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Bioinspired Hydrogels to Engineer Cancer Microenvironments*

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Abstract

Recent research has demonstrated that tumor microenvironments play pivotal roles in tumor development and metastasis through various physical, chemical, and biological factors, including extracellular matrix (ECM) composition, matrix remodeling, oxygen tension, pH, cytokines, and matrix stiffness. An emerging trend in cancer research involves the creation of engineered three-dimensional tumor models using bioinspired hydrogels that accurately recapitulate the native tumor microenvironment. With recent advances in materials engineering, many researchers are developing engineered tumor models, which are promising platforms for the study of cancer biology and for screening of therapeutic agents for better clinical outcomes. In this review, we discuss the development and use of polymeric hydrogel materials to engineer native tumor ECMs for cancer research, focusing on emerging technologies in cancer engineering that aim to accelerate clinical outcomes.

Keywords

polymeric hydrogels; engineered tumor models; tumor microenvironments; cancer research

1. INTRODUCTION

In developing new cancer therapeutics, there is a great need for in vitro platforms that will allow better screening of novel treatments. The US National Institutes of Health (NIH) (1) have estimated that in 2016, 1.68 million people will be diagnosed with cancer and 595,690 people will die of it. The average cost of a new therapy is around US$2.6 billion (2). Two reasons for these high costs are research and development expenses and the extensive animal testing necessary to comply with US Food and Drug Administration (FDA) guidelines. Moreover, these therapies also have a high failure rate because current in vitro and even in

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vivo models are unable to predict their efficacy and effects in humans. At present, the likelihood that a drug will complete all stages of clinical trials is 12%; 67% of drugs fail Phase 2 clinical trials (3). Therefore, we need to better understand the mechanisms of action underlying potential targets of therapeutics, and to better predict the clinical outcomes of such potential therapeutics. To this end, researchers must gain a better understanding of the cancer microenvironment in order to enable the development of research tools that more accurately portray the pathophysiological aspects of the tumor and its surroundings. Such research and development tools could be used for diagnostic applications. The average cost of a cancer therapy is $8,500 per patient per month, and global spending on cancer therapy is estimated to be $91 billion (4). In addition, many patients have to go through multiple rounds of treatment to eradicate their cancer. Thus, the technologies aiming to better mimic the tumor microenvironment in order to study and screen potential therapeutics could be adapted for diagnostic and preventive applications.

The tumor microenvironment is an intricate and dynamic milieu containing supporting cells, blood vessels, and extracellular matrix (ECM). It is also characterized by a complex combination of chemical, physical, and biological cues that regulate cancer progression, such as stiffness of the ECM, pressure of the growing tumor mass, secreted and sequestrated cytokines and growth factors, oxygen (O_2), pH, and glucose concentration. All of these cues help determine whether cancer cells grow or migrate, contributing to cancer development and metastasis. These environmental cues are present in a three-dimensional (3D) environment, which is drastically different from the conventional two-dimensional (2D) culture regimes used to study mechanisms that regulate cancer growth. These in vitro 2D culture regimes have minimal physiological relevance, as they do not accurately portray the complexity of the tumor environment. As a result, current studies using 2D culture regimes have shown limited translational impact, as well as a low screening ability for successful therapies.

A potential approach is to develop polymeric hydrogel materials that recapitulate the 3D cancer microenvironment. Hydrogels are cross-linked polymeric networks that are extensively swollen with water (5). These polymer networks can be fabricated from a wide variety of materials, including natural, synthetic, and semisynthetic polymers (6). Hydrogel properties such as viscoelasticity, swelling, and degradation, as well as cell-adhesion domains, can be engineered to tune a hydrogel for use with a targeted cell type (7). Such platforms can be used for the study of mechanisms underlying cancer development, growth, and metastasis, as well as to screen cancer therapeutics. A better understanding of the tumor microenvironment must be established in order to improve the design of these novel hydrogel materials.

In this review, we discuss the development and use of polymeric hydrogel materials to engineer native tumor microenvironments for cancer research, focusing on emerging technologies in cancer engineering to accelerate clinical outcomes.

2. PHYSICOCHEMICAL PROPERTIES OF TUMOR MICROENVIRONMENTS

The native tumor microenvironment is composed of a complex structure of ECM with varying chemical and physical gradients that lead to the migration and proliferation of cancer cells. The extracellular environment provides many cues that result in cancer development and metastasis. In the following subsections, we focus on key aspects of the tumor microenvironment that regulate cancer cell fates, including ECM composition, matrix remodeling, O_2, pH, cytokines, and matrix stiffness.

2.1. Extracellular Matrix

The ECM is the acellular component of a tissue made of a meshwork of highly cross-linked biomacromolecules (8). The ECM is composed of a wide variety of proteins and polysaccharides, such as fibronectin, collagen, laminin, glycoprotein, hyaluronic acid (HA), chondroitin sulfate, heparan sulfate, and so forth (8). Collagen is the most abundant protein in the ECM; it constitutes up to 30% of the total protein in the body and 90% of the ECM (9, 10). Collagen is a substantial structural feature of the ECM that regulates tensile strength and cell adhesion (11). Collagen molecules are made of three α-helical structures that together form a fibril structure. These fibers form larger supramolecular structures that create the backbone of collagen bundles (10). These fibrous bundles are modified through the hydroxylation of proline and lysine resides, allowing for the strengthening and cross-linking of collagen fibers (12). Another major ECM component is fibronectin. Fibronectin is made from a dimer that is joined by C-terminal disulfide bonds. These bonds can bind to other fibronectin dimers, to collagen, and to cell integrin receptors (13). Laminin is another crucial ECM protein. This molecule is important for cell attachment to the matrix (14), and it is known to increase migration in breast carcinomas (15, 16). All of these proteins in conjunction create a fibrillar network. This fibrillar network morphology can be imaged using second harmonic generation (SHG) microscopy (17, 18).

2.2. Matrix Remodeling

Within the ECM are cancer-associated fibroblasts (CAFs), which modify the ECM, leading to fibrillar ECM (19). Specifically, CAFs contribute to the synthesis of type I, type III, and type IV collagen, fibronectin, and laminin (20–22). An increased number of fibroblasts leads to an increase in type I collagen as well as to remodeling of the matrix, both factors that contribute to tumorigenesis (23).

Both CAFs and cancer cells secrete a wide variety of proteases and ECM modifiers. Two of the most-studied enzymes are matrix metalloproteinase (MMP) and thrombospondin motifs (ADAMTSs), which specialize in degrading the ECM (23). There are approximately 23 members of the MMP family (24). MMPs target a large range of extracellular proteins, including MMP-1, which targets collagen; MMP-2, which targets gelatin (denatured collagen); and MMP-7, which targets fibronectin (25, 26). These MMPs are of two types: soluble (secreted-type) and membrane-bound MMPs (27, 28). Soluble MMPs are secreted from the cell and are made up of pro-MMPs (29). In the prodomain of the soluble MMPs resides a cysteine residue, which ligates catalytic zinc (29). This cysteine–zinc interaction is disrupted in a two-step reaction. The reaction starts by cleaving the propeptide region to

remove the N-terminal polypeptide. The second step occurs through an autoproteolytic reaction, which generates an active enzyme (30). Membrane-type MMPs are anchored to the membrane and are activated by proprotein convertases (27). ADAMTSs share a similar structure to MMPs in that both have prodomains that need to be activated, degrading the surrounding matrix. The difference between MMPs and ADAMTSs is that ADAMTSs have a thrombospondin domain instead of a cytosine-rich domain (31).

