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Lessons from patient-derived xenografts for better in vitro modeling of human cancer☆,☆☆

Stephen Yiu Chuen Choi [a,c,1], Dong Lin [a,c,1], Peter W. Gout [a], Colin C. Collins [b,c], Yong Xu [d,**], Yuzhuo Wang [a,b,c,*]

[a] Department of Experimental Therapeutics, BC Cancer Agency, Vancouver, BC, Canada
[b] Department of Urologic Sciences, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada
[c] Vancouver Prostate Centre, Vancouver, BC, Canada
[d] Department of Urology, Second Affiliated Hospital of Tianjin Medical University, Tianjin, P.R. China

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ABSTRACT

The development of novel cancer therapeutics is often plagued by discrepancies between drug efficacies obtained in preclinical studies and outcomes of clinical trials. The inconsistencies can be attributed to a lack of clinical relevance of the cancer models used for drug testing. While commonly used in vitro culture systems are advantageous for addressing specific experimental questions, they are often gross, fidelity-lacking simplifications that largely ignore the heterogeneity of cancers as well as the complexity of the tumor microenvironment. Factors such as tumor architecture, interactions among cancer cells and between cancer and stromal cells, and an acidic tumor microenvironment are critical characteristics observed in patient-derived cancer xenograft models and in the clinic. By mimicking these crucial in vivo characteristics through use of 3D cultures, co-culture systems and acidic culture conditions, an in vitro cancer model/microenvironment that is more physiologically relevant may be engineered to produce results more readily applicable to the clinic.

Contents

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* Correspondence to: Y. Wang, Department of Experimental Therapeutics, BC Cancer Agency — Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada. Tel.: +1 604 675 8013; fax: +1 604 675 8019.

** Correspondence to: Y. Xu, Department of Urology, Second Affiliated Hospital of Tianjin Medical University, Tianjin, P.R. China. Tel./fax: +86 22 88326723.

E-mail addresses: schoi@bccrc.ca (S.Y.C. Choi), dlin@bccrc.ca (D. Lin), pgout@bccrc.ca (P.W. Gout), ccollins@prostatecentre.com (C.C. Collins), xymnwk@163.com (Y. Xu), ywang@bccrc.ca (Y. Wang).

[1] The authors contributed equally to this work.

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1. Introduction

Despite improvements in our understanding of the mechanisms of cancer pathogenesis and the continuous development of novel therapeutics, advanced cancers are in general still not curable. There is therefore a critical need for more effective treatments to improve disease management and patient survival. Use of in vitro cancer models has provided valuable information onthe understanding of cancer development and mechanisms of therapeutic action as they allow detailed analysis of these subjects under controlled conditions. As well, cancer cells grown in suspension culture, or as monolayers on plastic surfaces, are commonly used as cancer models in preclinical drug efficacy screenings. Major deficiencies of such models, however, include the lack of heterogeneity reflective of the original malignancy as well as an improper microenvironment, both of which are identified as major factors influencing cancer development and treatment resistance [1–3]. The poor resemblance of these in vitro models to human cancers and their microenvironments is considered a major reason why many preclinical findings fail to translate directly into clinical applications and the basis of the lack of predictive power of cultured cell-based models for drug efficacy and toxicity in humans [4]. As such, clinical tumor physiology, in addition to molecular and cellular biology, should be considered in the development of improved experimental cancer models.

To improve the clinical relevance of in vitro cancer models, it appears imperative to (i) use clinically relevant cancer tissue/cells that better represent the heterogeneity and complexity of cancers and (ii) mimic the tumor microenvironment more accurately. Although significant progress has been made over the past decade in the design of such models, current approaches still need further refinements that will allow reliable high-throughput analyses. In this review, we will discuss considerations regarding the use of in vitro systems of cancer cells/tissue, and then focus on critical microenvironmental factors observed in patient-derived xenografts and in the clinic that are worth contemplating. While it is expected that it will not be feasible to design in vitro systems that perfectly mimic the malignancy and its microenvironment, since that would likely lead to their loss of simplicity and ease of use, improvements in certain crucial aspects of cancer biology may be considered for the construction of clinically more relevant in vitro cancer models.

2. Tumor heterogeneity and model fidelity

The cellular and molecular heterogeneity of human cancers is well accepted. Tumor heterogeneity presents one of the greatest obstacles in model-based development of cancer therapeutics. Established human cancer cell lines can provide simplified cancer models and are commonly used in the preclinical studies of the disease. Such cell lines are valuable for basic studies but, unfortunately, have limited ability for predicting anti-cancer drug efficacy in the clinic [5]. One reason for this shortcoming is the relatively high homogeneity of established cell lines, a consequence of clonal selection during culturing, which is in contrast with the cellular heterogeneity of the parental tumors (Fig. 1). Furthermore, in vitro culture conditions can introduce additional evolutionary pressures such as oxidative stress [6], leading

