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Cancer Lett. 2016 September 28; 380(1): 319–329. doi:10.1016/j.canlet.2015.12.003.**Recapitulation of complex transport and action of drugs at tumor microenvironment using tumor-microenvironment-on-chip****Bumsoo Han^{1,2,3}, Chunjing Qu⁴, Kinam Park^{2,3}, Stephen F. Konieczny⁴, and Murray Korc⁵**¹ School of Mechanical Engineering, Purdue University, West Lafayette, IN, USA.² Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN, USA.³ Birck Nanotechnology Center, Purdue University, West Lafayette, IN, USA.⁴ Department of Biological Science, Purdue University, West Lafayette, IN, USA.⁵ Departments of Medicine, Biochemistry and Molecular Biology, Indiana University School of Medicine, and the Pancreatic Cancer Signature Center at Indiana University Simon Cancer Center, Indianapolis, IN 46202, USA.**Abstract**

Targeted delivery aims to selectively distribute drugs to targeted tumor tissue but not to healthy tissue. This can address many of clinical challenges by maximizing the efficacy but minimizing the toxicity of anti-cancer drugs. However, complex tumor microenvironment poses various barriers hindering the transport of drugs and drug delivery systems. New tumor models that allow for the systematic study of these complex environments are highly desired to provide reliable test beds to develop drug delivery systems for targeted delivery. Recently, research efforts have yielded new *in vitro* tumor models, the so called tumor-microenvironment-on-chip, that recapitulate certain characteristics of the tumor microenvironment. These new models show benefits over other conventional tumor models, and have the potential to accelerate drug discovery and enable precision medicines. However, further research is warranted to overcome their limitations and to properly interpret the data obtained from these models. In this article, key features of the *in vivo* tumor microenvironment that are relevant to drug transport processes for targeted delivery was discussed, and the current status and challenges for developing *in vitro* transport model systems was reviewed.

Keywords

Drug transport; Targeted delivery; Stromal Tissue; Microfluidics; Nanoparticles

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1. Introduction

Many promising anti-cancer drug candidates have been identified in the last several decades. However, only a handful have exhibited therapeutic efficacy on human patients. This is largely due to the limited delivery of drugs to target tumors, which can result in unwanted accumulation of compounds to non-targeted healthy tissues and organs, and ultimately to systemic toxicity. Targeted delivery, which aims to selectively distribute drugs to targeted tumor tissue but not to healthy tissue, can address many of these difficulties. Such targeted delivery, however, is very difficult to achieve [1]. Thus, the term “targeted” is used in this article refers to the preferential delivery of drugs to the tumor site. It should be distinguished from “targeted therapy” which refers to drugs interfering with specific molecular targets in cancers.

Recent developments in the field of nanotechnology enables the synthesis of a wide variety of nanoparticles (NPs), whose size and surface properties can be designed to serve as effective vehicles for targeted delivery. These nanostructures include liposomes, polymer micelles, dendrimers, drug nanocrystals, magnetic nanoparticles, gold nanoparticles/nanoshells, nanorods, nanotubes, and drug-polymer conjugates (all of which will be collectively referred to as NPs). Research aimed at controlling the size and surface properties of these NPs to be responsive to the tumor microenvironment has been performed as reported elsewhere [2-5]. Even though improvements in the delivery efficacy have been shown, the majority of administered NPs fail to reach target tumors. One of the biggest benefits of using NP formulations is to avoid non-aqueous solvents for administering hydrophobic drugs to patients, resulting in fewer side effects, while maintaining the same efficacy. The success of Abraxane® (nanoparticle albumin-bound paclitaxel) and Doxil® (PEGylated liposome formulation), in large part, relies on delivering anticancer drugs without using organic solvents. In order to maximize the therapeutic outcomes, however, drug accumulation as well as penetration into the targeted tumors should be improved. The challenge before us is to achieve effective delivery to the cancer cells since it is significantly hindered by various barriers engendered by the complex tumor microenvironment (TME).

After being administered into a patient's blood stream, the drugs (for brevity, the term “drug” is used to refer both drug and drug delivery system including NPs) are thought to be subjected to complex and multi-faceted transport processes prior to reaching the cancer cells as reviewed previously [6-10]. These include – i) blood flow-driven transport to the tumor vasculature, ii) transvascular transport (i.e., extravasation), iii) interstitial transport, and iv) cellular uptake and metabolism as illustrated in **Figure 1**. Excess drugs often occupy the interstitial space or are transported through the lymphatic vessels. These transport phenomena are governed by diffusion and convection processes, and the significance of each process is dependent on both drugs and the biophysical conditions of TME. The drug dependent properties are the size and surface properties, and the TME dependent ones include leakiness of the blood vessel wall, interstitial fluid pressure gradient, and the extracellular matrix (ECM) microstructure within the tumor interstitium. These processes and physiological conditions are highly dynamic, interconnected and vary spatiotemporally.

Besides these biophysical barriers, the TME also poses biochemical and biological complexities. Typically, tumor tissues consist of cancerous cells as well as stromal components that consist of various stromal cells including cancer-associated fibroblasts (CAF), diverse immune and inflammatory cell types and rich extracellular matrix components, such as type I collagen [11, 12]. In addition to the highly heterogeneous cancer cell populations, i.e. intra-tumoral heterogeneity, the complex stromal tissue acts as a repository for various growth factors and cytokines that can dramatically influence tumor growth and drug response, as well as creating a hypoxic environment. Thus, it is important to understand the TME to design and develop effective targeted drug delivery systems. New tumor models that allow for the systematic study of these complex environments are highly desired and will provide reliable test beds to characterize and optimize the design of drugs.

Several tumor models are available but they do not adequately address this challenge. Conventional static *in vitro* systems, including cell suspensions and cell monolayers, are not sufficient to study these complex *in vivo* transport processes because the model systems lack dynamic interactions among the cells, ECM, interstitial fluid and NPs. Animal models can provide a TME with all of these dynamic interactions, but such models are limited to systematically studying the effects of these dynamic interactions. Recently, research efforts have yielded new *in vitro* tumor models, the so called tumor-microenvironment-on-chip (T-MOC), that recapitulate certain characteristics of the TME. Although various configurations have been developed, T-MOCs are basically microfluidic platforms where cancer cells are cultured within the ECM under perfusion conditions. These new models show benefits over other conventional tumor models, and have the potential to accelerate drug discovery and patient-specific personalized treatment planning. However, the TME is extremely complex and there remain significant limitations to overcome. In this article, key features of the *in vivo* TME that are relevant to drug transport processes for targeted delivery are reviewed, and the current status and challenges for developing transport model systems are discussed.

