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Cancer-on-chip technology: current applications in major cancer types, challenges and future prospects.

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Abstract

Conventional 2D cell cultures are widely used for the development of new anticancer drugs. However, their relevance as *in vitro* models is increasingly questioned as they are considered too simplistic compared to complex, three-dimensional *in vivo* tumors. Moreover, animal experiments are not only costly and time-consuming, but also raise ethical issues and their use for some applications has been restricted. Therefore, it becomes crucial to develop new experimental models that better capture the complexity and dynamic aspects of in vivo tumors. New approaches based on microfluidic technology are promising. This technology has indeed been used to create microphysiological systems called "organ-on-chip" (OOC) which simulate key structural and functional features of human tissues and organs. These devices have further been adapted to create cancer models giving rise to the "cancer-on-chip" (COC) concept. In this review, we will discuss the main COC models described so far for major cancer types including lung, prostate, breast, colorectal, pancreatic, and ovarian cancers. Then, we will highlight the challenges that this technology is facing and the possible research perspectives that can arise from them.

Key words: Cancer-on-chip (COC), *in vitro* tumor models, microfluidic technology, nanomedicine.

1. Introduction

Cancer is a major public health issue all over the world. According to the International Agency of Research on Cancer (IARC), over 19.3 million new cancer cases were reported and about 10 million people died of cancer in 2020[1]. Moreover, cancer incidence is expected to highly increase during the next years especially in the developing countries[2]. Developing new

anticancer therapies that are more effective with less side effects is thus becoming an emergency. Many efforts are being undertaken in this field but the failure rate of new anti-cancer molecules remains very high (about 90%)[3]. This is partly attributed to the lack of reliable preclinical models that can successfully reproduce *in vivo* drug responses[4], complex tumor micro-environment and cancer related pathophysiological events[5] such as cancer cell-stroma interactions, angiogenesis and metastasis. Indeed, current cancer studies are largely based on the use of two-dimensional (2D) cell culture[6]. Although easy to set up and well standardized, 2D culture models are far from reflecting the *in vivo* complex system, where cells closely interact with each other and with the extracellular matrix (ECM)[7]. Important differences can thus be noticed between *in vivo* tumors and 2D culture models where cells experience unnatural growth kinetics[8]. Moreover, it has been reported that the cytoskeleton of cells in 2D cultures undergoes specific modifications in response to the mechanical constraints imposed by the solid growing surface[9]. This may lead to an unnatural cell polarization with altered gene expression and protein synthesis[9]. Another important issue of the current preclinical practices in new drug development is the systematic and intensive use of animal experiments. Although animal models have been found to be important for assessing drug efficiency and toxicity, they have several drawbacks. Besides being costly and time consuming, they have important anatomic, physiologic and genetic differences from the human body[10]. Ethical issues related to animal use have also to be considered. For circumventing these problems and having more reliable results in early stages drug development, researchers have been seeking to develop new preclinical models that can better simulate physiological and pathophysiological processes taking place in the human body. Three dimensional (3D) cell culture approaches seem to be promising tools to reach this goal, due to their ability to better simulate cell-cell and cell-ECM interactions than classical 2D models[9]. Thus, many 3D technology based models have been reported[11] such as spheroids and organoids[12]. These structures mimic the oxygen and nutrient gradients in human tissues but do not simulate *in vivo* dynamic conditions such as blood flow or breathing motions. Another milestone was reached through the adaptation of microfluidic technology to create microphysiological systems reproducing key dynamic phenomena of the human body[13]. The so called organ-on-chip (OOC) systems have been adapted to simulate large variety of organs and diseases including cancer (leading to the rise of the “cancer-on-chip” (COC) concept[14–18]. Many applications of these innovative technologies have been described in the literature, but

several challenges still remain to be overcome before these models enter routine use for new drug development.

The main purpose of this review is to highlight the potential of COC technology in cancer modeling and to show its ability to match the anatomic and pathophysiological features of different cancer types. We illustrate this through the analysis of COC application in the most frequent and lethal cancers[1] (lung cancer, prostate cancer, breast cancer, colorectal cancer, pancreatic cancer, ovarian cancer, brain tumors and hepatic cancers). We will first discuss the emergence and development of the OOC approach, and then describe in more details its applications in the selected cancer types. Finally, we will discuss the main challenges that arise from this technology and the resulting research opportunities.