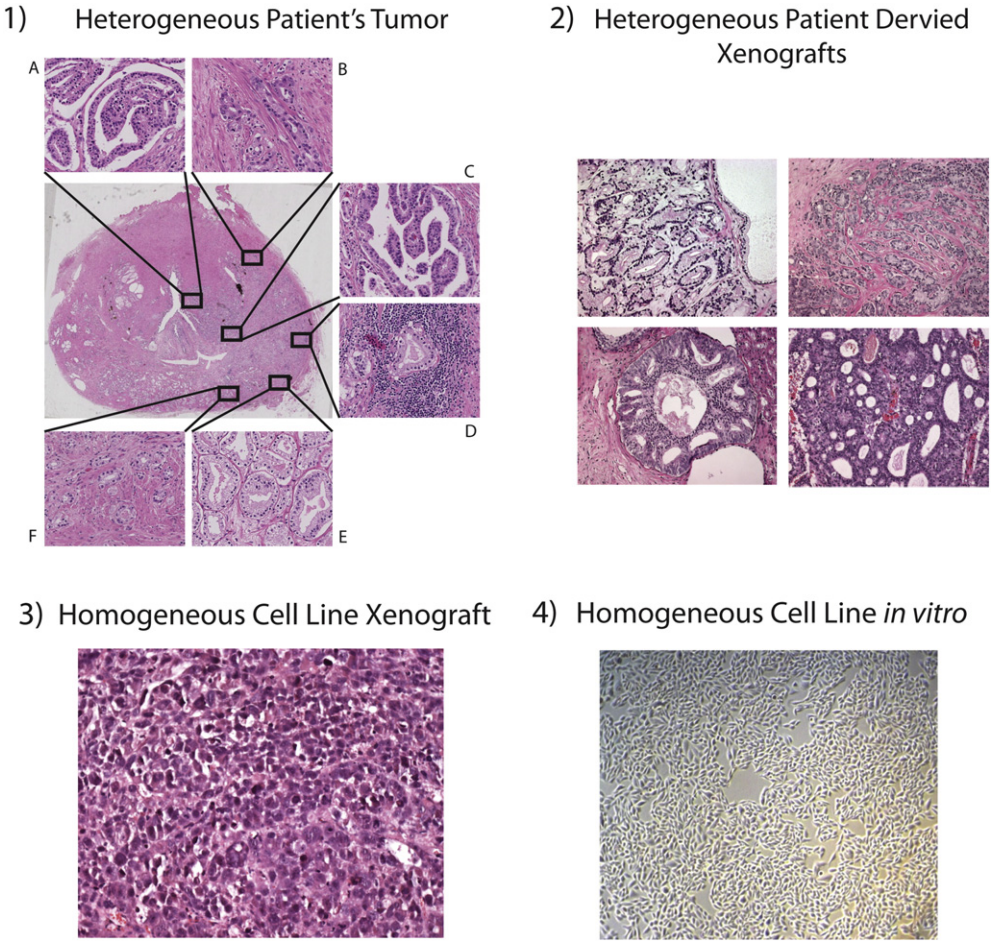

Fig. 1. Heterogeneity of a patient's tumor compared to homogeneity of cell line models. A sectioned whole-mount patient's prostate imaged at different cancerous regions (panel 1) show highly heterogeneous morphology. A–C: pattern of high Gleason grade (Grade 4); D–F: pattern of low Gleason grade (Grades 2–3). While this heterogeneity can be mostly recapitulated in patient-dervied xenograft models (panel 2), it is lost when using a cell line model in vivo (panel 3: image of PC3 prostate cancer cell line tumor grown in vivo) or in vitro (panel 4: image of PC3 prostate cancer cell culture).

to genetic and phenotypic changes that accumulate with repeated subculturing [7–10]. The clonal selection and long-term passage of cells in vitro thus result in homogeneous cancer cell populations adapted to particular culture conditions that greatly differ from cancer cell populations present in patients' tumors.

2.1. Patient-derived xenograft (PDX) models

More realistic preclinical cancer models are thought to be provided by transplantable, patient-derived cancer tissue xenograft (PDX) lines based on the grafting of fresh cancer tissue specimens subcutaneously, orthotopically or under the kidney capsules of immunodeficient mice (e.g., NOD-SCID, NSG mice). At the histopathological level, the PDX models retain, especially initially, the architecture and stromal components of the original tumor and therefore are thought to more accurately represent the complex biochemical and physical interactions between the cancer cells and their microenvironment [11,12]. At the cellular level, PDX models also preserve the inter-tumoral and intra-tumoral heterogeneity, as well as the phenotypic and molecular characteristics of the original cancer, including chromosomal copy number variants [13–15], single-nucleotide polymorphisms [16,17], and gene expression profiles [13,17–20]. In view of this, cancer tissues, or cells, derived from PDX models that mimic the biological and molecular characteristics of the original cancer with high fidelity could be employed to provide clinically more relevant in vitro cancer modeling systems. More specifically, such PDX tissues could be used to represent primary tumor samples from patients, either through the maintenance of tissue explants in vitro [21,22] or through establishment of primary cell cultures [23–25]. In addition to better mimicking the heterogeneity and biological characteristics of the original malignancy, use of cancer tissue xenografts has the advantage that the original tissue can be serially propagated in vivo. This results in more materials being available for repeated experimentation and potentially alleviates limitations pertaining to the inconvenience of patients' samples. To more fully represent the range of biological and molecular characteristics of patients' tumors, a panel of established xenograft lines covering multiple cancer tissues of origin and subtypes is required for the selection of the most appropriate model to address particular experimental problems.

2.2. Humanized in vivo models

In addition to the use of PDX models, another strategy used in vivo to improve the clinical relevance of cancer models is the use of humanized chimeric animals. While traditional in vivo studies often make use of human cancers transplanted into immunocompromised rodents, the incompatibilities between tumor and host often result in disruptions of critical native processes requiring species-specific interactions [26]. Efforts have therefore been made to incorporate additional human components into host animals to mitigate these differences and to better mimic the human disease, either through genetic engineering approaches to introduce human versions of certain genes [27], the seeding of human immune cells or stromal components [28,29], or through implantation of human organoids [30–32]. More importantly, observations from such studies have demonstrated that tumor microenvironmental factors can greatly impact certain aspects of cancer cell behavior. For example, while bone metastasis is a common clinical presentation of various malignancies such as breast and prostate cancer, the mechanisms involved still remain largely unknown due to the lack of appropriate in vivo models. However, it has been shown that by humanizing the primary tumor site [31,33], or by using human or tissue-engineered bone grafts as target metastatic sites [30,34,35], the osteotropic phenotype can be recapitulated to study the underlying factors involved. The application of such in vivo models is not limited to cancer and has also been used to study various aspects of immunology and toxicology [36,37]. Fundamentally, these studies serve to demonstrate that the microenvironment of the cancer is of critical importance and can greatly influence experimental outcomes, thus highlighting the need for better mimicking of the tumor microenvironment in order to improve the clinical relevance of in vitro models.

3. Tumor microenvironmental factors

The tumor microenvironment is the cellular and molecular environment in which the tumor exists. In addition to cancer cells, the microenvironment includes surrounding lymphatic and blood capillaries, immune cells such as lymphocytes and macrophages, fibroblasts, other normal cells, the extracellular matrix (ECM), and a variety of signaling molecules. Alterations to microenvironmental conditions may promote unrestrained cell proliferation [38], facilitate tumor initiation [39], and direct metastasis [40]. For example, as tumors develop, a shift in tissue dimensionality can occur through the loss of epithelial polarity [41] and alignment of ECM fibers [42,43]. The resulting changes in cell–cell contact can alter paracrine signaling and significantly contribute to disease progression [44]. Furthermore, aberrant tumor vascular function, including irregular and premature vascular networks, inadequate microcirculation and high vascular permeability, also contributes to the development of an adverse pathophysiological cancer microenvironment [45,46]. It can lead to hypoxia, upregulation of glycolytic capacity and lactate accumulation, extracellular acidosis, nutrient deprivation and energy depletion, and ATP hydrolysis and adenosine accumulation [47–50]. Hypoxia, in particular, is a commonly witnessed condition in most tumor masses and often contributes to mutagenesis, suppression of apoptosis, and epithelial-to-mesenchymal transition, among other effects on tumor biology [47,48]. Moreover, exosomes produced by cancer cells have been demonstrated to serve as an active mechanism of communication between the tumor and its microenvironment and could play a role in treatment resistance, metastasis and immune suppression [51,52].

Given the highly complex nature of the tumor microenvironment and its components, it is likely that many microenvironmental factors can contribute to cancer growth and progression. Rather than exhaustively defining every possible one, a few crucial but relatively underappreciated characteristics of the in vivo tumor microenvironment, drawn from our experience with PDX models, are highlighted below for consideration. They include the tumor architecture and its pathophysiological features, cell–cell interactions within the tumor, and high acidity of the tumor microenvironment. While other components of the tumor microenvironment are also highly relevant, extensively describing each element and fully acknowledging all contributors to the various fields are regretfully beyond the scope of this review. A summary of the various components as well as sources for additional information have been listed (Table 1).