2. Tumor microenvironment: A complex and chaotic bed for tumor growth

The tumor microenvironment is a complex and adverse environment for drug transport and action. It comprises a highly heterogeneous mixture of tumor and stromal cells embedded in an extracellular matrix that also includes cytokines, growth factors, inflammatory cells and macrophages. Together, the TME poses multi-faceted barriers including biological, chemical and physical hindrances to drug transport and actions. These barriers are highly dynamic and often interconnected. Their interactions and relative significance with respect to drug delivery and therapeutic efficacy vary drastically depending on the cancer type, stage and organs. The current difficulty in developing new anticancer drugs and drug delivery systems partly stems from the lack of a clear understanding of the delicate interplay of these barriers at the TME [13-15]. Thus, instead of providing a generic description on these hindrances, it is more relevant to collectively discuss the interplays that are associated with one type of cancer. Here, our discussion will be focused on pancreatic cancer and its associated TME unless mentioned otherwise.

Pancreatic ductal adenocarcinoma (PDAC) is a significant clinical challenge due to its poor prognosis and extremely low (7%) five-year survival rate [16]. Its extensive TME presents

many key features relevant to discussing the hindrances and resistance of drug transport and actions. One of the most notable characteristics of PDAC is its marked desmoplasia. The desmoplastic stroma of PDAC is composed of CAFs, various immune and inflammatory cell types and a dense extracellular matrix [11, 12], as illustrated in **Figure 1**. Moreover, PDAC is poorly vascularized and has extremely high interstitial fluid pressure (IFP) [17-19]. In this complex 3D TME, highly intricate and multifaceted interactions occur among pancreatic cancer cells (PCC), CAFs, tumor-associated macrophages (TAMs) and other immune cells. The biochemical and biophysical interactions occurring among the cells within the TME are poorly understood.

PDAC is a complex, heterogeneous and genetically unstable disease which is caused by prolonged accumulation of mutations in key oncogenes and tumor suppressor genes [13,47,113]. These include activation of the *Kras2* oncogene, inactivation of the tumor suppressor gene *Cdkn2A/Ink4A*, and finally, inactivation of the tumor suppressor genes *TP53* and *Dpc4/Smad4* [25,68]. Recent studies indicate that PCCs carry an average of 63 genetic alterations per cancer, which can be grouped into 12 core signaling pathways [20]. PDAC is thought to arise from precursor lesions such as pancreatic intraepithelial neoplasia (PanIN). These lesions develop into invasive carcinoma through a multistep carcinogenic process (illustrated in **Figure 2**).

The complex TME of PDAC poses multiple barriers that inhibit transport and action of drugs. First, the TME serves as a biophysical barrier that impedes effective transport of drugs to target cancer cells or associated stromal cells. After administration, drugs are subject to complex transport processes to reach the cancer cells, including blood flow-driven transport, drug-endothelium interactions, extravasation, interstitial transport and cellular uptake [6, 7]. Although the drugs are thought to preferentially extravasate more in tumors than in normal tissues via the so called enhanced permeation and retention (EPR) effect [24, 25], the hypovascularity of PDAC may limit the benefit of EPR effects [26, 27]. Nonetheless, a certain percentage of PDACs exhibit a strong angiogenic gene signature and areas of increased microvessel density [28], raising the possibility that drug delivery may be more efficient in this patient subgroup. However, even after the drugs preferentially extravasate into the PDAC TME, the drugs encounter a very dense stroma and significantly elevated IFP. The dense stroma is attributed to activated CAF, inflammatory immune cells and an excessive deposition of a complex ECM that includes dense collagen type I and III bundles, hyaluronic acid, fibronectin and desmin [17, 18, 29], as illustrated in **Figure 3**. The dense ECM microstructure and cell packing of stromal tissue significantly hinders interstitial transport in conjunction with elevated IFP [18, 27, 30-33]. These features are compounded by hypoxia and infiltration of growth promoting inflammatory cells at the TME.

Second, increasing evidence has shown that various TME components, including the ECM, soluble cytokines, growth factors, the MMP family of proteases, and immunosuppressive and pro-tumorigenic immune cells, contribute to extensive tumor promoting properties. Pancreatic stellate cells are activated into CAFs, which produce and deposit fibronectin and collagens, whereas inflammatory cells and macrophages produce chemokines and cytokines. Thus, the TME of PDAC is rich in growth factors, including fibroblast growth factors (FGFs), epidermal growth factor (EGF) receptor ligands, transforming growth factor- β

(TGF- β) isoforms, and connective tissue growth factor (CTGF) [19, 34, 35]. These chemical environments facilitate not only stroma production, but also enhance PCC proliferation, epithelial-mesenchymal-transition (EMT), metastatic potential, and importantly, therapeutic resistance through molecular interactions between cancer cells and CAFs. The combination of these complex biochemical environments with hypoxia and heterogeneous genetic mutations makes PDAC incredibly resistant to therapeutics.

The development of genetically engineered mouse models (GEMMs) of PDAC has greatly aided fundamental studies of the interactions between PCCs and stromal cells. For example, PDAC CAFs express vitamin D receptors, and activation of these receptors by the calcitriol analogue suppresses deleterious immunological cell infiltrates in mouse PDAC (mPDAC) [36]. Conversely, CAF depletion in mPDAC has been associated with altered immune gene expression and altered infiltrating immune cell populations, including decreased CD4⁺ effector T cells, increased CD4⁺Foxp3⁺ regulatory T cells (Treg), and decreased cytotoxic CD8⁺/Treg and CD3⁺/CD11b⁺ ratios [37]. *Ctla4* expression was also increased, meaning that treatment of CAF-depleted mice with a CTLA-4 blocking antibody attenuated PDAC progression, improved overall survival, induced tumor clearance in up to 25% of the pancreas, and reprogrammed the transcriptome to a pattern that resembled control (CAF-competent) tumors. Similarly, deletion of *Shh* in cancer cells to suppress mPDAC stroma led to more frequent PanIN and ADM lesions at a young age, an earlier appearance of loss of differentiation of mPDAC, increased metastasis, and more rapid death [37]. There was also enhanced cancer cell proliferation and angiogenesis, increased *Zeb1* and *Slug* expression consistent with EMT, and reduced CD45⁺ myeloid cells and F4/80⁺ monocytes infiltration. Similar results were observed when animals were treated with a Smoothed inhibitor. Genetic *Shh* deletion or pharmacological targeting of *Shh* signaling pathways attenuates stroma formation but leads to more aggressive mPDAC. Thus, depending on the strategy, stroma depletion can cause beneficial or deleterious effects in GEMMS of PDAC.

