013). MCF-7 breast cancer cells cultured in collagen gels exhibited stronger invasive and metastatic propensities than did those in 2D cultures (Chen et al., 2012), as evidenced by the increased expression of pro-angiogenic growth factors, matrix metalloproteinases (MMPs) and epithelial mesenchymal transition (EMT) markers (Chen et al., 2012). Although collagen gels can be manufactured in a more reproducible manner than Matrigel™, their microstructure nevertheless is affected by a number of factors, including tissue source, assembly conditions and crosslinking density. These variations may render inconsistent results (Hutmacher et al., 2010). Most importantly, the hydrogel properties cannot be altered easily to interrogate the contributions of specific environmental cues to tumor assembly.

Owing to its unique mechanical properties, good biocompatibility, well-controlled degradability and versatile processability, silk fibroin biomaterials have been designed for tissue regeneration (Kundu et al., 2013). In a recent study, non-mulberry *A. mylitta* silk fibroin protein scaffolds, prepared by surfactant assisted dissolution, followed by dialysis and lyophilization,

were used for 3D culture of breast cancer cell line MDA-MB-231 cells (Talukdar and Kundu, 2012). The study showed that the silk-based 3D microenvironment provided a biocompatible niche for cancer cell attachment, growth and tumor tissue formation. The engineered tumor exhibited tissue-like heterogeneity with various zones of cell proliferation.

Various biopolymers originally developed for tissue engineering applications also have been employed for the *in vitro* construction of tumor models. In the presence of divalent cations (e.g. Ca^{2+}), alginates form hydrogels with controlled mechanical properties and pore sizes (Fischbach et al., 2009; Xu et al., 2013). Calcium crosslinked alginate gels degrade slowly under physiological conditions through the gradual exchange of calcium (Shoichet et al., 1996). Because alginate is typically non-adhesive to cells, RGD peptides have been conjugated covalently to the polymeric matrix to facilitate cell adhesion (Fischbach et al., 2009; Ruoslahti and Pierschbacher, 1987). Oral squamous cell carcinoma cells (OSCC-3) cultured in RGD modified, alginate hydrogels secreted a higher level of interleukin 8 (IL-8), presumably as a consequence of integrin engagement. In contrast, substrate adhesion did not significantly affect the cellular production of vascular endothelial growth factor (VEGF). This study highlights the importance of tumor microenvironment in regulating cancer cell angiogenic signaling (Fischbach et al., 2009).

Hyaluronic acid (HA) is another biopolymer widely exploited for the construction of 3D tumor models (Dicker et al., 2013), and unlike alginate, is structurally identical to its human counterpart that is intimately involved in cancer growth and progression (Dicker et al., 2013). HA is highly expressed in tumors and is a necessary component of the microenvironment of cancer cells (Franzmann et al., 2003; Kosaki et al., 1999; Lokeshwar et al., 1997; Lokeshwar et al., 2001). The metastasis of tumor cells can be directed by the amount of HA on the cell surface (Naor et al., 1997; Zhang et al., 1995). Through the interaction with its cell surface receptors, HA can alter the biological activity of cancer cells by triggering transforming growth factor β (TGF- β), Rho GTPase, and FAK signaling pathways (Bourguignon et al., 2003;

Bourguignon et al., 2002; Fujita et al., 2002). In tumor tissues, HA facilitates the migration of invasive cancer cells through the expansion upon hydration and their interaction with HA via certain cell surface receptors (Toole and Hascall, 2002). The biodegradation of HA by hyaluronidase (HAase) also helps tumor cells to escape from the primary tumor mass and metastasize to secondary sites (Stern, 2008b). Inhibition of hyaluronidase in 3D HA hydrogels is shown to block the formation of “invadopodia” (see below) (Gurski et al., 2012). HA oligomers trigger angiogenesis and induce inflammatory cytokine production, which activates various signaling mechanisms for cancer progression (Franzmann et al., 2003). Hence, tumor progression and angiogenesis depend on HA and hyaluronidase levels, as well as the degradation profile of HA (Stern, 2008a; b; Toole and Hascall, 2002). High concentrations of HA sometimes are observed at tumor invasion sites, and it has been shown that the HA coating around tumor cells effectively protects these cells from immune system surveillance (Lokeshwar et al., 1997).

We have developed several types of chemically crosslinked HA hydrogels that are hierarchically structured, mechanically robust and biologically active (Xu et al., 2012a). These synthetic matrices have been used to investigate prostate cancer progression and metastasis (Gurski et al., 2009; Gurski et al., 2012; Xu et al., 2012a; Xu et al., 2014). Hydrogels with controllable mechanical stiffness (140 – 230 Pa) and pore sizes (70-100 nm) were prepared using HA derivatives carrying complementary reactive groups (Gurski et al., 2009; Xu et al., 2012b). Of note, the storage modulus of the elastic-solid-like hydrogel is comparable to that of the native lymph tissue through which PCa frequently metastasizes (storage modulus: 330 Pa) (Levental et al., 2007), and the pore size of the hydrogel is also relevant to the spacing (20-130 nm) found in tumor ECMs (Pluen et al., 2001). When cultured in 3D HA hydrogel environments, prostate cancer cells formed distinct clustered tumoroid structures that grew and merged, reminiscent of native tumors. Compared to cells cultured on 2D, the engineered tumoroids in 3D significantly increased the expression of pro-angiogenic factors and MDR proteins, both at

mRNA and protein levels. To mimic the tumor/stroma interaction, a HA-based bilayer hydrogel system that not only supports the tumoroid formation from prostate cancer (PCa) cells, but also simulates their reciprocal interactions with the tumor-associated stroma, was engineered (Xu et al., 2012a). As shown in Fig. 2C, PCa cells embedded in the bottom hydrogel layer received the stromal growth factor signals from the top, and in response formed enlarging tumoroids that exhibited biological features reminiscent of native tumor tissues (Xu et al., 2012a). Our HA hydrogel system also provides a flexible, quantifiable, and physiologically relevant platform to study prostate cancer metastasis. Metastatic prostate cancer cells in these hydrogels develop fingerlike structures, "invadopodia", consistent with their invasive properties. The number of invadopodia, as well as cluster size, shape, and convergence, can provide a quantifiable measure of invasive potential. Prostate cancer cell invasion through the HA hydrogel depends on HA interaction with its cell surface receptor RHAMM/CD168 and requires hyaluronidase activity. The engineered PCa model is amenable to dissection of biological processes associated with cancer cell motility through HA-rich connective tissues (Gurski et al., 2012).

Using adipic acid dihydrazide-crosslinked HA hydrogels, David et al. investigated the invasive behaviors of a total of thirteen different cancer cell lines (from primary tumor or metastases) (David et al., 2004). Similar to our observations, the invasiveness of the cancer cell is related to its production of hyaluronidase, the expression of HA binding receptors and the cellular secretion of HA. Moreover, optimal colonization occurred when cells produced hyaluronidase, but did not possess HA-binding sites and did not secrete HA. Separately, Pedron et al. revealed the pivotal roles of HA in regulating the invasion and malignancy of human glioblastoma cells in a 3D biomimetic environment using HA-integrated, radically polymerized gelatin hydrogels. Multicellular tumor-like structures were only observed in HA containing hydrogels, and a biphasic relationship between cellular malignancies and HA content in the hydrogels was discovered (Pedron et al., 2013). Overall, systematic manipulation of the local

ECM microenvironment surrounding cancer cells enables cancer researchers to study the early growth of tumors.

3.2. Synthetic matrices

Synthetic hydrogels and scaffolds have been developed and extensively explored in tissue engineering applications to closely mimic the key features of the native ECM environment, owing to their flexible biochemical and biophysical characteristics (Drury and Mooney, 2003). These biomaterials are attractive alternatives to naturally derived matrices described above. Well-defined synthetic, yet biomimetic, matrices are attractive because they offer significantly improved batch-to-batch consistency with more controllable and reproducible characteristics, such as matrix mechanical properties, porosity and degradation profiles (Hutmacher et al., 2010).

Poly(ethylene glycol) (PEG)-based hydrogels, prepared by chemical crosslinking of the functionalized polymers, have been used widely as artificial matrices for 3D cell culture purposes (Lin and Anseth, 2009). While PEG is not a physiologically relevant molecule, biomimetic PEG hydrogels can be synthesized readily via the covalent incorporation of specific biological functionalities (e.g. functional peptides). Moreover, the mechanical and biological properties of PEG hydrogels can be tuned to profoundly influence the functions of the resident cells (Hutmacher et al., 2010). Taking advantage of the biocompatible nature of enzyme-catalyzed crosslinking reaction, Loessner et al synthesized PEG-based hydrogels using complementary peptide-functionalized multiarm-PEG as substrates for thrombin-activated factor XIII (FXIII) (Loessner et al., 2010). The design flexibility enabled the incorporation of arginine-glycine-aspartate (RGD) peptides for integrin-mediated cell adhesion, and a matrix metalloproteinase (MMP) sensitive peptide for protease-based matrix degradation. The authors demonstrated that both cell proliferation and spheroid formation from ovarian cancer cells in 3D

depended on the integrin engagement and the ability of cells to proteolytically remodel their extracellular microenvironment.

In general, synthetic ECM can be designed to provide a biomimetic cell growth niche with precise control over both the physical and biological characteristics of the matrix for tumor tissue formation. In a recent study, researchers utilized a versatile PEG-peptide hydrogel system to investigate the influence of matrix properties and the inhibition of epidermal growth factor receptor (EGFR) on the growth of a pancreatic ductal adenocarcinoma cell line (PANC-1). The hydrogel was prepared via thiol-ene photoclick chemistry using 8-arm PEG-norbornene and MMP-sensitive peptide with terminal cysteines. These pathophysiologically-relevant hydrogels were designed to exhibit a wide range of stiffness (~1-18 kPa), with soft gels matching that of normal pancreatic tissue, and stiff gels reflecting the mechanical features of the tumor tissue. PANC-1 cells encapsulated in relatively soft hydrogels (G' similar to 2 kPa) retained high initial viability and formed clusters after prolonged culture, whereas cells encapsulated in stiff hydrogels (G' similar to 12 kPa) exhibited lower initial viability and reduced proliferation. Immobilization of an EGFR peptide inhibitor in soft hydrogels did not increase cell death, but the same peptide immobilized in stiff hydrogels induced significant cell apoptosis, owing to the reduced cellular expression of EGFR and reduced Akt activation in stiff hydrogels. These findings underscore the potential effects of matrix properties on the efficacy of antitumor drugs (Ki et al., 2013).

Porous scaffolds, widely used in tissue engineering applications, also have been adopted for tumor engineering purposes. Because scaffold preparation requires harsh conditions and organic solvents, cells must be introduced after the scaffold has been prepared. Mooney and colleagues reported the utility of polymeric scaffolds, prepared from poly(lactide-co-glycolide) (PLGA) via a gas foaming-particulate leaching process (Harris et al., 1998), for 3D culture of OSCC-3 cells (Fischbach et al., 2007). The angiogenic characteristics of the tumor cells were altered dramatically upon 3D culture in the porous scaffold, and corresponded much

more closely to tumors formed *in vivo*. In another study (Kim et al., 2011), PLGA-based scaffolds, fabricated by a solvent casting and particulate leaching technique, were used for 3D culture of human glioma cells. Similar to other 3D culture studies, cells in this model were less sensitive to chemotherapy as evident by a lower caspase-3 activity, and a higher expression of anti-apoptotic proteins. Such a scaffold, although entirely synthetic, provides a useful 3D platform to study the effect of microenvironmental conditions on tumor progression *in vitro*.

To foster more intimate cell-cell and cell-matrix interactions, researchers resorted to electrospun, microfibrinous meshes for 3D culture of cancer cells. In one example, porous electrospun poly(ϵ -caprolactone) (PCL) scaffolds were used for the culture of Ewing sarcoma cells in 3D (Fong et al., 2013). Cancer cells cultured in the 3D environment not only were more resistant to traditional cytotoxic drugs than were cells in 2D monolayer culture, but also exhibited remarkable differences in the expression pattern of the insulin-like growth factor-1 receptor / mammalian target of rapamycin pathway (Fong et al., 2013). Of note, since drug transport and interaction with the scaffold may have an impact on its efficacy, the decreased drug sensitivity found in 3D PCL scaffold may be partially attributed to the physical adsorption of the drug onto the scaffold. Overall, the reported PCL-based tumor model has the potential to provide a platform for the mechanistic studies of bone sarcomas and the evaluation of anticancer drug candidates for these malignancies (Fong et al., 2013). Modification of electrospun scaffolds with bioactive ECM-derived peptides also can be used to study pharmacokinetic properties of various drugs targeting cancer cells (Hartman et al., 2010). To gain mechanistic understandings of cancer metastasis, Sieh et al. cultured PCa cells on a tissue engineered bone constructs, fabricated by wrapping a human osteoblast cell sheet around a cell-seed, bone-mimetic composite scaffold (Sieh et al., 2010). Consistent with reported *in vivo* studies, the authors found that the intercellular and PCa cell-bone matrix interactions contributed to the observed increase in the expression level of various biomarkers associated with PCa bone metastasis.

