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Functionalizing the Tumor Microenvironment with Microfluidics for Anti-cancer Drug Development

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Abstract

Cancer is the leading cause of death worldwide. The complex and disorganized tumor microenvironment makes it very difficult to treat this disease. The most common *in vitro* drug screening method now is based on 2D culture which poorly represents actual tumors. Therefore, many 3D tumor models which are more physiologically relevant, have been developed to conduct *in vitro* drug screening and alleviate this situation. Among all these methods, microfluidic tumor model has the unique advantage of recapitulating tumor microenvironment in a comparatively easier and representative fashion. While there are many review papers available on the related topic of microfluidic tumor model, in this review we aim to focus more on the possibility of generating "clinically actionable information" from these microfluidic systems, besides scientific insight. Our topics cover the tumor microenvironment, conventional 2D and 3D cultures, animal model, and microfluidic tumor model, emphasizing their link to anti-cancer drug discovery and personalized medicine. We hope it can guide new researchers into this area to find their direction as well as showcase recent progress to readers of interest.

KEYWORDS Tumor-On-Chip, Cancer, Drug Discovery, Personalized Medicine, Tumor Model, Microfluidics, Three Dimensional Models

1. Introduction

Cancer is the second leading cause of death in the world, in both developed countries and less developed countries [1]. According to data published in GLOBOCAN by the IARC, there were an estimated 14.1 million new cases and 8.2 million deaths worldwide in 2012, which made cancer the second leading cause of death globally [2]. One of the primary reasons for the high mortality rate of cancer is due to cancer metastasis; the process where tumor cells acquire the ability to intravasate into the nearby blood or lymphatic system, travel through the circulatory system and extravasate to other parts of the patient [3]. Studies have estimated that 90% of deaths for patients with solid tumors can be attributed to metastasis [4, 5]. Over the years, research has demonstrated that the tumor microenvironment plays a key role in this process [3, 6, 7]. Specifically, the tumor microenvironment has also been shown to induce tumor heterogeneity and promote the growth of a tumor itself [8, 9].

To understand the underlying mechanisms through which the tumor microenvironment impacts cancer progression and prognosis, researchers have been trying to create a model that can more accurately represent the *in vivo* tumor microenvironment. There is much value in doing so because the tumor heterogeneity confers unique advantages for the tumor, such as an inherent resistance to therapy [10, 11]. Since different sub-populations of a tumor can respond differently to therapy, this makes it difficult for clinicians to treat cancer effectively. This heterogeneity for cancer can be further broken down into two sub-categories, intra-tumor and inter-tumor. Both categories of heterogeneity refer to different subpopulations of existing cancer cells. Intra-tumor refers to cancer cells within a tumor while inter-tumor heterogeneity refers to cancer cells between different tumors, tumors of different tissue types or between patients who are afflicted with the same cancer type. Such heterogeneity is observed in the cohort of circulating tumor cells (CTCs) [12, 13], which can be isolated from the peripheral bloodstream or cultured for further analyses. The exact mechanism through which tumor heterogeneity can be attributed to, cancer stem cells or clonal evolution, is still under debate. However, this heterogeneity will manifest both chemically and biologically which will then give rise to genetic and phenotypic variations seen in tumors.

Most current reviews either focus on specific fields such as cell migration and 3D cell culture or highlight the biological applications of *in vitro* tumor models [14-16], with limited emphasis on their contribution to anti-cancer drug screening and development. This review aims to provide readers with a detailed discussion on the current state-of-art microfluidics-based tumor models, with a special focus on their application in anti-cancer drug screening and the engineering approaches to recapitulate the tumor microenvironment (**Figure 1**). First, we provide a broad overview of the tumor microenvironment in terms of its physical, chemical and biological composition, to enable readers to better understand the underlying design principles behind tumor-on-a-chip models. Next, we look at the non-microfluidic tumor models which have been developed. Lastly, we discuss the tremendous potential of microfluidic tumor models and its ability to replicate the tumor microenvironment in a physiologically relevant manner which is comparatively easier to implement. Through the development of an *in vitro* model which can accurately represent the *in vivo* conditions of the tumor microenvironment, this would help not only researchers develop better drugs but could also pave the way for clinicians to personalize cancer therapy for individuals.

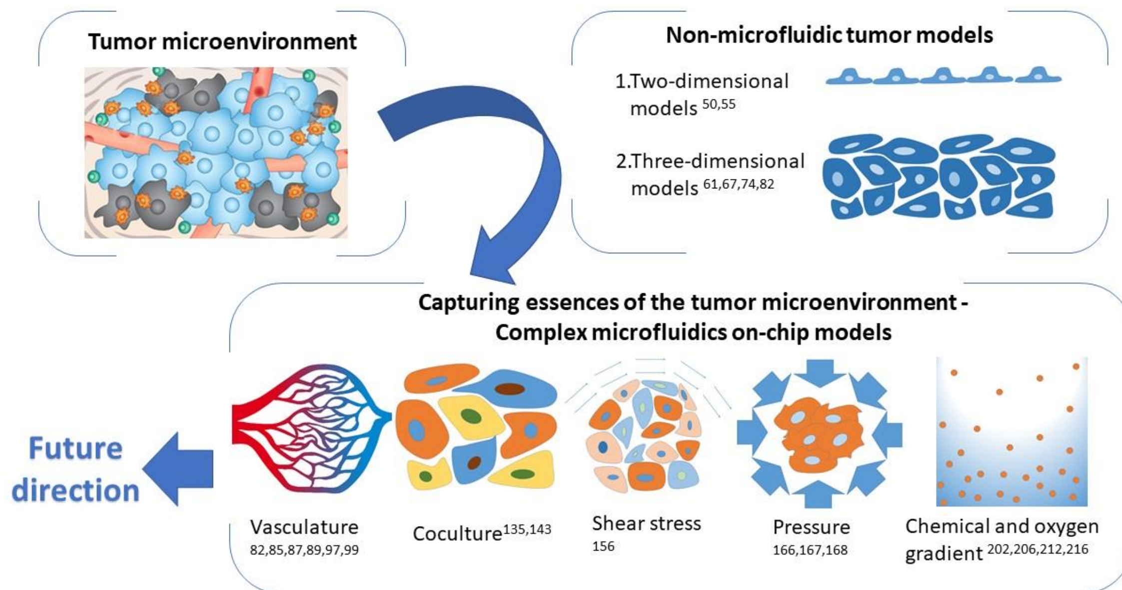

Figure 1: Overview of this review. Schematics showing how microfluidics can play a critical role in capturing the essence of the tumor niche: from replicating complex vasculature architecture, co-culturing of various cells to mimicking cell-cell interactions more accurately. Others include *in vitro* models to mimic the shear stress, pressure or biophysical and biochemical gradients which the tumor is exposed to.

2. Tumor microenvironment

On a macroscopic level, the solid tumor microenvironment is very similar to that of an organ, where it comprises specialized cells being sustained by blood vessels, with each performing a different role in a tumor [17]. For instance, apart from the malignant cells, the tumor microenvironment also includes several different immune cell types, cells from blood vessels (pericytes and adipocytes) and fibroblasts, each interacting and exhibiting dependencies on each other. As a result, solid tumors are inherently heterogeneous and structurally complex [18].