3. Transport barriers of TME and drug resistance

Pathophysiological characteristics of tumors present multiple levels of transport barriers to targeted delivery of drugs. These include leaky and chaotic vasculature of the tumor, increased IFP, less functional lymphatic vessels, dense ECM microstructure and high cell packing density [24, 25, 31-33], as illustrated in **Figure 4A**. These TME characteristics are highly dynamic, interconnected and vary spatiotemporally [13, 26], and the compounding effects of all of these physiological parameters on drug transport are not yet fully understood yet. In this section, the complex transport processes of how drugs reach to the targeted tumor will be discussed with relevant transport barriers posed by the TME.

Although it is not directly associated with TME, one of the most critical steps of drug transport is blood flow-driven transport. After being administered intravenously, the drugs first circulate in the blood stream consisting of complex cells and plasma proteins. During blood circulation, a significant portion of the drugs are taken up by the immune cells in the blood stream including monocytes, leukocytes, and dendritic cells; and in tissues by resident phagocytes, i.e., by the reticuloendothelial system (RES) of the spleen, liver, and lungs [38, 39]. This clearance significantly reduces the amount of the drugs available in the blood

stream to reach their intended target. Thus, the surface of many drug delivery systems, particularly NPs, is PEGylated in order to decrease the uptake by the RES and prolong the circulation of the NPs [8, 38, 40]. Besides the uptake by the immune system, the NPs also interact with other components within the blood [40, 41]. These often can result in hemolysis, which refers to red blood cell destruction and degradation of NP integrity. Ultimately, this results in premature release of the encapsulated drugs.

Once the drugs reach the vicinity of their target site after escaping being cleared from the circulation, they are transported across the endothelium of the tumor vasculature to enter the tumor interstitium (i.e., extravasate). The tumor microvasculature is characterized by a highly disorganized network of blood vessels whose endothelium exhibit enlarged intercellular gaps, known as fenestrations, that are heterogeneously distributed across the tumor vasculature [43]. The size of these intercellular gaps, so called vascular pores, for different tumors have been reported to be between 300 and 700 nm, and in rare occasions can be up to 2 μm , which are significantly larger than those found within the normal tissue (typically smaller than 20 nm) [44-46]. These values should be interpreted with caution though, since many of these were measured using xenograft models and very limited information is available for human. Further research is warranted to validate whether these values are applicable to various cancer types, stages and organs in human patients.

Transvascular transport is thought to be extravasation of drugs by convection driven by the pressure gradient across this porous endothelium and diffusion by the concentration gradient. Thus, the transvascular permeability (i.e., measure of transvascular transport at a given set of pressure and concentration gradient) depends on the size of both the drugs and vascular pore. For small macromolecules with hydrodynamic diameters that are much smaller than the pore size, transvascular permeability is observed to be quite insensitive to the pore size [44]. However, for NPs that are larger in size, e.g. 50-200 nm, the effect of the pore size on transvascular permeability becomes more profound. Transvascular permeability also depends on the developmental state of the cancer. Transvascular permeability was found to be 2-fold greater in primary sites of breast cancer tumors (in the mammary fat) when compared to that at a metastatic sites, while the metastatic site was associated with a greater level of vascularization [47]. Yuan et al. [46] experimentally investigated the effect of molecular size on transvascular transport, and provided a transport property database of various molecules. Monsky et al. [48] illustrated that transvascular transport of macromolecules could be enhanced using vascular endothelial growth factor. Netti et al. [49, 50] investigated transvascular transport enhancement by modulating tumor microvascular pressure using periodic or continuous injection of angiotensin II. Recently, a strategy to normalize the tumor vasculature to achieve improved drug delivery throughout the tumor tissue has been proposed [51, 52]. This approach aims to remodel the tumor vasculature to functional vasculature of the healthy tissues using antiangiogenic factors which bind to VEGF receptors of tumor-associated endothelial cells.

After extravasation, the drug transport through the tumor interstitial space against elevated tumor IFP and abnormal ECM structure [30, 33, 53]. IFP of a solid tumor stays at an elevated level and sharply decreases at the periphery of the tumor. Due to its importance during drug delivery, physiological changes by elevated tumor IFP have been studied by

many researchers [32, 50, 53]. The IFP of various tumor types varies from 4 to 50mmHg with an approximate average of 20mmHg, which is much higher than the IFP of normal tissues, approximately 2mmHg [32, 54]. Drastically higher IFPs of 75~130 mmHg are reported for PDACs [18]. This elevated IFP is thought to result from anomalous characteristics of tumor vascular structure including high vascular permeability and the lack of a well-developed lymphatic system. The elevated IFP adversely affects the transport of therapeutic agents at several different levels - i) less extravasation of the agents and ii) radially outward interstitial fluid movement at the periphery of tumor [8]. Consequently, the elevated IFP contributes to insufficient delivery of drugs to the interior of tumors. Moreover, higher collagen content and consequent dense organization of collagen fibrils results in lower diffusivity of drugs. Thus, transport of drugs is significantly limited in the tumor interstitial space [33, 55-57]. A wide variety of methods have been proposed and investigated to enhance the interstitial transport, but the main underlying strategies are either lowering tumor IFP [58-60], or modulating tumor ECM structure [33, 61]. However, due to the complex interaction involving various physiological parameters, the control or manipulation of the tumor IFP and ECM structure still warrants further research.

