

Opinion

Beyond Tissue Stiffness and Bioadhesivity:
Advanced Biomaterials to Model Tumor
Microenvironments and Drug ResistanceAnkur Singh,^{1,2,3,*} Ilana Brito,¹ and Jan Lammerding^{1,4}

Resistance to chemotherapy and pathway-targeted therapies poses a major problem in cancer research. While the fields of tumor biology and experimental therapeutics have already benefited from *ex vivo* preclinical tissue models, these models have yet to address the reasons for malignant transformations and the emergence of chemoresistance. With the increasing number of *ex vivo* models poised to incorporate physiological biophysical properties, along with the advent of genomic sequencing information, there are now unprecedented opportunities to better understand tumorigenesis and to design therapeutic approaches to overcome resistance. Here we discuss that new preclinical *ex vivo* models should consider – in addition to common biophysical parameters such as matrix stiffness and bioadhesivity – a more comprehensive milieu of tissue signaling, nuclear mechanics, immune response, and the gut microbiome.

Chemoresistance and Changes in Biophysical Factors

Therapeutic resistance in cancer often arises through genetic mutations that enhance drug metabolism, inactivate apoptotic pathways, and activate prosurvival signals [1–3]. The underlying genetic mutations are accompanied and sometimes preceded by changes in the biochemical and biophysical properties of the surrounding tissue. The biophysical factors, such as bioadhesivity, porosity, confinement, and stiffness, have been extensively studied as the response of individual cells to these factors and are vital for cellular functioning and tissue development. Cells cope with biophysical stimuli through integrated mechanosignaling by physically interconnected proteins starting from extracellular matrix (ECM) adhesion molecules (integrins), focal adhesion plaques, actin fibers, and structural components of the cells' nuclei, among others. The mechanotransduction response includes the activation of mechanosensitive transcription factors and downstream genes as well as the rearrangement of cellular structure and organization to adjust to the physical environment [4,5]. Mutations in cellular proteins and alterations in the cellular microenvironment aberrantly engage mechanosignaling networks in cancer cells, either by perturbing the mechanical input or by altering the signaling network itself, which can promote cell growth, invasion, migration, and probably chemoresistance. For example, integrin signaling has been shown to increase epidermal growth factor secretion and receptor tyrosine-protein kinase erbB-2 (ERBB2) clustering in breast cancer cells, resulting in resistance to the ERBB2 inhibitor trastuzumab [6]. Increased tumor and stroma stiffness has also become a hallmark of cancer, as evident from the use of palpation for detection of breast tumors and cancerous lymph nodes in the case of lymphomas. Increased tissue stiffness in the liver, pancreas, prostate, and lung has also been shown to be a positive indicator of disease progression in the corresponding cancers [7–10]. Nevertheless, how chemoresistance and changes in biophysical and biochemical factors relate to one another is poorly understood.

Highlights

Matrix stiffness influences the phenotype and epigenetics of tumor cells and influences chemoresistance across solid, palpable, and liquid tumors.

Biomaterials that independently modulate matrix stiffness from composition and architecture reveal that, in normal mammary epithelial cells, increasing matrix stiffness alone induces malignant phenotypes.

Healthy and malignant cells migrating through narrow confinements undergo nuclear deformation, which can result in transient loss of nuclear envelope integrity, herniation of chromatin across the nuclear envelope, DNA damage, and redistribution of mobile nuclear proteins.

An immune-privileged microenvironment could selectively impair the recognition of tumor antigens by cytotoxic T cells.

A commensal microbiota promotes the efficacy of cancer therapies.