The major component of any cellular microenvironment is the extracellular matrix (ECM) (**Figure 2**). Beyond providing structural support, the ECM also plays an important role in the architecture. The ECM is known to influence cell behavior in terms of cell migration, and anchorage, by serving as a medium to transmit external forces from environmental cues and even cell signaling throughout the entire lifespan of the cell and at critical phases in their lives [19, 20]. The ECM is composed of a large collection of biochemically distinct components including proteins, glycoproteins, proteoglycans, and polysaccharides [21, 22]. When these components are placed in a specific arrangement, they grant the cells which adhere to it via the focal adhesion points various biochemical, biomechanical and physical properties. As the ECM plays a part in almost all cellular behavior and is indispensable to all cells, it is always tightly regulated.

However, in the ECM of a tumor niche, changes in the amount, composition or topography of the ECM can lead to changes in the properties of the ECM itself. For instance, various collagens, including collagen I, II, III, V, and IX, have increased deposition rates during tumor formation [23-25]. The overexpression of these collagens leads to the formation of a stiffer ECM which

in turn promotes the formation of a tumorigenic microenvironment [26]. By doing so, cell growth is enhanced by stiffening and cell-cell junction integrity is compromised, impeding lumen formation. Over time, non-polarized, disorganized and invasive colonies that lack cell-cell junction proteins with irregular cell shapes are formed, which is one of the hallmarks of cancer [3].

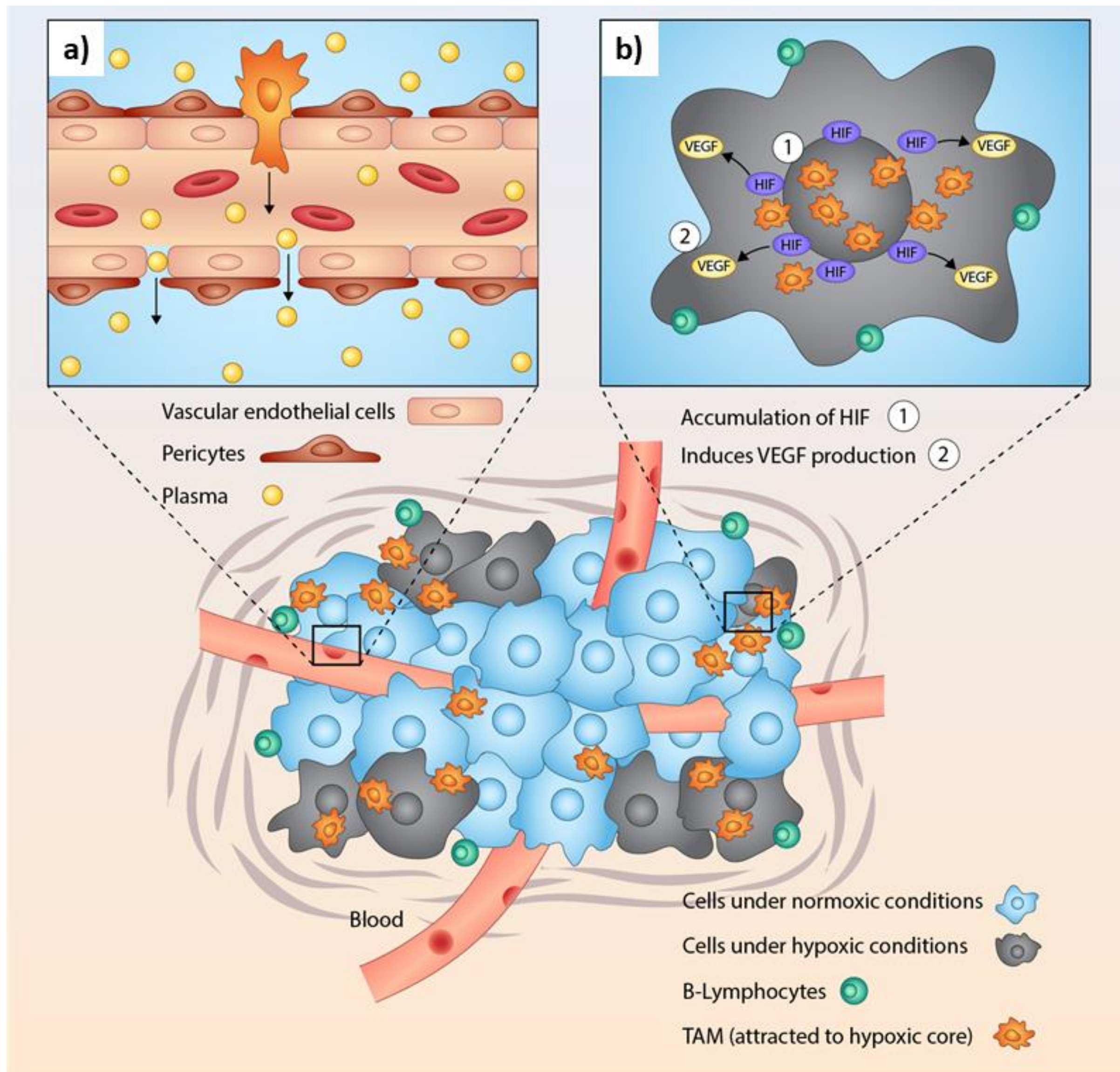

Figure 2: Overview of the tumor microenvironment. Cells in the tumor microenvironment comprising different subpopulations of cancer cells, lymphocytes, and macrophages. This makes it a highly complex environment with each element exhibiting some form of dependency on the other, of which only a few are illustrated. (a) Diagram of the blood vessel in a tumor with gaps indicating leakiness in the blood vessel. This, coupled with the fact that a tumor is typically larger in size than healthy tissues, leads to the formation of hypoxic regions. (b) Hypoxia then results in a cascade of events which is initiated when the secretion or breakdown of various growth factors is disrupted [25-29].

Apart from physical changes, chemical changes within the niche will also occur. A prominent example is represented by the hypoxia-inducible factor-1 (HIF-1), a type of transcription factor, which response to a decrease in available oxygen in the cell. HIF-1 is always present in healthy cells and degrades rapidly under normoxia by the von-Hippel-Lindau (VHL) tumor suppressor protein in normal tissues [27]. However, due to the enlarged size of a tumor when the cells start multiplying uncontrollably, some cells especially those near the periphery of a tumor do not have access to a blood vessel. As a result, these cells enter a state of hypoxia and HIF-1 rapidly accumulates as VHL is unable to break it down. This increased expression of HIF-1 has also been demonstrated to drive angiogenesis by promoting the formation of vessels in the tumor microenvironment through the upregulation of the vascular endothelial growth factor (VEGF) protein [28]. While the hypoxic conditions in a tumor may be thought to be alleviated with the formation of new blood vessels, this is not the case. In fact, the hypoxic conditions in a tumor are further exacerbated as the newly formed vessels are leakier and more dilated (**Figure 2a**). This further reduces the ability of the vascular system to deliver blood into a tumor [29]. As VEGF is also a form of chemoattractant, the hypoxic regions in a tumor will also be associated with a substantial increase in the number tumor-associated macrophages (TAMs) of up to 50% of the cell tumor mass (**Figure 2b**) [30, 31]. It is important to note here that the upregulation of VEGF is only one of the pathways through which angiogenesis is induced. Alternatively, through the expression of oncogenes such as such *Ras* or *Myc* inside tumor cells, angiogenesis can also be induced in a tumor. Moreover, VEGF is expressed in varying quantities within a tumor. As the expression of VEGF promotes neo-vascularization in tumors, this results in a network of blood vessels within a tumor which cannot be replicated or predicted easily, thereby making the tumor microenvironment highly complex.