Because of the defined amino acid composition and the structural and mechanical similarity to natural ECM, self-assembled peptide hydrogels have emerged as attractive 3D culture platforms (Loo et al., 2012). Gelation occurs via a process that involves concerted action of weak and non-covalent interactions, triggered by altering the composition of the aqueous media. The synthetic nature of the peptide building blocks allow for facile incorporation of specific biological relevant ligands to achieve desired biological functions (Ulijn and Smith, 2008; Zhang et al., 2005a). These physical gels contain entangled, amyloid-like fibers of ~ 10 nm thick and interstitial pores between 5 and 200 nm (Goldberg et al., 2007). Using RADA16-I peptide hydrogel, 3D models of ovarian cancer were created. Cells cultured in the peptide hydrogels not only exhibited strong invasion potentials, but also displayed significantly higher anticancer drug resistance than did cells cultured on the conventional 2D petri dish (Yang and Zhao, 2011). Hydrogels assembled from a rationally designed h9e peptide (Huang et al., 2011) containing sequences from an elastic segment of spider silk and a trans-membrane segment of human muscle L-type calcium channel, were used for 3D culture of human epithelial cancer cells (MCF-7). MCF-7 cells residing in the peptide hydrogel grew into 3D tumor-like clusters and responded to cisplatin treatment in a dose- and time-dependent manner (Huang et al., 2013). Gelation by supramolecular interactions through the entanglement of fibrillar structures is an attractive feature of self-assembled peptide hydrogels. Long term stability, however, needs to be improved.

4. 3D tumor models for drug testing

Although rapid advances in drug design methodology have led to dramatic increases in the discovery of both candidate drug compounds and their screenable drug targets (Dobson, 2004; Tan, 2005), a commensurate increase in the number of approved drugs has not been seen (LaBarbera et al., 2012). The high failure rate of drug candidates can be attributed in part to the usage of 2D monolayer cultures as the initial screening method that frequently produces

inaccurate and unreliable results and does not predict chemoresistance (van de Waterbeemd and Gifford, 2003). As discussed above, the same drug treatment elicits distinctly different responses from cells in 3D and on 2D. The ability of 3D systems to recapitulate tumor-like microenvironment suggests the potential of engineered tumor models to provide more accurate and reproducible toxicity information for the early-stage drug discovery (Lee et al., 2008).

4.1. High-throughput screening of drug candidates

Adaptation of 3D tumor models to high-throughput drug screening systems are highly desirable because they can provide predictable results in an efficient manner, thereby enabling the prioritization of candidate compounds for further development in the *in vivo* studies (Friedrich et al., 2009). For use as *in vitro* drug testing platforms, the 3D models must recapitulate the pathophysiological features of the tumor microenvironment so that *in vivo* drug efficacy can be reliably predicted (Mueller-Klieser, 2000). For high-throughput purposes, the implementation of the 3D tumor models into drug evaluation routines requires an experimental design that guarantees tumor constructs of the same cell type cultured under the same external conditions are essentially identical in morphology, structure, microenvironment and cellular physiology (Friedrich et al., 2009). Routine high-throughput drug screening protocols based on various 3D systems (Kim, 2005) have been developed (Friedrich et al., 2009).

Using ultra-low attachment 96-well round-bottomed plates (Vinci et al., 2012), researchers demonstrated that tumor cells formed uniform, single and centrally located spheroids of reproducible size within each well. The resultant MCTS were subjected to automated multiparametric analyses, including measurements of spheroid diameter, perimeter and area, using a Celigo™ cytometer. This platform not only enabled reliable assessments of key hallmarks of cancer, i.e. cell motility, matrix invasion and tumor angiogenesis, but also facilitated evaluation of various types of cell growth inhibitors. Not surprisingly, 2D and 3D cultures exhibited differential sensitivities to targeted agents, and in general, tumor cells were

less sensitive to soluble compounds in 3D than in 2D monolayer cultures. The utility of some agents in treating metastatic disease also has been identified. Other 3D culture techniques amenable to high throughput screening include liquid overlay (Li et al., 2011), hanging drop (Tung et al., 2011), microarray template (Hardelauf et al., 2011), microencapsulation (Zhang et al., 2005b) and magnetic cell levitation (Souza et al., 2010).

The differential drug sensitivity observed between 2D and 3D cultures can be attributed to decreased compound access or reduced drug sensitivity in response to hypoxic or more slowly cycling cells under 3D culture conditions (Fong et al., 2013; Minchinton and Tannock, 2006). It is also reported that cell-cell contact plays a role in drug resistance found in 3D. A negative correlation between cell-cell contact and drug sensitivity was observed in a PCL scaffold-based 3D tumor model (Fong et al., 2013). However, the exact signaling mechanism implicated in drug resistance in 3D is still unclear. Current work is focused on the study of the interplay among various factors (e.g. intracellular changes, paracrine signaling, modifications in the supporting matrix) that may contribute to the reduced drug sensitivity in 3D (Saraswathy and Gong, 2013).

Tumor models fabricated using matrix-assisted approaches have not yet been widely exploited for high throughput screening purposes because of the additional variables introduced by the matrix and challenges associated with manipulating and fabricating hydrogels or scaffolds in a high throughput fashion. If the matrix is simple, however, adaptation can be achieved. For example, using collagen or alginate gels, 3D tumor cell-culture array (Data Analysis Toxicology Assay Chip or DataChip) has been developed for the high-throughput toxicity evaluation of both the drug candidates and their cytochrome P450-generated metabolites (Lee et al., 2008). Specifically, cancer cells encapsulated within collagen or alginate gels with the volume as low as 20 nL, were spotted onto the surface of functionalized glass slides of the DataChip. One single DataChip contains 1,080 of this kind of individual microscale cell/gel tumor constructs. The system was used for spatially addressable screening against

multiple anti-cancer compounds. In conjunction with the complementary human P450-containing microarray, the device provided the toxicity results for the metabolites of the compound from three human P450 isoforms (CYP3A4, CYP1A2, and CYP2D6), which were designed to mimic the function of human liver.

4.2. Evaluation on drug delivery systems

Over the past few decades, concerted efforts have been made for the development of nanotechnology-based cancer therapeutics, with the promise of potentially prolonging patients' life expectancy, at the same time reducing treatment-related side effects (Byrne et al., 2008; Kumar et al., 2013; Peer et al., 2007). The engineered nano-formulations improve the pharmacological properties of drugs, and also potentially increase the drug efficacy through the enhanced permeability and retention (EPR) effect or the active targeting strategies (Byrne et al., 2008; Kang et al., 2010). The typical size of the developed nano-therapeutics for clinical applications ranges from 2 nm to 200 nm (Kamaly et al., 2012). For effective treatment of solid tumor, it is desirable that the nanomedicine can reach as many cells within the tumor parenchyma as possible (Kamaly et al., 2012). However, the penetration of the nano-therapeutics can be hindered by the elevated levels of interstitial fluid pressure (IFP), tortuosity and interstitial matrices of the tumor tissue (Wong et al., 2011). Moreover, the increased IFP limits the transport of nanomedicine to the poorly perfused regions of tumor tissues where access is dominated only by diffusion (Kim et al., 2010). The rate of diffusion and the penetration depth depend on the size of the drug carrier and the interactions between the carrier and the interstitial matrices (Kim et al., 2010).

Three-dimensional avascular tumor models have been used to assess the diffusion, distribution and drug efficacy of nano-therapeutics. Using a 3D MCTS model, Pun and coworkers reported that polystyrene nanoparticles (NPs) with an average diameter of 20 and 40 nm penetrated the MCTS, whereas larger NPs (100 or 200 nm) were restricted to the periphery

of the spheroids (Goodman et al., 2007). When collagenase was immobilized on the particle surface, the penetration depth of the NPs was enhanced significantly. Therefore, incorporating ECM-modulating enzymes in drug carriers may improve NP penetration in solid tumors. In a separate investigation, researchers discovered that doxorubicin (Dox)-loaded nanoparticles (Dox-NPs) with an average diameter of 37 nm penetrated into the core of 400 μm human cervical tumor spheroids in 30 min. Compared to free Dox, Dox-NPs not only exhibited a greater tumor penetration capacity, but also suppressed tumor growth more efficiently in the MCTS model (Kim et al., 2010). Similar observations were found with regard to the transport property of a pseudo-peptide-based drug delivery system using a Hela MCTS model (Ho et al., 2011). Overall, NP penetration depended on both the carrier concentration and the incubation time.

Three-dimensional tumor models assembled in hydrogel matrices also have been used for the evaluation of nanoformulations. The hydrogel network, with optimized stiffness and mesh size, was designed to simulate the ECM barrier of tumor tissues. Using a PCa tumor model created using an HA hydrogel, Xu et al discovered that Nile Red (NR)-loaded polymeric NPs with an average hydrodynamic size of 54 nm had a diffusion coefficient of 9.21 $\mu\text{m}^2/\text{s}$ and 4.04 $\mu\text{m}^2/\text{s}$ in water and the HA network, respectively. Dox-NPs introduced to the cell/gel constructs reached the embedded tumoroids within 2 hours, homogeneously distributed in the tumoroids and were internalized by tumor cells through caveolae-mediated endocytosis and macropinocytosis pathways. A comparison of intracellular drug localization revealed that on 2D cultures, free Dox was transported to the cell nuclei [Fig. 3A(a)] whereas Dox-NPs were excluded from the nuclei and distributed predominantly in the cytoplasm [Fig. 3A(b)]. Interestingly, under 3D culture conditions, both free Dox and Dox-NPs localized predominantly in the cytoplasm of individual cells in the tumoroids [Fig. 3A(c) and 3A(d)]. Finally, as indicated from the dose response curves and the IC₅₀ values (Fig. 3B and 3C), tumor cells cultured as multicellular aggregates in HA hydrogel were more resistant to both free Dox and Dox-NP treatments compared to 2D cultures. The drug resistance profile observed in 3D has been

attributed to the elevated expression of MDR proteins (multidrug resistance protein 1 and lung resistance-related protein) by LNCaP PCa cells cultured in HA hydrogels (Xu et al., 2014). It has been reported that primary cultures of PCa cells derived directly from the patients expressed significantly elevated levels of MDR proteins than PCa cell lines (i.e. LNCaP, PC3 and DU145 cell lines). As a result, patient-derived cells displayed lower drug sensitivity (Sanchez et al., 2009). The HA-based 3D tumor models closely mimic the *in vivo* tumor growth condition, and the increased expression of MDR proteins suggested a MDR cell phenotype. Therefore, the drug response from cells cultured in the 3D HA hydrogel should be more physiologically relevant than that from cells cultured on 2D.

The Zaman and Grinstaff team created 3D models of osteosarcoma and breast adenocarcinoma by first allowing the cancer cells (U2OS and MDA-MB-231) to aggregate in an agarose well, then encapsulating the spheroids in collagen gels (Charoen et al., 2014). Using the engineered tumor models, the researchers characterized the chemotherapeutic responses from paclitaxel, delivered in bolus as free drug or controlled release using an expansile nanoparticle (eNP). The eNPs were designed to release the drug payload via a pH mediated particle expansion within the cell endosome (Zubris et al., 2013). Confocal microscopy imaging showed that the eNPs, with an average diameter of ~100 nm (Colby et al., 2013), dispersed throughout the spheroid after diffusing through the collagen and into the spheroid. Compared to the bolus delivery, the eNPs delivery provided a significantly more pronounced inhibition in cell growth, in accordance with the *in vivo* observations from a murine xenograft model. Contrarily, no difference in response between bolus and eNP delivered paclitaxel was detected in 2D monolayer (Liu et al., 2013). These findings highlight the importance of physiologically relevant tumor models for generating clinically predictable assessments on drug formulations.

5. Summary and outlook

Engineered 3D systems provide a realistic and controllable environment for the incorporation of specific cells, ECM molecules, growth factors and other biochemical cues to better simulate the native tumor microenvironment that favors tumor growth and progression. These models will likely be of significant use in delineating the biological mechanisms that govern the pathological abnormalities observed in cancer. They also will serve as more reliable platforms for generating predictive results on *in vivo* evaluation of chemotherapeutic agents and their delivery systems.

Despite substantial and continued success in creating biomimetic 3D tumor models, major challenges and limitations remain associated with existing models. First, many models are created using long term 2D culture adapted cancer cell lines that may no longer accurately reflect the pathology of the initial disease. The establishment of therapeutically relevant treatment predictions relies on the availability of patients' cells, expanded in xenografts, with characteristic heterogeneity of the disease and different metastatic potentials. Successful culture of these cells *ex vivo* will provide access to a higher level of accuracy both in probing the biological underpinnings of metastasis, and in correctly identifying the susceptibility to pathway-targeted drugs (Fong et al., 2014). Equally important is the validation of the engineered tumor models, at least by the source xenografts, and ideally matched to each patient's deidentified history of drug therapies to guide and refine the current model.