3.1. Tumor architecture

One advantage of the PDX models is the retention of the original tumor architecture. While cancer cell lines are frequently transplanted into animals as a method of establishing tumors in vivo, the resultant tumors do not exhibit the distinct histological characteristics observed in the clinic (Fig. 1). One important consideration in this regard is that cells within tissues are surrounded by the ECM, a meshwork of proteins and proteoglycans such as laminin, collagen and fibronectin. It provides structural support, stability, flexibility and shape for the tissue and also mediates cell polarity, intracellular signaling and cell migration [53,54]. Inside the cells, ECM-induced signaling pathways are transmitted mainly through integrin molecules (i.e. heterodimeric transmembrane receptors that mediate cell adhesion to the ECM) and molecules of the immunoglobulin superfamily. Integrins act as bridges between the ECM and the internal cytoskeleton by transducing key intracellular signals through associating with clusters of kinases and adaptor proteins in focal adhesion complexes [55]. These interactions have been found to be distinctly mediated by specific integrin heterodimers

Table 1
Summary of various important components/factors of the tumor microenvironment and additional references for more detailed information.

Relevant factors of the tumor microenvironment	Summary	In vitro mimics	References for additional information
ECM alterations	Tumors have increased collagen I deposition. Furthermore, it has been shown that tumors may have altered expression of collagen-modifying enzymes that ultimately affect cell invasive and metastatic potentials through increased matrix stiffness and alignment of fibers at the invasive edge.	Use of different ECM compositions in 3D cultures	[278]
Immunosuppressive microenvironment	Localized suppression of the anti-cancer immune response occurs through various mechanisms, such as expression of immunosuppressive cytokines, recruitment of regulatory immune cells, or expression of immune inhibitory signaling molecules.	Co-culture systems with immune cells Addition of cytokines to culture medium	[279–282]
Acidic tumor microenvironment	Results from altered cancer metabolism and the overproduction of lactic acid. Alternatively, overexpression of other pH modulatory proteins such as NHEs and V-ATPases has also been implicated. Promotes various cancer characteristics such as angiogenesis, tissue invasion, therapy resistance, and immunosuppression.	Acidic culture conditions	[236,267,283,284]
Hypoxia	Oxygen tension within the tumor often fluctuates between normoxic and hypoxic conditions due diffusion limitations and abnormal vasculature. Poor oxygenation is particularly pertinent to radioresistance. Although expression of oxygen responsive genes is primarily mediated through HIF-1α, additional signaling pathways such as mTOR, Myc, and Notch have also been implicated. Furthermore, low oxygen levels can also alter various tumor stromal components.	Environmentally controlled chambers for culturing in hypoxic conditions Chemical inducers of hypoxic response	[47,278,285–287]
Necrosis/necroptosis	Cell death characterized by ruptured membranes and spilling of cytoplasmic content into the extracellular space. Recent studies have shown that necrosis is a regulated process much like apoptosis (referred to as programmed necrosis, or necroptosis) involving TNFR, RIPK and calcium signaling. This mode of death in the tumor, particularly following therapy, results in the release of pro-inflammatory damage-associated molecular patterns into the tumor microenvironment and may initiate an anti-cancer immune response.	Death receptor ligation with caspase inhibitors Apoptosis-deficient cell models	[288–291]
Exosomes	Emerging as an important mechanism of cell–cell communication. Cancer-derived exosomes have been shown to contain a large array of proteins and RNAs that help survival, drive progression, establish premetastatic niches, and modulate anti-cancer immunity.	Exosome isolation from culture supernatant or patient serum	[51,52]

binding to individual ECM components [56]. The signals are transmitted via the cytoskeleton to the nuclear matrix and affect gene expression [57]. As such, highly specific and localized signaling cascades can be activated by the ECM in association with various available growth factors through sequences of reactions involving networks of proteases and sulfatases [58].

The biophysical interplay between the cell and the ECM also establishes a dynamic mechanical reciprocity as its maintenance and homeostasis involve a tight balance between the biosynthesis, three-dimensional (3D) organization, cross-linking, and degradation of ECM proteins [58–60]. Furthermore, cell behavior within tissues is largely based on the 3D interactions of cells with the ECM and other cells, and on the responses to various mechanical stimuli. The correct architecture/structure is therefore another critical factor to consider in addition to the composition of the ECM. In fact, dimensional changes can influence tumor growth [61–63], cell migration [64], signaling [65] and drug response [66] independent of other phenotypic changes. This may partially explain frequent discrepancies between bench top and clinical efficacy of new therapies [67].

It has been reported that the architectural, biochemical and physical properties of cancer ECM are different from normal tissue. For example, breakdown of the epithelial basal membrane is a histologic hallmark of malignant tumors, and there is evidence to support that subtle changes in ECM preceding its complete breakdown can actively contribute to cancer initiation and progression [68]. Furthermore, tumor stroma is usually stiffer than normal, which enhances cell growth and survival and promotes cell migration [69]. There is also an increase in collagen gene expression and deposition along with an overproduction of heparin sulfate proteoglycans during tumor formation [70–72]. Additionally, cancer-associated blood coagulation forms fibrin clots within or adjacent to cancer tissues, which are subsequently replaced by collagenous stroma in a process similar to that in normal wound healing, further contributing to the abundant tumor stroma in highly invasive tumors [73,74]. It has also been reported that collagen I in cancer tissue is highly linearized and oriented adjacent to the epithelium, projecting perpendicularly into the tissue instead of forming relaxed non-oriented fibrils [69,75]. Increased expression of many ECM remodeling enzymes including matrix metalloproteinases (MMPs), urokinase, heparanases, and cysteine cathepsins is also often observed in a number of cancers [76,77]. The deregulation and disorganization of the ECM in cancer may greatly impact various signaling pathways and can promote malignant transformation, survival, and proliferation of cancer cells [78]. In view of these findings, using the correct type and composition of ECM in vitro to mimic altered tumor matrix could allow for analysis of cancer cell behavior in a more clinically relevant environment.

3.1.1. ECM components and 3D cultures

Various natural, semisynthetic and synthetic materials have been used to simulate tumor ECM in cultured cell systems, and important insights into tumorigenesis have been gained from their use. In particular, Matrigel, a basement membrane matrix preparation derived from murine sarcoma, and ECM components, derived from animal tissues such as collagen-I, laminin and fibronectin, are widely accepted in view of their intrinsic cytocompatibility, allowance for cell adhesion and ability to modify gel properties, such as fiber structure and porosity, by altering gelling conditions (e.g., temperature, gel concentration, pH and media components) [79]. However, the barrier properties of the natural basement membrane are dependent on covalent cross-links between collagen type IV molecules, which are destroyed in the isolation and solubilization processes of Matrigel [80]. Therefore, mechanistic studies of epithelial migration through soluble Matrigel may not be able to fully identify the molecular and cellular components involved in tumor progression and tissue invasion. For collagen matrices, a critical determinant of cell migration is the formation of fibers [81], which differs between pepsin- and acid-solubilized collagen. While acid-soluble collagen fibers reflect the natural structure of collagen in vivo, it contains telopeptides that may negatively affect biocompatibility [82]. Studies using collagen matrices should hence be carefully

interpreted in the proper context, taking the isolation techniques used into consideration [44]. One possible alternative is the use of cell-derived matrices (CDM), which contain ECM components naturally produced when certain types of cells (e.g. fibroblasts) are cultured at high density in vitro. It has been shown that CDM closely mimics the chemical and physical properties of in vivo matrix in terms of diversity, spatial heterogeneity, flexibility, and stiffness [83]. Recent studies have also shown that the CDM produced by cancer-associated fibroblasts recapitulated the architecture and content observed in parental tissue stroma [84,85]. In addition, cancer-associated CDM was sufficient and necessary to promote desmoplastic differentiation of normal fibroblasts and trigger tissue invasive behavior of breast cancer cells [85,86]. As such, CDM can be considered a more complex, physiologically relevant alternative to purified protein matrices.