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While genomic studies have benefitted from direct patient sample analysis, exploring the role that the stiffness of the microenvironment plays in cellular function has become possible only through the use of atomic force microscopy (AFM), microindenters, and engineered tissues. *Ex vivo* preclinical models that recapitulate the tumor microenvironment have been critical in improving our understanding of tumorigenic growth and resistance. In the case of mammary tumors, changes in tissue stiffness are associated with increased deposition and crosslinking of collagen type I, and the stiffness can increase from 100–400 Pa up to 1–5 kPa when comparing normal and cancerous mammary tissue [9,11]. It is now well accepted that matrix stiffness perturbs epithelial morphogenesis by clustering integrins to enhance extracellular signal-regulated kinase (ERK) activation and increase Rho-associated protein kinase (ROCK)-generated contractility and focal adhesions. Integrin signaling and stiffness are involved not only in chemoresistance in solid tumors but also in palpable lymphoid malignancies, as shown by us [12], as well as in liquid tumors [13]. Recent work from Shin and Mooney demonstrated that matrix softening leads to resistance against standard chemotherapy in myeloid leukemias [13]. More recently, matrix softness was shown to influence the histone methylation and epigenetics of tumor-repopulating cells [10], which exhibit high chemoresistance to conventional chemotherapeutic drug treatment. To better understand the role of tissue stiffness in cancer, we refer the reader to excellent recent reviews [11,14]. Nonetheless, these *ex vivo* models have yet to successfully address the reasons for the emergence of tumor resistance. This is because most *ex vivo* tissues focus on bioadhesive signaling and stiffness. Although extensively investigated, cell adhesion- and stiffness-mediated drug resistance is not the only factor that contributes to chemoresistance *in vivo*. Here we discuss that, in addition to matrix stiffness, cellular, biochemical, and biophysical parameters such as stress relaxation, adhesion, spatiotemporal protein signaling, and porosity/confinement need to be