Secondly, current 3D systems remain relatively simplistic and do not faithfully represent the native tumor microenvironment. Many engineered models appear similar to native tumor tissues only morphologically. Phenotypic similarity and heterogeneity need to be recaptured. When synthetic matrices are employed, detailed signaling studies of cells in the engineered microenvironment is lacking. A future trend is to improve the physiological complexity, which includes cell types, matrix composition, as well as temporal and spatial presentation of soluble factors employing innovative materials chemistry and engineering designs. The recreation of the complex, tumor-associated vascular system *in vitro* is another challenge task but necessary to

be addressed because these abnormal blood vessels not only influence cancer progression, but also greatly affect drug transport within the tumor tissues (Jain, 2012).

Finally, as the tumor models become more and more sophisticated, computational modeling (Shirinifard et al., 2009), system biology approaches (Deisboeck et al., 2011; Rajagopalan et al., 2013) and *in situ* real time imaging, detection and analysis modalities are sorely needed (Zaman, 2013). For example, crosslinking of hydrogel precursors in the presence of homogeneously dispersed cancer cells results in the entrapment of cells in a porous network with mesh size in the micron to submicron scale (Nicodemus and Bryant, 2008; Xu et al., 2014). To form large tumor-like aggregates, cells initially trapped at a single cell state must proliferate within the matrix, at the same time migrate through the network, via amoeboid and/or mesenchymal movement (Wolf and Friedl, 2006). There is a need for quantitative understanding of the collective movement of aggregated cancer cells in the 3D environment. Overall, with the advances in 3D culture techniques (Hutmacher et al., 2010), cancer genomics (Chin et al., 2011) and mathematical modeling (Haeno et al., 2012), promising 3D engineered tumor models will serve as the bridge between 2D monolayer culture and cancer xenografts to accelerate the translation of novel therapeutics to the clinic.

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Figure Caption

Fig. 1. Schematic illustration of a typical tumor microenvironment. Cancer cells reside in a complex microenvironment containing various supporting cells, extracellular matrix (ECM) and a suite of signaling molecules. These environmental components collectively contribute to the tumor-stromal interaction and tumor progression. Adapted from (Joyce and Pollard, 2009; Koontongkaew, 2013).

Fig. 2. Representative examples of culture platforms employed for the growth of 3D tumoroids. (A) Rotating wall vessel (RWV) bioreactors (i, ii) have been used for the 3D culture of human breast ductal carcinoma cells (T-47D, iii) and glioblastoma cells (labeled with green fluorescent protein, iv). Cells cultured in a state of free fall with minimal shear (ii) organized into millimeter sized aggregates with multiple layers of cells (iii and iv, scale bar: 200 μm). Reproduced with permission (Becker and Souza, 2013), Copyright 2013, Macmillan Publishers Limited. (B) A microfluidic device (i–iii) has been designed for the 3D culture of PC-3 prostate cancer cells. The device is composed of upper and lower channels separated by a non-adhesive, semi-permeable membrane. PC-3 cells, mixed with pre-osteoblasts and endothelial cells and introduced to the device as a monolayer, self-organized into 3D spheroids in one day (iv: optical image; v: fluorescent image, red-PC-3 cells, green-live cells; scale bar: 200 μm). Reproduced with permission (Hsiao et al., 2009), Copyright 2009, Elsevier Ltd. (C) A bilayer hydrogel platform (i) for the 3D culture of prostate cancer cells. LNCaP cells culture in the bottom layer, receiving heparin-binding epidermal growth factor-like growth factor (HB-EGF) released from the top layer, grew into spheroids (ii, iii, nuclei: blue) displaying cortical F-actin (green) and expressing E-cadherin (red). Reproduced with permission (Xu et al., 2012), Copyright 2012, Elsevier Ltd.

Fig. 3. Comparison of intracellular localization and cell-killing capacity of free Dox and Dox-NPs in 2D and 3D cultures of LNCaP prostate cancer cells. HA-based hydrogels were used for 3D cultures. (A) Confocal images show differential localizations of Dox and Dox-NPs in LNCaP cells after 2 h of drug exposure. Dox is inherently fluorescent and the internalized Dox or Dox-NPs were detected using a Zeiss510 NLO confocal microscope (excitation wavelength at 488 nm with a band pass filter of 565-615 nm). (B) Dose-dependent cell death induced by Dox or Dox-NPs on 2D and 3D cultures of LNCaP prostate cancer cells. Cell apoptosis analysis was performed using Cell Death Detection ELISA^{PLUS}. (C) Summary of the IC₅₀ values for various drug/culture combinations. (*, significant difference compared to the IC₅₀ value of 2D Dox condition, p < 0.05). Reproduced with permission (Xu et al., 2014), Copyright 2014, Elsevier Ltd.

Three-Dimensional *In Vitro* Tumor Models for Cancer Research and Drug Evaluation

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Abstract: Cancer occurs when cells acquire genomic instability and inflammation, produce abnormal levels of epigenetic factors/proteins and tumor suppressors, reprogram the energy metabolism and evade immune destruction, leading to the disruption of cell cycle/normal growth. An early event in carcinogenesis is loss of polarity and detachment from the natural basement membrane, allowing cells to form distinct three-dimensional (3D) structures that interact with each other and with the surrounding microenvironment. Although valuable information has been accumulated from traditional *in vitro* studies in which cells are grown on flat and hard plastic surfaces (2D culture), this culture condition does not reflect the essential features of tumor tissues. Further, fundamental understanding of cancer metastasis cannot be obtained readily from 2D studies because they lack the complex and dynamic cell-cell communications and cell-matrix interactions that occur during cancer metastasis. These shortcomings, along with lack of spatial depth and cell connectivity, limit the applicability of 2D cultures to accurate testing of pharmacologically active compounds, free or sequestered in nanoparticles. To recapitulate features of native tumor microenvironments, various biomimetic 3D tumor models have been developed to incorporate cancer and stromal cells, relevant matrix components, and biochemical and biophysical cues, into one spatially and temporally integrated system. In this article, we review recent advances in creating 3D tumor models employing tissue engineering principles. We then evaluate the utilities of these novel models for the testing of anticancer drugs and their delivery systems. We highlight the profound differences in responses from 3D *in vitro* tumors and conventional monolayer cultures. Overall, strategic integration of biological principles and engineering approaches will both improve understanding of tumor progression and invasion and support discovery of more personalized first line treatments for cancer patients.

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1. Introduction

Cancer is the major cause of death worldwide, and one in every four deaths in the United States is due to cancer-related diseases (Siegel et al., 2012). While cells in normal tissue reside in defined locations and maintain steady numbers, cancer cells remove these constraints through mutations in oncogenes and tumor suppressor genes (Esmaeilsabzali et al., 2013; Joyce and Pollard, 2009). Consequently, cells in the tumor tissues can sustain proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis (Hanahan and Weinberg, 2011). During cancer progression and metastasis, malignant cells maintain their close interactions with surrounding cells and the stromal extracellular matrices (ECM) (Fig. 1) (DelNero et al., 2013; Hanahan and Weinberg, 2011; Infanger et al., 2013; Nyga et al., 2011; Seo et al., 2013). Numerous stromal cells, including endothelial cells of the blood and lymphatic circulation, stromal fibroblasts, and innate and adaptive infiltrating immune cells together comprise the complex tumor microenvironment (Hanahan and Weinberg, 2011; Joyce and Pollard, 2009; Koontongkaew, 2013). The stromal ECM is composed of complex assemblies of collagens, glycosaminoglycans and proteoglycans and the molecules that bind to them (Jain, 1999; 2012). Tumor cells interact with those stromal components dynamically through growth factor-mediated tumor-stromal cell crosstalk (Murata et al., 2011) and integrin-mediated tumor-ECM interactions (Desgrosellier and Cheresh, 2010). Moreover, these interactions evolve along with the progression of the disease (Tlsty and Coussens, 2006), where the stromal microenvironment can initially exert inhibitory effects on even aggressive malignant tumor cells (Bissell and Hines, 2011; Joyce and Pollard, 2009; Xu et al., 2012a). However, as the disease progresses, cancer cells exploit and modify their surroundings to facilitate the inappropriate growth, angiogenesis, invasion and ultimately metastasis in a secondary site (Chung et al., 2012; Joyce and Pollard, 2009; Psaila and Lyden, 2009). In general, tumor growth and progression requires intricate interactions between cancer cells and their surrounding microenvironment.

In vitro studies aimed at gaining molecular understanding of cancer progression or the identification of effective anti-cancer therapeutics rely on the availability of a versatile platform that closely recapitulates pathophysiological features of the native tumor tissue and its surrounding microenvironment. Conventional two dimensional (2D) platforms (Hutmacher et al., 2010) are well established and straightforward to use. However, the absence of the third dimension can obscure the experimental observations, generating misleading and contradictory results (Hutmacher, 2010; Hutmacher et al., 2010). Additionally, screening in 2D may miss promising lead compounds whose actions are suppressed when cells are adhered to plastic. Often, promising results obtained from 2D cannot be translated similarly into *in vivo* settings (Goodman et al., 2008). Whereas cells on 2D are exposed to a uniform environment with sufficient oxygen and nutrients, cells in solid tumors are exposed to gradients of critical chemical and biological signals (Mehta et al., 2012), which can exert both stimulatory and inhibitory effects on tumor progression (Mehta et al., 2012). Intriguingly, certain tumor cells from cancer patients are intrinsically resistant to a broad spectrum of chemotherapeutic drugs without any previous exposure to those cytotoxic agents (Sanchez et al., 2009; Zhu et al., 2005; Zhu et al., 2012). This intrinsic drug resistance has been attributed to the overexpression of the multidrug resistance (MDR) proteins by tumor cells (Sanchez et al., 2009; Wartenberg et al., 1998; Zhu et al., 2012). Characteristics of the tumor tissue, namely hypoxia (Milane et al., 2011; Zhu et al., 2012), low nutrient supply (Zhu et al., 2012) and low pH (Webb et al., 2011; Wei and Roepke, 1994; Xu et al., 2014), all have been suggested to upregulate the expression of MDR proteins through specific cellular signaling pathways. Although one can partially recreate a MDR-conducive environment in 2D cultures, the lack of a three dimensional (3D) architectural context precludes the recapitulation and the maintenance of MDR behaviors (Correia and Bissell, 2012; Faute et al., 2002). Finally, the lack of the complex 3D ECM network structures in monolayer cultures can affect drug testing results. While anti-cancer agents applied to a monolayer cell culture typically reach cells without physical barriers, the same therapeutics delivered *in vivo*

encounter an entirely different environment that significantly restricts the partition of the drugs throughout the entire tumor (Goodman et al., 2008). The 3D organization of the tumor mass, as well as the associated stroma, fundamentally alters the diffusion profile for drugs, both through the cell-cell contacts and cell-matrix interactions (Chauhan et al., 2011). Detailed descriptions on physicochemical properties of native tumor microenvironments including cell-cell and cell-matrix interactions, tissue structure and mechanics, as well as juxtacrine and soluble factor signaling, can be found in recent reviews by Fischbach and coworkers (DeINero et al., 2013; Infanger et al., 2013; Seo et al., 2013).

Realizing the limitations of monolayer cultures, and inspired by the complexity of the native tumor microenvironment, researchers have developed various 3D models that recapitulate certain features of solid tumor tissues, such as tumor morphology (Gurski et al., 2012), gradient distribution of chemical and biological factors (Fracasso and Colombatti, 2000), expression of pro-angiogenic and MDR proteins (Fischbach et al., 2009; Xu et al., 2014), dynamic and reciprocal interactions between tumor and its stroma (Xu et al., 2012a). Moreover, compared to 2D monolayer cultures, cells in 3D generally exhibit a reduced sensitivity to some chemotherapeutic agents (Fong et al., 2013). This review adds to the existing literature by summarizing recent advances in *ex vivo* assembly of pathologically-relevant 3D tumor models using custom-designed culture devices and biologically-derived or biomimetic matrices. Critical assessments are provided to highlight the applications of these models toward a mechanistic understanding of cancer biology and in therapeutic evaluations of anticancer drugs, both free and nanoencapsulated. Possible mechanisms for the altered drug sensitivity observed in 3D culture conditions as compared to the corresponding 2D systems also are discussed.

2. Device-assisted assembly of tumor models

Three-dimensional multicellular tumoroids can be grown using engineered devices that maximize cell-cell interactions and solute transport without the undesirable interference from

scaffolding materials. Outlined below are two types of devices employed in cancer research: tissue engineering bioreactors and microfluidic systems.

2.1. Tissue engineering bioreactors

The multicellular tumor spheroid (MCTS) model, developed by Sutherland and collaborators (Sutherland et al., 1970) in the 1970s, has become a classic model system for cancer research. The straightforward procedure and the reliable reproducibility have contributed to the success and wide-spread usage of this model (LaBarbera et al., 2012). Using traditional cell culture plates or spinner flasks, MCTS can be produced by various techniques, such as liquid overlay, hanging drop or suspension culture (Page et al., 2013). Depending on the cell types, their substrate consumption rates and cell packing densities, tumor spheroids as large as 600 μm can be generated (Kunz-Schughart et al., 1998) and under certain conditions, MCTS with a necrotic core and gradient distributions of critical metabolites and growth factors have been obtained (Grimes et al., 2014).