Apart from changes in a tumor which are driven by external cues, biological changes can also be induced by changes in the DNA. For instance, a missense mutation in the p53 transcription factor, which can be held accountable for tumor initiation, development, and growth in more than half of solid tumor cancers [32]. Briefly, the p53 protein acts as a tumor suppressor as it induces apoptosis when cellular mutations occur and initiate DNA repairs in healthy cells [32]. However, when mutations occur in the p53 protein, in addition to the loss of tumor-suppressing functions of the cell, many of these mutations also cause active promotion of tumor development indirectly. This is because the mutation changes the DNA binding ability of the protein. Consequently, this affects the ability of the protein to interact with other proteins or transcription factors [33]. When the effects of all these aberrations in a tumor are added together, the resultant is a highly heterogeneous tumor. This can be explained by how the regional differences in a tumor arising from chemical and biological aspects of the niche will result in changes in the physical aspect of the niche. For instance, leaky blood vessels developed in a tumor will give rise to different sub-populations of cancer cells within a tumor as different hypoxic and pressure differences will be created in the niche (**Figure 2**). Due to this difference, the cells in a tumor respond differently to these cues and in the process, creates tumor heterogeneity. Hence, it is imperative that researchers create a suitable tumor model. A brief introduction to these models will be given in the following section.

3. Non-microfluidic tumor models

Over the past two decades, many models have been created to mimic this microenvironment and various techniques involving small animal models or two-dimensional (*2D*) *in vitro* cancer models have been developed for drug testing and screening [34, 35]. Broadly, these methods can be grouped into three main classes: tumor tissue explant, animal-based models, and cell

line-based tumor models. A short introduction to the first two methods will be included in this section, together with a discussion of the key advantages and disadvantages.

Tumor tissue explants are the most direct and rudimentary form of culturing tumors. In this procedure, the tumor is first removed from the patient after a solid biopsy is performed. The extracted tumor is then processed by removing the necrotic parts before it is then cut into small pieces and washed with cell media. The tumor is then seeded into collagen gel which has been pre-embedded on a culture dish and then cultured. As explants utilise similar techniques and equipment as those used in conventional cell culture work, this makes it easy for researchers to adopt tumor tissue explants in their research [36, 37]. Moreover, since the tumor explant is derived from the original patient sample, the tumor architecture is largely preserved, making this a representative model. Conversely, the reproducibility of a tumor also becomes an issue since a tumor is highly heterogeneous and varies not only between patients but also between different tumors or even within the same tumor inside an individual patient [38]. This implies that once the tumor explant has been used in an experiment, it may almost be impossible to obtain another explant with the same features to test the repeatability of the results.

There are also animal-based models which have been developed. Animal-based models typically involve mouse models. In this, two sub-types of mice are typically used: transgenic mice carrying cloned oncogenes or knockout mice lacking tumor suppressor genes. Even though a tumor is essentially being cultivated in a surrogate human model, this method has demonstrated some success over the years such as the discovery of APC gene suppression which initiates tumor formation in an animal mouse model of human familial adenomatous polyposis [39]. As a tumor will be cultivated *in vivo* within these animals, it is also able to better mimic the molecular and histopathological features of cancer [40, 41]. Indeed, this has made mouse models highly popular with researchers (65% of 949 oncology papers published in 2016 utilized a mouse tumor model [42]). However, as mice can tolerate higher drug concentrations than human patients, questions about their effectiveness in using them for drug tests will remain even if a representative *3D* model is to be created. As such, to further improve on animal-based models, other animals such as dogs and cats have been increasingly used in the search for new cancer treatments. These animals are selected because they possess genes which are more like humans than mouse genes. Studies conducted in recent years have demonstrated that the same tumor oncogenes and suppressor genes contribute to the development of cancer in humans and dogs [43]. Major molecular targets such as PTEN, BRAC1 or p53 have demonstrated sequencing identities that are more similar to humans as compared to those from a mouse, thereby indicating that a more accurate model can potentially be established [34]. However, for all the benefits that an animal-based model confers, they still have underlying ethical issues and have very strict regulations regarding the use of them which restricts their use to only very specific purposes such as in a drug test. With increasing recognition of this problem such as the report set out by the National Research Council (US) [44], the conventional position that animals can be used as they are not sentient is now weakened. Moreover, it is important to note that successful translation from animal models to clinical cancer trials only stand at 8% [45]. This discrepancy may be attributed to a variety of reasons ranging from the tangible, such as the lack of a standardized protocol for animal testing unlike in human trials [42], to the intangible such as how stress experienced in mice has been demonstrated to have a substantial impact on the experimental results [46]. It is for these reasons that researchers seek to build tumor models using methods which have a higher throughput and less batch-to-batch variation than those mentioned above.

3.1 Two-dimensional models

In *2D* models, the cells are typically cultured and grown as a monolayer on flat surfaces such as in cell culture flasks or wells. Here, the cell culture can be performed either as a monoculture or as co-culture with different cell types. While monocultures are well-established methods of culturing cells in an *ex vivo* environment and are optimal for obtaining cells, which are of the same phenotype, monocultures are typically not used in tumor models because they are too simple. Given that a tumor comprises various cell types, the resulting cell-cell communication and interaction cannot be replicated in a culture consisting of only a single cell type. Furthermore, these interactions cannot be ignored as they could lead to a cascade of whole other events. For instance, cancer metastasis which is the leading cause of death in cancer patients occurs through a series of mechanisms (most prominently the epithelial to mesenchymal transition [EMT]). EMT is initiated when the cells lose adhesion and polarization while acquiring migratory properties as part of its interaction with other cells in a tumor [47]. As a result, monocultures are inherently limited in what they can achieve in terms of creating a suitable environment for researchers to study the tumor.

Techniques which involve co-culturing of cells have been developed in order to overcome this limitation. Co-cultures, as the name suggests, comprises two or more monocultures of cells in a single culture dish or well. Fundamentally, co-cultures are very similar to monocultures in terms of how they are both still *2D* cultures which can be cultured with similar experimental techniques. This provides researchers with a quick, simple and reliable way to understand the interactions between cells. For example, by studying the genetic alterations in the tumor cells, the events through which the stroma affects cancer cells can be elucidated [48]. Co-cultures are typically achieved via two methods: direct or indirect. In a direct co-culture, the cells are layered on top of one another and are in physical contact with each other. However, in an indirect co-culture, the cells are cultured using well-inserts [49]. In the former, due to the physical contact between cells, this allows for the study of adhesion molecules such as N-cadherins [50]. In the latter, signaling pathways between the different types of cells such as cancer cells and fibroblasts can be better studied [51]. In general, there is a need to ensure that the cells of the co-culture require similar media and that extra care must be taken during the cell culture, such as the need to seed one cell type at a time in order to improve on reliability. However, the main advantage is that the *2D* co-culture model offers a quick and simple method to mimic specific aspects of the tumor microenvironment.