It has been reported, however, that culturing cells on ECM protein-coated polymer surfaces does not necessarily lead to in vivo-like cell behavior. It has become apparent that traditional two-dimensional (2D) culture approaches are inadequate in mimicking the 3D space of the original cancers. In view of this, 3D cultures are more suitable for modeling the tumor microenvironment in vitro since they can simulate the dimensional aspects of tumors in a controlled fashion. Merely switching culture dimensionality from 2D to 3D radically affects protein expression [87], cell proliferation [88], differentiation [89], and metabolism [90]. 3D in vitro models are here only briefly discussed since they have been thoroughly reviewed elsewhere [91–94] and are not the main focus of this review.

3D cultures bridge the gap between traditional 2D cultures and in vivo animal models. In the last decade, increased use of 3D models that mimic specific tissues have significantly improved the understanding of the mechanisms of tumor development and facilitated the development and screening of new therapeutics. 3D models allow rapid experimental manipulations and their format can be tailored to suit particular scientific problems and downstream uses. On the other hand, it should be noted that 3D cultures do not fully reproduce the in vivo tumor microenvironment, as systemic effects are notably absent. Also, establishing these models can introduce alternate factors that may modulate cell behavior, such as cellular confinement affecting cellular stiffness responses, and steric hindrance altering diffusion of secreted molecular signals [44,95,96]. Therefore, the results generated in 3D culture systems must be interpreted cautiously and need to be further validated in vivo. While the advantages and limitations of various 3D culture systems have been well reviewed by others [92,97–99], a few major points are worth noting. Although the concentration of collagen matrices can be adjusted to mimic the density of a particular tissue under study, these matrices lack other ECM components and may have altered covalent crosslinks [92]. Alternatively, Matrigel may mimic the basement membrane as it contains basement membrane components, but its presence as a 3D gel is vastly different from the thin layer found separating and supporting the epithelium [97]. Cell-derived matrices can have lower amounts of collagen and larger internal spaces [83]. Finally, in addition to considering the properties of the various available matrix materials and their potential effects on cell behavior, it is also important to consider the matrix characteristics of the specific tissue of origin. The proper considerations pertaining to particular cancer types are discussed in greater detail by other reviews in this issue.

3.2. Cell–cell interactions

Early seminal experiments have demonstrated that malignant properties of cells isolated from cancers can be reversed when they are placed in the midst of the normal tissue of origin [100]. Such discoveries bring into question the very nature of cancer cells and the details regarding their existence in the first place. While contradictory to the commonly accepted somatic mutation model, a proposed tissue organization field theory (TOFT) suggests that cancer is a tissue-based disease generated as naturally proliferating cells are liberated from restraints imposed by their microenvironment. Environmental factors disrupting the communication between cells and their extracellular matrix could be the driving force behind the development of neoplasia [101–103]. Additionally, it is well documented that cell–cell interaction plays an important role in this process. Many cell types, including fibroblasts, smooth muscle cells, immune and inflammatory cells, endothelial cells, pericytes, and adipocytes are present in the tumor microenvironment, further contributing to its complexity. Two of these cell types are of particular interest, namely cancer-associated fibroblasts (CAFs, also referred to as tumor-associated fibroblasts in the literature) and regulatory immune cells.

3.2.1. Cancer-associated fibroblasts (CAFs)

In addition to ECM components of a patient's tumor, the PDX tumor architecture also contains, especially initially, stromal cells of the original tumor, in particular CAFs. They form an important component of the tumor microenvironment [1,104]. Studies with tumor xenografts, based on co-implantation into mice of either normal fibroblasts or CAFs together with cancer cells, have been performed independently by many groups. They consistently demonstrate significantly increased tumor growth-promoting potential of CAFs compared to normal fibroblasts [105–111]. CAFs are able to produce tumor-supportive ECM and secrete growth factors and chemokines that further alter the ECM and generate oncogenic signals [112]. They therefore play key roles in tumor transformation, cell proliferation and tissue invasion [113,114]. Recently, CAFs have also been found to play an important role in the development of resistance to chemotherapy based on reduced intratumoral drug delivery [115–118]. However, it is worth noting that a recent clinical trial of pancreatic cancer using IPI-926, a Hedgehog signaling inhibitor shown to deplete tumor-associated stroma [116], has failed, suggesting that a better understanding of the intricate tumor–stromal interactions is imperative.

Substantial evidence in vivo has shown that CAFs are critical drivers of tumor progression in a number of organs. In the prostate, CAFs are able to stimulate transformation of immortalized prostate epithelial cells whereas normal fibroblasts cannot [105]. Studies based on xenograft models of human cells have shown that recombination of CAFs with non-tumorigenic epithelial cells results in their permanent malignant transformation [105,119]. Soluble factors secreted by CAFs such as TGFβ, IGF1, HGF/scatter factor and other chemokines act in paracrine fashion on adjacent epithelial cells and work in coordination with other signaling molecules, such as ECM and integrins, to facilitate carcinogenesis and cancer progression [120–123]. In addition, WNT16B secreted by CAFs promotes an epithelial-to-mesenchymal transition (EMT) and survival of cancer cells after cytotoxic therapy [118]. In the case of breast cancer, there is evidence showing that molecular alterations of tumor stromal cells alone can significantly alter the behavior and progression of tumors [124,125]. Also, CXCL12 and CXCL14, secreted by CAFs in breast tumors, can increase the proliferation, tissue invasion and metastasis of breast cancer cells [106,126]. These data suggest that CAFs form a crucial component of the tumor microenvironment and play an important role in tumorigenesis and cancer progression.

3.2.1.1. Co-culturing with CAFs. The presence of non-cancerous cell types in the tumor microenvironment can, in part, be mimicked through co-culturing cancer cells with cell types of interest. As the most abundant cell type in the tumor stroma, and given their tumor-promoting abilities, CAFs are frequently used in co-culture systems. Furthermore, fibroblast and cancer cell co-culture systems have been used in combination with 3D culture techniques and have provided more reliable in vitro models to mimic stromal–epithelial interactions in the tumor. The ability of CAFs to promote tumorigenesis and support cancer development and tissue invasion has been observed in such models [127–129].