Culturing cancer cells in an aggregated state statically does not always lead to the rapid formation of complex 3D structures. In tissue engineering applications, bioreactors are designed to overcome mass transfer limitations associated with traditional static cultures (Wendt et al., 2009) and/or to introduce physiologically relevant biomechanical and biophysical stimulations to cultured cells (Tandon et al., 2013; Tong et al., 2013; Tong et al., 2014). By maintaining culture parameters with tissue-specific biological, chemical, or physical cues, these bioreactors (Martin et al., 2004) have facilitated the engineering of complex and functional tissue constructs (Wendt et al., 2009). In the context of cancer progression, invasion and metastasis, bioreactors have been utilized both to miniaturize the natural counterparts, to introduce relevant forces or to create a controlled environment to foster the assembly tumor-like tissues (Hutmacher et al., 2010).

To facilitate accelerated cell growth and to maintain unrestricted cell-cell interactions, a rotating wall vessel (RWV) bioreactor, originally designed to recapitulate certain conditions that occur in the microgravity environment of space, was adapted for the culture of cancer cells, with the goal of generating 3D tumor aggregate structures that simulate the native tumor tissue (Becker and Souza, 2013; Zhau et al., 1997). As shown in Fig. 2A, RWV is designed as a horizontally rotating vessel without internal mechanical agitator. Additionally, the vessel is completely filled with cell culture media; therefore there is no air-liquid interface in the device (Ingram et al., 1997). Within the RWV, microcarrier beads are utilized to provide the solid support for adherent cells to attach and aggregate. As the RWV rotates, culture fluid can reach a near laminar flow condition and the cellular aggregates cultured in the fluid are in a state of free fall, but never reach the bottom of the vessel because of the constant rotation of the device (Becker and Souza, 2013; Goodwin et al., 1993). As the tumor aggregates grow, multiple cell-covered microcarrier beads coalesce, undergo cellular bridging and as a result, form high-density, 3D aggregate structures. Of note, when microcarrier beads are included as an integral component of a RWV bioreactor, the resultant tumor construct will contain a non-degradable, bio-inert core that compromises the overall biological integrity of the engineered tumor. The removal of the solid core is not straightforward and can result in the dissociation of the cell aggregates (Skardal et al., 2010).

The RWV cell culture technique has enabled the production of large batches (10 to 500 mL) of 3D multicellular aggregates (Hammond and Hammond, 2001) in millimeter scale (Goodwin et al., 1992). Tumor agglomerates cultured in this type of bioreactor develop small areas of necrosis during continued growth (Becker and Souza, 2013). Therefore, these tumor constructs can be used to model bulky tumors with regions of low proliferative activity and increased drug resistance (Becker and Souza, 2013). Various tumor models, including hepatocellular carcinoma (Chang and Hughes-Fulford, 2009), neuroblastoma (Redden and Doolin, 2011), breast adenocarcinoma (Kaur et al., 2011), and melanoma (Marrero et al., 2009),

have been successfully engineered using RWV-based bioreactors. This type of bioreactor also has been utilized for the *ex vivo* culture of tissue explants. For example, bone marrow biopsies from multiple myeloma patients cultured in an RWV bioreactor exhibited viable myeloma cells inside the bone microenvironment and overall well preserved histo-architecture. This system then was applied successfully to evaluate the cytotoxic effects exerted by a proteasomal inhibitor not only on myeloma cells but also on angiogenic blood vessels (Ferrarini et al., 2013).

While the RWV system minimizes physical forces, other bioreactors are designed to model naturally occurring forces in the tumor microenvironment. Owing to tumor-associated angiogenesis and lymphangiogenesis, as well as to changes in the tumor stroma, tumor tissues exhibit elevated interstitial fluid flow (Munson et al., 2013; Shields et al., 2007) Using a model system that incorporates Matrigel™ with entrapped cancer cells and interstitial flow, established by a pressure head of 1 cm water at an average velocity of 0.2 $\mu\text{m/s}$, researchers examined effects of flow on tumor cell migration, with and without lymphatic endothelial cells. Overall, physiological levels of interstitial flow strongly enhance tumor cell polarization and migration. Moreover, tumor cells utilize interstitial flow to create and amplify autologous transcellular chemokine gradients and thus are chemotactic toward the draining lymphatic (Shieh and Swartz, 2011).

Bioreactors also have been designed to enable the investigation of cancer cell interactions with stromal cells (Krishnan et al., 2011; Yates et al., 2007). For example, a compartmentalized bioreactor was utilized for the growth of osteoblastic tissue (OT) and the co-culture of OT with metastatic breast cancer cells (Dhurjati et al., 2008). The bioreactor contains two compartments separated by a dialysis membrane (6-8 KDa cutoff) (Dhurjati et al., 2006). One of the compartments was used for cell culture and the other one as a medium reservoir. The purpose of the dialysis membrane is to retain all the growth factors and cytokines (with molecular weights larger than the dialysis membrane cutoff) that are secreted into the cell growth compartment, because it is known that the spatially and temporally ordered sequence of

those secreted growth stimuli is closely aligned with specific stages in both the osteoblast development and the metastatic cancer colonization. The bioreactor-based co-culture system allows for the mechanistic study of the early stages of metastatic colonization of breast cancer cells to the bone tissue. In this work, sequential stages of the interaction between invasive cancer cells and the OT, including cancer cell adhesion, penetration and colony formation, reflected some of the features involved in breast cancer bone metastasis as observed in clinic (Dhurjati et al., 2008).

2.2. Microfluidic devices

Microfluidic technology was introduced as a tool for biological analysis in the early 1990s (Hong and Quake, 2003). It processes or manipulates small (10^{-9} to 10^{-18} liters) amounts of fluids, using microchannels with dimensions of 1 to 1000 micrometers (Zhang and Nagrath, 2013). In such regime, fluid flow within the microfluidic devices is strictly laminar, therefore, the concentrations of molecules can be well controlled both in space and time (Whitesides, 2006). Microfluidic technologies offer a number of useful capabilities for analysis including the ability to use very small quantities of sample and reagent and to perform experiments with short processing times, high resolution and sensitivity (Whitesides, 2006). Recently, microfluidics also has been used to simulate the physiological cues in cellular environment by precise spatial and temporal control over gradients of soluble biological factors and cell-cell contacts (El-Ali et al., 2006). Components for downstream cellular analysis (e.g. imaging or molecular characterization) also can be connected with the microfluidic cell culture device to form an integrated system (Zhang and Nagrath, 2013). These attractive features make microfluidics a powerful tool with which to study tumor progression, invasion and angiogenesis (Zhang and Nagrath, 2013).

For instance, a two-layer microfluidic system was developed for the culture of 3D multi-cell type spheroids of fluorescently labeled metastatic prostate cancer cells (PC-3 cell line),

osteoblasts and endothelial cells (Fig. 2B) (Hsiao et al., 2009). The system enables the formation of uniformly-sized spheroids, and also ensures the uniform distribution of PC-3 cells as well as the other two co-cultured cell types across all spheroids within the device. The engineered 3D microfluidic tumor model mimics the bone microenvironment in which the metastatic prostate cancer cells reside. This microfluidic device maintained high cell viability and permitted prostate cancer cells to grow at a physiologically relevant rate (Hsiao et al., 2009). These 3D microscale tumor tissue constructs have been used both for drug testing and for gaining in-depth understanding of cancer biology (Liu et al., 2010; Wu et al., 2008).

In another example, a microfluidic system was developed for the co-culture of tumor and endothelial cells under varying flow shear stress conditions (Buchanan et al., 2014). The system enables the study of the shear force effect on endothelial organization and paracrine signaling during tumor angiogenesis. Specifically, in this study, a central microchannel (711 μm) embedded within a collagen I-based hydrogel matrix serves as a neovessel through which tumor-relevant hydrodynamic stresses are introduced. Green fluorescent protein (GFP)-labeled breast cancer cells (MDA-MB-231) were seeded in the bulk of the collagen hydrogel, which surrounded endothelial cells lining the luminal surface of the central microchannel. The authors demonstrated that endothelial cells assemble a confluent endothelium on the microchannel lumen, maintaining the channel integrity under physiological flow shear stresses. The cancer cells co-cultured with endothelial cells under low flow conditions significantly enhanced the expression of pro-angiogenic factors (Buchanan et al., 2014).

In the native tumor microenvironment, an intricate network of chemokines and growth factors facilitates the crosstalk between cancer cells and stromal cells. Such intimate communications are conducive to cancer progression, through the recruitment of the immune system and new blood vessels (Mantovani et al., 2010; Roussos et al., 2011). Both tumor cells and stromal cells exhibit directional migration towards a chemokine source during disease progression and tumor metastasis (Mantovani et al., 2010; Roussos et al., 2011). To investigate

cancer migration and invasion, researchers have relied on microfluidic devices to generate stable and quantifiable concentration gradients of chemokines. For example, U87MG brain cancer cells seeded on channels in a microfluidic device were found to invade a type I collagen-based matrix in response to a gradient of epidermal growth factor (EGF). Specifically, cancer cells were guided towards higher EGF level, and the directional bias relied on both the gradient magnitude and EGF concentration (Zervantonakis et al. , 2010) In addition to providing a versatile and powerful tool for cancer research, microfluidic technology also has been utilized for the isolation of circulating tumor cells (Nagrath et al., 2007), molecular diagnosis (Pekin et al., 2011) and high-throughput screening of anti-cancer drugs (Kim et al., 2012). The readers are referred to a recent review article for in-depth discussions of these applications (Zhang and Nagrath, 2013).

3. Matrix-assisted assembly of 3D tumor models

The intricate molecular network of tumor-associated stromal ECM is an important component of the tumor microenvironment, and plays crucial roles in cancer progression and invasion (Dutta and Dutta, 2009; Weaver et al., 1997). Studies have shown that the blockage of ECM-integrin interactions led to death of highly metastatic breast cancer cells, thereby restoring morphologically normal breast structures (Wang et al., 2002). In general, association of cancer cells with the ECM produce survival or death signals at cellular levels to influence the efficacy of chemotherapeutic agents (Fracasso and Colombatti, 2000). Because tumor-associated stromal cells exerts either positive or negative effects on tumor growth and propagation, the absence of the stromal components in MCTS models limits the utility of such models in cancer research (Joyce and Pollard, 2009). Advanced technologies developed in the field of tissue engineering have enabled cancer researchers to create matrix-derived 3D tumor models that more closely recapitulate pathophysiological features of native tumor tissues (Hutmacher et al., 2010). These 3D systems have improved our understanding of the dynamic and reciprocal interactions

between the solid tumor and their surrounding microenvironment, including the stromal ECM molecules, immune systems, stromal cells, as well as growth factors and cytokines (LaBarbera et al., 2012). In this section, we summarize various types of matrices (Table 1) that have been utilized for the assembly of 3D tumor models.

3.1. Naturally derived matrices

Three-dimensional tumor models have been created using fibroblast-derived matrices (Lee et al., 2011). This matrix, produced predominately by cancer associated fibroblasts, resembles the mesenchymal microenvironment typically associated with advanced carcinomas *in vivo* (Serebriiskii et al., 2008). Compared to 2D plastic culture, cancer cells cultured in the fibroblast-derived matrices exhibited distinctly different cell morphology, aggregation pattern, proliferation profile and invasive potential (Serebriiskii et al., 2008). Disadvantages of this type of cell-derived matrices include lengthy sample preparation and batch-to-batch composition variations. In addition, the membrane-like structures have limited thickness and cannot be physically manipulated without compromising their structural integrity. Finally, fibroblast associated matrices do not fully represent the composition and structure of the tumor microenvironment (Nyga et al., 2011).

Separately, basement membrane extracts have been widely utilized in cancer research (Hutmacher et al., 2010; Poincloux et al., 2011). The utility of reconstituted basement membranes from mouse tumor as cell culture substrate was first demonstrated in the mid-1980s (Kleinman et al., 1986). The product, known as Matrigel™ or Cultrex® (Fridman et al., 2012; Sasser et al., 2007) consisting of collagen type IV, perlecan/HSPG2 and laminin, has been considered for a long time as the material of choice for 3D cancer cell culture. For example, Price et al (Price et al., 2012) demonstrated the influence of Matrigel™ culture on the expression of microRNA (miRNAs), non-encoding RNAs thought to be involved in cancer cell adhesion, proliferation and invasion. These authors discovered that tissue-like miRNA

expression levels and patterns were found when colon cancer cells were cultured in Matrigel™, but not on 2D plastic (Price et al., 2012). Although critical microenvironmental cues are restored in Matrigel™, this type of matrix often contains residual heparan sulfate growth factors, lacks human motifs, and contains undefined substances, and suffers from considerable batch-to-batch variation (Zaman, 2013). These shortcomings make it difficult to compare and analyze results from various research groups (Hutmacher et al., 2010). The identification of specific matrix components and soluble factors that contribute to the observed cell behaviors also is challenging.