Cancer cells also modify the ECM to facilitate migration. Because of the prevalence of interstitial collagen in the tumor microenvironment, the ECM is subject to numerous posttranslational modifications (32). Collagen cross-linking is mediated primarily by lysyl oxidase (LOX) and LOX-like enzymes. LOX cross-linking increases both cancer cell migration and ECM stiffness (33, 34). Baker et al. (35) have shown that this increase in stiffness (due to LOX cross-linking) leads to an increase in colorectal cancer migration. In breast cancer, LOX cross-linking induces β_1-integrin clustering, PI3K (phosphatidylinositol 3-kinase) signaling, and focal adhesions (36). Another key collagen modifier is procollagen-lysine 2-oxoglutarate 5-dioxygenase (PLOD2), which initiates lysine hydroxylation of collagen (37–39). These cross-links are crucial for the formation of mature collagen fibers, are regulated by hypoxia, and cause an increase in tumorigenesis (40, 41).

2.3. O_2 and pH

O_2 and pH are two important factors that regulate cell growth. Changes in these factors have been suggested to lead to changes in cancer cell growth and migration (42, 43). The relative distance to a blood vessel determines O_2 tension and pH in the tumor microenvironment. Datta et al. (44) have shown that changes in the O_2 and pH gradients around the tumor can be observed depending on the state of the vasculature. As the distance from the blood vessel to the tumor increases, O_2 concentration decreases to levels below 2%, a state known as hypoxia (45, 46). Hypoxia causes an increase in angiogenesis (47), metastatic potential (48, 49), and DNA replication (48, 50) and reduces protein synthesis (51).

The O_2-sensing mechanism occurs through transcription factors termed hypoxia-inducible factors (HIFs). HIFs are composed of α and β subunits (52). HIF1α is accumulated in severe hypoxic conditions, typically below 1.5% O_2 (53), and is rapidly degraded in a nonhypoxic environment. Less is known about HIF2α and HIF3α; whereas HIF1α is constantly expressed ubiquitously in most tissues, HIF2α and HIF3α are found in only a subset of tissues (54–56). Hypoxia in general causes upregulation of a variety of factors (Figure 1), from proangiogenic to ECM modifiers (57). Moreover, cells in a hypoxic environment are usually far away from blood vessels and therefore less exposed to drugs, and they have lower proliferation rates and thus less sensitivity to radiotherapy and p53-mediated apoptosis (58). Recently, our lab developed an in vitro platform to better screen potential cancer therapeutics in a hypoxic cancer tumor microenvironment (42).

Hypoxia also affects cell metabolism. The normal metabolism and generation of adenosine triphosphate (ATP) are highly sensitive to O_2 concentration. Hypoxia causes a substantial decrease in ATP production (59). Normally, cancer cells have to change their metabolism to rapidly produce ATP (60). Under hypoxia, cancer cells switch from their normal oxidative phosphorylation to glycolysis. In order to maintain high levels of ATP, these cells drastically

increase their glucose uptake. By using glycolysis, cancer cells can generate ATP even faster than conventional oxidative phosphorylation. However, this type of metabolism causes the pH in the extracellular environment to fall from a physiological value of 7.2 to an acidic value of between 5.6 and 6.8 (46).

2.4. Stiffness

Stiffness of the ECM is a crucial characteristic of the tumor microenvironment, with the majority of the ECM composed of collagen type I. As described in detail above, the density of ECM in the tumor microenvironment increases as a result of new ECM secretion and upregulation of cross-linkers by CAFs and cancer cells. In fact, an increase in collagen cross-linkers such as LOX and PLOD2 facilitates tumor progression (61). Cancer cells respond to an increase in LOX secretion by clustering nonreceptor tyrosine kinases, specifically the focal adhesion kinase (FAK). Paszek et al. (34) have shown that 3D matrices with a Young's modulus of 400 Pa enable a significant increase in the size of colonies compared with colonies cultured in 3D matrices with a Young's modulus of 160–170 Pa. They linked the increase in size to clustering of $\alpha_5\beta_1$-integrin, which caused stimulation of FAK phosphorylation, activated RhoA, and resulted in cytoskeletal contractility.

Another important factor of the tumor microenvironment is the porosity (specifically, pore size) of the surrounding matrix. Pathak & Kumar (62) have shown that ECM matrix stiffness and confinement play a role in cancer cell migration, demonstrating that cells in stiffer and narrower channels migrate faster. Wong et al. (63) demonstrated on polyacrylamide pillars that cells will spread more quickly on stiffer substrates. Haeger et al. (64) explored the concepts of confinement and cell–cell junctions in collagen gels. They concluded that, depending on the type and density of the ECM, the mode of migration changes; cells in denser matrixes show "follow-the-leader"-type migration, whereas less porous matrices demonstrate single-cell migration.

ECM stiffness also increases the differentiation of epithelial cancer cells to a more mesenchymal phenotype. Wei et al. (65) recently reported that nuclear translocation of TWIST1 (twist family basic helix–loop–helix transcription factor 1) leads to an increase in mesenchymal transition via this mechanotransduction pathway.

3. TUMOR ANGIOGENESIS

Angiogenesis is the growth of new capillaries from existing vasculature. Tumors have a high demand for nutrients due to an increase in the generation of ATP. This high demand for O_2 and glucose via the vascular network results in upregulation of proangiogenic signals that stimulate blood vessel growth and increased blood supply to the tumor.

3.1. Endothelial Recruitment

In order to meet the demand for nutrients, tumors have developed a variety of strategies for releasing cytokines and growth factors to activate the quiescent endothelial cells (ECs) around them to generate new blood vessels. Unique features of the tumor's environment such as O_2 and glucose deprivation affect blood vessels, leading to migration of the ECs

(66). Comprehensive reviews by Carmeliet and Jain explore the complex mechanism of tumor angiogenesis (67–69).

A key mechanism underlying tumor angiogenesis is the response of ECs to vascular endothelial growth factor (VEGF), which induces their sprouting and proliferation (70). A secondary mechanism involves platelet-derived growth factor (PDGF), which activates perivascular cells to enable migration of ECs. Perivascular cells usually provide stability and vessel maturation. However, because of the constant release of cytokines in the tumor environment, vessels can never become truly mature. Angiogenic factors are also upregulated because of the hypoxic environment caused by the irregular blood supply (67).

CAFs further help the migration of blood vessels by secreting MMPs that degrade the surrounding matrix. Throughout this process, inflammatory cells infiltrate the site, which can positively or negatively increase the migration speed of ECs. This constant inflammatory response has given rise to an analogy between tumors and wounds—a tumor is “the wound that never heals” (Figure 2) (71).

3.2. Vascular Structure

In healthy tissue, there is an orderly and efficient network of blood vessels that provides nutrients to tissue. These vessels are kept in balance due to a cocktail of pro- and antiangiogenic factors and a systematic network of lymphatic vessels to drain fluid and metabolic waste. In tumors, the exact opposite is observed. Due to the high levels of VEGF and other proangiogenic factors, tumor vasculature is immature, tortuous, and hyperpermeable (72). These vessels have an uneven diameter and blind ends that can lead to turbulent blood flow. Moreover, lymphatic vessels are highly disorganized and dilated (72). Due to these abnormalities, the efficiency of the vasculature in delivering nutrients and removing waste is greatly reduced.

The ECs within the tumor’s vessels are also deformed. Tumor ECs have long, ruffled margins and fragile cytoplasmic projections (73). The tips of the tumor ECs can branch into the lumen, creating intercellular gaps in the vessel wall. These gaps lead to leaky blood vessels, a condition known as the enhanced permeability and retention (EPR) effect. The gaps between the ECs are ~1.5 μm wide (73), causing blood leakage and reduced blood flow to the tumor site.

3.3. Vascular–Extracellular Matrix Microenvironment

The tumor vasculature contains key integrins that interact with the primary ECM proteins within the tumor (collagen and laminin). ECs express numerous integrins, including $\alpha_1\beta_1$, $\alpha_2\beta_1$ $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_8$, and $\alpha_5\beta_1$ (74). These integrins, especially $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are specific for type I collagen, have been the targets of some current therapies.

Another key characteristic of the tumor ECM is its relatively organized, aligned fibrous structure. Aligned fibrous collagen increases the migratory potential of ECs (75). Levels of VEGF and VEGF receptor (VEGFR) are increased through the interaction between ECs and basement membrane proteins such as laminin and collagen IV (76, 77). These, in turn,

stimulate the angiogenesis of vessels, thereby causing the secretion of MMPs, such as MMP-8 and MMP-9 (78), to enable the intravasation of cancer cells.