Although *2D* monolayers of cells can be successfully cultivated on cell culture plates and serve as a method for researchers to study various aspects of the tumor environment in detail, *2D* monolayers are still found to be lacking. For instance, *2D* models cannot be used in the testing of drug efficacy as previous studies have shown that the efficacy still varies quite substantially between *in vivo* as compared to *2D* monoculture [52, 53]. This may be attributed to how *2D* layers are still spatially irrelevant given that actual tumors are *3D* in nature. Specifically, the location of focal adhesions and the various cell surface proteins such as integrin and their binding has been demonstrated to be markedly different from their original *3D* form [54, 55]. Furthermore, 2D cultured cells are artificially polarized when their cytoskeleton rearranges as they are stretched on the culture plate, which in turn causes these cultured cells to express genes and proteins which are atypical [56]. Hence, there is a need for 3D models in order to obtain more information such as drug penetration or to aid in understanding the tumor niche better. Given the different benefits and drawbacks of the two models (2D vs. 3D), one should first understand what questions need to be addressed in order to pick the most appropriate model.

3.2 Three-dimensional models

Conventional *in vitro* culture methods could also generate three-dimensional tumor structure which is more physiologically relevant than *2D* models and more cost-efficient than animal models [57]. These methods can be divided into four categories: suspension culture, non-adherent surface methods, hanging drop methods, and scaffold-based culture.

Suspension culture prompts aggregate formation by agitating fluid and preventing cells from adhering to container surface [58]. There are mainly two types of approaches to keep cells suspending in the culture medium. One is to use a stirrer, like a spinner flask bioreactors [59-61]. The other approach is to rotate the container wall. Representative devices are rotary cell culture system [62, 63]. Suspension culture generally has high throughput but lack the ability to control the size and uniformity of the spheroids formed [64, 65].

Instead of agitating fluid, the non-adherent surface method prevents cell adherence to the container surface and promotes spheroid formation directly through the use of antifouling surface coatings [66]. Many different material coatings, such as agarose, poly-HEMA and pluronic acid coatings have been proved to work well in forming viable cellular spheroids [67-70]. Although the size of spheroids generated using this method varies in a large range, this problem can be overcome by using microarrays instead [66].

Hanging drop methods force suspended cells to aggregate and then develop into regular and tight spheroids at the bottom of small hanging droplets (typically $20\mu l$) [71]. The hanging drops are generated at the underside of a flat surface as a result of surface tension and gravity [66]. This technique has been widely used in cancer research and many improvements have been developed in the past few years [72-74]. It has high reliability and reproducibility but fluid volume is limited and the method is not good for long-term culture due to difficulty in refreshing the culture medium [58, 75].

In scaffold based cultures, the tumor cell suspension is usually mixed together with a hydrogel or a solid scaffold to develop a three-dimensional structure [63, 76]. Many different materials such as chitosan-alginate, collagen, Matrigel and Puramatrix hydrogel, were capable of forming such a scaffold structure [77-81]. An arguable advantage of this approach is that the scaffold could mimic ECM and better recapitulate the *in vivo* microenvironment. However, scaffold base culture also has drawbacks of batch variance, limited throughput, non-uniform spheroid size and distribution [63].

4. Capturing essences of the tumor microenvironment - Complex microfluidics on-chip models

Conventional *in vitro* tumor models have revealed much basic information about malignant cells. However, these models cannot predict tumor cell response accurately due to lack of a physiologically relevant microenvironment. On the other hand, although animal models have been used to recapitulate the human body environment, it is a time-consuming and expensive approach. In addition, the deviation in animal models caused by non-human genetic material seems impossible to be overcome. In contrast, a microfluidic tumor-on-a-chip can mimic the human body environment without the presence of foreign environmental factors. It also has the advantage of being cost-effective while maintaining a high throughput. Although it can be very challenging to fully regenerate the *in vivo* microenvironment in a microfluidic system, researchers have been able to capture some key features in the tumor to recapitulate the process of tumor growth and use it to better predict drug response.

Table 1 Summary of microfluidic devices mimicking tumor vasculature

Approaches	Media refreshing method	2D culture	3D culture
Endothelial cells monolayer	Perfusion	[82]: Primary BAOEC [83]: HDMECs, MDA-MB-231	[81]: T47D, PC9, HMVEC [84]: HUVEC, A549, T24 [85]: HUVEC, MDA-MB-231, MCF-7
Circular endothelial cells tube	Perfusion		[86]: HUVEC, HCCLM9 [87]: MDA-MB-23), TIME [88]: MDA-MB-231, HT-1080, HUVEC, HMVEC [89]:HUVECs, rat aortic SMCs, 3T3 [90]: P53LMACO1, HH [91]: HMVEC-d, HMVEC-dLy
Vascular network	Direct exchange		[92]: HUVEC, NHLFs, HL-60, U87MG [93]: HUVEC, NHLFs [94]: HUVEC, dermal fibroblast, human lung fibroblast, hPC-PL [95]: iPSC-EC [96]: fibroblast, HUVEC [97]: HUVEC
	Perfusion		[98]: primary hBM-MSCs, osteo-differentiated (OD) primary hBM-MSCs, HUVECs, MDA-MB-231 [99]: ECFC-ECs, NHLFs [100]: HUVECs [101]： HBTAEC, MCF-7, MDA-MB-231 [102]: human iPSCs (WTC11, C2A), HUAEC, HUVEC, HDLEC, NHLF

4.1 Vasculature

Vasculature plays an essential role in the growth and metastasis of malignant tumors [103]. Malignant cells exchange nutrient and waste through vascular networks. Without recruiting new blood vessels, the size of a tumor is limited to a few millimeters in diameter [104]. The blood circulation system is also a key component in metastasis because many malignant cells migrate as circulating cancer cells through blood vessels [105]. Therefore, many attempts have been made to mimic blood vessels in microfluidics devices in recent years (**Table 1**), ranging from a planer layer of endothelial cells [81-85] to circular functional and perfusable vascular network consisting of various cell types [92, 98, 101].

Endothelial cells monolayer is the most direct approach to mimic blood vessel function in a microfluidic chip. This monolayer approach is very effective in applications where cylindrical vessel geometry of blood vessels is not very essential to the study, such as in the testing of drugs which prevent cancer cell migration because this method is high throughput and simple to fabricate [106]. As compared to the traditional transwell method, the monolayer design also

has the added advantage of easily generating shear stress. Shear stress helps cells arrange themselves to establish the barrier function [106]. As shown in **Figure 3a**, there are generally two different methods to construct these endothelial cells monolayer inside a microfluidic chip. One is to culture endothelial cells on a porous membrane [81-83]. As the endothelial monolayer becomes confluent, poles on the porous layer will be covered by cells. When fresh medium flows on one side of the membrane, it can then diffuse through the cellular monolayer to the other side of the membrane, mimicking *in vivo* blood perfusion. For example, a device was designed to incorporate a monolayer of human dermal blood microvascular endothelial cells (HMVEC) on a porous PDMS layer to mimic blood vessel in their drug screening platform [81]. It was shown that human non-small cell lung cancer cells (PC9) cultured in their devices had stronger drug resistance to four different apoptotic inducers than two-dimensional cultured PC9 cells. Instead of using PDMS monolayer for cell attachment, the other approach employs the use of hydrogels [84, 85]. In that approach, the hydrogel not only works as a scaffold for the growth of endothelial cells monolayer but also serves a function of mimicking the ECM for tumor spheroid formation.