Collagen type I, a native ECM component produced by cells in the stromal environment, influences the growth and metastasis of resident cancer cells (Moss et al., 2009). Collagen can be collected from various biological sources including bovine skin, rat tail and human placenta (Elsdale and Bard, 1972; Pacak et al., 2014). Collagen-based hydrogels have been widely used for the construction of 3D tumor models (Chen et al., 2012; Ingber, 2008; Szot et al., 2011; Yip and Cho, 2013). MCF-7 breast cancer cells cultured in collagen gels exhibited stronger invasive and metastatic propensities than did those in 2D cultures (Chen et al., 2012), as evidenced by the increased expression of pro-angiogenic growth factors, matrix metalloproteinases (MMPs) and epithelial mesenchymal transition (EMT) markers (Chen et al., 2012). Although collagen gels can be manufactured in a more reproducible manner than Matrigel™, their microstructure nevertheless is affected by a number of factors, including tissue source, assembly conditions and crosslinking density. These variations may render inconsistent results (Hutmacher et al., 2010). Most importantly, the hydrogel properties cannot be altered easily to interrogate the contributions of specific environmental cues to tumor assembly.

Owing to its unique mechanical properties, good biocompatibility, well-controlled degradability and versatile processability, silk fibroin biomaterials have been designed for tissue regeneration (Kundu et al., 2013). In a recent study, non-mulberry *A. mylitta* silk fibroin protein scaffolds, prepared by surfactant assisted dissolution, followed by dialysis and lyophilization,

were used for 3D culture of breast cancer cell line MDA-MB-231 cells (Talukdar and Kundu, 2012). The study showed that the silk-based 3D microenvironment provided a biocompatible niche for cancer cell attachment, growth and tumor tissue formation. The engineered tumor exhibited tissue-like heterogeneity with various zones of cell proliferation.

Various biopolymers originally developed for tissue engineering applications also have been employed for the *in vitro* construction of tumor models. In the presence of divalent cations (e.g. Ca^{2+}), alginates form hydrogels with controlled mechanical properties and pore sizes (Fischbach et al., 2009; Xu et al., 2013). Calcium crosslinked alginate gels degrade slowly under physiological conditions through the gradual exchange of calcium (Shoichet et al., 1996). Because alginate is typically non-adhesive to cells, RGD peptides have been conjugated covalently to the polymeric matrix to facilitate cell adhesion (Fischbach et al., 2009; Ruoslahti and Pierschbacher, 1987). Oral squamous cell carcinoma cells (OSCC-3) cultured in RGD modified, alginate hydrogels secreted a higher level of interleukin 8 (IL-8), presumably as a consequence of integrin engagement. In contrast, substrate adhesion did not significantly affect the cellular production of vascular endothelial growth factor (VEGF). This study highlights the importance of tumor microenvironment in regulating cancer cell angiogenic signaling (Fischbach et al., 2009).

Hyaluronic acid (HA) is another biopolymer widely exploited for the construction of 3D tumor models (Dicker et al., 2013), and unlike alginate, is structurally identical to its human counterpart that is intimately involved in cancer growth and progression (Dicker et al., 2013). HA is highly expressed in tumors and is a necessary component of the microenvironment of cancer cells (Franzmann et al., 2003; Kosaki et al., 1999; Lokeshwar et al., 1997; Lokeshwar et al., 2001). The metastasis of tumor cells can be directed by the amount of HA on the cell surface (Naor et al., 1997; Zhang et al., 1995). Through the interaction with its cell surface receptors, HA can alter the biological activity of cancer cells by triggering transforming growth factor β (TGF- β), Rho GTPase, and FAK signaling pathways (Bourguignon et al., 2003;

Bourguignon et al., 2002; Fujita et al., 2002). In tumor tissues, HA facilitates the migration of invasive cancer cells through the expansion upon hydration and their interaction with HA via certain cell surface receptors (Toole and Hascall, 2002). The biodegradation of HA by hyaluronidase (HAase) also helps tumor cells to escape from the primary tumor mass and metastasize to secondary sites (Stern, 2008b). Inhibition of hyaluronidase in 3D HA hydrogels is shown to block the formation of “invadopodia” (see below) (Gurski et al., 2012). HA oligomers trigger angiogenesis and induce inflammatory cytokine production, which activates various signaling mechanisms for cancer progression (Franzmann et al., 2003). Hence, tumor progression and angiogenesis depend on HA and hyaluronidase levels, as well as the degradation profile of HA (Stern, 2008a; b; Toole and Hascall, 2002). High concentrations of HA sometimes are observed at tumor invasion sites, and it has been shown that the HA coating around tumor cells effectively protects these cells from immune system surveillance (Lokeshwar et al., 1997).

We have developed several types of chemically crosslinked HA hydrogels that are hierarchically structured, mechanically robust and biologically active (Xu et al., 2012a). These synthetic matrices have been used to investigate prostate cancer progression and metastasis (Gurski et al., 2009; Gurski et al., 2012; Xu et al., 2012a; Xu et al., 2014). Hydrogels with controllable mechanical stiffness (140 – 230 Pa) and pore sizes (70-100 nm) were prepared using HA derivatives carrying complementary reactive groups (Gurski et al., 2009; Xu et al., 2012b). Of note, the storage modulus of the elastic-solid-like hydrogel is comparable to that of the native lymph tissue through which PCa frequently metastasizes (storage modulus: 330 Pa) (Levental et al., 2007), and the pore size of the hydrogel is also relevant to the spacing (20-130 nm) found in tumor ECMs (Pluen et al., 2001). When cultured in 3D HA hydrogel environments, prostate cancer cells formed distinct clustered tumoroid structures that grew and merged, reminiscent of native tumors. Compared to cells cultured on 2D, the engineered tumoroids in 3D significantly increased the expression of pro-angiogenic factors and MDR proteins, both at

mRNA and protein levels. To mimic the tumor/stroma interaction, a HA-based bilayer hydrogel system that not only supports the tumoroid formation from prostate cancer (PCa) cells, but also simulates their reciprocal interactions with the tumor-associated stroma, was engineered (Xu et al., 2012a). As shown in Fig. 2C, PCa cells embedded in the bottom hydrogel layer received the stromal growth factor signals from the top, and in response formed enlarging tumoroids that exhibited biological features reminiscent of native tumor tissues (Xu et al., 2012a). Our HA hydrogel system also provides a flexible, quantifiable, and physiologically relevant platform to study prostate cancer metastasis. Metastatic prostate cancer cells in these hydrogels develop fingerlike structures, "invadopodia", consistent with their invasive properties. The number of invadopodia, as well as cluster size, shape, and convergence, can provide a quantifiable measure of invasive potential. Prostate cancer cell invasion through the HA hydrogel depends on HA interaction with its cell surface receptor RHAMM/CD168 and requires hyaluronidase activity. The engineered PCa model is amenable to dissection of biological processes associated with cancer cell motility through HA-rich connective tissues (Gurski et al., 2012).

Using adipic acid dihydrazide-crosslinked HA hydrogels, David et al. investigated the invasive behaviors of a total of thirteen different cancer cell lines (from primary tumor or metastases) (David et al., 2004). Similar to our observations, the invasiveness of the cancer cell is related to its production of hyaluronidase, the expression of HA binding receptors and the cellular secretion of HA. Moreover, optimal colonization occurred when cells produced hyaluronidase, but did not possess HA-binding sites and did not secrete HA. Separately, Pedron et al. revealed the pivotal roles of HA in regulating the invasion and malignancy of human glioblastoma cells in a 3D biomimetic environment using HA-integrated, radically polymerized gelatin hydrogels. Multicellular tumor-like structures were only observed in HA containing hydrogels, and a biphasic relationship between cellular malignancies and HA content in the hydrogels was discovered (Pedron et al., 2013). Overall, systematic manipulation of the local

ECM microenvironment surrounding cancer cells enables cancer researchers to study the early growth of tumors.

3.2. Synthetic matrices

Synthetic hydrogels and scaffolds have been developed and extensively explored in tissue engineering applications to closely mimic the key features of the native ECM environment, owing to their flexible biochemical and biophysical characteristics (Drury and Mooney, 2003). These biomaterials are attractive alternatives to naturally derived matrices described above. Well-defined synthetic, yet biomimetic, matrices are attractive because they offer significantly improved batch-to-batch consistency with more controllable and reproducible characteristics, such as matrix mechanical properties, porosity and degradation profiles (Hutmacher et al., 2010).

Poly(ethylene glycol) (PEG)-based hydrogels, prepared by chemical crosslinking of the functionalized polymers, have been used widely as artificial matrices for 3D cell culture purposes (Lin and Anseth, 2009). While PEG is not a physiologically relevant molecule, biomimetic PEG hydrogels can be synthesized readily via the covalent incorporation of specific biological functionalities (e.g. functional peptides). Moreover, the mechanical and biological properties of PEG hydrogels can be tuned to profoundly influence the functions of the resident cells (Hutmacher et al., 2010). Taking advantage of the biocompatible nature of enzyme-catalyzed crosslinking reaction, Loessner et al synthesized PEG-based hydrogels using complementary peptide-functionalized multiarm-PEG as substrates for thrombin-activated factor XIII (FXIII) (Loessner et al., 2010). The design flexibility enabled the incorporation of arginine-glycine-aspartate (RGD) peptides for integrin-mediated cell adhesion, and a matrix metalloproteinase (MMP) sensitive peptide for protease-based matrix degradation. The authors demonstrated that both cell proliferation and spheroid formation from ovarian cancer cells in 3D

depended on the integrin engagement and the ability of cells to proteolytically remodel their extracellular microenvironment.

In general, synthetic ECM can be designed to provide a biomimetic cell growth niche with precise control over both the physical and biological characteristics of the matrix for tumor tissue formation. In a recent study, researchers utilized a versatile PEG-peptide hydrogel system to investigate the influence of matrix properties and the inhibition of epidermal growth factor receptor (EGFR) on the growth of a pancreatic ductal adenocarcinoma cell line (PANC-1). The hydrogel was prepared via thiol-ene photoclick chemistry using 8-arm PEG-norbornene and MMP-sensitive peptide with terminal cysteines. **These pathophysiologically-relevant hydrogels were designed to exhibit a wide range of stiffness (~1-18 kPa), with soft gels matching that of normal pancreatic tissue, and stiff gels reflecting the mechanical features of the tumor tissue.** PANC-1 cells encapsulated in relatively soft hydrogels (G' similar to 2 kPa) retained high initial viability and formed clusters after prolonged culture, whereas cells encapsulated in stiff hydrogels (G' similar to 12 kPa) exhibited lower initial viability and reduced proliferation. Immobilization of an EGFR peptide inhibitor in soft hydrogels did not increase cell death, but the same peptide immobilized in stiff hydrogels induced significant cell apoptosis, owing to the reduced cellular expression of EGFR and reduced Akt activation in stiff hydrogels. These findings underscore the potential effects of matrix properties on the efficacy of antitumor drugs (Ki et al., 2013).

Porous scaffolds, widely used in tissue engineering applications, also have been adopted for tumor engineering purposes. Because scaffold preparation requires harsh conditions and organic solvents, cells must be introduced after the scaffold has been prepared. Mooney and colleagues reported the utility of polymeric scaffolds, prepared from poly(lactide-co-glycolide) (PLGA) via a gas foaming-particulate leaching process (Harris et al., 1998), for 3D culture of OSCC-3 cells (Fischbach et al., 2007). The angiogenic characteristics of the tumor cells were altered dramatically upon 3D culture in the porous scaffold, and corresponded much

more closely to tumors formed *in vivo*. In another study (Kim et al., 2011), PLGA-based scaffolds, fabricated by a solvent casting and particulate leaching technique, were used for 3D culture of human glioma cells. Similar to other 3D culture studies, cells in this model were less sensitive to chemotherapy as evident by a lower caspase-3 activity, and a higher expression of anti-apoptotic proteins. Such a scaffold, although entirely synthetic, provides a useful 3D platform to study the effect of microenvironmental conditions on tumor progression *in vitro*.