One challenge in the engineering of CAF co-cultures is the heterogeneity of CAFs found in cancer tissues. CAFs can originate from resident

fibroblasts, epithelial cells, endothelial cells or mesenchymal cells. CAFs can also be sorted, based on their histopathological features, into myofibroblasts, which are activated fibroblasts expressing α-smooth muscle actin (α-SMA), and non-myofibroblasts. Thus CAFs show a high degree of heterogeneity due to their various origins. Studies using cancer xenograft models and genetically engineered mouse models further suggest that different subpopulations of CAFs may play distinct roles in tumor progression [110,130–132]. In view of this, CAFs derived from human tumor tissues via primary cultures, containing various CAF subpopulations, likely form a clinically more relevant supply for co-culture systems than commonly used established fibroblast cell lines. However, significant differences in fibroblast-derived paracrine effects on cancer cells have been observed using CAFs from different patients, indicating an inter-tumoral heterogeneity of CAFs [133]. Therefore, data obtained from a single CAF/cancer co-culture system may need to be interpreted with caution. Multiple co-culture systems based on different CAF sources may provide data that are clinically more relevant.

3.2.2. Regulatory immune cells

Despite the advantage of PDX models in maintaining the original tumor histology, the most commonly noted drawback is the requirement for an immunodeficient host. While the broadly immunosuppressive environment through lack of functional immune cells is essential for tumor implantation and growth, it is also not reflective of the intricacies of different cell types and cytokines found in the patient's tumor microenvironment. As such, this puts major limitations on the use of xenograft models in assessing efficacy of immunotherapies and immunomodulatory properties of certain chemotherapeutics [134,135]. This serves to highlight, somewhat paradoxically, the immune system as an important component of the tumor microenvironment.

Not only is there increasing clinical evidence that infiltration of immune cells into the tumor is prognostically significant [136–138], but the avoidance of immune destruction is considered an emerging hallmark of cancer [139]. Current opinions suggest that there are at least two ways in which the immune system is involved in cancer. As cancers progress and accumulate genetic alterations, the abnormalities are often reflected in ways that are detectable by the immune system. Elevated expression of tumor antigens [140] and secretion of danger signals following chemotherapy and radiation [141,142] mark cancer cells for elimination. The cytotoxic arm of the immune system, comprising mainly of cytotoxic $CD8^+$ T cells and NK cells, is thought to be responsible for cancer elimination and is aided by secretion of pro-inflammatory cytokines (such as IL-2, IFNγ, TNFα) from various additional helper cell types (Table 2). Alternatively, the regulatory, immunosuppressive arm of the immune system is thought to inhibit normal effector cell function in the tumor microenvironment, causing a localized immune deficiency where cancer cells can no longer be effectively eliminated and thus are allowed to progress [143,144].

The immunosuppressive arm of the immune system is of particular interest, as it is thought to be the predominant arm active in the tumor microenvironment. The importance of this is exemplified in considering the challenges of eliciting anti-cancer immunity through immunotherapeutic approaches. One major hurdle involves overcoming the initial immunosuppressive tumor microenvironment [145,146] and could be achieved through lymphodepletion prior to adoptive cell transfer or vaccination. The depletion of host lymphocytes (either through chemotherapy or whole body radiation) followed by hematopoietic stem cell transfer has been shown to increase the effectiveness of eliciting anti-cancer immune responses [147–150]. In particular, this approach has been shown to alter the hematopoietic stromal components through decreasing the abundance of suppressive cell types and abolishing the cytokine sink previously established in the tumor microenvironment [151,152]. Furthermore, repopulation of immune cells following transplantation is then skewed towards expansion of anti-cancer immune cells [153] and improves effector immune cell

Table 2
Summary of major immune cell types found in the tumor microenvironment and their role in anti-cancer immunity or immune suppression.

Immune cell subtypes	Summary of functions	References
Anti-cancer cell types		
$CD8^+$ cytotoxic T cells	Considered the predominant cell type involved in anti-cancer immunity. Recognizes tumor antigens through MHC–TCR interactions and are activated in the presence of costimulatory molecules. Can directly eliminate cancer cells through granzymes and death ligand signaling.	[292–296]
Natural killer (NK) cells	Eliminates cancer cells using mechanisms similar to cytotoxic T cells. Are activated by engagement of various NK receptors, such as NKG2D, expressed on the cell surface.	[293,297,298]
T_H1 helper T cells	Upon recognition of tumor antigens, T_H1 helper T cells can secrete pro-inflammatory cytokines such as IFNγ, TNFα, and IL-2 that aid in the activation and expansion of cytotoxic cell types.	[296,299,300]
iNKT cells	Expresses a mix of cytotoxic T cell and NK cell receptor molecules and are activated by lipid antigens. Upon activation, can both directly and indirectly (via activation of dendritic cells) activate cytotoxic T cell and NK cell function.	[301,302]
Classic (M1) macrophages	Activated by IFNγ and releases high levels of pro-inflammatory cytokines (such as IL-12 and TNFα) and reactive oxygen/nitrogen species. Are generally considered tumoricidal through activation of downstream cytotoxic cells and amplification of the T_H1 response.	[163,303,304]
T_H17 cells	Characterized by the production of IL-17 family of cytokines, their involvement in cancer immunity is still under investigation. They seem to play a role in anti-tumor immunity through recruitment of other anti-cancer cell types, such as T_H1 cells and NK cells. However, they have also been reported to have pro-tumor effects through promoting tumor vascularization and differentiating into regulatory T cells.	[305–308]
Suppressive cell types		
Regulatory T cells (Tregs)	Considered the primary suppressive cell type in the tumor microenvironment. Inhibits anti-cancer immunity through release of anti-inflammatory cytokines IL-10 and TGFβ, suppression of antigen-presenting cells through CTLA-4 and LFA-1 signaling, and active killing of effector cells by granzyme B and perforin.	[160,161,309–311]
Type 2 (M2) macrophages	Suppresses effector immune cell activity through IL-10, TGFβ, IDO, arginase, and prostaglandins. Actively promotes tumor development through inducing new blood and lymph vessel formation, degrading extracellular matrix via matrix metalloproteinase expression, and promoting metastasis.	[162,312–316]
Plasmacytoid dendritic cells (pDCs)	Decreases proliferation of effector T cells and increases generation of regulatory T cells through expression of immunosuppressive cytokines and cell surface molecules such as ICOS-L.	[175,177,317]
Myeloid derived suppressor cells (MDSCs)	Depletes arginine and sequesters cysteine to deprive effector T cells of essential nutrients required for proliferation, produces reactive nitrogen and oxygen species to disrupt antigen recognition and induce T cell apoptosis, and inhibits L-selectin expression on $CD8^+$ and $CD4^+$ T cells to prevent their extravasation at the tumor site.	[184,318–321]
T_H2 helper cells	Characterized by production of IL-4, IL-5, IL-6, IL-10, and IL-13. Is generally considered a tumor promoting cell type through diminishing cytotoxic capacities in T cells and skewing macrophage development to the M2 phenotype.	[279,322,323]

trafficking to the tumor [154]. As such, better mimicking of the regulatory arm of the immune system may be worth of further consideration in creating a more relevant in vitro microenvironment.