To study the formation of tumor vasculature, researchers have been using Matrigel, a mixture containing mouse sarcoma ECM as the substrate to culture and form EC networks. An important drawback of Matrigel is that, because it is generated from mouse sarcoma, it is completely undefined and has significant batch-to-batch variation (79). The main components of Matrigel are laminin, type IV collagen, and enactin. Although it mimics the basal lamina, this composition does not accurately mimic the complex tumor ECM. To address this shortcoming, investigators have developed biomaterials to study cancerous ECM–EC interactions.

3.4. Trends in Targeting of Tumor Angiogenesis

The role of tumor vasculature in cancer growth, development, and metastasis has prompted the development of a wide variety of therapeutics. One of the first was an anti-VEGF inhibitor called bevacizumab (80). This drug is a humanized neutralizing antibody that blocks VEGF-A or inhibits the activation of VEGFR2. Another strategy has been to target tyrosine kinase, which participates in many proangiogenic pathways. Drugs such as sunitinib, sorafenib, pazopanib, and axitinib have been approved for cancer treatment. These drugs simultaneously target multiple cell types, such as cancer cells, pericytes, and ECs, through the inhibition of PDGF and VEGF signaling pathways. However, these drugs show minimal efficacy in the clinic. Therefore, combination therapies of these antiangiogenic inhibitors and standard chemotherapeutic agents are being considered. For example, Heist et al. (81) have shown an increase in survival of patients with non-small-cell lung cancer when they are treated with the chemotherapeutics carboplatin and paclitaxel following anti-VEGF therapy. The main reason for this increase in efficacy has been hypothesized to be due to the restoration of normal vasculature (69). This blood vessel restoration prevents the EPR effect and enables better drug delivery and an increase in O_2 supply. This increase in turn improves the efficacy of conventional drugs. Drugs such as doxorubicin have significantly decreased efficacy in a hypoxic environment (82), and their use in combination with an anti-VEGF therapy could dramatically increase their efficacy.

4. POLYMERIC HYDROGELS AS ENGINEERED THREE-DIMENSIONAL MICROENVIRONMENTS

Polymeric hydrogels, which are composed of a 3D hydrophilic network, are a promising material for regenerative medicine and pharmacological applications due to their multitunable properties and structural similarity to native ECM (83–85). Their physicochemical and biological properties, such as mechanical strength, degradation behavior, nutrient transport, and spatiotemporal topography, can be regulated by use of polymers with different compositions, by their cross-linking density, and by tailoring with various bioactive molecules (such as cell-adhesion ligands or proteolytic degradation sites). In addition, these matrices are utilized as artificial cellular microenvironments for recapitulating ECM in soft tissues due to their high swelling properties and mechanical resemblance. In particular, in situ cross-linkable hydrogels, which undergo a phase transition

from solution to gel through various cross-linking chemistries, have attracted substantial attention as engineered cellular microenvironments and drug-delivery carriers because they are minimally invasive and amenable to encapsulation of therapeutic agents during hydrogel formation under mild conditions (86, 87). Advances in materials engineering have prompted the development of various in situ–forming hydrogels and cross-linking strategies to create engineered microenvironments for supporting cell growth and directing cell fate (Figure 3) (88). In this section, we discuss the various types of polymeric hydrogels, and their cross-linking chemistries, that are used to create 3D hydrogel matrices for a broad range of biomedical applications.

4.1. Natural Hydrogel Matrices

Collagen type I is a well-known ECM protein that provides the structural frame required to support cell growth and adhesion through cell-to-cell and cell-to-matrix interaction through its various binding sites that interact with cell surface receptors and other ECM components (e.g., fibronectin) (89, 90). Collagen type I also includes proteolytic degradable sites for ECM remodeling within cellular microenvironments, which is critical for regulating cell behavior in these microenvironments (90). Generally, collagen hydrogels are prepared via physical cross-linking by pH and temperature sensitivity, which can provide 3D fibrillar matrices recapitulating the ECM in vivo. Increasing the pH and temperature of the acidic collagen precursor solution induces the formation of fibrillar hydrogel matrices (91). Because of its unique properties, collagen has been widely used as a scaffold for tissue engineering and regenerative medicine, as well as in other biomedical applications. However, collagen has critical disadvantages, namely weak mechanical properties and batch-to-batch variation. In an effort to overcome these limitations, investigators have developed many chemical cross-linking methods, including chemical glycation (92) and the enzyme-mediated cross-linking reaction, to generate stiffer and more stable collagen hydrogels through chemical cross-linking. For example, Vorp and colleagues (93) fabricated chemically cross-linked collagen hydrogels by using transglutaminase that catalyzes amide cross-linking from specific functional groups (e.g.,γ-carboxamide and primary amine in collagen chains). Using this approach, these authors created more collagen hydrogels that were more stable than physically cross-linked collagen hydrogels. Li and colleagues (94) utilized a different cross-linking condition by using genipin, an excellent natural cross-linker for proteins with low cytotoxicity (e.g., collagen and gelatin). The mechanical properties of the collagen hydrogels can be controlled by varying genipin concentrations, ranging from 2 to 50 kPa of compressive strength. However, a disadvantage of this system is its slow chemical reaction rate (more than 24 h to fully cross-link the networks). More recently, Kong and colleagues (95) developed a novel strategy to create more stable and multitunable collagen hydrogels by using poly(ethylene glycol) conjugated to di(succinic acid *N*-hydroxysuccinimide ester) (PEG-diNHS); these hydrogels induce interconnection between collagen fibers through cross-linking of primary amines in polymer backbones. Using the engineered collagen hydrogels, these authors investigated the effect of matrix stiffness on the behavior of cancer spheroids of hepatocarcinoma cells, demonstrating that carcinoma cells embedded in soft hydrogels form malignant cancer spheroids, whereas cells encapsulated in stiff hydrogels form compact hepatoids with suppressed malignancy.

Although many approaches have been developed to enhance the stability of collagen matrices, advanced engineering technologies are still required to improve these materials' cytocompatibility and multitunable properties. Fibrin hydrogels have been used in a wide range of biomedical applications due to their biocompatibility, biodegradability, and ease of fabrication (96). Fibrin hydrogels are fabricated by the cross-linking of fibrinogens through thrombin-mediated ionic interactions (97, 98). In this reaction, thrombin cleaves fibrogen's N-terminal end, which can induce polymerization of fibrin into fibrous hydrogel matrices (97, 98). These fibrin matrices contain various cellular responsible domains, including integrin, heparin, and fibronectin binding sites, that are critical in regulating cellular activities in wound healing and tissue regeneration (99). Because of their unique properties, fibrin gels have been utilized as therapeutic implants and as vehicles for regenerative medicine and drug delivery, as well as for clinical treatments. Compared with collagen hydrogel, fibrin gels have a relatively higher and broader range of matrix mechanical properties (approximately 1 to 30 kPa) because of variations in their fibrinogen and thrombin concentrations. However, fibrin gels degrade quickly because of the tissue factors and other enzymes secreted by cells, limiting their ability to support cell growth. Therefore, many studies have focused on improving fibrin gel stability toward the goal of long-term cell culture, which will lead to a better understanding of the effect of 3D matrices on cellular activities. The main goal of these studies is to incorporate functionalized PEG as a cross-linker, defined as a PEGylation. Del Bufalo et al. (100) utilized PEG–fibrin hydrogels to provide artificial tumor microenvironments for cancer research. They prepared PEGylated fibrin hydrogels by simply mixing fibrinogen and functionalized PEG in order to induce cross-linking between the polymer backbones through chemical reactions. Using the stable matrices, these authors investigated the effect of 3D microenvironments on the growth of cancer cells (e.g., lung adenocarcinoma cell lines, A549, and H1299), demonstrating that the 3D matrices supported tumor growth similar to that in in vivo models.