To foster more intimate cell-cell and cell-matrix interactions, researchers resorted to electrospun, microfibrinous meshes for 3D culture of cancer cells. In one example, porous electrospun poly(ϵ -caprolactone) (PCL) scaffolds were used for the culture of Ewing sarcoma cells in 3D (Fong et al., 2013). Cancer cells cultured in the 3D environment not only were more resistant to traditional cytotoxic drugs than were cells in 2D monolayer culture, but also exhibited remarkable differences in the expression pattern of the insulin-like growth factor-1 receptor / mammalian target of rapamycin pathway (Fong et al., 2013). Of note, since drug transport and interaction with the scaffold may have an impact on its efficacy, the decreased drug sensitivity found in 3D PCL scaffold may be partially attributed to the physical adsorption of the drug onto the scaffold. Overall, the reported PCL-based tumor model has the potential to provide a platform for the mechanistic studies of bone sarcomas and the evaluation of anticancer drug candidates for these malignancies (Fong et al., 2013). Modification of electrospun scaffolds with bioactive ECM-derived peptides also can be used to study pharmacokinetic properties of various drugs targeting cancer cells (Hartman et al., 2010). To gain mechanistic understandings of cancer metastasis, Sieh et al. cultured PCa cells on a tissue engineered bone constructs, fabricated by wrapping a human osteoblast cell sheet around a cell-seed, bone-mimetic composite scaffold (Sieh et al., 2010). Consistent with reported *in vivo* studies, the authors found that the intercellular and PCa cell-bone matrix interactions contributed to the observed increase in the expression level of various biomarkers associated with PCa bone metastasis.

Because of the defined amino acid composition and the structural and mechanical similarity to natural ECM, self-assembled peptide hydrogels have emerged as attractive 3D culture platforms (Loo et al., 2012). Gelation occurs via a process that involves concerted action of weak and non-covalent interactions, triggered by altering the composition of the aqueous media. The synthetic nature of the peptide building blocks allow for facile incorporation of specific biological relevant ligands to achieve desired biological functions (Ulijn and Smith, 2008; Zhang et al., 2005a). These physical gels contain entangled, amyloid-like fibers of ~ 10 nm thick and interstitial pores between 5 and 200 nm (Goldberg et al., 2007). Using RADA16-I peptide hydrogel, 3D models of ovarian cancer were created. Cells cultured in the peptide hydrogels not only exhibited strong invasion potentials, but also displayed significantly higher anticancer drug resistance than did cells cultured on the conventional 2D petri dish (Yang and Zhao, 2011). Hydrogels assembled from a rationally designed h9e peptide (Huang et al., 2011) containing sequences from an elastic segment of spider silk and a trans-membrane segment of human muscle L-type calcium channel, were used for 3D culture of human epithelial cancer cells (MCF-7). MCF-7 cells residing in the peptide hydrogel grew into 3D tumor-like clusters and responded to cisplatin treatment in a dose- and time-dependent manner (Huang et al., 2013). Gelation by supramolecular interactions through the entanglement of fibrillar structures is an attractive feature of self-assembled peptide hydrogels. Long term stability, however, needs to be improved.

4. 3D tumor models for drug testing

Although rapid advances in drug design methodology have led to dramatic increases in the discovery of both candidate drug compounds and their screenable drug targets (Dobson, 2004; Tan, 2005), a commensurate increase in the number of approved drugs has not been seen (LaBarbera et al., 2012). The high failure rate of drug candidates can be attributed in part to the usage of 2D monolayer cultures as the initial screening method that frequently produces

inaccurate and unreliable results and does not predict chemoresistance (van de Waterbeemd and Gifford, 2003). As discussed above, the same drug treatment elicits distinctly different responses from cells in 3D and on 2D. The ability of 3D systems to recapitulate tumor-like microenvironment suggests the potential of engineered tumor models to provide more accurate and reproducible toxicity information for the early-stage drug discovery (Lee et al., 2008).

4.1. High-throughput screening of drug candidates

Adaptation of 3D tumor models to high-throughput drug screening systems are highly desirable because they can provide predictable results in an efficient manner, thereby enabling the prioritization of candidate compounds for further development in the *in vivo* studies (Friedrich et al., 2009). For use as *in vitro* drug testing platforms, the 3D models must recapitulate the pathophysiological features of the tumor microenvironment so that *in vivo* drug efficacy can be reliably predicted (Mueller-Klieser, 2000). For high-throughput purposes, the implementation of the 3D tumor models into drug evaluation routines requires an experimental design that guarantees tumor constructs of the same cell type cultured under the same external conditions are essentially identical in morphology, structure, microenvironment and cellular physiology (Friedrich et al., 2009). Routine high-throughput drug screening protocols based on various 3D systems (Kim, 2005) have been developed (Friedrich et al., 2009).

Using ultra-low attachment 96-well round-bottomed plates (Vinci et al., 2012), researchers demonstrated that tumor cells formed uniform, single and centrally located spheroids of reproducible size within each well. The resultant MCTS were subjected to automated multiparametric analyses, including measurements of spheroid diameter, perimeter and area, using a Celigo™ cytometer. This platform not only enabled reliable assessments of key hallmarks of cancer, i.e. cell motility, matrix invasion and tumor angiogenesis, but also facilitated evaluation of various types of cell growth inhibitors. Not surprisingly, 2D and 3D cultures exhibited differential sensitivities to targeted agents, and in general, tumor cells were

less sensitive to soluble compounds in 3D than in 2D monolayer cultures. The utility of some agents in treating metastatic disease also has been identified. Other 3D culture techniques amenable to high throughput screening include liquid overlay (Li et al., 2011), hanging drop (Tung et al., 2011), microarray template (Hardelauf et al., 2011), microencapsulation (Zhang et al., 2005b) and magnetic cell levitation (Souza et al., 2010).

The differential drug sensitivity observed between 2D and 3D cultures can be attributed to decreased compound access or reduced drug sensitivity in response to hypoxic or more slowly cycling cells under 3D culture conditions (Fong et al., 2013; Minchinton and Tannock, 2006). It is also reported that cell-cell contact plays a role in drug resistance found in 3D. A negative correlation between cell-cell contact and drug sensitivity was observed in a PCL scaffold-based 3D tumor model (Fong et al., 2013). However, the exact signaling mechanism implicated in drug resistance in 3D is still unclear. Current work is focused on the study of the interplay among various factors (e.g. intracellular changes, paracrine signaling, modifications in the supporting matrix) that may contribute to the reduced drug sensitivity in 3D (Saraswathy and Gong, 2013).

Tumor models fabricated using matrix-assisted approaches have not yet been widely exploited for high throughput screening purposes because of the additional variables introduced by the matrix and challenges associated with manipulating and fabricating hydrogels or scaffolds in a high throughput fashion. If the matrix is simple, however, adaptation can be achieved. For example, using collagen or alginate gels, 3D tumor cell-culture array (Data Analysis Toxicology Assay Chip or DataChip) has been developed for the high-throughput toxicity evaluation of both the drug candidates and their cytochrome P450-generated metabolites (Lee et al., 2008). Specifically, cancer cells encapsulated within collagen or alginate gels with the volume as low as 20 nL, were spotted onto the surface of functionalized glass slides of the DataChip. One single DataChip contains 1,080 of this kind of individual microscale cell/gel tumor constructs. The system was used for spatially addressable screening against

multiple anti-cancer compounds. In conjunction with the complementary human P450-containing microarray, the device provided the toxicity results for the metabolites of the compound from three human P450 isoforms (CYP3A4, CYP1A2, and CYP2D6), which were designed to mimic the function of human liver.

4.2. Evaluation on drug delivery systems

Over the past few decades, concerted efforts have been made for the development of nanotechnology-based cancer therapeutics, with the promise of potentially prolonging patients' life expectancy, at the same time reducing treatment-related side effects (Byrne et al., 2008; Kumar et al., 2013; Peer et al., 2007). The engineered nano-formulations improve the pharmacological properties of drugs, and also potentially increase the drug efficacy through the enhanced permeability and retention (EPR) effect or the active targeting strategies (Byrne et al., 2008; Kang et al., 2010). The typical size of the developed nano-therapeutics for clinical applications ranges from 2 nm to 200 nm (Kamaly et al., 2012). For effective treatment of solid tumor, it is desirable that the nanomedicine can reach as many cells within the tumor parenchyma as possible (Kamaly et al., 2012). However, the penetration of the nano-therapeutics can be hindered by the elevated levels of interstitial fluid pressure (IFP), tortuosity and interstitial matrices of the tumor tissue (Wong et al., 2011). Moreover, the increased IFP limits the transport of nanomedicine to the poorly perfused regions of tumor tissues where access is dominated only by diffusion (Kim et al., 2010). The rate of diffusion and the penetration depth depend on the size of the drug carrier and the interactions between the carrier and the interstitial matrices (Kim et al., 2010).

Three-dimensional avascular tumor models have been used to assess the diffusion, distribution and drug efficacy of nano-therapeutics. Using a 3D MCTS model, Pun and coworkers reported that polystyrene nanoparticles (NPs) with an average diameter of 20 and 40 nm penetrated the MCTS, whereas larger NPs (100 or 200 nm) were restricted to the periphery

of the spheroids (Goodman et al., 2007). When collagenase was immobilized on the particle surface, the penetration depth of the NPs was enhanced significantly. Therefore, incorporating ECM-modulating enzymes in drug carriers may improve NP penetration in solid tumors. In a separate investigation, researchers discovered that doxorubicin (Dox)-loaded nanoparticles (Dox-NPs) with an average diameter of 37 nm penetrated into the core of 400 μm human cervical tumor spheroids in 30 min. Compared to free Dox, Dox-NPs not only exhibited a greater tumor penetration capacity, but also suppressed tumor growth more efficiently in the MCTS model (Kim et al., 2010). Similar observations were found with regard to the transport property of a pseudo-peptide-based drug delivery system using a Hela MCTS model (Ho et al., 2011). Overall, NP penetration depended on both the carrier concentration and the incubation time.

Three-dimensional tumor models assembled in hydrogel matrices also have been used for the evaluation of nanoformulations. The hydrogel network, with optimized stiffness and mesh size, was designed to simulate the ECM barrier of tumor tissues. Using a PCa tumor model created using an HA hydrogel, Xu et al discovered that Nile Red (NR)-loaded polymeric NPs with an average hydrodynamic size of 54 nm had a diffusion coefficient of 9.21 $\mu\text{m}^2/\text{s}$ and 4.04 $\mu\text{m}^2/\text{s}$ in water and the HA network, respectively. Dox-NPs introduced to the cell/gel constructs reached the embedded tumoroids within 2 hours, homogeneously distributed in the tumoroids and were internalized by tumor cells through caveolae-mediated endocytosis and macropinocytosis pathways. A comparison of intracellular drug localization revealed that on 2D cultures, free Dox was transported to the cell nuclei [Fig. 3A(a)] whereas Dox-NPs were excluded from the nuclei and distributed predominantly in the cytoplasm [Fig. 3A(b)]. Interestingly, under 3D culture conditions, both free Dox and Dox-NPs localized predominantly in the cytoplasm of individual cells in the tumoroids [Fig. 3A(c) and 3A(d)]. Finally, as indicated from the dose response curves and the IC50 values (Fig. 3B and 3C), tumor cells cultured as multicellular aggregates in HA hydrogel were more resistant to both free Dox and Dox-NP treatments compared to 2D cultures. The drug resistance profile observed in 3D has been

attributed to the elevated expression of MDR proteins (multidrug resistance protein 1 and lung resistance-related protein) by LNCaP PCa cells cultured in HA hydrogels (Xu et al., 2014). It has been reported that primary cultures of PCa cells derived directly from the patients expressed significantly elevated levels of MDR proteins than PCa cell lines (i.e. LNCaP, PC3 and DU145 cell lines). As a result, patient-derived cells displayed lower drug sensitivity (Sanchez et al., 2009). The HA-based 3D tumor models closely mimic the *in vivo* tumor growth condition, and the increased expression of MDR proteins suggested a MDR cell phenotype. Therefore, the drug response from cells cultured in the 3D HA hydrogel should be more physiologically relevant than that from cells cultured on 2D.

The Zaman and Grinstaff team created 3D models of osteosarcoma and breast adenocarcinoma by first allowing the cancer cells (U2OS and MDA-MB-231) to aggregate in an agarose well, then encapsulating the spheroids in collagen gels (Charoen et al., 2014). Using the engineered tumor models, the researchers characterized the chemotherapeutic responses from paclitaxel, delivered in bolus as free drug or controlled release using an expansile nanoparticle (eNP). The eNPs were designed to release the drug payload via a pH mediated particle expansion within the cell endosome (Zubris et al., 2013). Confocal microscopy imaging showed that the eNPs, with an average diameter of ~100 nm (Colby et al., 2013), dispersed throughout the spheroid after diffusing through the collagen and into the spheroid. Compared to the bolus delivery, the eNPs delivery provided a significantly more pronounced inhibition in cell growth, in accordance with the *in vivo* observations from a murine xenograft model. Contrarily, no difference in response between bolus and eNP delivered paclitaxel was detected in 2D monolayer (Liu et al., 2013). These findings highlight the importance of physiologically relevant tumor models for generating clinically predictable assessments on drug formulations.

5. Summary and outlook

Engineered 3D systems provide a realistic and controllable environment for the incorporation of specific cells, ECM molecules, growth factors and other biochemical cues to better simulate the native tumor microenvironment that favors tumor growth and progression. These models will likely be of significant use in delineating the biological mechanisms that govern the pathological abnormalities observed in cancer. They also will serve as more reliable platforms for generating predictive results on *in vivo* evaluation of chemotherapeutic agents and their delivery systems.