Once the drugs are transported through the tumor interstitial space, these should act on tumor cells, but their efficacy may also be limited due to complete or partial drug resistance [62-65]. Multidrug resistance (MDR) is thought to be caused by a group of membrane proteins that extrude cytotoxic molecules, thus maintaining the intracellular drug concentration below effective levels. These proteins belong to the ATP binding cassette (ABC) superfamily of membrane transporters [66], most of which use the energy of ATP hydrolysis for the efflux of drugs (i.e. active transport). This family includes the well-characterized P-glycoprotein (Pgp) encoded by the MDR-1 gene [67-72], the multidrug resistance protein (MRP) [73-77] and the mitoxantrone resistance protein (MXR), also known as the breast cancer resistance protein (BCRP) [78-80]. Numerous clinical data imply that MDR phenotypes in tumors are associated with the overexpression of these transporters. Since these transporters have wide recognition patterns of substrates, the overexpression of these proteins will result in multidrug resistance. In addition to the over-expression of these transporter proteins, cellular drug resistance also appears to be mediated by the binding of tumor cells to the ECM [81, 82].

The most extensively studied strategy for efficient drug delivery and efficacy is to inhibit drug efflux by modulating the activities of the MDR-associated proteins. This can be achieved by the co-application of MDR modulators with anti-cancer drugs. A wide variety of compounds have been identified as MDR modulators. For example, verapamil, cyclosporine and their derivatives have been investigated in preclinical studies and in some cases have resulted in increased intracellular drug concentration [83-85]. Besides these chemosensitizers, monoclonal antibodies have been studied as potential MDR modulators [86, 87]. In addition to the MDR-associated proteins, the membrane lipid has also been investigated as a target for manipulation, as reviewed elsewhere [88]. The alteration of membrane biophysical properties, including membrane fluidity and permeability, could increase or decrease cellular uptake of drugs [89-91]. Polymeric excipients [92] and transcriptional regulators [93] have also been studied. Although heat shock has been reported to induce MDR in some cancer cells [94, 95], an increase in cellular drug uptake

and cytotoxicity by ultrasound-induced hyperthermia was reported [96, 97]. Unfortunately, delivery of these modulators to the target tumor is as challenging as the drug delivery obstacles we face.

To achieve effective targeted drug delivery, various strategies have been proposed to exploit these pathophysiological characteristics of the TME. Currently, many drug delivery systems, primarily NP-based systems, are designed based on so-called “passive” and “active” targeting strategies, which rely on increased extravasation and ligand-receptor interactions, respectively [98]. The passive targeting is based on the fenestration and prolonged circulation by PEGylation. This is often called the EPR effects since it is caused by the increased vascular permeability of tumor vasculature [24, 25]. However, it has not been shown whether such EPR effect exists in human tumor. The term “active targeting” is used to describe a strategy to attach ligands on the surface of NPs so that the NPs selectively bind to the target tumor cells or endothelium. Clearly, active targeting becomes effective only after the NPs reach the vicinity of the target tumors. These strategies can result in improved accumulation of NPs at the tumor, but the *in vivo* efficacy of NPs and NP-mediated drug delivery is still significantly impaired [1, 10, 38]. Only about 5% of the administered dose ends up at the target tumors. Although this may be 5-fold increase compared with drug molecules, the remaining significant portion of the NPs is still taken up by the RES of the spleen, liver, and lungs as illustrated in **Figure 4B**.

4. Tumor models to simulate TME

Due to the multifaceted complexities of the TME ranging from molecular, genetic, and biological, to chemical and physical parameters, it is very difficult to interpret the efficacy and resistance of drugs and drug delivery systems. All TME parameters are highly dynamic, interconnected and vary spatiotemporally, and may adversely affect the extravasation and interstitial transport of drugs and the subsequent action [13, 26]. In order to achieve effective treatment, both drug and delivery system should be designed to properly transport through, and act on, target cells in this complex environment. One critical bottleneck to developing effective targeted delivery systems is a limited quantitative understanding of the *in vivo* transport and action of drugs due to a lack of versatile models capable of rapid systematic study [7, 99].

Most widely used tumor models are two dimensional (2D) cell monolayers, often consisting of human cancer cell lines on a substrate. Although these cell lines are valuable tools, their 2D culture environment does not mimic the TME. Thus, the outcome from traditional 2D cell cultures often fails to be indicative of *in vivo* or clinical outcomes. A growing number of studies reported that the physical, chemical and mechanical microenvironment of cancer cells significantly affects cellular behaviors [100-106]. These include changes in cell morphology, gene expression and drug responses. Moreover, interactions of cancer cells with other cell types, ECM molecules, and the interstitial fluid in TME, should be properly represented. In order to address these deficiencies, three-dimensional (3D) models including spheroids and engineered tissue scaffolds have been developed [107, 108]. The 3D microenvironment and architectural structure provided by these models induce cell morphology, signaling, and gene expression similar to *in vivo* TME.

The simplest *in vitro* 3D tumor model is multicellular tumor spheroid, whereas tumor cells spontaneously aggregate and form spheroid structure [108-110]. Spheroids can be prepared by several methods including hanging drop method, liquid-overlay cultures, and dynamic bioreactors. By hanging drop method, cells are formed within small droplets of cell suspensions (approximately 20 to 50 μ L) [111]. Due to the surface tension, liquid can maintain droplets when the lid is inverted and cells settled at the bottom of the droplet, air-liquid interface. Liquid-overlay culture prevents cell adhesion to the culture substrates to induce cells to adhere to each other and form aggregates. To prevent adhesion to the culture vessel, the surface can be coated with agarose, poly-2-hydroxyethyl methacrylate (poly-HEMA), or Matrigel inducing cellular aggregations [110, 112]. Spheroids can also be formed by culturing in bioreactors, which can provide dynamic conditions preventing cell-substrate contact and promoting cells to form aggregates by either stirring or rotating bioreactors [113]. Nonetheless, these are convenient and relatively simple techniques to simulate *in vivo* solid tumor. Cells within spheroids display cell-cell interactions and produce *in vivo* tumor-like biochemical responses compared to 2D cell culture. However, the lack of interstitial fluid dynamics, which is highly relevant to drug transport at TME, is one of the major limitations [114, 115].

