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3D *in vitro* bioengineered tumors based on collagen I hydrogels

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ABSTRACT

Cells cultured within a three-dimensional (3D) *in vitro* environment have the ability to acquire phenotypes and respond to stimuli analogous to *in vivo* biological systems. This approach has been utilized in tissue engineering and can also be applied to the development of a physiologically relevant *in vitro* tumor model. In this study, collagen I hydrogels cultured with MDA-MB-231 human breast cancer cells were bioengineered as a platform for *in vitro* solid tumor development. The cell–cell and cell-matrix interactions present during *in vivo* tissue progression were encouraged within the 3D hydrogel architecture, and the biocompatibility of collagen I supported unconfined cellular proliferation. The development of necrosis beyond a depth of ~150–200 μm and the expression of hypoxia-inducible factor (HIF)-1α were demonstrated in the *in vitro* bioengineered tumors. Oxygen and nutrient diffusion limitations through the collagen I matrix as well as competition for available nutrients resulted in growing levels of intracellular hypoxia, quantified by a statistically significant ($p < 0.01$) upregulation of HIF-1α gene expression. The bioengineered tumors also demonstrated promising angiogenic potential with a statistically significant ($p < 0.001$) upregulation of vascular endothelial growth factor (VEGF)-A gene expression. In addition, comparable gene expression analysis demonstrated a statistically significant increase of HIF-1α ($p < 0.05$) and VEGF-A ($p < 0.001$) by MDA-MB-231 cells cultured in the 3D collagen I hydrogels compared to cells cultured in a monolayer on two-dimensional tissue culture polystyrene. The results presented in this study demonstrate the capacity of collagen I hydrogels to facilitate the development of 3D *in vitro* bioengineered tumors that are representative of the pre-vascularized stages of *in vivo* solid tumor progression.

1. Introduction

Cancer biologists, biomedical researchers, and oncologists have long relied on two-dimensional (2D) Petri dish studies and small animal models to study the complex tumorigenic mechanisms of angiogenesis, invasion, and metastasis. However, these models of tumor development have thus far been inadequate for cultivating the discovery of definitive cancer termination and prevention treatments. 2D cell culture models lack the structural architecture necessary for proper cell–cell and cell-matrix interactions and are therefore incapable of replicating an *in vivo* phenotype [1–5]. Small animal models are the current gold standard for conducting cancer research, even though there are considerable differences between cancer progression in humans and animals [3,6]. Additionally, animals intrinsically contain many uncontrollable factors, including host cells, an immune response, hemodynamics, and endogenous growth factors. These variables complicate isolating the impact of specific stimuli, such as cellular, chemical, and mechanical cues, during therapeutic testing [7]. Recently, some promising three-dimensional (3D) cell culture models have been developed for studying tumor progression *in vitro*. Results in the literature show that these models are beginning to restore the cellular morphologies and phenotypes seen during *in vivo* tumor development [8–13].

Ghajar and Bissell recently defined Tumor Engineering as "the construction of complex culture models that recapitulate aspects of the *in vivo* tumor microenvironment to study the dynamics of tumor development, progression, and therapy on multiple scales [14]." This burgeoning field of research is rapidly evolving the study of cancer progression *in vitro* [5,15]. Fischbach and colleagues have engineered an array of 3D *in vitro* tumor models using both synthetic and natural polymeric scaffolds to demonstrate angiogenic factor secretion and drug responsiveness [8], the effects of tumor oxygen tension and 3D cell-extracellular matrix (ECM) interactions on angiogenic potential [12], and endothelial cell remodeling of dense collagen I matrices in response to potential secretion of angiogenic factors from underlying cancer cells [9]. Nelson and Bissell have highlighted the importance of developing

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functional 3D *in vitro* models of mammary gland acini for advancing breast cancer research [10] and have fabricated 3D epithelial culture models using lithography [11]. Our group has reported previously on the potential use of nanofibrous scaffolds, such as bacterial cellulose and electrospun polymer composites, for tissue engineering *in vitro* tumor models [16].

The pre-vascularized stages of solid tumor growth can be characterized by identifiable criteria within the tumor microenvironment, including an uninhibited 3D proliferative capacity [17], regions of hypoxia surrounding a necrotic core [18,19], and activation of genetic factors that lead to the recruitment of local endothelial cells for self-sustaining angiogenesis (Fig. 1a) [17,20]. Uninhibited 3D proliferative capacity is a trait that cancer cells achieve during *in vivo* tumor development following a series of mutations that cause growth signal autonomy, insensitivity to antigrowth signals, and resistance to apoptosis [17]. Cancer cell lines *in vitro* maintain this phenotype, demonstrating limitless proliferation within the confines of their environment. Culturing cancer cells in 3D scaffolds has been shown to foster this proliferative potential, allowing for growth of clinically relevant tumor masses [8]. However, within an *in vivo* tumor microenvironment, there are restrictions on tumor growth enforced by oxygen and nutrient diffusion limitations through tissue. Hypoxia, a state of limited oxygen availability, occurs within 100–200 μm of the closest vasculature. Cancer cells that cannot adjust to the oxygen and nutrient deficiencies at the core of a growing tumor mass cede to cell death through either apoptosis or necrosis. A key marker for identifying hypoxia is hypoxia-inducible factor (HIF)-1α, a heterodimeric transcription factor protected from degradation when the surrounding oxygen tension is at a hypoxic level [18,19]. Solid tumors evolve from an avascular to a vascular state by responding to this microenvironmental hypoxic stress and initiating an angiogenic response from the host vasculature. This process is instigated by the cancer cells, which secrete growth factors and cytokines that interact with local endothelial cells, promoting vascular sprouting and neovascularization [21]. Vascular endothelial growth factor A (VEGF-A), a heparin-binding homodimeric glycoprotein, plays a major role in initiating this process through stimulating vascular permeability and endothelial growth [22]. Activation of VEGF-A gene transcription occurs in direct response to the development of hypoxia and HIF-1α expression [23].