Despite substantial and continued success in creating biomimetic 3D tumor models, major challenges and limitations remain associated with existing models. First, many models are created using long term 2D culture adapted cancer cell lines that may no longer accurately reflect the pathology of the initial disease. The establishment of therapeutically relevant treatment predictions relies on the availability of patients' cells, expanded in xenografts, with characteristic heterogeneity of the disease and different metastatic potentials. Successful culture of these cells *ex vivo* will provide access to a higher level of accuracy both in probing the biological underpinnings of metastasis, and in correctly identifying the susceptibility to pathway-targeted drugs (Fong et al., 2014). Equally important is the validation of the engineered tumor models, at least by the source xenografts, and ideally matched to each patient's deidentified history of drug therapies to guide and refine the current model.

Secondly, current 3D systems remain relatively simplistic and do not faithfully represent the native tumor microenvironment. Many engineered models appear similar to native tumor tissues only morphologically. Phenotypic similarity and heterogeneity need to be recaptured. When synthetic matrices are employed, detailed signaling studies of cells in the engineered microenvironment is lacking. A future trend is to improve the physiological complexity, which includes cell types, matrix composition, as well as temporal and spatial presentation of soluble factors employing innovative materials chemistry and engineering designs. The recreation of the complex, tumor-associated vascular system *in vitro* is another challenge task but necessary to

be addressed because these abnormal blood vessels not only influence cancer progression, but also greatly affect drug transport within the tumor tissues (Jain, 2012).

Finally, as the tumor models become more and more sophisticated, computational modeling (Shirinifard et al., 2009), system biology approaches (Deisboeck et al., 2011; Rajagopalan et al., 2013) and *in situ* real time imaging, detection and analysis modalities are sorely needed (Zaman, 2013). For example, crosslinking of hydrogel precursors in the presence of homogeneously dispersed cancer cells results in the entrapment of cells in a porous network with mesh size in the micron to submicron scale (Nicodemus and Bryant, 2008; Xu et al., 2014). To form large tumor-like aggregates, cells initially trapped at a single cell state must proliferate within the matrix, at the same time migrate through the network, via amoeboid and/or mesenchymal movement (Wolf and Friedl, 2006). There is a need for quantitative understanding of the collective movement of aggregated cancer cells in the 3D environment. Overall, with the advances in 3D culture techniques (Hutmacher et al., 2010), cancer genomics (Chin et al., 2011) and mathematical modeling (Haeno et al., 2012), promising 3D engineered tumor models will serve as the bridge between 2D monolayer culture and cancer xenografts to accelerate the translation of novel therapeutics to the clinic.

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Figure Caption

Fig. 1. Schematic illustration of a typical tumor microenvironment. Cancer cells reside in a complex microenvironment containing various supporting cells, extracellular matrix (ECM) and a suite of signaling molecules. These environmental components collectively contribute to the tumor-stromal interaction and tumor progression. Adapted from (Joyce and Pollard, 2009; Koontongkaew, 2013).

Fig. 2. Representative examples of culture platforms employed for the growth of 3D tumoroids. (A) Rotating wall vessel (RWV) bioreactors (i, ii) have been used for the 3D culture of human breast ductal carcinoma cells (T-47D, iii) and glioblastoma cells (labeled with green fluorescent protein, iv). Cells cultured in a state of free fall with minimal shear (ii) organized into millimeter sized aggregates with multiple layers of cells (iii and iv, scale bar: 200 μm). Reproduced with permission (Becker and Souza, 2013), Copyright 2013, Macmillan Publishers Limited. (B) A microfluidic device (i–iii) has been designed for the 3D culture of PC-3 prostate cancer cells. The device is composed of upper and lower channels separated by a non-adhesive, semi-permeable membrane. PC-3 cells, mixed with pre-osteoblasts and endothelial cells and introduced to the device as a monolayer, self-organized into 3D spheroids in one day (iv: optical image; v: fluorescent image, red-PC-3 cells, green-live cells; scale bar: 200 μm). Reproduced with permission (Hsiao et al., 2009), Copyright 2009, Elsevier Ltd. (C) A bilayer hydrogel platform (i) for the 3D culture of prostate cancer cells. LNCaP cells culture in the bottom layer, receiving heparin-binding epidermal growth factor-like growth factor (HB-EGF) released from the top layer, grew into spheroids (ii, iii, nuclei: blue) displaying cortical F-actin (green) and expressing E-cadherin (red). Reproduced with permission (Xu et al., 2012), Copyright 2012, Elsevier Ltd.

Fig. 3. Comparison of intracellular localization and cell-killing capacity of free Dox and Dox-NPs in 2D and 3D cultures of LNCaP prostate cancer cells. HA-based hydrogels were used for 3D cultures. (A) Confocal images show differential localizations of Dox and Dox-NPs in LNCaP cells after 2 h of drug exposure. Dox is inherently fluorescent and the internalized Dox or Dox-NPs were detected using a Zeiss510 NLO confocal microscope (excitation wavelength at 488 nm with a band pass filter of 565-615 nm). (B) Dose-dependent cell death induced by Dox or Dox-NPs on 2D and 3D cultures of LNCaP prostate cancer cells. Cell apoptosis analysis was performed using Cell Death Detection ELISA^{PLUS}. (C) Summary of the IC₅₀ values for various drug/culture combinations. (*, significant difference compared to the IC₅₀ value of 2D Dox condition, p < 0.05). Reproduced with permission (Xu et al., 2014), Copyright 2014, Elsevier Ltd.

Figure 1

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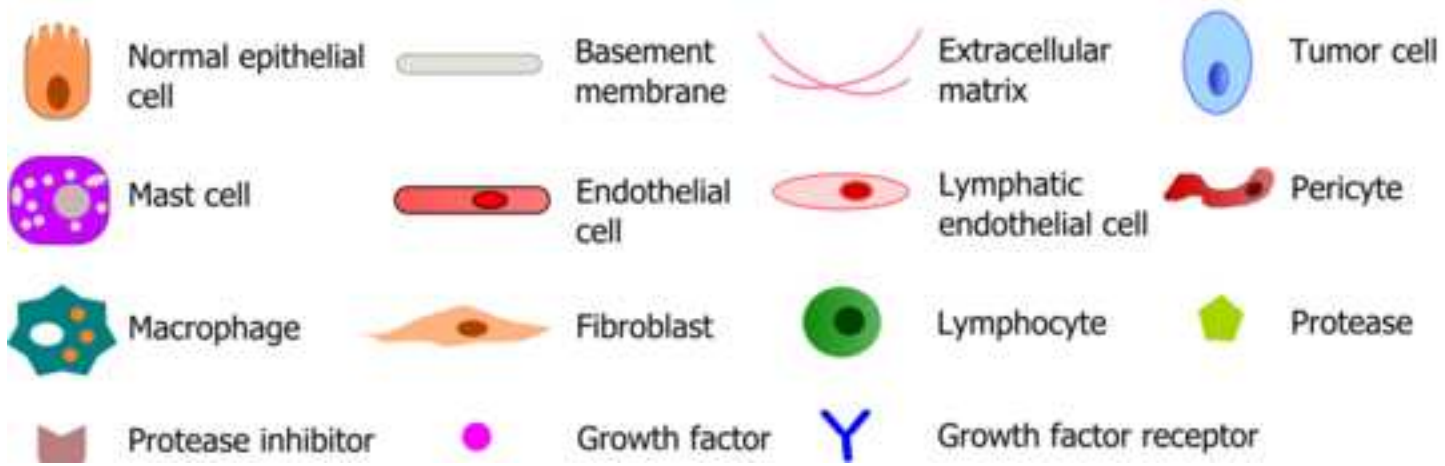
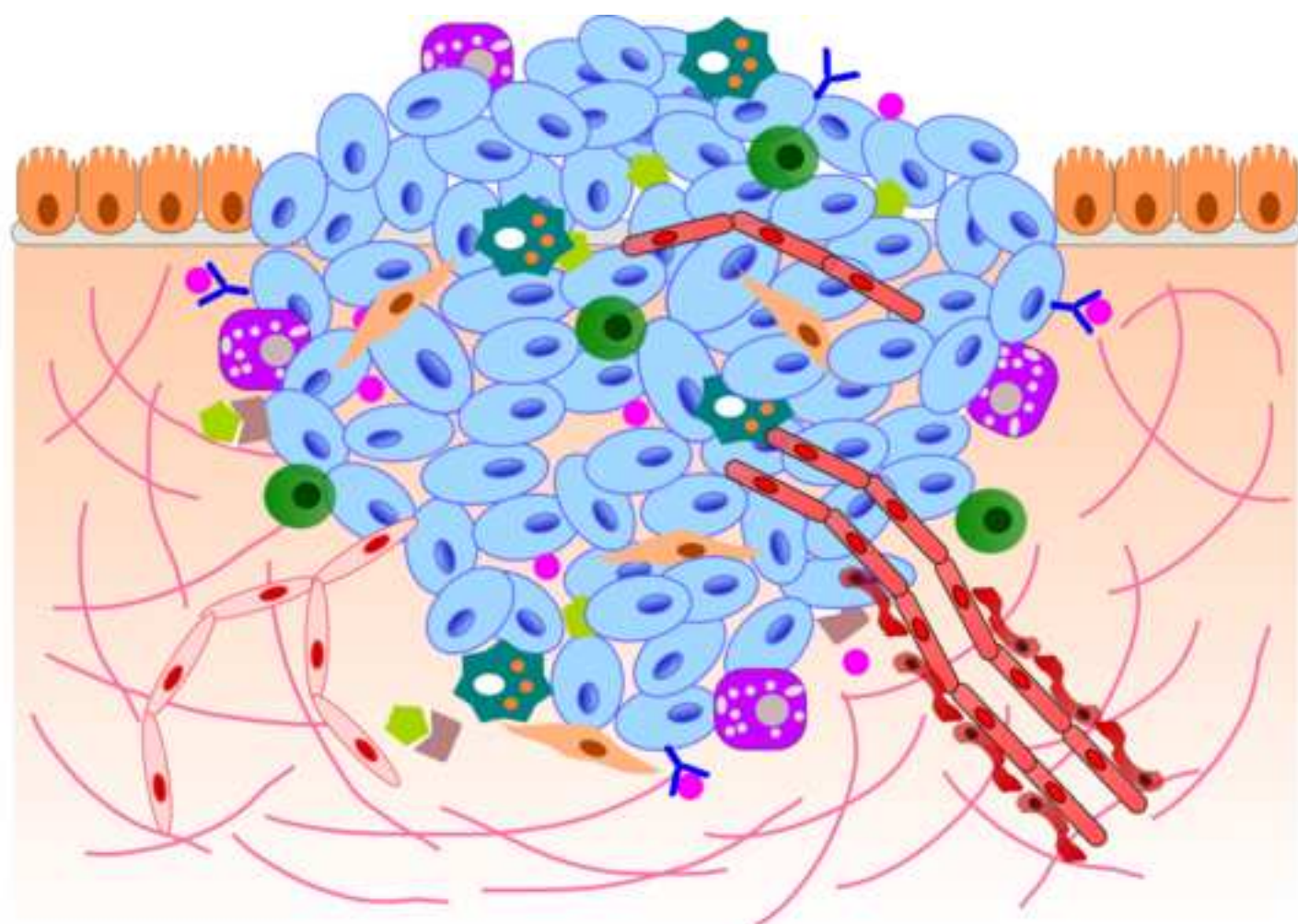