Of the many currently known suppressive cell types (Table 2), regulatory T cells are the best characterized and affect a wide range of immune cells [155,156]. The association of regulatory T cells with cancer and the tumor microenvironment is also well established. An increased number of regulatory T cells in the peripheral blood of patients and a high regulatory T cell density in tumor tissue are generally correlated with poor patient outcomes in many cancer types [157–159]. Their immune suppressive properties are conferred by the transcription factor FOXP3, and they promote tumor development by inhibiting the cytotoxic anti-cancer response, most notably through the release of anti-inflammatory cytokines IL-10 and TGFβ [160,161].

The M2 macrophages form a second subtype of regulatory immune cells closely associated with tumor development. In addition to suppressing the anti-cancer immune response, M2 macrophages are known to actively promote tumor development through mechanisms involved in tissue repair. They have been associated with the ability of cancers to induce new blood vessel formation, remodel stromal tissue, and dissociate connective tissue for metastasis [162,163]. The importance of M2 macrophages in cancer progression has been highlighted in a number of studies [164–167]. Furthermore, the presence of M2 macrophages is also correlated with other signs of tumor progression, such as elevated regulatory T cell counts, induction of angiogenesis, and increase in lymph node metastasis [168–170]. A number of experimental approaches targeting M2 macrophage involvement in cancer have shown initial efficacy in vivo and further validate the importance of their presence in the tumor microenvironment [171–174].

Other immunosuppressive immune cell populations closely associated with developing tumors are the plasmacytoid dendritic cells (pDCs) and the less-characterized myeloid-derived suppressor cells (MDSCs). The immunosuppressive function of pDCs is thought to be mediated by their ability to reduce effector T cell populations and induce regulatory T cell development and expansion [175–177]. A number of studies have confirmed the presence and function of pDCs in various cancers [178–181]. MDSCs can further contribute to immune suppression at the tumor site by exhibiting T cell-suppressing properties [182]. This is accomplished by both recruiting regulatory T cells and impairing effector T cell functions through depleting arginine and sequestering cysteine, both of which are essential nutrients required for proper T cell proliferation [183,184]. In concordance with the previously mentioned cell types, association of MDSCs with poor prognosis and various aspects of cancer progression is also well supported by a number of studies [185–189].

Collectively, the evidence strongly indicates an important functional role for immune cells in the tumor microenvironment. In particular, the immunosuppressive regulatory subtypes seem to play an important role in facilitating tumor progression. While most xenograft models mimic an immunosuppressive tumor microenvironment through the use of immunocompromised hosts, the complexity of the interactions and balance between anti-cancer and immunosuppressive cell types in the tumor microenvironment are often incompletely reflected. A useful alternative to this would be the use of syngeneic tumor models or other transgenic models of spontaneous cancers. Furthermore, as certain therapeutic compounds may have immunomodulatory properties [190–195], the use of an immune-intact system could help elucidate whether such secondary effects would enhance or diminish treatment efficacy through stimulating or further suppressing the anti-cancer immune response.

3.2.2.1. Co-cultures with immune cells. Given the critical importance of an immunosuppressive tumor microenvironment in cancer development and progression, another cell type worth considering for in vitro co-culture systems would be immune cells. Because immune cell activities are often responses to external stimulation by other cell types, co-cultures of cancer cells and immune cells are frequently used to assay specific immune cell activities, including changes in cytokine production and release [196–198], anti-cancer cytotoxic activities [199–201], and immune cell maturation and differentiation [202,203]. Interestingly, the communication between cancer cells and immune cells appears to be bidirectional, with immune cells also modulating cancer cell characteristics such as migratory potentials [204,205] and chemokine production [206].

One challenge to co-culturing cancer cells and immune cells on a regular basis in vitro is the vast diversity of the immune subtypes potentially present in the tumor microenvironment. This problem is further compounded by the fact that most tumor-associated immune cells do not function in isolation, but rather act through intricate communication with other immune subtypes. As such, determining the exact cell types in the correct proportions needed to mimic the tumor microenvironment is challenging. However, immune functions are often modulated through secreted cytokines, and it is well documented that certain immunosuppressive cytokines are predominantly present in the tumor microenvironment [207–209]. More importantly, it has been demonstrated that immunosuppressive cytokines such as TGFβ and IL-10 can play a significant role in promoting cancer progression through mechanisms acting directly on cancer cells and the surrounding stromal cells. For example, while TGFβ has been shown to have cytostatic and anti-proliferative effects in maintaining mature epithelial tissue homeostasis [210,211], its deregulated signaling has been shown to aid cancer development, particularly through promoting epithelial-to-mesenchymal transition [212–214], angiogenesis [215], metastasis [216], and stromal remodeling [217]. The presence of IL-10 has been shown to confer resistance to apoptosis [218,219]. As such, the addition of classic immunosuppressive cytokines to the cell culture media could be one strategy to mimic the effects of an immunosuppressive tumor microenvironment on cancer cells. Interestingly, additional cytokines commonly present in the tumor microenvironment, such as IL-6 and IL-17, could also directly act on cancer cells and otherwise impact cancer development. IL-6 has been shown to induce STAT3- and NFκB-mediated signaling cascades in cancer cells [220], increasing cell proliferation and protecting against apoptosis [221]. Furthermore, it has the potential to induce chronic, cancer-promoting inflammation [222,223]. Although the functional roles of IL-17 in the tumor microenvironment remain to be further elucidated, it has been reported to promote angiogenesis [224], cancer cell growth and survival [225]. While there are recent advances in in vitro culture models of cancer–immune interactions, particularly through inclusion of immune cells in various 3D model systems [226], the supplementation of such cytokines to the culture medium in vitro, both for traditional and emerging co-culture studies, could potentially yield more clinically relevant results.

3.3. Acidic tumor microenvironment

One final and highly critical characteristic of the tumor microenvironment is its relatively high acidity. While an acidic tumor microenvironment is commonly observed in patients as well as in PDX models, its importance appears to be rather underappreciated in vitro. The tumor microenvironment has elevated extracellular acidity (pH ~6.5 compared to a physiological pH of 7.4) as a result of altered energy metabolism. Cancer cells have long been known to exhibit an altered glucose metabolism compared to normal cells. This phenomenon was observed as early as the 1920s, when Otto Warburg noted that cancer cells derive the majority of their energy through fermentation and production of lactic acid rather than through respiration, even under conditions of abundant oxygen (aerobic glycolysis) [227]. The associated higher consumption of glucose and also glutamine, utilization of glycolysis as the predominant method of catabolism, excessive lactic acid generation, and other alterations to normal metabolic pathways are collectively considered another hallmark of cancer, common to a vast majority of malignancies [139]. While lactic acid has previously been considered

an inconvenient waste product associated with altered energy metabolism [228], there is growing evidence that increased lactic acid secretion plays an active role in shaping the tumor microenvironment to support cancer survival.