considered. For *ex vivo* models, it will thus be important to incorporate these various biophysical parameters, ideally in a modular fashion to maximize control of cell fate and the drivers of oncogenic transformations. We propose several new areas of technological advancement needed for the building of better *ex vivo* cancer models to understand tumor resistance. These topics cover the integration of biomaterials-based engineering with emerging forefronts of tissue mechanics, nuclear mechanics, the immune response, and the gut microbiome.

Integrating Independent Control of Biomechanical and Spatiotemporal Signaling of Tumors

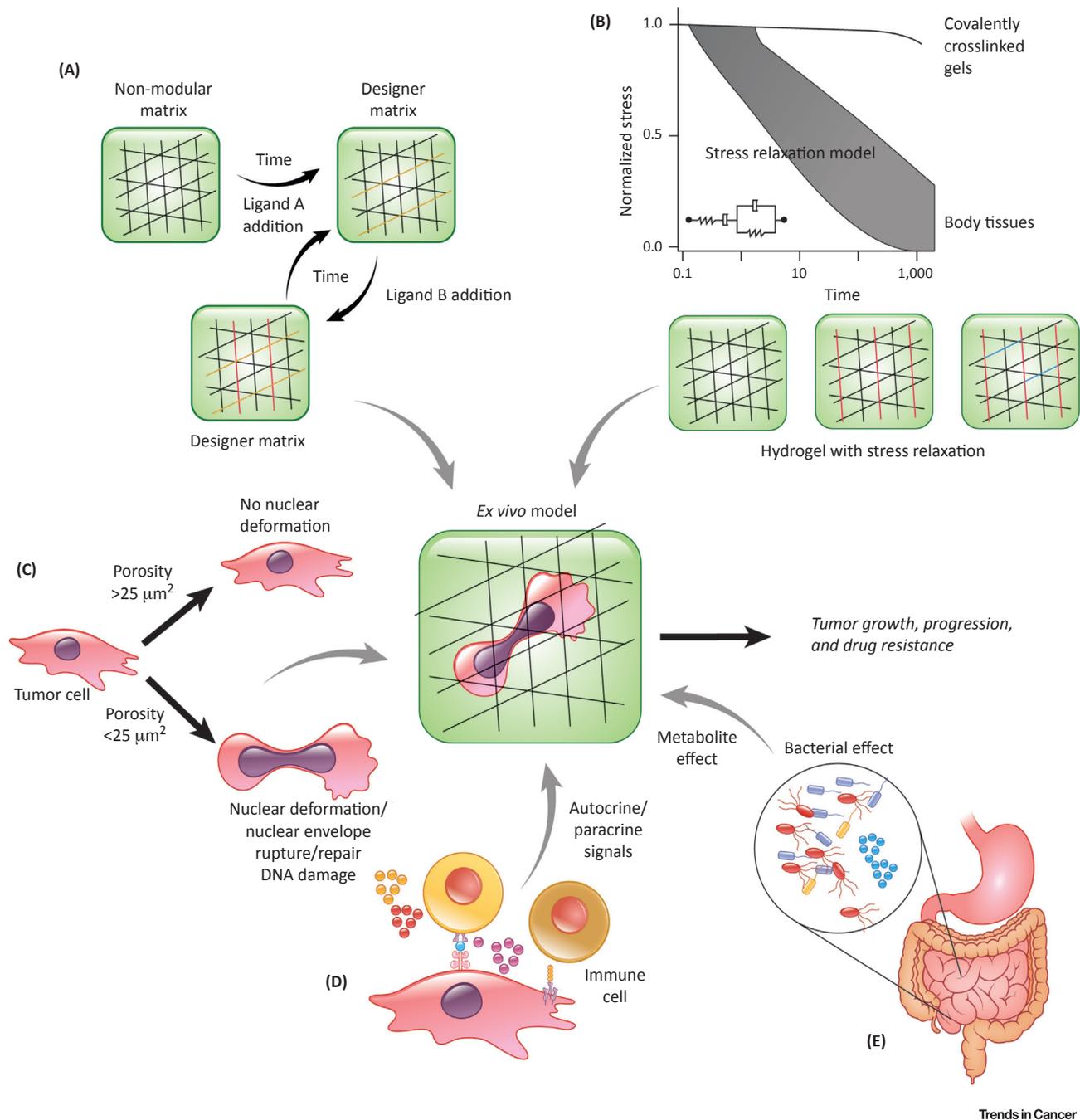
The plasticity of cancer cells to evolve different drug-resistant phenotypes is encoded by the organization and spatiotemporal dynamics of signal transduction networks. This plasticity allows them to adapt to challenging microenvironments, remodel them in their own favor, and withstand highly toxic therapeutic assaults. A recent review discusses the rich molecular signaling dynamics and their impact on cancer cell proliferation, survival, invasiveness, and drug resistance [15]. Most prior studies in cancer 3D modeling used hydrogels or scaffolds as hydrated networks of motif-containing bulk proteins or their peptidomeric forms. These peptides are either short peptides that represent adhesive binding motifs (e.g., fibronectin- or vitronectin-derived RGD) or hydrogel crosslinking peptides that are matrix metalloproteinase (MMP) degradable and allow matrix remodeling. While numerous studies have shown the utility of these hydrogels in spread, mesenchymal-like cells, we have engineered modular hydrogels to show that integrin ligands and matrix degradability can serve as prosurvival signals in B and T cell lymphomas, modulating their 3D aggregation and response to therapeutics [12]. Using RGD-presenting hydrogels and complementary studies in patient-derived xenograft mouse models, we have further shown that integrin $\alpha v \beta 3$ acts as a membrane receptor for thyroid hormones to mediate angiogenesis in malignant T cells [16]. This particular study led us to discover a novel mechanism for the endocrine modulation of T cell lymphoma pathophysiology.