A large number of studies have historically relied on mouse xenograft and allograft (heterotopic and orthotopic) tumor models to test the efficacy of anti-tumor cytotoxic agents against established cancer cell lines. Although these studies have advanced the field and our knowledge of tumor biology, they are not ideal approaches because the tumors and TME that develop have limited similarity to the human disease. A better approach is to utilize GEMMs that initiate cancer in the correct cell type and at the correct time, to generate a tumor that more closely recapitulates the human TME. However, it is still challenging to obtain mechanistic information regarding drug and NP transport and action in GEMMs. More detailed reviews on animal models used in pre-clinical drug testing can be found elsewhere [116]. The TME of the animal models has many key features that are lacking in 2D and 3D *in vitro* models. However, even animal models often fail to simulate human *in vivo* environments and to provide a mechanistic explanation of the *in vivo* behavior of NPs [99]. This is because of (i) the unknown scaling factors necessary to extrapolate from animal models to human subjects [117], (ii) the mismatch between human cancer cells and mouse matrix environments [118, 119], (iii) the difficulties to simulate the heterogeneity of tumor microenvironmental parameters [120, 121], and (iv) the inability to independently control these parameters in GEMMs. Thus, a new model system is greatly desired, in which the TME parameters can be systematically and independently controlled, but at the same time the dynamic interactions among the fluids, ECM, cells and NPs are maintained.

In order to address the limitation of *in vitro* static cell culture and mimic a more *in vivo*-like environment, various new cancer cell cultures on microfluidic platforms have been developed as reviewed elsewhere [122-124]. Since this review is focused on drug transport at TME, several models relevant to study drug transport and action are presented in **Figure 5**. Most of them are based on microfluidic technology and often called “tumor-on-chip” or “tumor-microenvironment-on-chip.” Although not shown, first generation microfluidic cancer cell cultures were 2D cell monolayers cultured on microchannels in the presence of

fluid flow exerting shear stress on the cells [125, 126]. Specifically, endothelial cells were cultured on a microfluidic channel where shear stress was applied at controlled manner to study the morphological changes *in vitro* [125]. Epithelial transport characteristics of multiple chemical compounds were also studied under both temporal and spatial chemical gradients [126]. The presence of cell-fluid interaction was a significant advance from the conventional 2D cell monolayer models, but the lack of 3D environment was the major limitation to be addressed since the cell physiology of 2D models is distinctively different from that of cells cultured in 3D matrices. To address this, 3D culture environments have been created by combining tumor spheroids and tissue engineering technology.

First, tumor spheroid models have been scaled up into a 384-well format hanging drop culture plate for high throughput assay of drug sensitivity as illustrated in **Figure 5A** [127]. This model significantly increased the throughput of screening of drug sensitivity for a given spheroid types, and also reported the differences in the drug response when the same types of cells were cultured in either 2D or spheroid format. In order to address the lack of interstitial fluid dynamics, recent studies tried to integrate spheroids into microfluidic platforms where spheroids were subject to interstitial fluid flow [128-130]. In these models, cancer cells or spheroids were cultured within polymeric matrices to mimic cell-matrix interactions *in vivo*, allowing for the generation of spatial gradients of growth factors and pH [100, 128, 131-135]. As illustrated in **Figure 5B**, Walsh et al. [128] reported that pH gradient of the perfused spheroid culture on a microfluidic platform, and visualized the doxorubicin diffusion through the tumor spheroids. However, this model was still limited to mimic elevated IFP, which is one of the key features of TME.

Although the interstitial fluid flow has been known to hinder drug transport as well as to affect the morphology and migration of cells, it is very difficult to recreate within *in vitro* tumor models, even in microfluidic platforms. Polacheck et al [136] developed a microfluidic platform to mimic stable pressure gradients and fluid flow across tumor interstitium as shown in **Figure 5C**. In this model, breast cancer cells (MDA-MB-231) were seeded in type I collagen matrix and cultured under the perfusion of interstitial fluid flow created by pressure difference across the matrix. By controlling the pressure of each media channel, the flow rate could be precisely controlled and its effects on cell migration behavior were studied. Cell migration relevant to metastasis and angiogenesis has also been studied using microfluidic platforms [137, 138]. In addition, a microfluidic platform has been proposed to mimic hypoxia [139].

All of these 3D tumor models show great promise for mimicking the *in vivo* TME, and ultimately engineered tumors [140, 141]. The most significant advantages of these microfluidic 3D models are flexibility and controllability to systematically study the effects individual TME parameters. However, microfluidics models still warrant further research to create directional cell-matrix and tissue-tissue interactions [142]. Since cells are typically seeded within polymeric scaffolds in these 3D models, their cell-matrix interactions are non-directional and this can greatly affect cell polarity differently from *in vivo* circumstances during cancer development [107, 143, 144]. Moreover, drug transport *in vivo* is greatly affected by the interfacial phenomena at the tissue-tissue interface, including endothelium-blood, endothelium-interstitium, and interstitium-lymphatics endothelium. These

interactions need to be present in the model in order to properly simulate the drug transport *in vivo*.

As shown in **Figure 5D**, a T-MOC platform has been developed to recapitulate the complex and multiple transport processes in the TME [145]. Rather than mimicking a whole solid tumor, this platform was designed to recapitulate tumor tissue placed between capillary and lymphatic vessels. It had a 3D structure formed by stacking two PDMS layers of microchannels with a porous membrane inserted between the layers. Endothelial cells were cultured on the porous membrane to mimic the endothelium of the capillary. Along the capillary channel, drug-suspended medium flowed at physiologically relevant velocity and pressure. After extravasation, drugs entered the center channel of the bottom layer, which simulates the tumor interstitium. In this tumor channel, cancer cells were cultured within a 3D collagen matrix, and the interstitial fluid flowed through the matrix and exerted elevated IFP. Then, the drugs were transported through this 3D tissue structure to reach the cancer cells, and remaining drugs might be drained to two side channels mimicking the lymphatics. Using this T-MOC platform, the transport of NPs and the effects of various TME parameters on the transport were systematically studied, including the vascular pore size, IFP, and collagen content and cell packing density of tumor tissue.