4.2. Synthetic Hydrogel Matrices

Synthetic polymers have several advantages over natural polymers, such as more controllable physicochemical properties. PEG is the representative biomaterial for medical applications. PEG is a bioinert and hydrophilic polymer used as both a surface-grafting material, to create an antifouling surface on biomaterials, and as a coating material, to enhance the lubricity of medical catheters. PEG hydrogels are used commercially as clinical tissue sealants, and PEG has been utilized as a hydrogel matrix for tissue regeneration and drug-delivery systems due to its biocompatibility and multitunable properties. However, PEG should be modified with bioactive molecules (e.g., cell-adhesion sites and MMP-sensitive moieties) to support cell growth within 3D microenvironments. The most common strategy is to fabricate PEG hydrogels through photopolymerization of diacrylated PEG (PEGDA) chains (101). During hydrogel formation, the bioactive molecules can be incorporated into the matrices. Anseth and colleagues (102) utilized photocurable PEG hydrogels as a template to study the effect of mechanical memory on stem cell fate, demonstrating that stem cells have mechanical memory that stores information from past physicochemical conditions and regulates their fate.

There are many other ways to create PEG hydrogels, including Michael-type addition reactions, thiolene reactions, and enzyme-mediated cross-linking reactions. Lutolf & Hubbell (103) developed a PEG hydrogel incorporating cysteine oligopeptides via Michael-type additions. The conjugative reaction occurs under mild conditions, which is critical for in vitro and in vivo applications. Segura and colleagues (104) developed similar PEG hydrogels through a thiol-maleimide Michael-type addition by using zinc, thereby demonstrating the multitunable properties of the hydrogel matrices, such as kinetics of hydrogel formation and matrix stiffness. Using this matrix, these authors investigated the impact of matrix stiffness on cellular activities of human dermal fibroblasts.

4.3. Semisynthetic Hydrogel Matrices

Although natural and synthetic hydrogels have been widely used as 3D matrices in tissue engineering and regenerative medicine, they have some limitations, such as matrix stability, batch-to-batch variation, and the challenge of accurately recapitulating native ECM. To overcome these limitations, investigators have developed chemically modified natural hydrogels (defined as semisynthetic hydrogels). There are various approaches to creating engineered polymeric hydrogels for tissue engineering and regenerative medicine.

Alginate is a naturally derived anionic polymer that is composed of repeating units of (1–4)-linked β-D-mannurinate (M units) and α-L-gluronate (G units) (105). Alginate hydrogels form in the presence of divalent cations (e.g., Ca^{2+} and Mg^{2+}) through ionic interactions between the G units in the polymer backbones. Due to its biocompatibility and easy fabrication process, alginate hydrogels have been used in various clinical applications, including wound dressing, drug delivery, and tissue regeneration, as well as in treatments of myocardial infraction (106, 107). Although alginate-based hydrogels are useful in various biomedical applications, improving their mechanical properties and bioactivity remains a challenge. To address these limitations, researchers have endeavored to chemically modify alginate chains. Dhoot et al. (108) have conjugated cell-adhesive sites [L-arginine, glycine, L-aspartic acid (RGD)] into alginate chains to enhance cellular activities. Zhu and colleagues (109) have developed an enzymatically cross-linked alginate hydrogel, which is composed of tyramine-conjugated alginate that can form chemically cross-linked hydro-gels through a horseradish peroxidase–mediated oxidative reaction. More recently, Mooney and colleagues (110) developed a novel strategy to create highly stretchable and tough alginate hydrogels through the formation of interpenetrating networks. To improve mechanical stiffness and hydrogel stability, these authors fabricated the hydrogels by hybridization of ionically cross-linked alginate networks using Ca^{2+} and photo-cross-linkable polyacrylamide gels, resulting in highly elastomeric alginate hydrogels. With advances in engineering approaches, alginate hydrogels with biological activities have the potential to provide 3D matrices for tissue regeneration and other clinical applications.

Gelatin, which is denatured collagen type I, has been widely used in tissue engineering and regenerative medicine because of its biocompatibility, biodegradability, ease of fabrication, and cost-effectiveness [reviewed by Klotz et al. (111)]. It also contains cellular responsive sites, such as integrin binding sites and proteolytically degradable domains, which play a critical role in regulating cellular activities within 3D microenvironments (112). In order for

gelatin to be used as a biomaterial, the polymer backbone must be chemically cross-linked to create stable gelatin matrices at body temperature. There are various ways to form covalently cross-linked gelatin networks, including enzyme-mediated cross-linking and photo-cross-linking reactions. Using transglutaminase and formaldehyde, one can prepare gelatin hydrogels with unmodified gelatin. However, the process must overcome certain critical limitations, such as slow phase transition and cytotoxicity. Toward that end, many approaches have been developed to create gelatin hydrogels using chemically modified gelatin. Khademhosseini and colleagues (113) created methacrylated gelatin (GelMA), which can form hydrogels through photopolymerization. These authors used the bioactive hydrogels to create various cell-laden matrices for regenerative medicine, as well as engineered tissue models (114, 115). Recently, they also developed grapheme oxide (GO)-incorporated GelMA hydrogels to enhance mechanical properties through additional physical interaction between GO and polymer chains (39). More recently, we developed a novel gelatin hydrogel composed of gelatin grafted with ferulic acid (FA) that formed hydrogel matrices with O_2 consumed in the laccase-mediated chemical cross-linking reaction (87). In this reaction, laccase catalyzes the cross-linking of FA with O_2 consumption, resulting in 3D hydrogel networks created through di-FA formations. We demonstrated that O_2-controllable hydrogels provide the artificial hypoxic microenvironment necessary to support enhanced vascular differentiation and tube formation of endothelial progenitor cells (EPCs). Using advanced hydrogel matrices, we are applying the engineered vasculatures to the treatment of ischemic tissues and are using engineered tumor models to study tumor development.

HA, an anionic linear glycosaminoglycan consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine, is commonly used in clinical applications as a soft tissue filler and an antiadhesion adjuvant, as well as a scaffold for tissue engineering and regenerative medicine (116, 117). Although HA generates very viscous solutions, it needs chemical cross-linking in order to create a stable hydrogel for in vitro and in vivo applications. HA hydrogels have been widely utilized as 3D cellular microenvironments to support vascular morphogenesis and cancer cell growth, as HA has several binding sites (e.g., CD44 and CD168) that play a critical role in regulating cellular signaling (118). However, HA–cell bonds are relatively weak, and there is no key cell-adhesion site in the HA backbone; therefore, cell-adhesion moiety should be incorporated into the hydrogel matrices to enhance cell binding affinity. Moreover, HA does not have proteolytic degradable sites (e.g., MMP-sensitive sites), which are crucial for matrix remodeling within cellular microenvironments. Thus, investigators should consider employing both cell-adhesion and MMP-sensitive sites when designing 3D cellular microenvironments using HA hydrogels.

There are many ways to engineer microenvironments using HA hydrogels. Burdick and colleagues (119) developed physically cross-linked HA hydrogels through host–guest interactions. They synthesized two kinds of precursor polymers: adamantine-modified HA (guest macromer) and β-cyclodextrin-modified HA (host macromer). The HA hydrogels are rapidly formed by simple mixing of the solutions, resulting in physically cross-linked HA networks through guest–host bonds. The same research group also developed photocurable HA hydrogels consisting of RGD-modified methacrylated HA (120). The functionalized HA hydrogels were formed by photopolymerization, and their mechanical properties and

degradation behaviors could be controlled by varying relevant parameters (e.g., cross-linking density, polymer concentrations, degree of substitution of methacrylate, MMP-sensitive sites). Using multitunable HA hydrogel matrices, these authors investigated the effect of matrix stiffness and degradation on stem cell fate. They demonstrated that the differentiation of human mesenchymal stem cells (hMSCs) was regulated by matrix degradation–mediated cellular traction, independently of cell morphology or matrix stiffness. Our group has used acrylated HA (AHA) hydrogels as 3D microenvironments to support vascular assembly of human pluripotent stem cell–derived early vascular cells (EVCs) to create stable engineered vasculatures (121). We encapsulated the EVCs within RGD-modified AHA hydrogels, as the EVCs can mature into ECs and pericytes to create multicellular vasculatures within 3D matrices. We also demonstrated that the engineered vasculatures connect to host vasculatures with blood perfusion following transplantation.