However, the flow of information between tumor cells and the surrounding ECM is bidirectional and functions in a spatiotemporal manner. The adhesion process involves dynamic interactions occurring over multiple time and length scales, from seconds for nanoscale integrin receptor–ECM ligand binding to days and weeks for meso/macroscopic ECM remodeling and cancerous tissue organization. Similar to the dynamic nature of cell–ECM interactions, the engineering of materials to elicit desired tumor cell responses will require precise and independent, multidimensional control over matrix spatiotemporal bioligand presentation, structural porosity, and the mechanical properties of the materials (Figure 1A, Key Figure). Recent biomaterials designs now allow spatiotemporal control, as reported by several research groups across various cell–tissue models [17–21]. Nonetheless, current hydrogels have yet to demonstrate that the same biomaterials can provide independent control over all of the bioadhesivity, spatiotemporal signaling, stiffness, and porosity of the material. In a major advance, chemical strategies that allow external manipulation of ligand presentation in real time were recently developed. A new class of hydrogels was reported that used an addition–fragmentation–chain transfer chemistry and permitted repeated exchange of biochemical ligands in a non-destructive manner [22]. Such advances afford powerful designer tools in material engineering to study cancer cell processes.

Another emerging trend is the understanding of stress relaxation *in vivo* and *ex vivo* and is increasingly becoming a crucial parameter for biomaterials design. Using bead displacement methods, Legant and colleagues showed deformations of 20–30% peak principal strain in the hydrogel surrounding the cell [78]. In 2D culture of cells on acrylamide gels, strains of 3–4% (ratio of traction to elastic modulus) are typically observed [23]. The ability of *ex vivo* scaffolds to either store (purely elastic) or dissipate (viscoelastic) forces generated by cells in contact with these surfaces can influence a cell's interaction with its surrounding [24] and we believe they may in turn regulate spread, growth, migration, and possibly chemoresistance. Stem cell growth and differentiation are enhanced in hydrogels with fast stress relaxation characteristics, as reported recently by Chaudhuri and colleagues [24]. Most non-degradable, synthetic hydrogels are purely elastic, whereas many naturally derived matrices and tissues are viscoelastic (Figure 1B), can be degraded/remodeled by cells, and often exhibit partial stress relaxation when a constant strain of 15% is applied [24]. For example, collagen and fibronectin matrices exhibit a decrease in the storage or elastic modulus over time when a constant strain is applied. This is likely to occur from the unbinding of weak hydrophobic and electrostatic interactions that hold the fibers in a network [25,26]. The elasticity of these materials is also nonlinear. On reconstituted natural matrices, the resistance to cellular traction forces is expected to relax over time due to flow and remodeling of the matrix, dissipating the energy that cell-generated forces impart into the material. Substrates with stress relaxation enhance cell spreading at a low initial elasticity, which is mediated through $\beta 1$ integrin, actin polymerization, and actomyosin contractility and is associated with increased Yes-associated protein (YAP) nuclear localization and proliferation [26,27]. This suggests that increased stress relaxation can compensate for matrices with a lower stiffness. Since several mechanosignaling network components are involved, we suspect that changes in stress relaxation and the resulting cellular response could be key to chemoresistance. Therefore, it is imperative that advanced tissue models for cancer (and also for regenerative medicine) research should consider stress relaxation beyond, and independent of, stiffness and bioadhesivity. Some of the questions to be considered are: can stress relaxation potentially program tumor cells into a more resistant phenotype? Can stress relaxation crosstalk with genetic mutations? What is the role of time-dependent viscoelastic properties in tumorigenesis and the infiltration of immune cells? What categories of tumors depend on stress relaxation? These questions can be answered by using engineered biomaterials that incorporate stress relaxation behaviors

Key Figure

Ex Vivo Cancer Tissues with Multidimensional Control of Biophysical and Biochemical Properties



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Figure 1. The schematic depicts the integration of new strategies into existing tumor tissue models. (A) Time-dependent, controlled, reversible exchange of biochemical ligands. (B) Stress relaxation, which models the viscoelastic behavior of tissues compared with current, covalently crosslinked matrices. Simple covalently crosslinked hydrogels (black-lined network) with stress relaxation can be designed by including ionic bridges (red lines) and chemical spacers (e.g., polyethylene glycol; blue lines). (C) 3D niche porosity to model the nuclear deformability of cancer cells. (D,E) Integrating the cell-cell and autocrine/paracrine effect of the immune system and the gut microbiome.

and comparing them with purely elastic materials. The challenge will be in developing such materials where stress relaxation, bioadhesivity, porosity, stiffness, and topography are independently tunable.

Designing 3D Niche Porosity to Accommodate Nuclear Deformability of Cells

In vivo cells often have to transit through narrow constrictions smaller than their nuclear diameter during migration; for example, when passing through interstitial spaces or endothelial layers during intra- and extravasation. This concept applies to all migrating cells, including immune cells, fibroblasts, invasive cancer cells, and possibly even tissue stem cells. Recent work suggests that the biophysical properties of the nucleus can play a crucial rate-limiting role during cell migration in 3D environments: For pores substantially smaller than the nuclear cross-section, migration efficiency decreases, and cells eventually stall completely when the pore size reaches the ‘nuclear migration limit’ [28]. The ability of cells to pass through such confined spaces is mostly determined by nuclear size, deformability, and cytoskeletal contractility, which are affected by nuclear and cytoskeletal composition and organization [28–31]. As these parameters can vary widely between cell types, porosity and confinement should be key considerations in engineered microenvironments. Although most engineered niches have considered the porosity of the scaffold from the crosslinking density perspective, better material designs are needed to account for nuclear deformability, as some applications may favor designs that prevent (specific) cells from entering while other designs may benefit from having large enough pores to allow cells to enter the material. In addition to limiting the motility of cells, moving a large cell nucleus through small pores can have other biological consequences. Migrating through such tight spaces places substantial physical stress on the nucleus, which can result in transient loss of nuclear envelope integrity, herniation of chromatin across the nuclear envelope, DNA damage, and redistribution of mobile nuclear proteins *in vitro* and *in vivo* [32–34]. Recent *in vitro* work by the Discher group further supports the concept that migration through tight spaces results in increased genomic stability [34,35], but direct observations of migration-induced DNA damage *in vivo* and resulting genomic instability remains outstanding. Nuclear deformation may also alter chromatin organization and gene expression, which could further modulate tumor progression and resistance to therapy. Nuclear envelope rupture and migration-induced DNA damage may emerge as a potential novel therapeutic approach to specifically target metastatic cells. Supporting this idea, combined inhibition of nuclear envelope repair and DNA damage repair resulted in significant death of cancer cells during migration through confined environments [32]. Finally, our recent work further links nuclear envelope rupture in micronuclei and an inflammatory response, explaining how chromosomal instability drives metastasis through a cytosolic DNA response [36].