More complex devices incorporating the tubular structure of blood vessels generally fall into two categories depending on whether the endothelial cells grow on a circular scaffold or in a cylindrical hydrogel channel (**Figure 3b**). In the former, endothelial cells or multiple types of cells are grown on a vessel-like scaffold and implanted. The resultant artificial blood vessel is then implanted into the microfluidic device. Although fabrication of the device is quite tedious, the artificial blood vessels generated by this method have relatively high mechanical strength and look like real blood vessels [86]. In one such example, an artificial blood vessel was constructed by growing human umbilical vein endothelial cells (HUVECs) in a tubular scaffold made of cellulose and collagen. By implanting three such vessels in one chip and culturing HCCLM9 cancer cells in between, it was shown that the endothelial monolayer and pulse flow could significantly delay tumor migration [86]. The other class of devices incorporating circular endothelial cell tube involves growing cells on the inner surface of a cylindrical hydrogel channel [87-91]. This method can effectively build a circular endothelial cell tube in a microfluidic device, but the co-cultured cancer cells must be fixed in the hydrogel first before construction of the endothelial cellular tube can begin. One example is a study of human breast cancer cell intravasation and extravasation in an artificial microvessel platform [88]. Human breast carcinoma cells MDA-MB-231 adenocarcinoma and HT-1080 fibrosarcoma cells were first cultured in a collagen hydrogel. A cylindrical channel was then drilled in the hydrogel using a metal rod. HUVECs were injected into the channel and gradually grown into a circular endothelial cell layer. In this study, the migration speed of cancer cells in the ECM was shown to be slower than that in ECM/vessel interface and predefined matrix tracks.

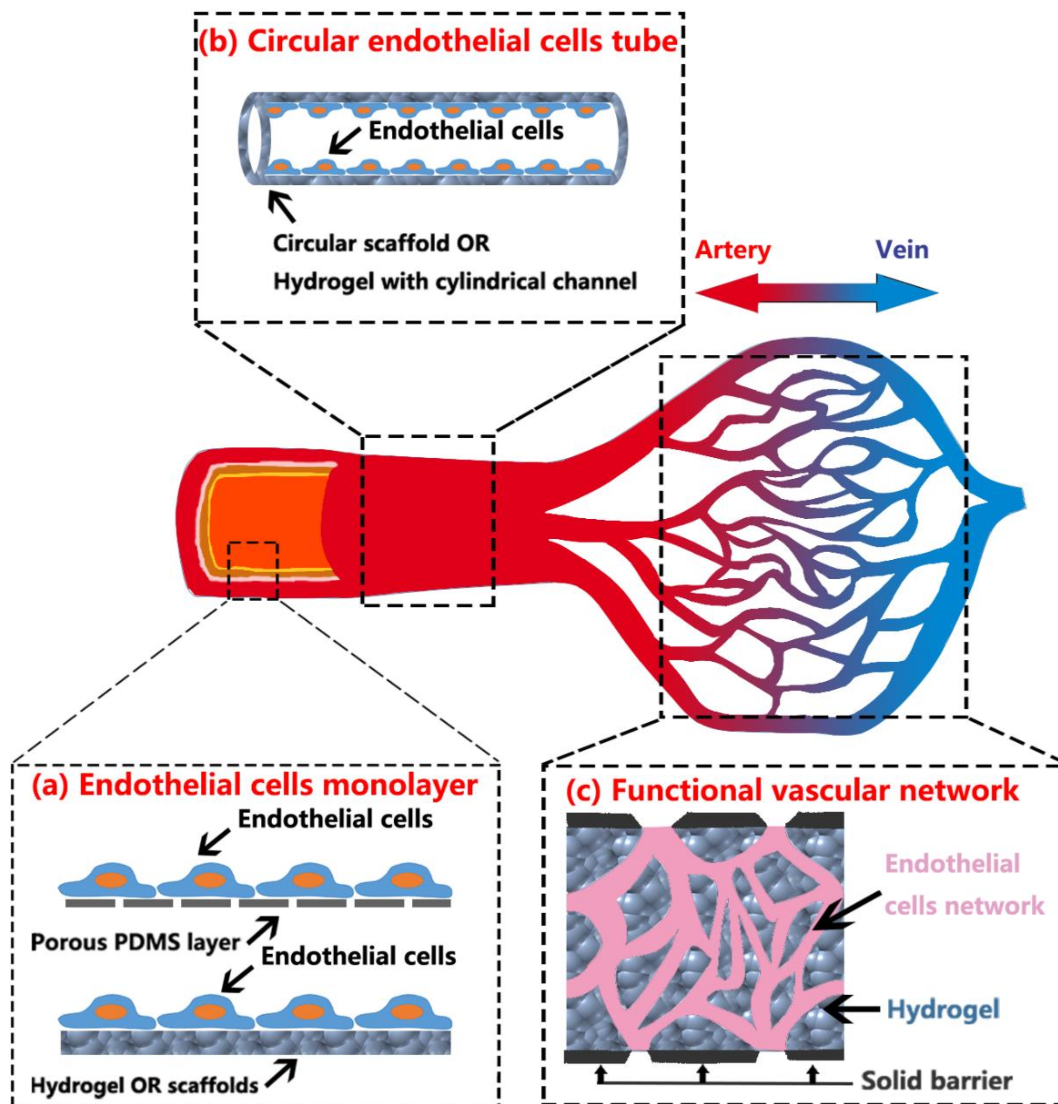

Figure 3 Microfluidic device mimicking blood vessels using (a) endothelial cells monolayer, (b) circular endothelial cells tube and (c) functional vascular network

Different from the previous two types of devices where the artificial blood vessel is formed on a well-defined surface, some devices prompt endothelial cells to sprout in the hydrogel to develop an irregular vascular network to mimic *in vivo* capillary vessel networks (**Figure 3c**). The blood vessels developed in this type of devices are cross-linked and perfusable, closely recapitulating the *in vivo* blood flow conditions [94-97, 107]. This is necessary for studies which require the crosslinking structure of the vascular network or when angiogenesis inhibitors are involved [92, 93, 97-102]. For example, fibroblasts and HUVEVs were used to generate a vascular network in fibrinogen gel in a microfluidic chip [96]. By using this device, it was found that high-density lipoproteins exhibit a bi-phasic effect on angiogenic sprout growth and inhibit TNF-α stimulated angiogenesis. In another example, human breast cancer cell MDA-MB-231 suspension was flowed into the vascular network to model the extravasation process of cancer cells [98]. By blocking A3 adenosine receptors, cancer cells were observed to have a reduced extravasation rate. Through this, it was shown that adenosine played an important role in promoting tumor cells extravasation.

The level of complexity increases as one progress from the use of endothelial cells monolayer to circular endothelial cells tube and to the functional vascular network. The more complicated devices are, the closer a representative of *in vivo* conditions can be achieved albeit with a lower throughput and becoming harder to operate. In recent years, techniques which can overcome the abnormal vasculature in the tumor, such as nanoparticle drug delivery and drugs normalizing vascular network, were shown to be able to greatly improve anti-cancer treatment [108-113]. For example, simultaneous Tie2 activation and Ang2 inhibition in mice was shown to normalize vasculature. This halved the lung tumor growth rate and extended survival time by more than 20% [109]. As such, the development of a more comprehensive microfluidic tumor model with better-mimicked vasculature will allow *in vitro* fast screening of such treatment and greatly contribute to the development of anti-cancer drugs.