With advances in biomaterials engineering, various polymeric hydrogels have been used as 3D matrices for a wide range of medical applications. Although these engineered matrices are promising materials, well-defined 3D cellular microenvironments are still required in order to accurately recapitulate the native cellular environments, which present myriad physical, chemical, and biological challenges, as well as spatiotemporal complexity.

5. ENGINEERING TUMOR MICROENVIRONMENTS

Tumor microenvironments, with their complexity, diversity, and dynamic nature, play a critical role in cancer development and progression (122). Thus, many researchers have endeavored to create artificial tumor microenvironments recapitulating native tumors by using various engineered biomaterials (123). Advances in materials engineering have enabled the use of various polymeric hydrogels as engineered 3D matrices that recapitulate the pathological tumor ECM to allow studies of basic cancer biology and screening of the efficacy of anticancer agents (124, 125). In this section, we discuss the most recently engineered tumor microenvironments aiming for a better understanding of cancer biology and emerging anticancer therapeutics. In particular, we focus on models of the tumor ECM used to study angiogenesis, invasion, and metastasis occurring in native tumor microenvironments, as well as screening of anticancer drugs.

Breast cancer is the most common and deadly tumor in women worldwide (126). Various polymeric hydrogels have been used to study the effect of numerous factors on breast cancer progression, including invasion, metastasis, and tumor-associated angiogenesis (127). These engineered platforms offer the ability to evaluate drug efficacy and screen newly developed anticancer drugs in order to obtain a better understanding of breast cancer biology and improved treatment outcomes.

Charoen et al. (128) utilized biomimetic 3D breast cancer models comprising collagen hydrogels encapsulating multicellular spheroids (MDA-MB-231) to evaluate the anticancer therapeutic effect of paclitaxel-loaded polymeric nanoparticles. They fabricated the multicellular spheroids by culturing the cells on the top of agarose hydrogels, to which the cells could not attach as they did not contain cell-adhesive motives. The authors controlled the size of the tumor spheroids (diameters ranging from 100 to 400 μm) by varying the cell-

seeding density. The cancer spheroids were encapsulated within collagen hydrogels by simple mixing of cell suspension and polymer solutions. Using the tumor models, these authors investigated the therapeutic efficacy of nanoparticles encapsulating paclitaxel versus delivery of the drug without carriers. To this end, they loaded the drug into pH-sensitive polymeric nanoparticles and treated the tumor models. Interestingly, the drug combination with nanoparticles had a greater therapeutic effect than the drug alone, suggesting that the engineered tumor models are a promising platform to evaluate drug candidates and delivery systems for new cancer therapies.

Shoichet and colleagues (129) utilized HA hydrogels as a platform for studying variations in matrix stiffness, MMP sensitivity, and the concentration of cell-adhesion sites in breast cancer cell invasion. They fabricated HA hydrogels with independently varying cross-linking (mechanical) and ligand (chemical) densities to study the effects of each factor on breast cancer invasion. They observed that soft hydrogels tailored with an MMP-sensitive moiety exhibited extensive cancer invasion compared with stiff matrices without MMP-sensitive sites. This finding demonstrates the importance of matrix stiffness and cell-mediated matrix degradation in breast cancer invasion. Interestingly, these authors also showed that cell-adhesive ligand density affects cancer cell proliferation, rather than migration.

Sarcomas are heterogeneous mesenchymal tumors diagnosed in more than 2,000 people worldwide each year (130). Anseth and colleagues (131) utilized PEG-based hydrogels in an investigation of the effect of biochemical and biophysical matrix properties on fibrosarcoma (HT1080 cell) migration. They prepared peptide-functionalized PEG hydrogels through thiolene photopolymerization; the hydrogel matrices could be spatially and temporally patterned using standard lithography. The authors used this technique to fabricate hydrogel matrices with mechanical gradients ranging from 60 to 260 Pa, and they examined the effect of matrix adhesion at different moduli on HT1080 cell migration. They demonstrated that HT1080 cells on soft matrices migrate faster and over longer distances than HT1080 cells on stiff matrices. The authors also found that cells tended to migrate from stiff to soft areas. These results suggest that the engineered tumor models are innovative tools for investigating several unresolved questions in tumor development, including the effect of tissue interfaces on tumor motility and invasion.

Our lab has studied sarcoma cell migration in an O_2-controllable hydrogel model (132). We generated a gelatin-based hydrogel cross-linked with O_2 consumption in a laccase-mediated reaction. This reaction created a hypoxia-inducible gel with O_2 tension ranging from 1% to 5%, providing a biomimetic environment of a 3D hypoxic O_2 gradient. Using this model, we showed that sarcoma cells migrate along increased O_2 tension via PLOD2 modification of secreted collagen. Cells in the hypoxic gradient hydrogels have a faster migration rate and migrate for longer distances, and they upregulate the expression of PLOD2, LOX, and type I collagen. This migration was halted when the cells were treated with a PLOD2 inhibitor, minoxidil. This finding demonstrates that O_2 plays a critical role as a 3D physicotactic agent in sarcoma metastasis through remodeling of collagen matrices.

Glioblastoma multiforme (GBM) is a common and deadly form of brain tumor that is aggressive and malignant (133). Various engineering approaches have been developed to recapitulate and model GBM tumor microenvironments in vitro in order to obtain a better understanding of GBM molecular and systems biology (134). Using PEG-based hydrogels, Wang et al. (135) bioengineered 3D brain tumor models to investigate the effects of matrix stiffness on GBM cells. The hydrogels were prepared through photopolymerization and incorporated RGD- and MMP-sensitive sites to support 3D cell growth and matrix remodeling. HA was also included in order to mimic its concentration in the brain ECM. To examine the effect of matric moduli on GBM cell fate in three dimensions, Wang et al. encapsulated U87 cells (a model GBM cell line) in soft (1-kPa) or stiff (26-kPa) hydrogels, mimicking the matrix stiffness of normal brain or GBM tissue. Interestingly, they found that increasing stiffness led to slower cell proliferation but that the cells formed denser tumor spheroids as well as upregulated matrix degradation (through HA synthase 1 and MMP-1). These results demonstrate the importance of ECM decomposition and remodeling during tumor invasion and metastasis. This bioengineered 3D hydrogel platform may provide an innovative brain tumor model for investigating the mechanisms underlying GBM progression, as well as for evaluating the efficacy of potential drug candidates for the treatment of GBM.

Prostate cancer is one of the most commonly diagnosed cancers in men worldwide (136). Many researchers have utilized advanced engineering platforms to determine the molecular mechanism underlying growth and metastasis (137). Fong et al. (138) utilized HA hydrogel–based 3D models using patient-derived prostate tumors for drug screening. As is well known, patient-derived xenograft (PDX) cells show poor cell viability in standard culture conditions. To overcome this limitation, these authors encapsulated the PDX cells in the HA hydrogel matrices and evaluated both cell viability and drug efficacy. They observed that most cells were viable within the matrices until day 14. Fong et al. also investigated the drug sensitivity of 3D tumor models from different patients. Interestingly, the 3D PDX cells were much more resistant to docetaxel compared with the cell lines. This result suggests that the primary culture system offers potential for direct culture of patients' tumor tissues for rapid drug screening, and thus may represent an innovative platform for personalized cancer therapeutics.