Although many epithelial tumors including PDAC and ductal carcinoma in situ (DCIS) originate from round epithelial duct where malignant cells acquire invasive properties and disrupt normal epithelial duct geometry. A T-MOC to mimic this directional cell-cell and cell-matrix interactions is shown in **Figure 5E** [146]. A lumen structure was generated along the microfluidic channel using a fluid dynamic phenomenon called “viscous fingering” [147]. First, collagen solution was filled along the microchannel and then culture medium droplets were placed at the inlet port to initiate the viscous fingering. As the lumen formed, the microfluidic chip was incubated to polymerize the collagen and fix the structure. Once the lumen structure was produced, cancer cells were seeded along the lining of the lumen by filling with cancer cell suspension. A micrograph of the cultured tumor with epithelial ductal geometry is shown.

5. Summary and conclusion

In order to achieve targeted drug delivery, the barriers posed by various aspects of the TME must be overcome to improve the delivery and efficacy of drugs. Identifying a molecular target for delivery systems is a good starting point, but it is not enough to guarantee efficient delivery. For example, to design targeted drug delivery systems, multifaceted aspects of TME should be considered including - i) the dense stroma, hypovascularity, and high IFP of the tumor which pose biophysical barriers to drug transport; ii) hypoxia, CAF-cancer cell interactions, and genetic instability which hinder the actions of drugs; and iii) substantial immunosuppression which present within the TME. These barriers are highly dynamic, interactive, and spatiotemporally vary during development, progression and treatment. Thus, a systematic study to establish quantitative knowledge of the effects of these parameters on the transport and action of drugs is a must. To mechanistically understand this complex environment, new tumor models are needed that provide systematic control of relevant parameters and rapid/high content analysis of multifaceted drug transport and actions.

In this context, recent efforts to develop new *in vitro* tumor models such as T-MOC will provide a robust and convenient platform to rapidly screen various drug formulations, and to develop new targeted delivery strategies. Although these platforms have been developed to recapitulate the complex TME by culturing cancer cells with stroma cells within 3D extracellular matrices under perfusion, it is still not realistic, nor possible, to design a generic system to fully replicate every aspect of the TME. In order to maximize the benefit of these platforms, the model should be developed to test a specific hypothesis or certain aspects of TME while systematically varying biological, physical and chemical characteristics. Thus, rather than creating another step for drug discovery and screening, it can be used to obtain knowledge and insights, which can be extrapolated to drug design for animals and humans.

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Highlights

- Targeted delivery aims to selectively distribute drugs to targeted tumor but not to healthy tissue.
- Targeted delivery is very difficult to achieve due to transport barriers engendered by tumor microenvironment.
- Tumor microenvironment comprises a heterogeneous mixture of tumor and stromal cells embedded in an extracellular matrix.
- New in vitro tumor models based on microfluidic technology have been developed to recapitulate the complex transport in the tumor microenvironment.

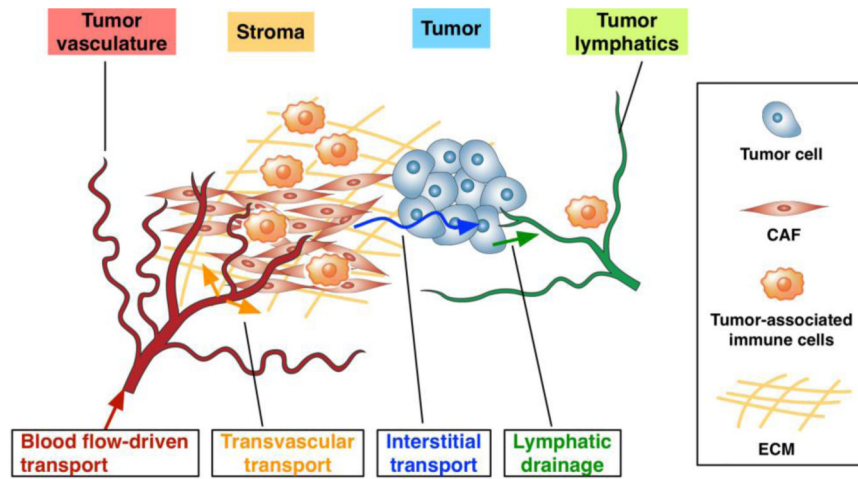


Figure 1. Complexity of the tumor microenvironment

TME poses multi-faceted barriers to drugs transport owing to the dense stromal tissue which is composed of collagens, fibronectin, and hyaluronan, an abundance of cancer-associated fibroblasts, and aberrant interactions between infiltrating tumor-associated immune cells, cancer cells, and CAFs.

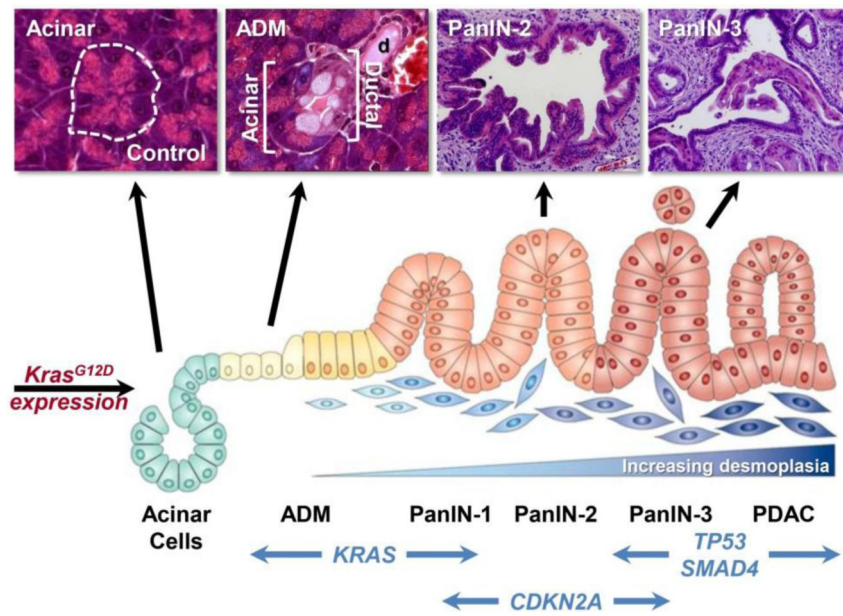


Figure 2. Schematic model of genetic alterations and histological examples of acinar cells, ADM (acinar-ductal metaplasia), PanIN, and PDAC progression as observed in a mouse model of PDAC
 Diagram adapted from Morris et al. [21]. Images from Zhu et al. [22] and Shi et al. [23].