Given the importance of cell migration in many biomedical applications, ranging from tissue engineering to prosthetic device coating and cancer therapy, these recent findings highlight the importance of considering pore sizes and the nuclear deformability of the relevant cells in the design process of biomaterials-based scaffolds. In general, when designing new biomaterials-based *ex vivo* tissue-engineered models for development, wound healing, tumors, and even the coating of prosthetic devices, one should consider tissue scaffold pore sizes of $<25 \mu\text{m}^2$ in cross-section (Figure 1C). These considerations will vary with regard to tumor type and patient-specific attributes. Infiltrating immune cells, such as leukocytes, neutrophils, and dendritic cells, can squeeze through much smaller pores, down to around $1\text{--}2 \mu\text{m}^2$. We suggest that the scaffold pore size is an important design parameter, especially since small pores could not only slow migrating cells but also induce nuclear rupture and deformation. We suspect that nuclear rupture and deformation can contribute to DNA damage and genomic instability, promoting

drug resistance through genomic rearrangements or mutations that increase cell proliferation and/or abnormal signaling. Nevertheless, a generalized approach in determining pore size is not feasible because of wide differences in the biophysical characteristics of tumor cells. Applications for different cells will require different considerations for pore size, and the degradability of the material must also be considered, as many cells can remodel the ECM, for example, by secretion of MMPs.

Integrating the Immune System and the Effect of the Gut Microbiome

In addition to changes in cell and tissue stiffness, other hallmarks of cancer include chronic inflammation and altered immune responses [37]. While modeling of the tumor immune interaction in engineered tissues is important, another point of view is that tumor-infiltrating immune cells differentiate into phenotypes that support each step of the metastatic cascade and thus are novel targets for therapy. *In vivo* tumor-infiltrating T cells (including regulatory T cells), B cells, immunosuppressive myeloid-derived suppressor cells (MDSCs), and macrophages continuously interact with tumor cells through direct cell contact or by the secretion of a milieu of proinflammatory cytokines and chemokines. This immune-privileged microenvironment could selectively impair the recognition of tumor antigens by cytotoxic T cells and also protect residual tumor cells against cytotoxic destruction [38–41]. Immune cell populations, like T cells and natural killer cells, can control metastases of cancer cells by either restricting them to the primary tumor niche or promoting migration away from the primary tumor site [42,43]. Even macrophages, the cells of the innate immune system, adapt to the tumor microenvironment and polarize to the M1 extreme and secrete high levels of interleukins (e.g., interleukin-8 and -10) and granulocyte colony-stimulating factor, which suppress immunity [44]. The secretion of cytokines is dependent on tumor subtype.

The introduction of these immune components and recapitulation of the immune–cancer interaction complex in an *ex vivo* engineered system is ideal but non-trivial (Figure 1D). One of the main challenges is that immune cells, when cultured *ex vivo*, undergo rapid apoptosis over time unless rescued by antiapoptotic signals and replenished with a fresh supply of immune cells [45]. Newer approaches that recapitulate the continuous replenishment of immune cells *in vivo* can be achieved by integrating microfluidic platforms [46] with engineered tissues, where immune cells could be added to the feeding lymphatic or vascular networks. Simple encapsulation of immune cells in collagen or Matrigel is suboptimal, as these biomaterials may not provide the necessary survival signals and functional immune cells. Alternatively, advanced biomaterials-based scaffolds that support immune cell growth [47–51] could be integrated with the existing tumor niches and other on-chip approaches [52]. The development of such tissues can further be applied to study the efficacy of immunotherapy and the effect of immunomodulatory drugs on immune cells.