Whereas prostate cancer is the most commonly diagnosed cancer in men, representing 29% of all incident cases, ovarian cancer accounts for only 3% of all incident cancer cases in women each year (139). It is challenging to model ovarian cancer because of its genetic complexity, diverse pathology, and the unique mechanisms underlying its metastasis. Thus, many studies have attempted to recreate sophisticated ovarian cancer microenvironments to study growing cancer spheroids and metastasis of ovarian cancer cells (137). Kaemmerer et al. (140) developed 3D ovarian cancer models by using GelMA hydrogels encapsulating a human epithelial ovarian cancer cell line (OV-MZ-6) derived from a patient. They investigated the effect of matrix stiffness, matrix degradation, and incorporation of ECM components (laminin-411 and HA) on cancer spheroid formation. The hydrogels' matrix stiffness was modulated by varying the polymer concentrations from 0.5 to 9.0 kPa. Hydrogels with medium stiffness (3.4 kPa) enabled the formation of tumor spheroids and led to higher proliferation compared with more or less stiff matrices. In addition, the researchers

noticed that inhibition of proteolytic degradation of matrices reduced the formation of spheroids and that incorporating laminin-411 and HA further facilitated spheroid growth within the gelatin-based hydrogels. These results demonstrate that semisynthetic gelatin hydrogels may provide alternative, bioengineered 3D ovarian cancer culture models in vitro.

6. EMERGING TECHNIQUES TO CREATE ENGINEERED TUMOR/TUMOR ANGIOGENESIS MODELS

Recently, advanced tools have been developed to accurately mimic natural tumor microenvironments composed of ECM, vasculature, and supporting stromal cells (141, 142). These innovative approaches include 3D coculture systems, micropatterning, microfluidics, and 3D bioprinting, which provide unique opportunities to recapitulate complex and sophisticated tumor microenvironments, such as multicellular microenvironments and spatiotemporal gradients of various physicochemical and biological cues. In this section, we discuss the most recent tools used to recreate tumor microenvironments using these advanced fabrication methods.

Our lab has evaluated the effect of hydrogel stiffness and O_2 tension on cancer cell fate and vascular cell invasion. We fabricated HA-based hydrogels tailored with RGD- and MMP-sensitive sequences through a Michael-type addition reaction to support the growth of 3D cancer cells (HT1080 cells) (143). The stiffness of the hydrogel matrices was modulated by varying the amount of cross-linkers (e.g., cysteine-containing peptides; GCRDGPQGWGQDRCG) ranging from 80 to 600 Pa. We observed different cell morphologies depending on the stiffness. While fibrosarcoma cells spread on relatively stiff hydrogels (300 Pa), they formed aggregates at lower stiffnesses, demonstrating that matrix stiffness influences sarcoma morphology. We also utilized coculture systems of HT1080 cells and vascular EPCs [e.g., endothelial colony-forming cells (ECFCs)] to study the effect of matrix stiffness and O_2 tension on vascular cell invasion of 3D tumor microenvironments (Figure 4a). Interestingly, we found that ECFCs cultured on top of the 3D tumor models under hypoxic conditions exhibited extensive vascular invasion of 3D tumor models at all levels of matrix stiffness, through MMP-mediated matrix degradation and upregulation of proangiogenic factors [e.g., VEGFs and angiopoietin-1 (ANG-1)]. This result clearly demonstrated that O_2 tension affects vascular invasion into the tumor microenvironment, suggesting that coculture models may provide a promising platform for basic studies of tumor biology, including the vascular invasion that selectively impedes cancerous processes.

Microfluidic 3D tumor models recreate the complex and heterogeneous aspects of the native tumor environment (144, 145). A critical advantage is the ability of the 3D microscale system to provide multifunctionality (e.g., spatial/temporal controls), including perfusable fluid systems that can accurately recapitulate the native tumor microenvironment (144). Jeon et al. (146) developed an in vitro model of tumor cell extravasation by using the microfluidic technique and collagen hydrogels. They created a microfluidic system consisting of three independently addressable media channels separated by ECM-mimicking chambers (Figure 4*b*). The inner channels are composed of an EC monolayer surrounding a collagen hydrogel, which allowed cancer cell migration (e.g., breast cancer cells, MDA-MB-231) from the

vessel-mimicking regions to the hydrogel matrices (defined as tumor extravasation). These authors evaluated the permeability of the endothelial monolayer, which showed excellent media permeability, then examined cancer extravasation into the collagen hydrogel matrices through the EC monolayers. The microscaled perfusable tumor models allowed the study of breast cancer extravasation, resulting in extravasation of 38.8% of the tumor cells within 1 day of their introduction. This result suggests that advanced tumor models present the opportunity to develop therapeutic strategies for the treatment of metastatic tumors, as well as personalized medicine applications.

3D bioprinting techniques have been widely used to create highly precise and complex 3D architectures with living cells. The key advantage of 3D printing is its ability to create complex architectures with high efficiency and customizability on a desktop printing scale. For example, multicellular 3D tissue constructs can be printed by use of a multichannel cartilage filled with bioinks encapsulating the targeted cells. Many researchers have used this innovation to fabricate sophisticated artificial tissues as well as tumor microenvironments (147). Sun and colleagues (148) fabricated in vitro cervical tumor models by using gelatin-based hydrogels (Figure 4*c*). They used these models to compare the cellular behavior in a 3D cervical tumor model with that in traditional 2D culture models, including cancer cell proliferation, MMP protein expression, and chemoresistance. Interestingly, they found that the HeLa cells in the 3D construct had a higher proliferation rate, resulting in tumor spheroid formation. They also found that the cells embedded into the 3D matrices showed higher MMP protein expression and chemoresistance than those in the 2D cultures. These findings suggest that this novel 3D printing technique may ultimately lead cancer studies toward better clinical outcomes.

7. FUTURE DIRECTIONS AND CONCLUSION

Significant recent advances in synthesis of hydrogel biomaterials and other 3D miniature devices have been explored for tissue regenerative applications, but they have not been fully taken advantage of in the field of cancer research. For example, following recent advances in organon-a-chip technologies, the next logical step would be to make a tumor on a chip or to create more physiologically relevant diseases on a chip. For example, Huh et al. (149) have developed a lung-on-a-chip model that can mimic the physiological environment of the lung. The next step for this technology should be to integrate cancer phenotypes in specific locations into the chip in order to mimic tumor metastasis. Many examples of angiogenic devices on a chip are beginning to be used in intravasation or extravasation of tumor cells into and out of existing vasculature (42). However, most of these devices use collagen gel as the 3D hydrogel portion of the device. The next generation of these approaches should incorporate advanced materials to augment these natural ECMs, thereby enabling more accurate mimicking of physiologically relevant chemical and cytokine gradients.

These technologies will also have to incorporate multiple cell types. As discussed above, the tumor microenvironment is a complex combination of ECM and multicellular cues. CAFs have been studied for their importance in tumor migration and ECM modification. Preliminary studies have incorporated stromal cells into systems to study changes in cancer migration. Moreover, CAFs change the barrier and structure of ECs and vasculature. In order

to culture multiple cell types that require different culture conditions and ECM components, investigators must develop more complex patterning of hydrogel networks and matrices. Our lab has shown that cancer and EC patterning can be controlled solely via ECM proteins (150). Technologies developed by Burdick and colleagues (151) take advantage of different properties of “self-healing” and shear-thinning gels to print patterns that could be adapted for multiple cell types and ECM coculture platforms.

The ultimate goal of most of these complex systems is to discover new mechanisms in cancer development, growth, and metastasis. However, due to the complexity of these devices, they have limited use outside bioengineering laboratories. Classical biological researchers do not have the instruments (e.g., 3D printers, syringe pumps, chemical equipment for biopolymer synthesis) to perform these assays. Efforts should be made to simplify the mode of operation for ease of use. Moreover, these devices can be employed for potential therapeutic evaluation and possible replacement of in vivo models. Such trials, though complex, are still less expensive and more controllable than current in vivo studies. Simplifying current technologies into one- or two-component devices would greatly enhance their translatability and promote their widespread use. If this task is accomplished, the transition from drug discovery to personalized medicine could be achieved.