The biocompatibility and 3D architecture of collagen I hydrogels are suitable properties for reproducing the microenvironmental conditions of a solid tumor. Collagen I is a frequently used substrate for cell culture and tissue engineering applications, because it contains the tripeptide RGD (Arg-Gly-Asp), a short amino acid sequence that preferentially binds to receptors on cell surfaces [24]. Cell-mediated degradation of collagen I through the secretion of cleaving enzymes allows for remodeling of the matrix during proliferation, migration, and infiltration [25]. Furthermore, hydrogel concentration, scaffold thickness, and cell seeding density can be tailored to stimulate specific cellular responses within the engineered microenvironment. We hypothesize that collagen I hydrogels can be used as 3D cell culture scaffolds for bioengineering tumors that mimic key characteristics of *in vivo* tumor progression.

While the influence of hypoxic oxygen levels and cell-matrix interactions on the angiogenic potential of cancer cells cultured *in vitro* has been documented [12], a 3D *in vitro* tumor microenvironment that inherently promotes a phenotype typical of the pre-vascularized stages of *in vivo* solid tumor progression has not been established. In this study, MDA-MB-231 human breast cancer cells

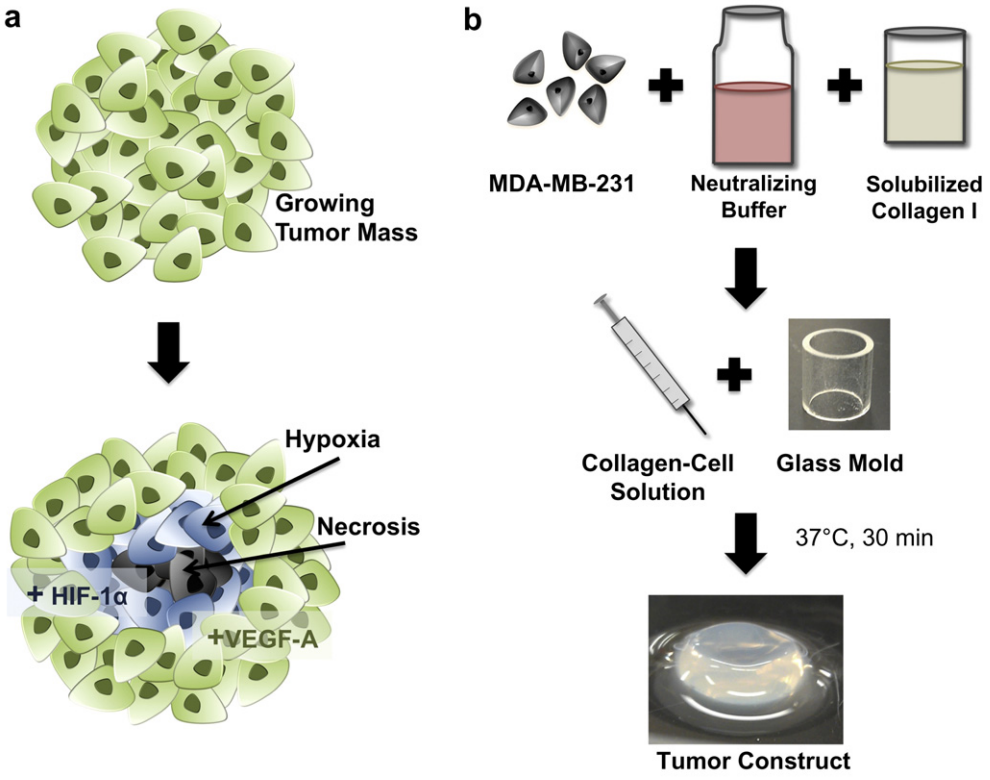

Fig. 1. (a) The pre-vascularized stages of *in vivo* solid tumor development can be characterized by identifiable criteria within the tumor microenvironment, including an uninhibited 3D proliferative capacity, regions of hypoxia surrounding a necrotic core, and activation of angiogenic growth factors, including VEGF-A. (b) Collagen I hydrogels cultured with MDA-MB-231 human breast cancer cells were bioengineered as a platform for *in vitro* solid tumor development.