2. Microfluidics technology

Microfluidics is defined as the science of systems that process or manipulate small quantities of fluids (10^{-9} to 10^{-18} L) using micrometer-sized channels[19,20]. Microfluidic technology is considered as an interdisciplinary research field combining several applications in chemistry, physics, biology, medicine and other disciplines[21,18]. In the early 1950's, microfluidics gained momentum in microanalytical, gas chromatography (GPC), high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE) methods[19]. Since the 1990's, microfluidics has become one of the most dynamic technological areas[22] with a revolutionary impact on a wide range of applications[20] especially in biology. Indeed microfluidic platforms enable experiments to be performed at a small scale[23] reducing the consumption of reagents and biological samples. This is particularly important when the study concerns, for example, new therapeutic molecules since they are synthesized in small and costly quantities in their early development stages. In addition, microfluidic platforms can be used to optimize biological studies by giving a more precise control over the experimental conditions such as pH, temperature, shear stress and chemical concentrations[23–25]. Recent manufacturing techniques based on the use of soft lithography can be employed to fabricate customized devices[26] that can generate physiological-like conditions including oxygen and nutrient gradients, fluid flow phenomena and other biochemical and biomechanical processes taking place in the human body[27]. Besides providing precise control over experimental conditions,

microfluidic platforms also show high analytical performance[28] with the integration of sensors in the microfluidic device[29]. Real-time analysis can thus be carried out. Microfluidic platforms are most commonly made from optically transparent materials such as polydimethylsiloxane (PDMS) or poly(methyl methacrylate) (PMMA), which allows performing real-time imaging procedures[30]. Moreover, research for new materials for use in developing OOC continues[31,32], and smart materials with various properties such as memory[33], responsiveness[34], and electroconductivity[35] represent attractive examples that can be employed for defined applications in future.

Because of these advantages, microfluidic devices have been successfully used since the early 1990's for developing many important bio-analytical processes such as DNA analysis[36], immunoassays[37,38] and protein studies[5,39]. In the frame of cell culture and disease modelling, the use of microfluidics started to emerge in the early 2000's. For example, in 2004, Leclerc *et al.* used a PDMS device for performing a large-scale culture of hepatocytes by associating many microfluidic culture chambers[40]. This device included oxygen supplying compartments which were shown to be crucial for a successful cell culture. In 2010, the emergence of the organ-on-chip (OOC) concept marked a major evolution in the use of microfluidics in the biological field. Indeed, by developing the first "lung-on-chip" device (described below), Huh *et al.*[41] demonstrated that microfluidics could be used not only to culture cells in an efficient way, but also to reproduce the human physiological aspects at tissue and organ levels. From there, many research efforts have been made to fully exploit the benefits of this technology.

In the following paragraphs, we will briefly describe the most important progress done in the OOC field, and we will discuss in more detail the adaptation of this technology to the modelling of cancerous pathologies.

3. Microfluidics applications for studying cancer: cancer-on-chip (COC) concept

Cancer is a very complex process that evolves over years or decades and it involves a variety of exogenous and endogenous factors[42]. Tumor behavior in the body depends partly on the genetic characteristics of the cancer cells[43]. However, these cancer cells interact also closely with other elements of the tumor microenvironment (TME) such as ECM molecules, stroma cells, tumor microvasculature, immune cells, and multiple signaling factors[44]. These

interactions between the tumor cells and their TME strongly influence cancer pathological processes such as angiogenesis[45], metastasis[44], and drug resistance[46]. It is thus crucial to take all these interactions into account to develop relevant *in vitro* models for cancer studying or anticancer drug testing.

Compared to conventional 2D cell culture, the introduction of microfluidics technology for cancer modelling has allowed to reach a higher level of complexity in creating *in vitro* systems that better mimic the *in vivo* diversity of the tumor microenvironment. This technology makes it possible to associate different cell types in a unique device with a controlled 3D architecture. More importantly, microfluidic systems can be used to reproduce *in vivo* chemical and mechanical cues that can influence the tumor behavior including

(i) oxygen and nutrient gradients,

(ii) fluid flow,

(iii) shear stress, and (iv) mechanical constraints related to physiological movements such as respiratory motions and digestive peristalsis.