A common perspective on the purpose of excessive lactic acid production by cancers is that altered energy metabolism confers a proliferative advantage to them [228–230]. While it is puzzling that cancer cells opt for aerobic glycolysis that generates ATP at an 18-fold lower efficiency than oxidative phosphorylation, the predominant theory suggests that the advantage lies in the incomplete utilization of glucose. This allows upstream intermediates to be redirected for biosynthesis and gives cancer cells abundant building blocks for the synthesis of cellular components [230]. Another explanation that has been offered is that aerobic glycolysis arose as an adaptation to hypoxic conditions commonly experienced in tumors, which often encounter fluctuating oxygen levels, periodically alternating between normoxic and hypoxic conditions [231,232]. A dependence on glycolysis regardless of oxygen levels could therefore confer a proliferative advantage to cancer cells, making them less susceptible to hypoxic stress during episodes of spontaneous hypoxia, even if such protection comes at the cost of reduced energy production during times of adequate oxygenation [228]. However, as the field is rapidly advancing, recent studies have indicated that alterations to cancer cell metabolism may actually be a result of oncogenic transformation. Genetic alterations to various classic cancer-associated pathways (such as Myc, PI3K, AMPK, and p53) have been shown to alter metabolic patterns in favor of glycolysis [233–236]. Furthermore, in addition to altering glucose metabolism, increased glutamine utilization in excess of nitrogen requirements has also been observed. This has been shown in particular to facilitate fatty acid biosynthesis through use of TCA cycle intermediates and also results in excessive lactate production [237,238]. Such alterations to glutamine metabolism could also modulate apoptosis, particularly in light of its importance in glutathione synthesis and maintenance of cellular redox states [239,240].

In addition to proliferation and survival, many other advantages affecting multiple aspects fundamental to cancer biology have been ascribed to increased production of lactic acid and subsequent acidification of the tumor microenvironment. One such aspect is the ability of cancer cells to invade local tissue and ultimately metastasize. It has been found that cancer-adjacent normal tissue is also subjected to increased acidity, making it more susceptible to cancer invasion [241]. The regions of highest tumor invasion corresponded to areas of lowest pH; tumor invasion did not occur in regions with normal or near-normal extracellular pH. Also, cancer cells in the invasive edge of tumors were found to upregulate proteins in acid-generating pathways such as the glucose transporter GLUT-1 and the sodium–hydrogen exchanger NHE-1 [242]. Furthermore, lactate transporters are confined to the leading edge of lamellipodia and play a role in human cancer cell invasiveness [243,244]. Additionally, studies have shown that an acidic extracellular environment can induce expression and secretion of various proteases commonly found in the tumor microenvironment associated with ECM degradation [245–247]. As such, an acidic tumor microenvironment is an important contributing factor to tissue invasion and metastasis of cancer cells via a combination of toxicity to adjacent normal cells, degradation of the ECM through secreted and activated proteases, and increased cancer cell motility.

The excessive lactic acid generation by cancer cells also has a profound effect on angiogenesis. It has recently been shown that extracellular lactate can stimulate endothelial cell growth through signaling mechanisms involving receptor tyrosine kinases [248]. This lends further evidence to claims that lactic acid is a driving force behind angiogenesis [249]. It has been shown that endothelial cells use lactate transporters to import lactic acid from the environment, causing an activation of HIF-1α and stimulation of the NFκB/IL-8 pathway for tumor angiogenesis [250,251]. Furthermore, lactic acid is also able to induce VEGF production in macrophages and endothelial cells to stimulate endothelial cell migration [252].

The effectiveness of certain chemotherapeutic agents is known to be altered in the acidic tumor microenvironment. In particular, the acidic gradient can negatively affect the efficacy of common weak base chemotherapies [253,254]. The semi-permeable nature of the cell membrane allows small, uncharged molecules to readily diffuse into the cell and excludes charged ionic species. For weakly basic therapeutics such as mitoxantrone, doxorubicin, and topotecan, the acidic extracellular environment in the tumor results in an accumulation of the charged species in the extracellular space and greatly decreases their efficacy [255,256]. Furthermore, the acidic microenvironment can also enhance the activity of known drug transporters such as p-glycoproteins and confer an additional mechanism of drug resistance through increased efflux [257,258]. However, the acidic tumor environment can also be exploited for therapeutic purposes. The uncharged form of weak acids is favored in an acidic environment and permeability through the cell membrane is improved. As such, an increased efficacy is observed for compounds such as chlorambucil and melphalan [259,260], and nanoparticle drug delivery systems engineered to release their contents in an acidic environment also have the potential for enhanced specificity for tumor tissue [261].

Finally, one largely ignored effect of the acidic tumor microenvironment is local suppression of the anti-cancer immune response. The immune system has long been known to be highly dependent on the surrounding environment for function, whether through the presence of cytokines or various helper cells. There is increasing evidence in the literature to suggest that alterations to the extracellular pH, particularly an increase in acidity, can greatly hamper normal immune cell functions [198,262–264]. It has been shown that under acidic conditions, tumor-infiltrating T lymphocytes became anergic and were unable to eliminate cancer cells both in vitro and in vivo. This inhibition, however, was found to be reversible as cytotoxic capacities were restored when the extracellular pH was reverted to physiological levels [198,265]. Furthermore, the presence of lactic acid also altered the differentiation and antigen presentation abilities of dendritic cells [266] and skewed the differentiation pattern of tumor-associated macrophages towards the suppressive M2 phenotype [264]. Considering the important role that the immune system plays in cancer development and progression as mentioned above, we recently summarized the evidence suggesting that the extracellular pH plays an equally important role in modulating immune function and proposed that cancer-generated lactic acid could be viewed as an immunosuppressive regulatory metabolite rather than a simple waste product [267].

Taken together, it is clear that an acidic tumor microenvironment has profound influences on multiple cancer characteristics including cell proliferation, tissue invasion/metastasis, angiogenesis, treatment resistance, and evasion of immune destruction. As such, it is crucial to take the effects of an acidic extracellular environment into account regardless of the aspect of cancer biology studied or the model system used.

3.3.1. Acidic culture conditions

While in vivo systems can approximate the acidic tumor microenvironment commonly seen in patients, most in vitro cell culture systems completely ignore this aspect of cancer biology. Cancer cells are routinely grown in a culture medium buffered to a physiological pH of 7.4. While such optimal pH conditions may be most beneficial to the health and proliferation of the cells, they do not reflect the environment of cancer cells in patients' tumors, which can be as acidic as pH <6.5. As mentioned above, the acidic tumor microenvironment induced by excessive lactic acid generation plays a critical role in multiple fundamental aspects of cancer biology. The use of a non-acidic condition in vitro could in part account for the discrepancies often observed between results obtained in vitro and in vivo.

The issue of the acidic tumor microenvironment becomes even more critical when we consider evidence from the literature documenting enormous changes to cancer cells when they are cultured under acidic

conditions (Fig. 2). In addition to boosting tissue invasiveness, migratory potential [245,268] and altering drug sensitivity [269,270], an acidic culture condition can have profound effects on other aspects of cancer. Changes to metabolic patterns have been observed when cancer cells are cultured in acidic conditions, with a switch from reliance on aerobic glycolysis and production of lactic acid under a physiological pH to a reversion back to oxidative phosphorylation in an acidic environment [271]. Furthermore, culturing cancer cells in an acidic environment have also been demonstrated to increase autophagic flux [272] and reduce cell sensitivity to radiation therapy, potentially through prolonging the G2/M cell cycle arrest and allowing more time for repairing radiation-induced damage [273,274]. The lowering of the extracellular pH also results in a decreased intracellular pH in cancer cells, resulting in global histone deacetylation and corresponding global alterations to gene expression patterns [275]. The vast changes observed by simply altering the extracellular pH are not limited to cancer cells. As previously mentioned, an increased extracellular acidity as a result of altered cancer metabolism can have profound effects on the angiogenic potentials of endothelial cells [250,251] as well as the anti-cancer function of tumor-infiltrating immune cells [198,267].