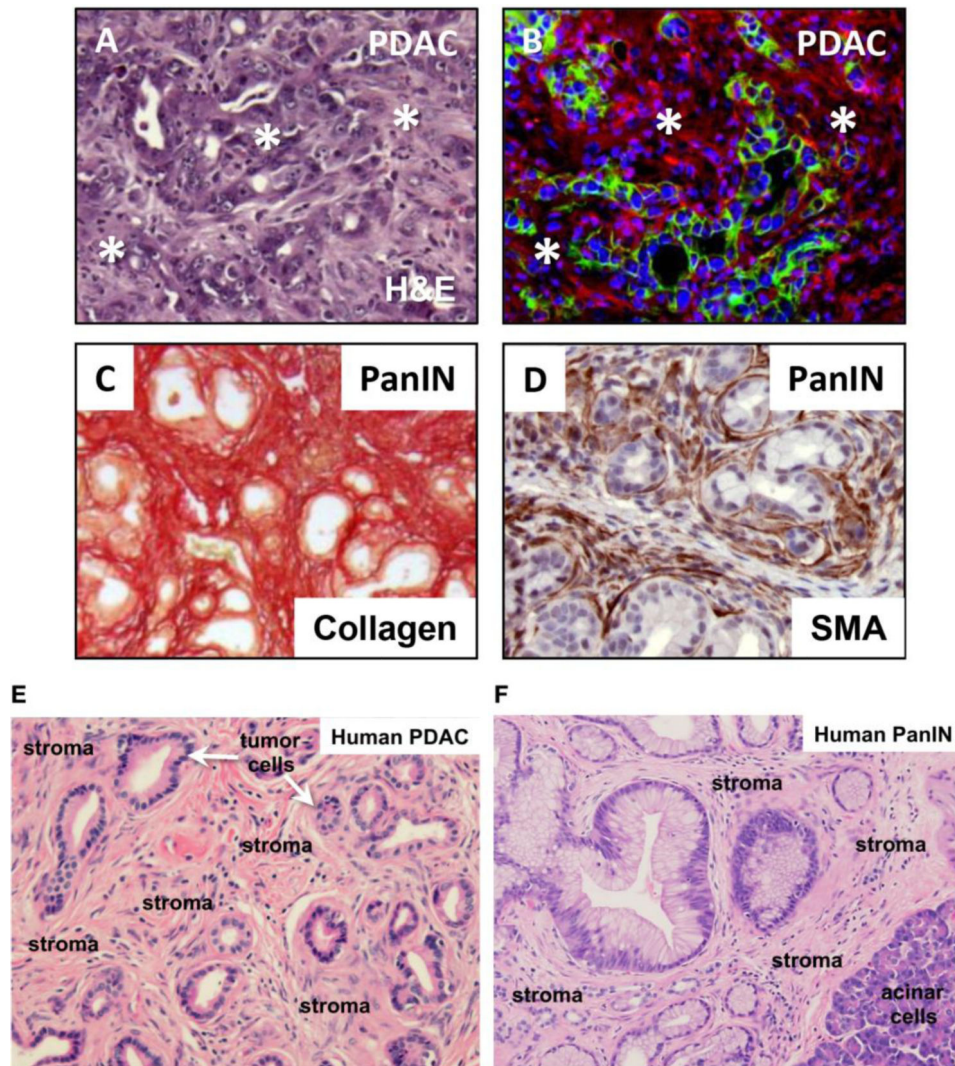


Figure 3. Stromal components in PDAC and PanIN

(A, B) Mouse PDAC from *elastase-CreER^{T2}/LSL-Kras^{G12D/+}/LSL-Tp53^{R172H/+}/R26^{mTmG/+}* mice. Upon tamoxifen treatment, *CreER^{T2}* becomes active in the acinar cell compartment, leading to activation of *KRAS^{G12D}*, *TP53^{R172H}* and mGFP expression. In (B) membrane localized tdTomato Red (mT) labels stromal cells whereas membrane localized GFP (mG) marks the tumor epithelial cells. The majority of PDAC tumor mass consists of stromal components (indicated by asterisks in A and shown in red in B). (C, D) Mouse PanIN lesions from *elastase-CreER^{T2}/LSL-Kras^{G12D/+}/LSL-Tp53^{R172H/+}* mice. Sirius Red staining identifies extensive collagen deposition in (C). Similarly, anti-smooth muscle actin (SMA) (brown stain) identifies cancer associated fibroblasts (CAFs) surrounding the transformed PanIN epithelial cells in PDAC samples (D). (E, F) Human PDAC and PanIN H&E stained sections. The predominant stromal components are noted.

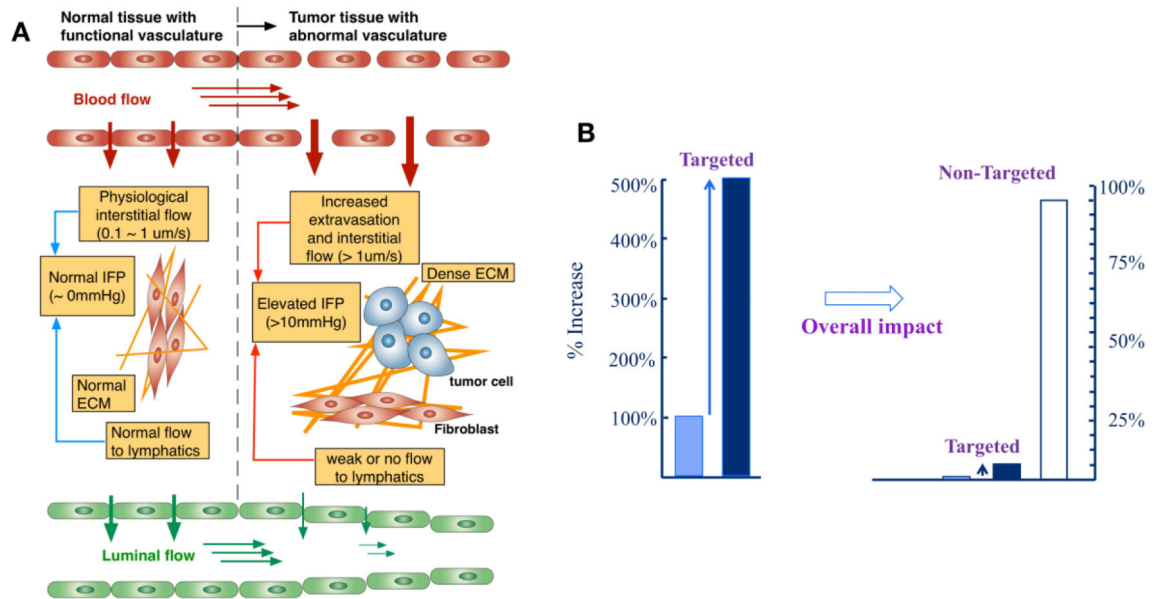


Figure 4. Transport barriers at the tumor microenvironment, and the outcome of typical targeted delivery