Personalized medicine is a new and exciting field in cancer research; several technologies in this area have made the transition from benchtop to clinic. Recently, Jonas et al. (152) demonstrated the use of a complex system that enables clinical testing of up to 16 different therapeutics in microdoses. The researchers created Delrin acetal resin blocks that can hold 16 different individual therapies by controlling the drug-release kinetics in the tumor microenvironment. This device is implanted via a biopsy needle into mice bearing human tumors, then extracted via a corning needle and evaluated histologically. More technologies like this one need to be adapted for use in a clinical setting and, more importantly, over a wide variety of cancer cell types. Other challenges include the inherent heterogeneity of tumors, with a wide variety of genomic changes, and the ability to keep tumor samples viable outside the body. Finally, these advanced tissue culture technologies have to be mass produced for broader application and ease of use. Mass production will enable researchers across disciplines to employ the latest technologies. Furthermore, mass production requires a more scalable synthetic design for wide-scale use. This may fulfill the need for controllable and reproducible systems that can be used for cancer diagnosis and treatment.

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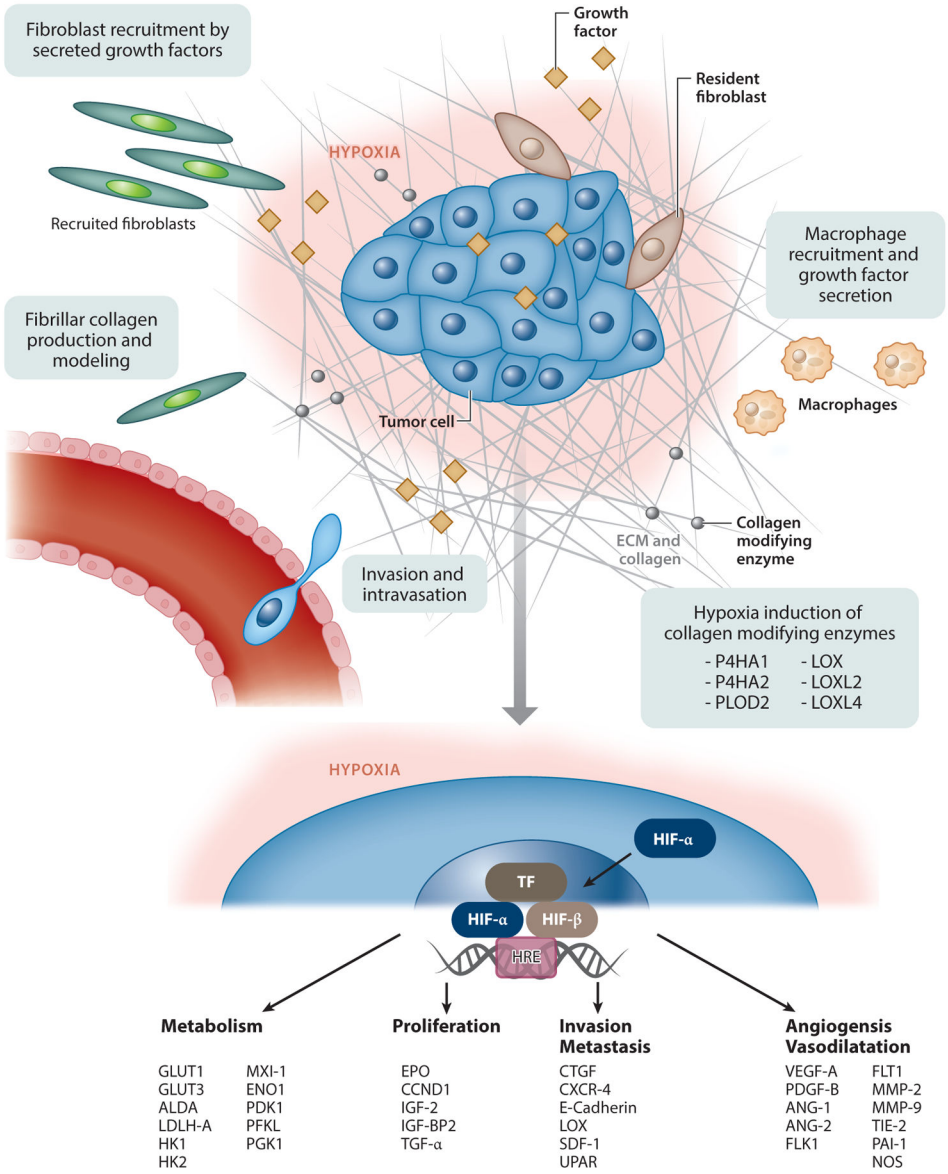

Figure 1.

Cancerous pathways affected by accumulation of hypoxia-inducible factor (HIF). Abbreviations: ALDA, aldolase A; ANG-1, angiopoietin 1; ANG-2, angiopoietin 2; BMDSC, bone marrow–derived stem cell; CCND1, cyclin D1; CTGF, connective tissue growth factor; CXCR-4, C-X-C chemokine receptor type 4; ECM, extracellular matrix; ENO1, enolase 1; EPO, erythropoietin; FLK1, VEGF receptor 2; FLT-1, VEGF receptor 1; GLUT, glucose transporter; HK, hexokinase; IGF-2, insulin growth factor 2; IGF-BP2, insulin-like growth factor–binding protein 2; LDHA, lactate dehydrogenase A; LOX, lysyl oxidase; miRNA, microRNA; LOXL, lysyl oxidase homolog 1; MMP, matrix metalloproteinase; MXI-1, max interactor 1; P4HA, prolyl 4-hydroxylase subunit α1; PAI-1, plasminogen activator inhibitor 1; PDGF-B, platelet-derived growth factor B; PDK1, pyruvate dehydrogenase kinase 1; PFKL, phosphofructokinase L; PGK1, phosphoglycerate kinase 1; PLOD, procollagen-lysine 2-oxoglutarate 5-dioxygenase; SDF-1, stromal cell–

derived factor 1; TF, transcription factor; TGF-α, transforming growth factor α; TIE-2, angiopoietin receptor 2; UPAR, urokinase plasminogen activator receptor; VEGF, vascular endothelial growth factor. Modified from Reference 46.