4.2 Coculture

Many different non-neoplastic cells exist in the tumor microenvironment and interact with tumor cells. Endothelial cells, fibroblasts, and immune cells are the three main cell types which appear in most cancers and are vital components in enhancing the drug resistance of tumors [103, 114-120]. Endothelial cells are one of the major components of blood vessels and play an important role in tumor angiogenesis [121]. Its interplay with malignant cells is also key to cancer metastasis [122]. Different from normal fibroblasts, cancer-associated fibroblasts proliferate faster, produce more extracellular matrix and secrete unique cytokine, which significantly enhances carcinogenesis [123, 124]. Immune cells were shown to both enhance and inhibit malignant cells proliferation, migration, and metastasis [125-128]. Therefore, mimicking cell-cell interactions is very necessary for the design of a comprehensive *in vitro* tumor model. Current microfluidic devices capable of culturing multiple cell types can be divided into two categories, mixed co-culture system and separate co-culture system [129, 130].

Mixed co-culture microfluidic devices mix different cell types and culture them inside one single chamber [131-138] (**Figure 4a**). Although this method can better recapitulate heterogeneous cell growth environment in the tumor, it can be quite challenging to distinguish different cell types in monitoring cellular behavior in such devices, especially when one is observing the activities of more than two cell types. A typical method to identify certain cell type in such a system is to use a different fluorescent label. However, due to the concern of cross-talking between fluorescent signals, no more than 4 fluorescent dyes are usually used simultaneously on a single device. This technique was demonstrated in a recent publication where lysed human blood was cultured in a microwell device to conduct drug screening [135]. CD45-allophycocyanin was used to distinguish WBCs from putative CTCs and viability of putative CTCs was assessed by using calcein-AM/EtBr. Formation of cell clusters in the microwells was shown to be inversely correlated with increased drug concentration and therapeutic treatment and this liquid biopsy technique can potentially be used to develop personalized medicine.

Instead of simultaneously culturing various cell types in the same chamber, a separate co-culture system grows each cell type in a separate chamber and all the cell-cell interaction is achieved by substance diffusion through the culture media between individual culture chambers [131, 139-148] (**Figure 4b**). For example, cancer-associated fibroblasts (CAFs) and NSCLC cells were co-cultured in separate chambers connecting by a fluid channel and cell behavior was observed under the microscope [143]. By using this microfluidic device, it was shown that CAFs might promote NSCLC cell invasion by up-regulation of GRP78 expression. As compared to a mixed co-culture, a separate co-culture can easily distinguish the different cell types cultured in the device without fluorescent staining. However, the main drawback is

that it is not able to mimic certain cell-cell interactions which cannot be transmitted through culture media such as the ECM interaction generated by fibroblasts and the immune response from WBCs.

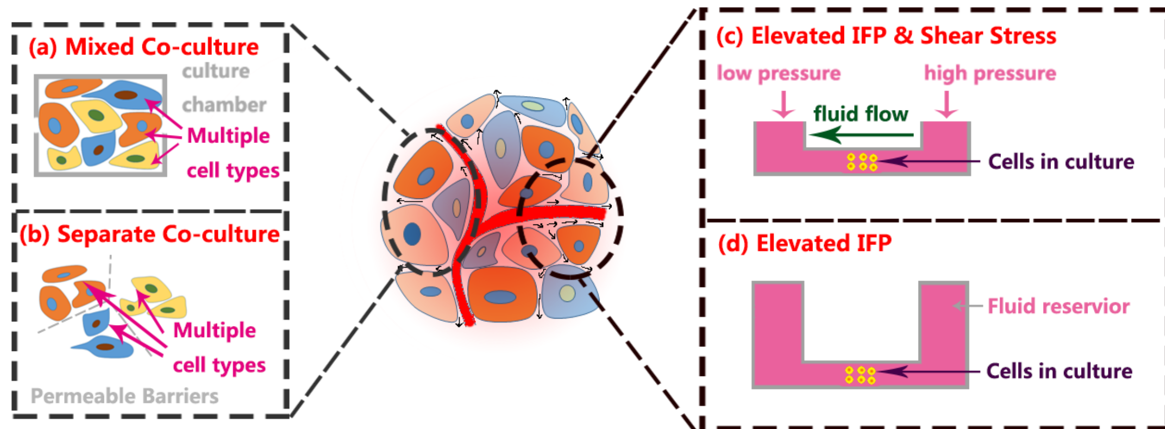

Figure 4 Microfluidic devices mimicking heterogeneous cell types and elevated IFP in tumor microenvironment using the approach of (a) mixed co-culture, (b) separate co-culture, (c) elevated IFP & shear stress and (d) elevated IFP.

4.3 Shear Stress

Due to the presence of interstitial flow in tumors, there is a corresponding fluid shear stress with a magnitude of approximately 0.1 dyn cm^{-2} [149]. Although this shear stress is very small when compared with the intravascular shear stress, it can still stimulate some oncogenic signaling pathways on cancer cells [150] as well as upregulate TGFβ and activate fibroblast contraction to stiffen the ECM [151]. In addition, this shear stress can also prompt vascular angiogenesis against the direction of interstitial flow [152]. Therefore, many *in vitro* researches have been conducted to mimic and investigate the influence of shear stress on tumor cells.
All current microfluidic studies generate the fluid shear stress by applying a continuous flow of culture medium in direct contact with target cells (Table 2). This flow is usually generated by a peristaltic pump [153], a syringe pump [154] or a gravitational force arising from the height difference between fluids at the inlet and outlet of the device [155]. Associated shear stress value is then estimated using numerical simulation software to make sure it is physiologically relevant. A significant difference of target cell behavior could be observed in the presence of shear stress. For example, a microfluidic device employing a straight channel design with a syringe pump generated medium flow studied the response of ovarian cancer cell spheroids to shear stress [156]. It was found that fluid shear stress could prompt ovarian cancer cell dissemination, enrich cancer stem cell population as well as enhance tumor's chemoresistance to anti-cancer drugs.
Almost all current microfluidic devices incorporating fluid flow can generate a shear stress with physiologically relevant value and can demonstrate the impact of shear stress in cultured tumor cells *in vitro* [157-160]. In current microfluidic models culturing three-dimensional tumors, fluid only flows around tumor spheroids and the quantified shear stress value is the shear stress on the outer surface of the tumor. This is, however, still different from the actual situation in the human body where shear stresses also exist inside the tumor [161].