In addition to immune cells, tumor progression and the efficacy of antitumor therapies are now known to be affected by the microbiome. For example, *Fusobacterium nucleatum*, among other bacteria, is enriched in colorectal cancer patients' microbiomes [53,54] and *Helicobacter pylori* has been shown to be abundant in patients with non-cardia gastric cancers [55]. These interactions can be local, as *Clostridium*, *Bifidobacterium*, and *Salmonella* (reviewed in [56]) have been shown to grow in the tumor microenvironment and elicit immune reactions that can assist in tumor suppression. Similarly, in the colon, microbial biofilms have been implicated in the progression of colon cancer [57]. Direct impacts of the microbiome may also be systemic. Recently, the production of a microbe-derived carcinogen, deoxycholic acid, was shown to directly contribute to liver cancer [58]. Although the mechanisms that causally link microbes and tumorigenicity have not been established [59], several mechanisms have been proposed,

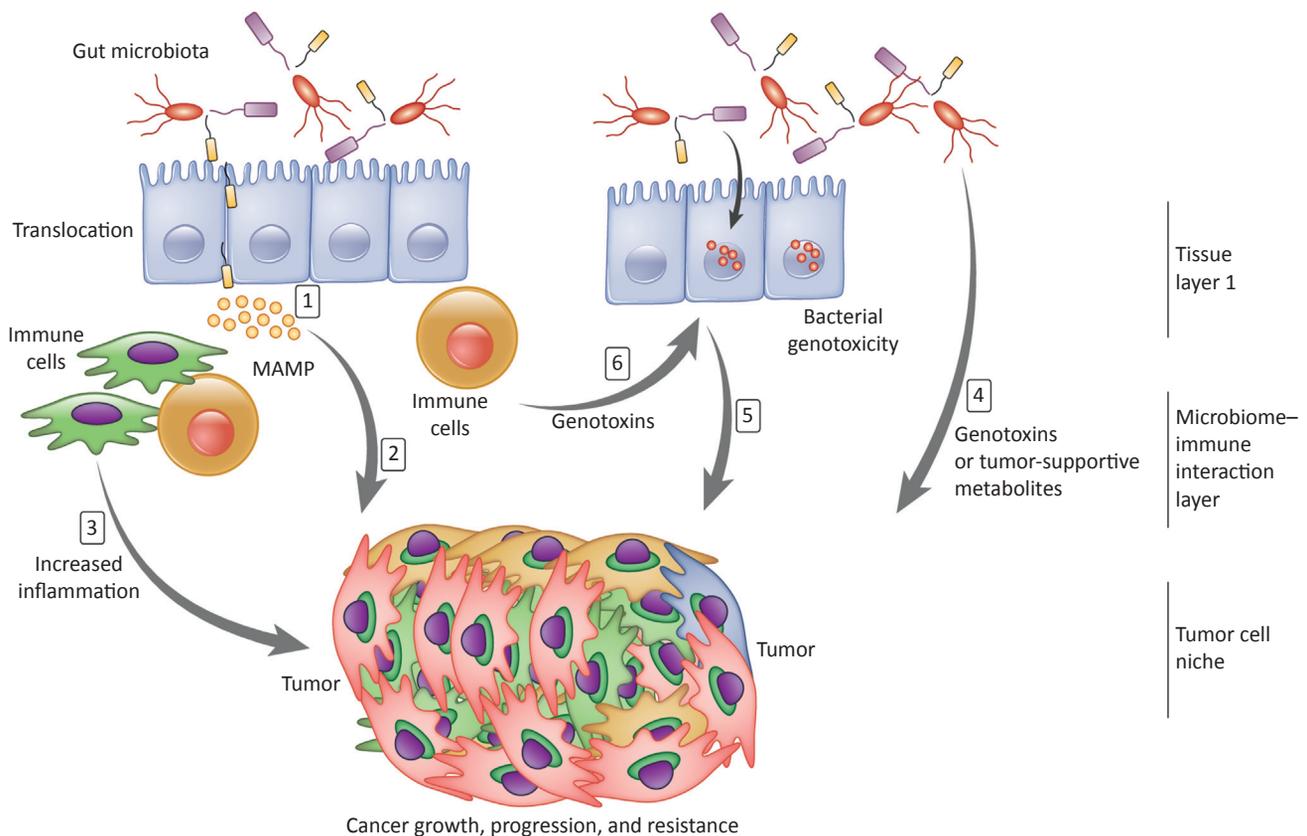
including metabolic signaling to promote proliferation or angiogenesis pathways [60]. Microbes may also produce reactive oxygen species, which may in turn contribute to DNA damage and, ultimately, tumor resistance. Manipulating these systems *in vivo* can be technically challenging due to the complexity of the communities and the difficulty of working with germ-free or monocolonized mice. An outstanding question is how to best integrate the effects of the microbiome into current *in vitro* engineered model systems for cancer and tumor resistance to create a more efficient cancer ecosystem (Figure 1E).