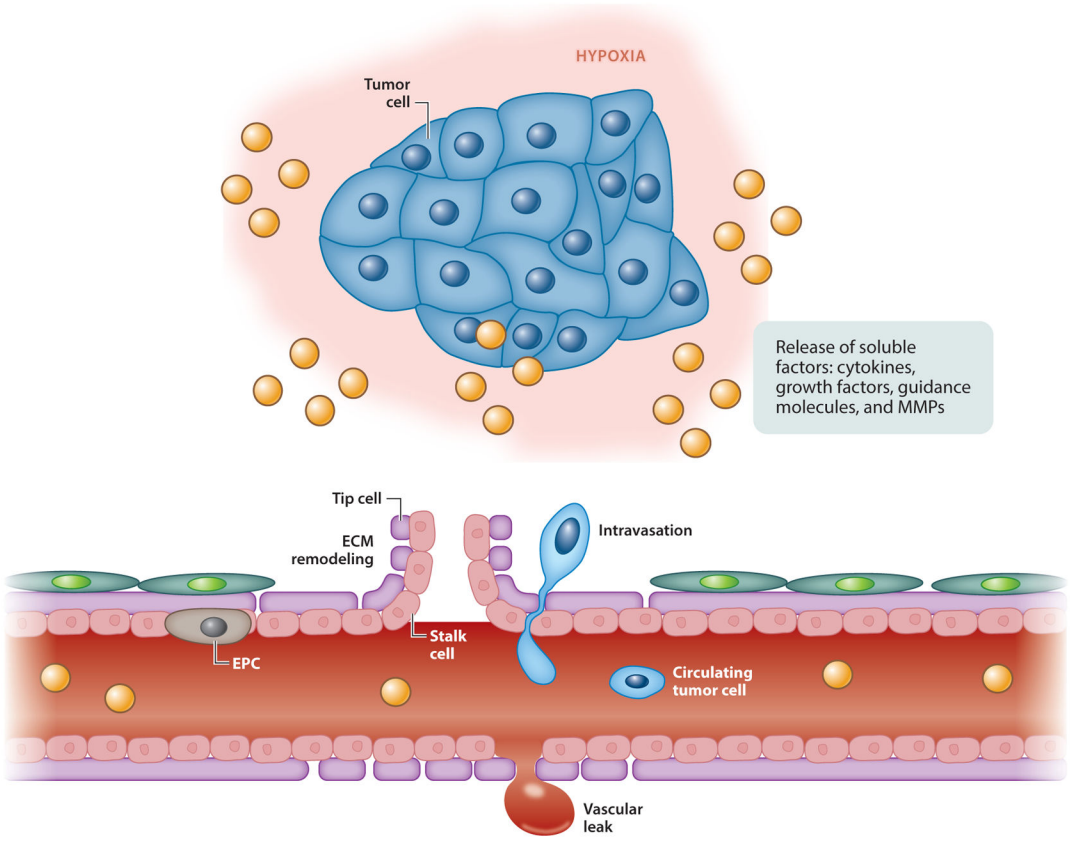

Figure 2.

Tumor angiogenesis, in which certain growth and environmental factors lead to the recruitment of endothelial cells (70). Abbreviations: ECM, extracellular matrix; EPC, endothelial progenitor cell; MMP, matrix metalloproteinase.

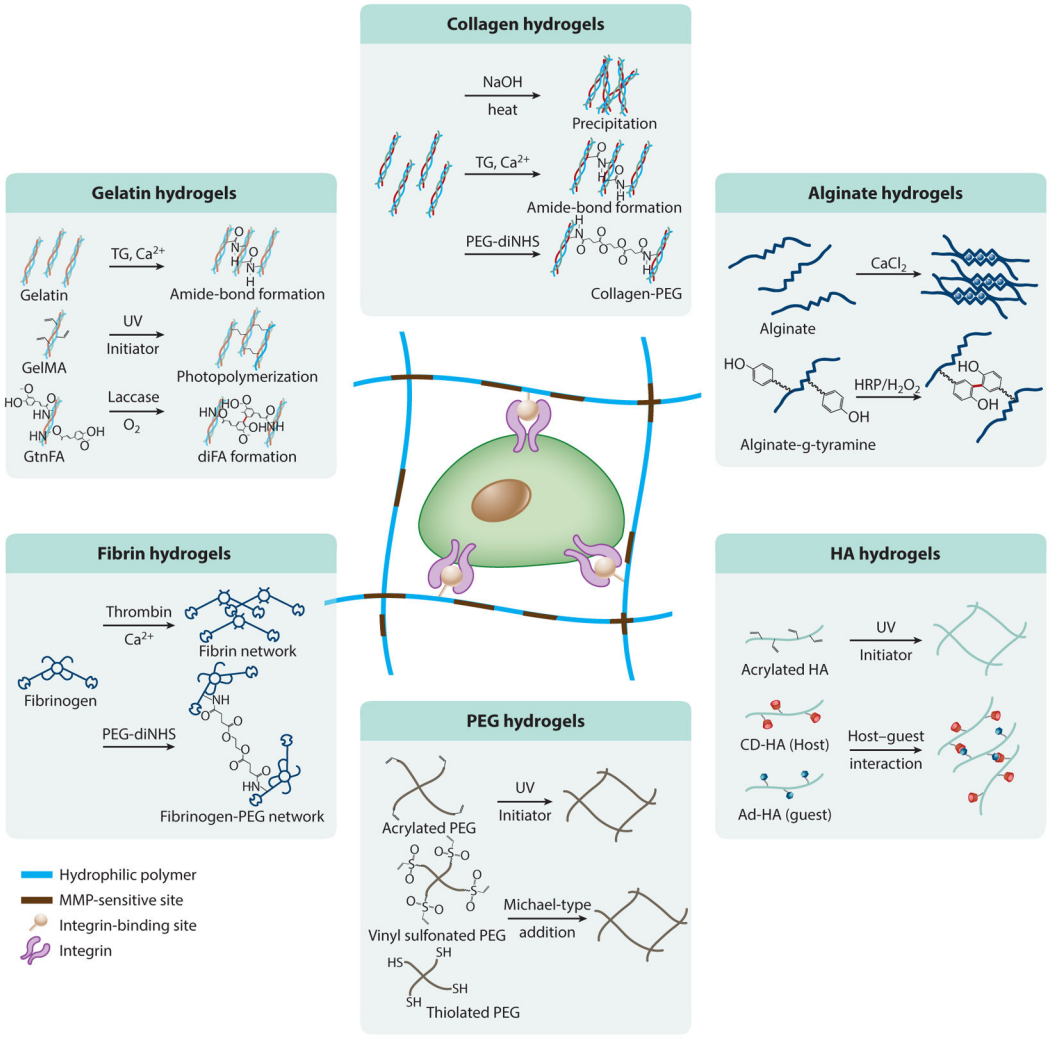

Figure 3.

In situ cross-linkable hydrogels as an artificial cellular microenvironment. Cross-linking strategies are detailed for each hydrogel system. Abbreviations: CD-HA, β-cyclodextrin-modified hyaluronic acid; FA, ferulic acid; GelMA, methacrylated gelatin; GtnFA, gelatin grafted with ferulic acid; HA, hyaluronic acid; MMP, matrix metalloproteinase; PEG, poly(ethylene glycol); PEG-diNHS, poly(ethylene glycol) conjugated to di(succinic acid *N*-hydroxysuccinimide ester).

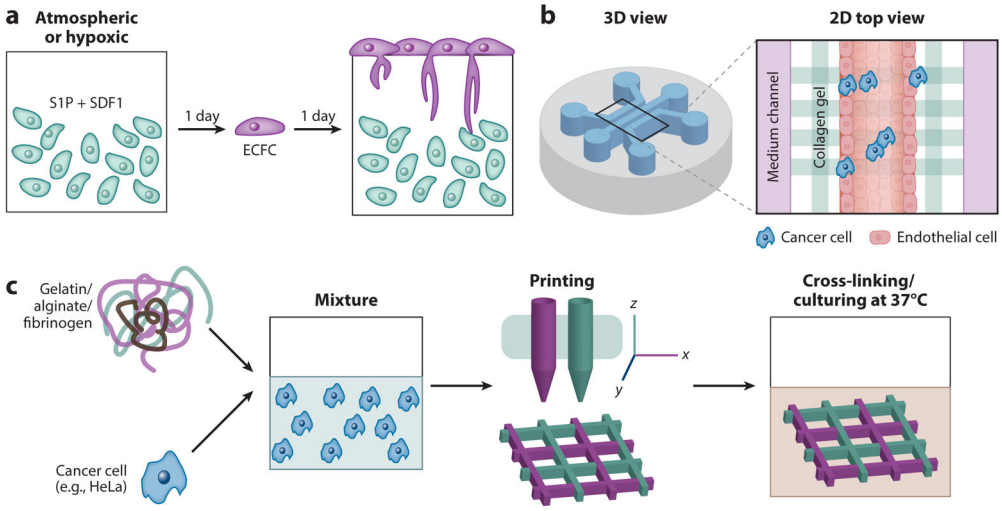

Figure 4.

Emerging techniques to create three-dimensional (3D) tumor microenvironments. (*a*) Strategy for studying vascular cell invasion into tumor microenvironments. (*b*) A microfluidic system consisting of three independently addressable media channels separated from extracellular matrix (ECM)-mimicking hydrogel matrices. (*c*) The printing process used to create 3D HeLa–hydrogel matrices. Abbreviations: ECFC, endothelial colony-forming cell; SDF-1, stromal cell–derived factor 1; S1P, sphingosine-1-phosphate; 2D, two-dimensional. Panel *a* modified from Reference 143. Panel *b* modified from Reference 146. Panel *c* modified from Reference 148.