(A) Schematic of vascular and tissue structure relevant to drug transport of normal and tumor tissues. In normal tissue, the endothelium is tightly packed and very low interstitial fluid flow presents. This fluid flows to the lymphatics through the normal ECM, and the IFP minimally builds up. On the contrary, the endothelium of tumor tissue is leaky and has large pores, which leads to high interstitial fluid flow and more extravasation of the NPs. In conjunction with less functional lymphatics and the dense ECM, this increased interstitial fluid flow results in elevated IFP, which adversely affects the extravasation. The compounding effects of the elevated IFP, leaky vasculature, and poor vascularization of the tumor are still not fully understood. Images from Ref [7]. (B) Relative NPs distribution at a target tumor site. Various NP-mediated drug delivery strategies have been reported to improve the drug accumulation at the intended target up to 5-fold increase. However, the majority of the administered drug ends up at non-targeted sites. Adapted from Ref [42].

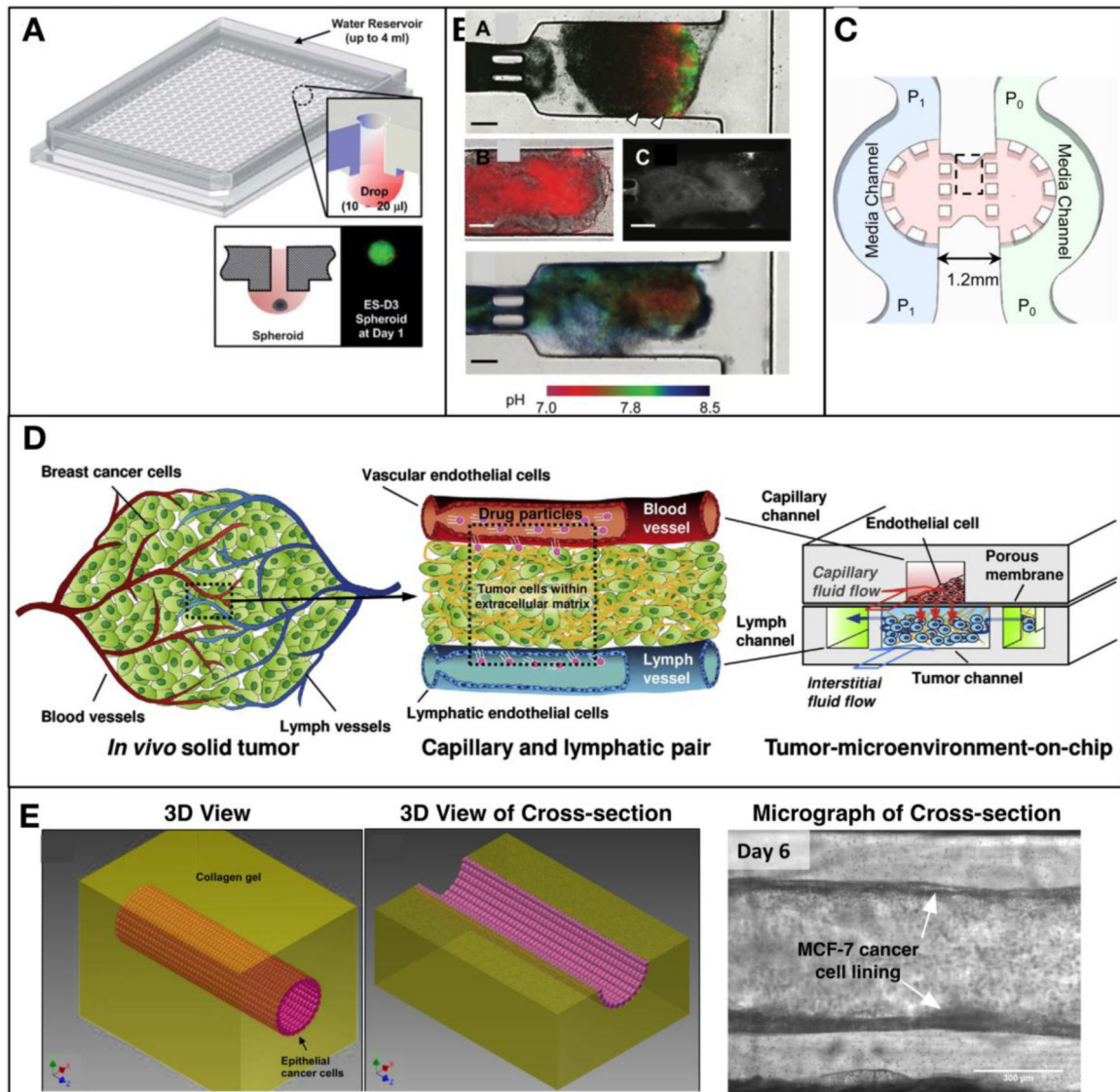


Figure 5. Recent development of in vitro tumor models

(A) Illustration of a high throughput hanging drop spheroid culture array plate, and its cross-sectional view. (inset) Cartoon of the hanging drop formation and a spheroid [127]. (B) A microfluidic platform to culture tumor cells capable of monitoring cell growth, apoptosis, and pH [128]. (C) A tumor-on-chip platform to study the effects of interstitial fluid pressure gradient [136]. (D) A platform to mimic multiple transport processes at tumor vasculature and interstitium [145]. (E) A platform to mimic epithelial tumor growth and metastasis from circular lumen structure [146].