Table 2 Summary of microfluidic co-culture systems and chips with mimicked shear stress or IFP

Mimic approach	Media exchange	2D culture	3D culture
Mixed co-culture	Direct exchange	[137]: Mouse fibroblast, natural killer cells	[162]: HepG2, 3T3, 4T1 [132]: MCF-7, HUVEC, NHF, RG2, H35 [135, 163]: MCF-7, Blood samples from breast cancer patients [134]: H1650, A549, cancer-associated fibroblasts, blood samples from early lung cancer patients
	Perfusion		[131]: HepG2, 4T1 [133]: SPCA-1, HFL1, cells from the fresh lung cancer tissues [136]: HMT-3522, HMF, breast DCIS [157]: ECFC-ECs, NHLFs [138]: Human pulmonary microvascular endothelial cells, NCI-H441
Separate co-culture	Direct exchange	[142]: HS5, HuH7 [145]: SKBR3, BT474, ThP1, HS5, MCF7 [147]: PBMC, MDA-MB-231 [164]: primary mouse spleen cells, B16-F10	[162]: HepG2, 3T3, 4T1 [139]: HT-29, BJ [140]: mouse smooth muscle precursor cells, hMVECs, MTLn3, MDA-MB-231 [141]: HCT116, primary rat liver microtissues [143]: A549, SPCA-1, WI38 [144]: MDA-MB-231, RAW 264
	Perfusion	[146]: T24, Raw 264.7, BJ-5Ta, HUVECs	[131]: HepG2, 4T1 [157]: ECFC-ECs, NHLFs
Shear stress	Perfusion	[158]: LS174T, Colo205, THP-1, HL-60 [153]: HCT116 [159]: JH-EsoAd1 [160]: HEK 293T, PANC-1, A549, HT-29 [165]: patient-derived xenograft with lung, melanoma and bladder cancers [155]: 22Rv1, PC3, TOV112D, OV90, tumor tissue from patient with benign prostatic hyperplasia	[154]: MDA-MB-231 [157]: ECFC-ECs, NHLFs [156]: SKOV-3
IFP	Direct exchange	[166]: A549, primary murine AEC	[167]: MDA-MB-231, PC-3

	Perfusion		[168]: MDA-MB-231 [169]: MDA-MB-231

4.4 Pressure

Elevated extracellular stresses have been observed in many tumors [170-178]. It enhances tumor cell proliferation and builds up a transport barrier to drug delivery, greatly lowering the efficacy of anticancer drugs and therapies. Extracellular stresses have two components, namely solid stress (SS) and interstitial fluid pressure (IFP). Abnormal SS is developed by the unregulated growth of tumorigenic cells and is transmitted by the extracellular matrix [179]. In addition to increasing drug resistance, a high SS could influence tumor cells' gene expression, comprise immune cells ability to kill cancerous cells or even turn macrophages to tumor-friendly cells, thereby enhancing the metastatic potential of cancer cells [180-184]. On the other hand, the IFP is a fluid related pressure, which includes the interstitial-fluid osmotic pressure and interstitial-fluid hydrodynamic pressure. It is usually close to zero in normal tissue. However, due to the impairment of blood and lymph vessels in a tumor, the IFP could rise up to more than 30mmHg at the center and drop drastically to the normal value at the periphery of a tumor[185]. The uniformly elevated IFP in tumor center is responsible for inhibiting drug delivery [172, 178, 186] while the steep pressure gradients at the periphery favors tumor growth and metastasis [154, 187-190].

A few *in vitro* models have been used to show the effect of SS on malignant cells proliferation, growth, and migration [180, 191, 192]. However, no microfluidic studies investigating the effect of SS on tumor cells have been reported until now. There are some microfluidic devices involving SS but stretching is usually the focus rather than compression. For example, the alveolar morphological and viability differences were studied using a microfluidic system in response to a combination of fluid and solid mechanical stress [166]. In this study, human alveolar epithelial cells A549 and primary murine alveolar epithelial cells were cultured on a deformable membrane. The solid mechanical stresses were then generated by stretching and release of the membrane with alveoli. The authors were able to conclude that stretching could prompt cell detachment from culture surface from that study.

As compared to SS, the impact of interstitial pressure on tumors is better studied using microfluidics. Most research is focused on how IFP prompts cancer cells migration and metastasis [157, 167-169]. Although many current microfluidic devices have conducted research on the effect of IFP on tumor cells, they inadvertently mixed up an elevated IFP with fluid shear stress when a pressure-driven fluid flow is used (**Figure 4c**). For example, a microfluidic platform utilized external media reservoirs to investigate the IFP gradient's influence on collagen-embedded human breast cancer cell line, MDA-MB-231 [168]. The pressure gradient was found out to induce cell adhesion activation, cell polarization along flow direction and migration towards regions with higher pressures. Instead of using external fluid reservoirs to generate pressure, another study utilized a microfluidic device which integrated a gasket to generate fluid pressure. The device was then used to study the molecular mechanism behind pressure induced tumor cell invasion in microfluidic tumor models [167].

All the present approaches that mimic IFP have an associated shear stress due to the presence of significant fluid flow. As such, it is hard to identify the effect of IFP on tumor alone. Therefore, mimicking static fluid pressure is key to understanding the role of IFP in drug resistance of tumor and whether it is necessary to be recapitulated in an in vitro drug screening platform (**Figure 4d**). Hence, more research needs to be done on tumor-on-a-chip devices.

4.5 Chemical and oxygen gradient

Many recent microfluidic devices were utilized to generate chemical or oxygen gradient in cancer research because chemical and oxygen concentrations have also been shown to greatly affect cancer progression and therapeutic efficacy [193, 194]. Chemical gradients play an important role in tumor cell invasion of metastatic cancer [195, 196] as it significantly directs the migration and differentiation of tumor cells. Hypoxic environment, on the other hand, is known to reduce cytotoxicity of many anticancer drugs, affect angiogenesis, promote metastasis and alter the metabolic activity of cancer cells [183, 197].

Microfluidic devices usually generate chemical gradient either by diffusion or convection [193]. Diffusion-based devices produce a continuous concentration gradient by allowing controlled diffusion of chemicals through a fluid or gel chamber [198-201]. By observing cell behavior in these devices, it was found that different types of cancer cells may react differently to the same chemical stimuli. This dynamic response was regulated by the β-catenin dependent Wnt signaling pathway [202]. Most convection-based devices are gradient generators in which several specific chemical concentrations are generated by mixing the different amount of fluid. [203-205] These are widely used in many drug screening systems to prepare drug gradients [135, 206]. There have also been some interesting microfluidic devices which adopt alternative approaches to generate these chemical gradients. For example, a thread network was shown to allow cell growth with different chemical concentrations by absorbing solutions of high and low chemical concentrations [207]. Electric fields can also be used to modulate the chemical gradient in a microfluidic device and this feature may be easily incorporated into existing devices [208].

There is usually a hypoxic core of solid tumors due to the abnormal vascular network and high cell density. A hypoxic environment can be easily incorporated into most existing systems by using a hypoxic incubator or a hypoxic workstation [194]. However, they are not able to generate oxygen gradient with spatial controls. Spatial control of oxygen concentration, which allows for the study of cancer cells respond to different oxygen levels, is important for it can reproduce oxygen distribution seen in actual tumors. To address this, many microfluidic systems with oxygen controls were developed [209]. Most of them control the oxygen levels in culture chambers and can be categorized into three groups; introducing oxygen scavenging chemicals [210, 211], gas supply channels adjacent to the cell chamber [212] and oxygen impermeable materials [213, 214]. Different from these engineering approaches, some recent works adopted *3D* tissue structures to reconstitute the oxygen gradient in the tumor [215, 216]. They rebuilt largely *in vitro 3D* tumor tissues and were able to generate a hypoxic environment in the inner core, which greatly recapitulated *in vivo* spatial oxygen distribution. In a very interesting study, cell layers were wrapped like a movie strip to generate a 200um-thick 3D tissue which allowed spatial analysis of metabolites at different depths [216]. They demonstrated the change in 88 metabolites during cellular adaptation to hypoxia gradient and found that cell metabolism was deregulated in the absence of HIF.

Current microfluidic devices are capable of recapitulating *in vivo* chemical and oxygen gradient independently. However, more studies should be done to simultaneously incorporate both features to better mimic actual tumor microenvironment and predict drug response [210, 216].