Use of co-cultures of cancer cells and cancer-associated cell types of interest in an optimal molecular environment, adjusted to be minimally disruptive to cell growth and proliferation, allows the introduction of many artificial changes. In view of the important role that an acidic tumor microenvironment plays in regulating multiple crucial aspects of cancer biology, lowering the pH of the culture medium to values observed in in vivo tumors could be a simple yet critical adjustment to making in vitro studies more reflective of real tumor conditions.

4. Conclusions and caveats

While in vitro cell culture models allow a more detailed and convenient analysis of cancer-related properties and processes, it is becoming increasingly evident that the use of simplified cell growth conditions may unwittingly lead to experimental observations that do not accurately reflect tumor physiology. There is abundant evidence in the literature suggesting that alterations to in vitro culture conditions can have drastic consequences for cancer cell biology and may lead to misinterpretations of the results. Given that PDX models often demonstrate the highly heterogeneous nature of patients' tumors, the use of the xenograft sublines that best meet the desired molecular and phenotypic characteristics for experimentation is of critical importance.

However, similar to any other cancer modeling system, PDXs have their inherent limitations and deficiencies. As mentioned previously, the lack of a fully functional human immune system presents a major challenge. To circumvent this limitation, more sophisticated PDX models (humanized models) should be developed via co-grafting of tumor tissue along with bone marrow stem cells of the same patient similar to approaches in developing humanized immune systems in mice [276,277]. This combination may reconstitute the human immune system and allow investigation into the role of the immune system in cancer metastasis as well as the efficacy of immune-based therapies.

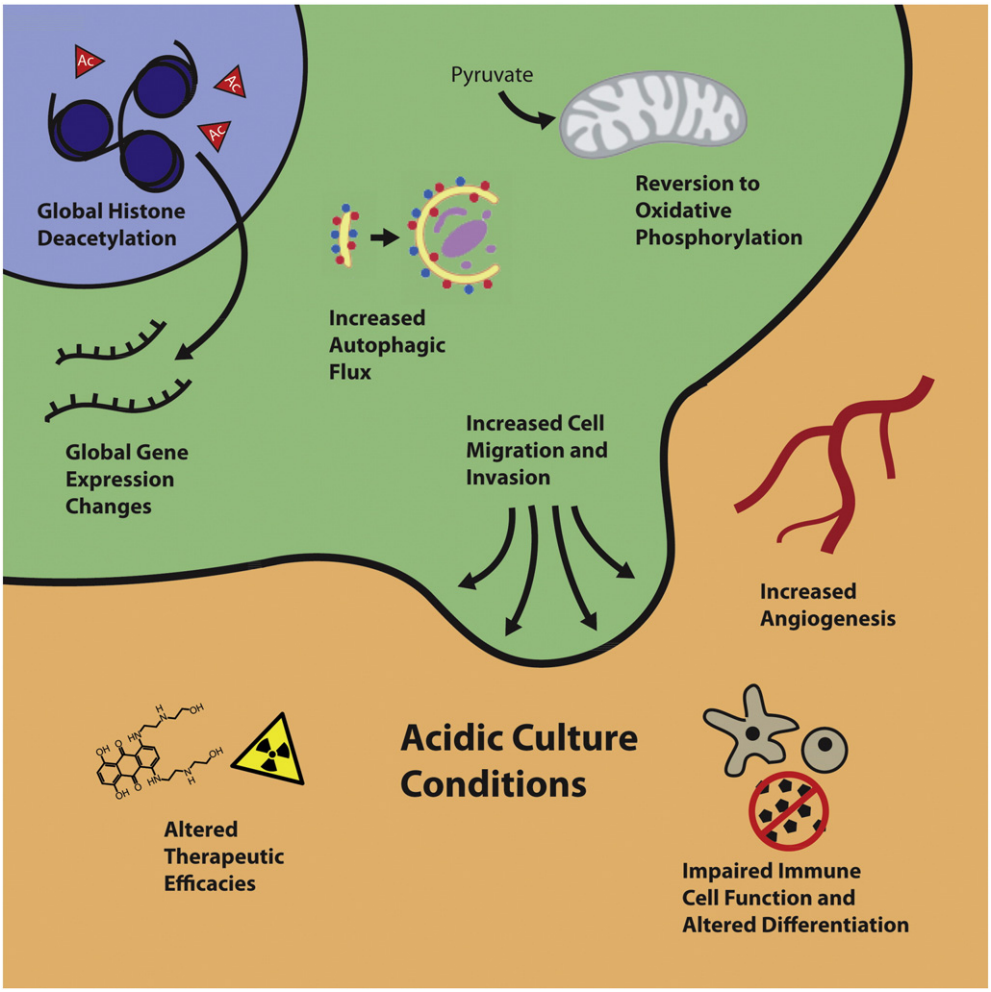

Fig. 2. Aspects of tumor biology affected by an acidic extracellular environment as mimicked by an acidic culture condition. Profound changes are observed in both cancer cells and tumor-associated stromal cells, including altered energy metabolism, increased tissue invasion, increased angiogenesis, altered therapeutic efficacies, and impaired immune cell functions. Such an acidic culture condition more closely reflects the tumor microenvironment and should be considered when engineering in vitro conditions that are more physiologically relevant.

Second, although PDX models are relatively stable, increasing histopathological and molecular differences between patient tumors and xenografts are foreseeable following continual passaging. It is therefore prudent to establish cryopreservation of PDXs at an early generation, ensuring the preservation of the cellular and molecular characteristics of the original tumor and an unlimited supply of a particular patient's tumor. Last but not least, the high cost and amount of human resources associated with their use, compared to traditional cell line-based systems, form another major factor hampering the widespread use of PDX models. However, these downsides are counteracted by the increased clinical relevance of the PDX cancer models, compared to other existing models, as it is a most critical requirement for cancer models in drug development.

We know that while many aspects of using in vivo animal cancer models are beneficial, there are also many inaccuracies associated with current approaches. Investigators have been very successful at "curing" mice from numerous cancers, but unfortunately these "cures" cannot always be repeated in humans during clinical trials. Clearly, a better pre-clinical balance between improved in vitro mimicry and selecting animal models that are more closely related to the clinical scenario is needed. As discussed above, the crucial aspects of the tumor microenvironment include tumor architecture, various cell–cell interactions, and an acidic microenvironment. By mimicking these fundamental components through 3D cultures, co-culture systems, and acidic culture conditions, in vitro models of tumor physiology may be improved to render results with increased clinical relevance.

Competing interests

The authors declare that they have no conflicting interests.

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