The longest-standing evidence for a link between the microbiome and cancer is in relation to colon cancer. *In vitro* models of colon cancer utilize organoid models, which are extremely useful for testing drug delivery, genetic manipulation, and the incorporation of biopsied tissue. Bacteria can be administered in these models by injecting them directly into the organoid cavity [61], but integration of biofilms into the organoid model of colon cancer remains to be done. An alternative to organoids for intestinal cancer models are 3D scaffold models of intestinal tissue. Several studies have been completed with co-culture of organisms on these scaffolds [62,63], but these experiments are limited in scope to a handful of organisms and are constrained by the difficulties of co-culturing intestinal cells with organisms that are normally found in an anaerobic environment. There are additional engineering challenges in introducing the range of biologically relevant cell types, which would both enhance mucous production and maintain barrier integrity. More attention is warranted in the areas of understanding the microscale interactions of these organisms and cancer cells and the shear stress caused by the laminar flow of intestinal contents, peristalsis, and large-scale mechanical reflexes and cellular apoptosis.

The mechanistic effects of the microbiome in colon cancer may be direct (e.g., signaling and small-molecule delivery directly to intestinal stem cells, affecting their proliferation) or indirect, via immune system function. The most striking example of this relates the microbiome and the efficacy of cancer immunotherapies in rodent models of colon cancer and melanoma [64,65]. It is currently thought that the microbiome alters the function of the immune system, specifically through changes in immune cell composition, maturation, and the inflammatory milieu. Several questions remain: do *in vitro* models of cancer lacking microbes adequately recapitulate *in vivo* malignancies or must we include microbes? Are there effects on tumors or the immune system that are specific to certain microbes or microbiome compositions? Are microbiome-mediated effects on cancer progression direct or predominantly mediated through the immune system? Can microbiome-mediated effects on cancer progression be modeled by simply introducing metabolites, cellular lysate, or supernatant to tumor cell culture or do we need to co-culture bacterial cells via *ex vivo* gut reactors integrated with immune and cancer tissues to elicit real-time feedback? Additionally, this feedback is likely to impact the composition of the microbiome as well as its function. The promise of the integration of complex microbial communities is that it will permit bidirectional analysis of microbiome effects on cancerous cells and the effects of tumorigenic cells on those bacteria. Single-cell analysis of individual bacteria [66] will provide information on selective pressures exerted on bacteria in the tumor microenvironment; metagenomic and strain-level analyses of microbial communities [67] co-cultured with tumor cells can be used to examine the function of these cells, including information on the metabolites they produce that might in turn influence tumorigenic progression and the formation of resistance. *In vitro* systems may prove fruitful in testing the effects of these different communities on immune or cancer tissue.

Last, because we know that the tumor microenvironment is permissive to bacterial colonization, can we study the effects of tumor-targeting synthetic bacteria *in vitro*? Din and colleagues

engineered a side-trap array microfluidic platform to co-culture human cervical cancer HeLa cells with *Salmonella typhimurium* [68]. Ingber and colleagues have also reported a simple biomimetic ‘human gut-on-a-chip’ microdevice that recapitulates epithelium polarization and villus-like folds and can be used for co-culture of a normal intestinal microbe (*Lactobacillus rhamnosus* GG) for extended periods of time (>1 week) without compromising epithelial cell viability [69]. Yissachar *et al.* [70] have taken an alternative approach whereby segments of intestinal tissue are excised and cultured *in vitro*, linking intestinal inputs and outputs directly to cultured immune and neuronal cells. This system may prove valuable in linking microbiome outputs to other *in vitro* models of tumor progression. The next challenge will be in determining how to integrate the diversity of the microbiome so that we can study its effect on overall tumor progression, tumor resistance, and drug targeting while modulating other aspects of the tumor microenvironment crosstalk (Figure 2).



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Figure 2. Ex Vivo Cancer Models with Possible Microbiome–Tumor Interactions. The figure shows an example of a three-layer *ex vivo* model. (1) Alterations in gut microbiota may result in increased bacterial translocation. (2) Increased abundance of microorganism-associated molecular patterns (MAMPs) directly influences tumor cells through local or distant mediators. (3) MAMPs stimulate Toll-like receptors on immune and other niche cells, leading to an increased tumor-supportive milieu of cytokines (e.g., interleukins) and growth factors. (4) The microbiota mediates tumor suppression through the generation of short-chain fatty acids and biological activation of cancer-preventing phytochemicals. (5) Bacterial genotoxins, after being delivered to the nuclei of host cells, actively induce DNA damage in organs that are in direct contact with the microbiome, such as the gastrointestinal tract. Other genotoxic components include reactive oxygen species, reactive nitrogen species released from inflammatory cells, and hydrogen sulfide from the microbiota. (6) Gut-mediated metabolites may result in: (i) activation of genotoxins such as acetaldehyde; (ii) activation of the metabolism of hormones; or (iii) alterations in the metabolism of bile acids.