Figure 2
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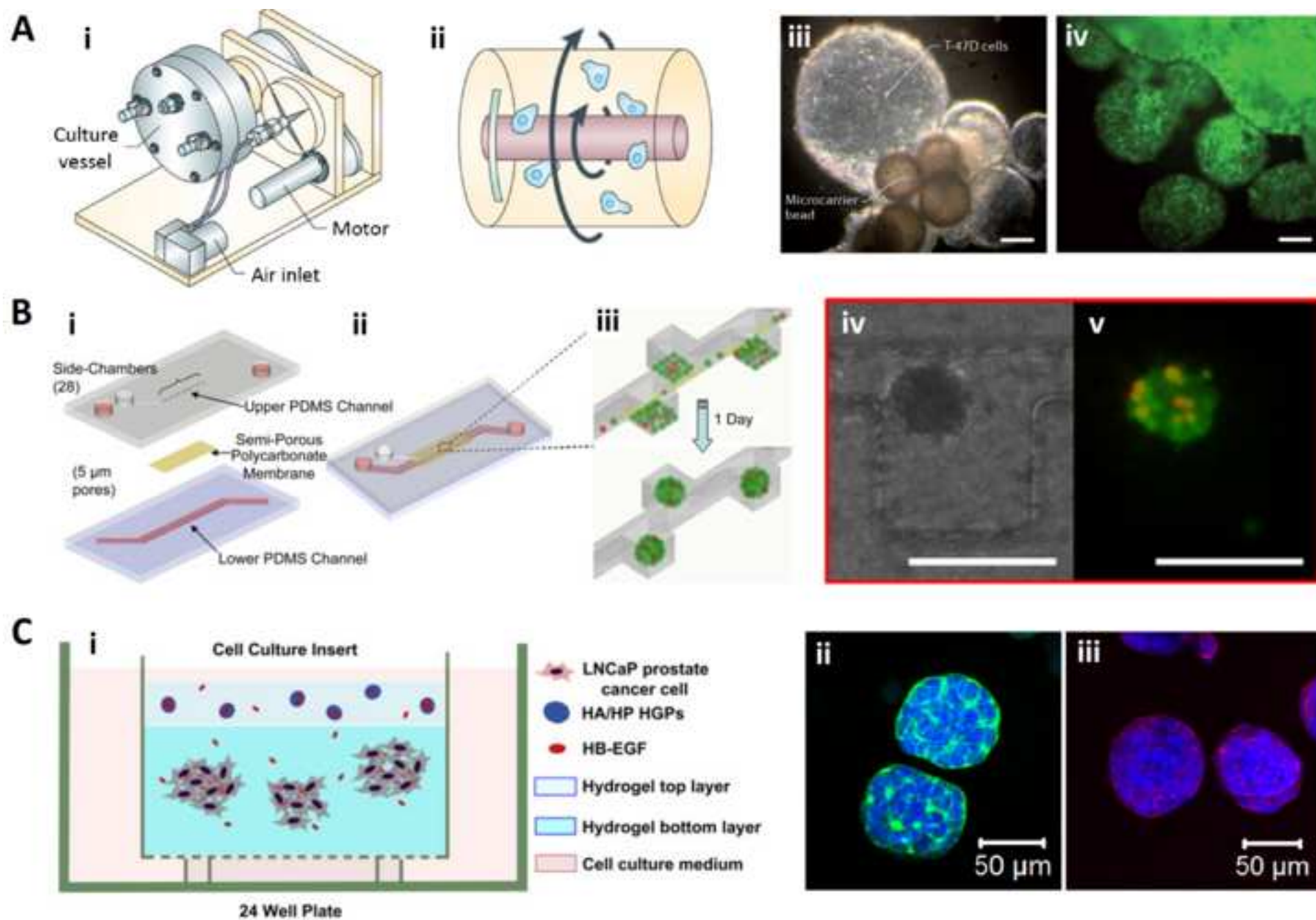


Figure 3A
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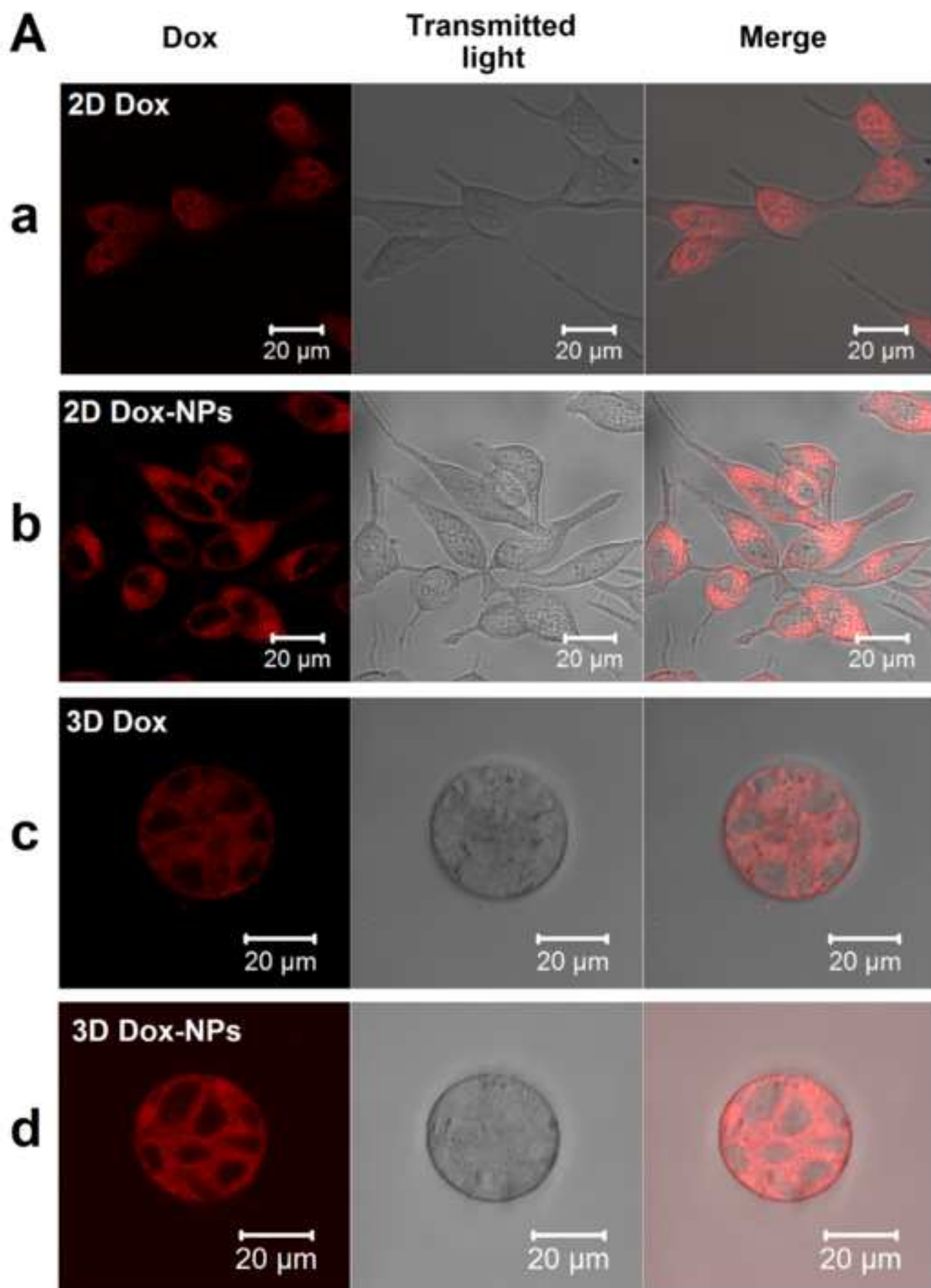
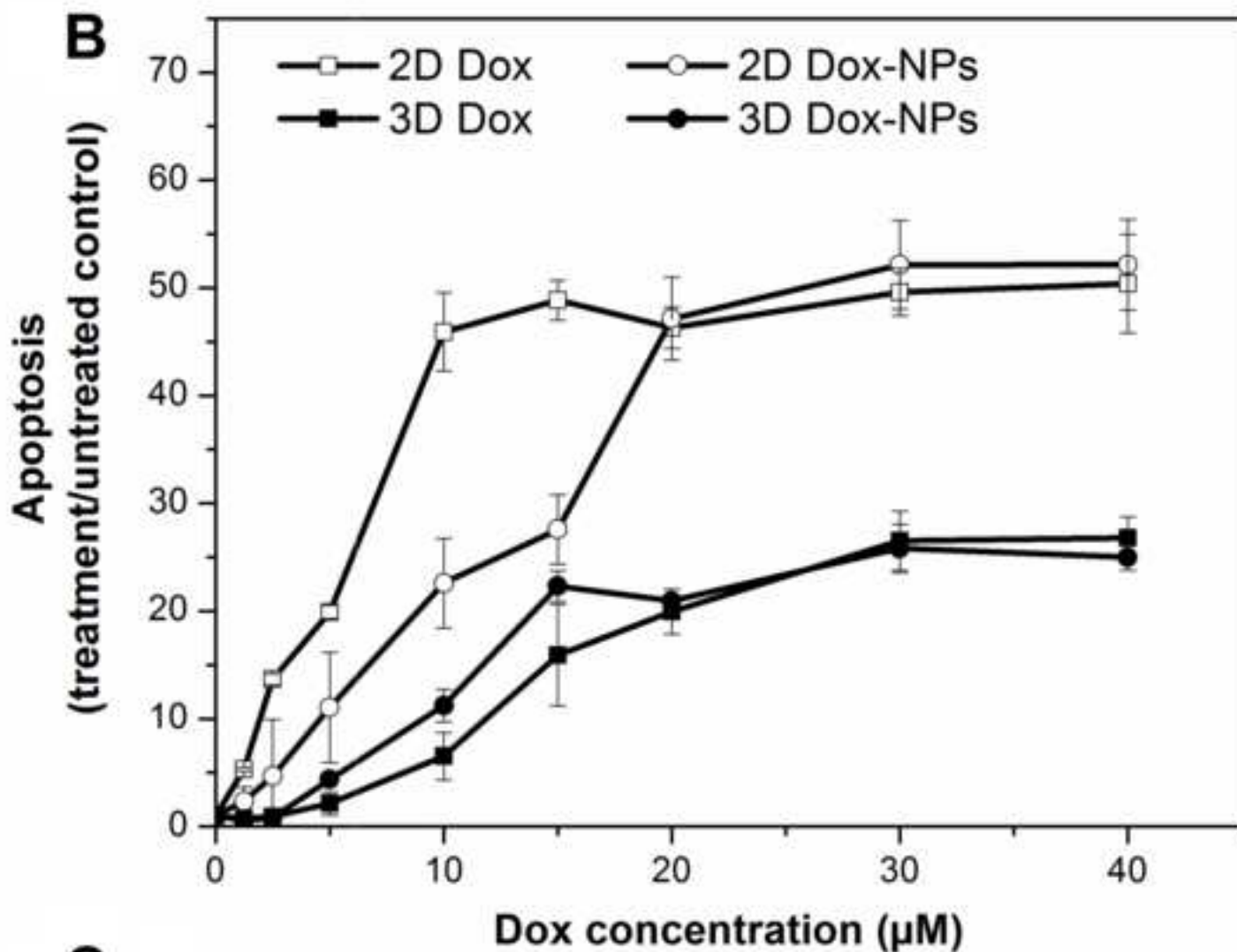


Figure 3B-C
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	2D Dox	2D Dox-NPs*	3D Dox*	3D Dox-NPs
IC ₅₀ (µM)	5.0 ± 0.1	11.0 ± 0.8	15.0 ± 0.4	12.3 ± 0.8

Table 1.

Naturally derived and synthetic matrices for 3D tumor engineering.

Type of materials	Properties	Tumor types / Cell lines	References
Fibroblast-derived matrices	Produced mainly by cancer associated fibroblasts. Mimic microenvironment in advanced carcinomas.	Human colorectal carcinoma / HCT116 Human pancreatic carcinoma / PANC-1	Serebriiskii et al. (2008)
Matrigel™ or Cultrex®	Basement membrane extracts consisting of collagen type IV, laminin, perlecan, etc.	Human colon adenocarcinoma / SW480 Human breast adenocarcinoma / MDA-MB-231	Price et al. (2012) Poincloux et al. (2011)
Type I collagen	Native ECM component produced by cells in the stromal environment. Influences the growth and metastasis of resident tumor cells.	Human breast carcinoma / MCF-7 Human hepatocellular liver carcinoma / HepG2	Chen et al. (2012) Yip and Cho (2013)
Silk fibroin	Unique mechanical properties, good biocompatibility, well-controlled degradability and versatile processability.	Human breast adenocarcinoma / MDA-MB-231	Talukdar and Kundu (2012)
Alginate	Biodegradable hydrogels with controlled mechanical properties and pore sizes. Non-adhesive to cells.	Human hepatocellular carcinoma / MHCC97L, HCCLM3 Oral squamous cell carcinoma / OSCC-3	Xu et al. (2013) Fischbach et al. (2009)
Hyaluronic acid	Essential ECM component in the tumor microenvironment. Interacts with its cell surface receptors, such as CD44 or	Human prostate cancer / LNCaP	Xu et al. (2012a) Xu et al. (2014)

	RHAMM. Involved in tumor expansion, angiogenesis and metastasis.	Human glioma / U87MG	Pedron et al. (2013)
PEG	Synthetic matrices with controllable biochemical and mechanical properties. Specific biological functionalities can be covalently incorporated into the PEG hydrogels.	Human epithelial ovarian cancer / OV-MZ-6	Loessner et al. (2010)
		Human pancreatic ductal adenocarcinoma / PANC-1	Ki et al. (2013)
PLGA	Porous biodegradable synthetic scaffolds. Convenient to handle and amenable to large-scale use.	Oral squamous cell carcinoma / OSCC-3	Fischbach et al. (2007)
		Human glioma / U251	Kim et al. (2011)
PCL	Biologically inert synthetic scaffolds. Medical grade PCL-tricalcium phosphate (mPCL-TCP) scaffolds can be used to study bone metastasis.	Ewing sarcoma / TC-71	Fong et al. (2013)
		Prostate cancer / PC3 and LNCaP	Sieh et al. (2010)
Synthetic peptides	Defined amino acid composition for facile incorporation of specific biological relevant ligands.	Human ovarian carcinoma / SK-OV-3	Yang and Zhao (2011)
		Human breast carcinoma / MCF-7	Huang et al. (2013)