5. Future directions

As discussed, current microfluidic tumor models have achieved much in showing the significance of incorporating the vascular system, co-culture with physiologically relevant cell types and mimicking elevated interstitial fluid pressures as well as shear stresses. However, the

artificial microenvironment in a microfluidic chip is still a long way from the human body in terms of blood vessel architecture, cell lines used in co-culture models and functionality of mimicked elements. Fully recapitulated tumor microenvironment *in vitro* is also impossible with current technologies. Therefore, the key to developing a successful microfluidic tumor model is to select and incorporate essential features which can facilitate better drug response prediction depending on the application.

Current microfluidic tumor models with mimicked vasculature have demonstrated that tumor cells exhibit increased drug resistance when artificial blood vessels were used [81]. Human endothelial cells can sprout in hydrogels to develop a vascular network with dimensions similar to real capillaries [96, 107]. Although many *in vitro* microvessel models have been developed, none of them can actually reproduce the architecture of capillaries seen in a tumor [217, 218]. While large artificial blood vessels generated by planar or circular channels can reproduce a physiologically relevant multilayer structure, which comprises of mainly endothelial cells, vascular smooth muscle, and connective tissue, these vessels are often too big to be implanted in a vascularized tumor. On the other hand, although the artificial vascular network has similar dimensions to normal capillaries and allows coculture of multiple cell types, building a vascularized artificial tumor remains a big challenge.

By using co-culture models, it was found that cancer-associated fibroblasts may promote cancer cell invasion [143]. Commercially available cell lines are commonly used in co-culture models. However, the relevance of using immortalized cells for biological studies is questionable with issues of mutation and contamination [219, 220]. Some microfluidic works have already demonstrated the feasibility of conducting patients' cell culture in microfluidic devices [133-135, 163]. More of such studies should be carried out to facilitate the development of better cancer therapeutic strategies and personalized medicine.

Some elements among the *in vivo* microenvironments such as IFP and shear stress have been mimicked in microfluidic devices, but their full functionality has not been reproduced yet. For example, current approaches with an elevated pressure chamber do not have a blood vessel component and the magnitude of pressure is less than 10% of actual IPF *in vivo* [166-169]. Another example is the culture of patient-derived tumor tissue [155, 165]. Due to the direct use of tumor tissue, this approach includes many key components in tumor such as heterogeneous cell types and abnormal blood vessel networks. However, nutrients and drugs are not delivered through the vascular network in these studies, which compromise the prediction of drug response in these models. In addition, the various types of cells in tumor tissues were all treated as tumor cells in these studies. This can be misleading in predicting tumor response *in vitro.* For example, the efficacy of some anticancer drugs can be overestimated when these drugs are able to kill non-tumor cells in the tumor tissue because the drug efficacy is assessed based on the general cell viability of the whole tumor.

6. Conclusion

Cell sources used in most *in vitro* tumor models described are cell lines derived from human patients. This is due to the difficulties in culturing primary tumor cells harvested from liquid biopsy or solid tumors and the heterogeneity in utilizing clinical samples. Besides, the current gold standard to validate proposed models still involves comparing the result with animal tests. This is primarily because animal tests are much more easily approved than clinical testing on human subjects. It is inevitable that the future generation of *in vitro* tumor models will need to use primary human cells with integrated *into vivo* conditions in order to generate predictions which are physiologically more relevant.

Nevertheless, rapid progress in different aspects of tissue engineering will greatly assist the development of *in vitro* tumor models that can recapitulate various physiologically relevant *in vivo* conditions. Multiple properties of human tissue will be incorporated into more comprehensive and sophisticated systems. The advances in microfluidic tumor models will continue to help researchers achieve a better understanding of cancer biology as well as develop novel therapeutic strategies and enabling personalized treatment.

7. Glossary

CTC: circulating tumor cell
ECM: extracellular matrix
IFP: interstitial fluid pressure
SS: solid stress
WBC: white blood cells

22Rv1: prostate cancer tumors
3T3: mouse fibroblast
4T1: breast cancer cells
A549: lung adenocarcinoma cell line
AEC: alveolar epithelial cell
B16-F10: melanoma cell line
BAOEC: bovine aortic endothelial cells
BJ: human foreskin fibroblasts
BJ-5Ta: fibroblasts
BT474: breast cancer epithelial cell lines
Colo205: human colorectal adenocarcinoma cell line
DCIS: ductal carcinoma *in situ*
ECFC-ECs: human endothelial colony forming cell-derived endothelial cells
H1650: non-small cell lung cancer cell line
H35: rat hepatoma cells
HBM-MSCs: human bone marrow-derived mesenchymal stem cells
HBTAEC: primary human breast tumor-associated endothelial cell
HCCLM9: hepatocellular carcinoma cell line
HCT116: human colorectal carcinoma cell line
HDLEC: human dermal lymphatic endothelial cells
HDMEC: human dermal microvascular cells
HEK 293T: human embryonic kidney epithelial cell line
HepG2: human hepatocytes
HFL1: human lung fibroblast cell line
HH: bovine vascular endothelial cell
HL-60: human promyelocytic leukemia cells
HMF: human mammary fibroblasts
HMT-3522: human mammary epithelial cell line
HMVEC: human dermal blood microvascular endothelial cells
HMVEC-d: human dermal microvascular endothelial cells
HMVEC-dLy: human lymphatic microvascular endothelial cells
hPC-PL: human placental pericytes
HS5: human bone marrow stromal cell line
HT-1080: fibrosarcoma cells
HT-29: human colorectal adenocarcinoma cells

HuH7: human hepatocarcinoma cell line
HUAEC: human umbilical arterial endothelial cells
HUVEC: human umbilical vein endothelial cells
JH-EsoAd1: esophageal adenocarcinoma cell line
LS174T: human colorectal adenocarcinoma cell line
MCF-7: human mammary epithelial cell line
MDA-MB-231: human breast adenocarcinoma cell line
MTLn3: mouse breast cancer cells
NCI-H441: Human alveolar epithelial cells from lung tumor
NHF: normal human fibroblasts
NHLF: normal human lung fibroblast
iPSC: induced pluripotent stem cell
iPSC-EC: induced pluripotent stem cell-derived endothelial cell
OV90: ovarian cancer ascites
P53LMACO1: mouse vascular smooth muscle cell
PANC-1: human pancreatic adenocarcinoma cell line
PBMC: human peripheral blood mononuclear cell
PC-3 human prostate cancer cells
PC9: human non-small cell lung cancer cell line
Raw 264.7: macrophages
RG2: rat glioblastoma cells
SMC: smooth muscle cells
SKBR3: breast cancer epithelial cell lines
SKOV-3: human ovarian carcinoma cell line
SPCA-1: human non-small cell lung cancer cell line
SW264: mouse leukemic monocyte-macrophage
T24: human bladder cancer cells
T47D: human ductal breast epithelial tumor cell line
ThP1: human monocytes
TIME: telomerase-immortalized human microvascular endothelial cell line
TOV112D: ovarian cancer cells
U87MG: human glioblastoma multiforme cells
WI38: human lung fibroblast

Author contributions

MLS, RHS, and BLK are involved in the writing (original draft, review/editing) and visualization of the manuscript. CTL and JH are involved in the writing (review/editing) of the manuscript. MLS, CTL, BLK, and JH are involved in the conceptualization of the manuscript.

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