Concluding Remarks

Engineered microenvironments have already emerged as important and useful tools to study tumor cells *in vitro*. A key advantage of *ex vivo* models is that the number of features (and correspondingly, the number of variables in the system) is constrained so that the effects of specific factors can be more clearly delineated. Although the most comprehensive models may not necessarily be the best model for a particular application, there remains a need to address unresolved questions of cellular, molecular, and microenvironment complexity (see Outstanding Questions). In addition to conventional bioadhesive matrices, decellularized matrices and glycosaminoglycans (GAGs) offer alternative choices for fine-tuning the 'right' characteristics in the model in terms of both pore size and matrix relaxation. Most previous *ex vivo* models have focused on stiffness and biochemical ligand presentation as the predominant design parameters and tumor invasion as the primary readouts. We propose that additional parameters should be considered in the design of such models, including viscoelastic properties, physical pore sizes, and the microbiome. Additional aspects of tumor microenvironments could include cancer-associated fibroblasts, stromal cells, immune cells, the role of fluid flow, matrix heterogeneity, and interactions with the vascular and lymphatic circulations [71–73]. In addition, genomic and epigenetic evolution of cancer cells should be considered as key drivers of therapy resistance, and the effect of the physical microenvironment on these processes should be investigated in more detail. We believe that careful modeling of *ex vivo* niches will result in improved understanding of epigenetic, metabolic, and signaling patterns in cancer, therefore leading to the development of new therapeutics [16,74–77]. Modular approaches that enable independent tuning of individual biophysical and biochemical parameters will facilitate more systematic studies. Technical advances that permit co-culture of bacteria with cancer cells can not only lead to better understanding of the tumor–microbiome interaction but also drive new directions to systemically deliver an antitumor toxin using synthetically engineered bacteria. Finally, newer *ex vivo* models that recapitulate complete selective aspects of the tumor immune microenvironment interactome are needed to maximize our effort towards a tumor microenvironment-driven precision medicine strategy and evaluate synergies between combination therapies to overcome resistance. Such innovative approaches will increase the 'predictive power' of preclinical inhibitors, provide potential biomarkers for correlative studies in new inhibitor clinical trials, and provide clues towards mechanisms that induce resistance to therapeutic inhibitors by more faithfully representing patient biological features and creating clinically relevant treatment regimens.

Acknowledgments

The authors acknowledge financial support from the National Institutes of Health (NIH) (1 R01 AI132738-01A1, A.S.; 1 R33 CA212968-01, A.S.; U54 CA210184, J.L.; R01 HL082792, J.L.), Department of Defense Career Development Award (W81XWH-17-1-0215, A.S.), the US Department of Defense Breast Cancer Research Program (Breakthrough Award BC150580, J.L.), and the National Science Foundation (NSF) CAREER Awards (DMR-1554275, A.S.; CBET-1254846, J.L.). The authors also acknowledge financial support from the Sloan Foundation Research Fellowship (I.B.) and the NSF (MCB-1650122, I.B.). This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs, through the Peer Reviewed Cancer Research Program, under Award No. W81XWH-17-1-0215 (A.S.). Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense, the NIH, the NSF, or other funding agency.

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Outstanding Questions

How do chemoresistance and changes in biophysical and biochemical factors relate to one another?

Can stress relaxation potentially program tumor cells into a more resistant phenotype? Can stress relaxation crosstalk with genetic mutations? What categories of tumors depend on stress relaxation?

What is the role of time-dependent viscoelastic properties in tumorigenesis and infiltration of immune cells?

How can we engineer *ex vivo* models with cell-specific consideration of pore size?

Is it sufficient to leave out the microbes from *in vitro* models of cancer? Are there effects of specific microbes or is it mainly the triggers they provide to the immune system?

Can microbiome-mediated effects on cancer progression be modeled by simply introducing metabolites, cellular lysate, or supernatant to tumor cell culture or do we need to co-culture bacterial cells via *ex vivo* gut reactors integrated with immune and cancer tissues to elicit real-time feedback?

How can we best integrate the effects of the microbiome into current *in vitro*-engineered model systems of cancer and tumor resistance to create a more efficient cancer ecosystem?

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