For example, Yi *et al*[47]. combined microfluidic technology and 3D bioprinting to develop a glioblastoma model for evaluating patient-specific cancer-cell responses to chemotherapy and radiations[47]. This model consisted of a compartmentalized cancer–stroma concentric-ring structure with a peripheric vascular compartment and a central cancer compartment. Through this configuration, the authors could simulate the oxygen gradient found in *in vivo* tumors and thus reproduce in vivo relevant drug responses[47]. Microfluidic platforms are indeed being increasingly used in oncology research[48] and their applications can be divided into three main goals:

- (i) improving our understanding of the pathophysiological processes involved in cancer and their possible therapeutic implications (cancer cell interactions with the tumor microenvironment elements[49–51], tumor angiogenesis[52–54], cancer cell invasion and metastases[55–59], isolation and enrichment of cancer stem cells[60]).
- (ii) performing anticancer drug screening[61–63].
- (iii) setting-up or improving diagnosis or prognosis applications such as the detection and isolation of circulating tumor cells (CTC)[64–66], the analysis of tumor cell exosomes

or nucleic acids[67] and the rapid performing of immunohistochemistry staining protocols[68].

In this section, we will describe the recent advances in microfluidic tumor modelling of the most common cancers to highlight the contribution of these new models to the development of cancer research and related therapeutics.

3.1 Lung cancer

Besides being the deadliest cancer[1], lung cancer is probably one of the best examples to illustrate the interest of using microfluidic tumor-on-a-chip devices as they are particularly suitable for reproducing the lung's complex environment. As we described before, microfluidic lung models (lung-on-a-chip) have allowed to effectively reproduce the essential structural and functional features of the alveolar-capillary membrane (ACM)[41] and the small airways[69]. These models were further adapted to study a variety of pathophysiological aspects of lung cancer. For example, Hassel *et al.* used microfluidic models of pulmonary alveolus and small airways to study the growth and drug responses of non-small cell lung cancer (NSCLC)[70]. In this study, a human NSCLC cell line was injected into the alveolus and the small airways models (Fig. 1A). It was shown that cancer cell proliferation was more important in the alveolus microenvironment, which corresponds to the *in vivo* observations. More interestingly, this study demonstrated that the application of mechanical constraints through the hollow side channels of the microfluidic alveolus model affected the cancer cell behavior. These breathing-like motions reduced the cell growth and invasion ability and modified their sensitivity to tyrosine-kinase inhibitors (TKI). While one limit of this model could be the lack of immune cells that influence NSCLC responses to anticancer therapies[71], this example clearly demonstrates the interest of using microfluidic models which open new research perspectives that remain inaccessible with classical 2D or 3D cell culture platforms. Besides creating orthotopic models, microfluidic devices are also increasingly being used to study lung cancer invasion and metastasis[72 73], which are directly linked to its prognosis. Xu *et al*. developed a multi-organ microfluidic device to simulate lung cancer metastasis in different organs including brain, liver and bone[74]. The lung-like part of the device was composed of an upper air channel and a lower medium channel separated by a microporous PDMS membrane coated

with ECM molecules. Bronchial epithelial cells and A549 cancer cells were cultured on the upper membrane surface while stromal cells (microvascular endothelial cells, fibroblasts, macrophages) were cultured on the lower membrane surface. The use of two lateral vacuum chambers allowed the application of cyclic stretching of the tissue layers. Lung chamber was connected through side channels to three other distinct chambers containing astrocytes, hepatocytes or osteoblasts to mimic lung cancer metastasis to the brain, liver or bone. After forming a primary tumor mass in the lung chamber, A549 cancer cells performed an epithelial-mesenchymal transition and spread to the secondary locations. However, one limit of this model was the absence of a functional endothelial barrier in the secondary sites making it impossible to reproduce cancer cell extravasation before creating secondary tumors. Liu *et al.* used a similar yet improved approach to investigate brain metastasis of non-small cell lung cancer[75]. In this study, the lung chamber was connected to a brain mimicking organ with a functional blood-brain barrier (BBB) structure (Fig. 1B). Different lung cancer cell subpopulations with variable metastatic potential were tested in this system. Authors demonstrated that cancer cells with high brain metastatic potential overexpressed Aldo-Keto Reductase family 1B10 protein (AKR 1B10). Moreover, silencing AKR 1B10 protein expression in these cells reduced their ability to cross the BBB and hence their metastatic potential. Similar results were obtained in *in vivo* using nude mice models of brain metastatic lung cancer[75]. This suggested that microfluidic devices can be reliable alternatives to *in vivo* animal experiments for lung cancer metastasis studies notably for the metastasis phenomena.

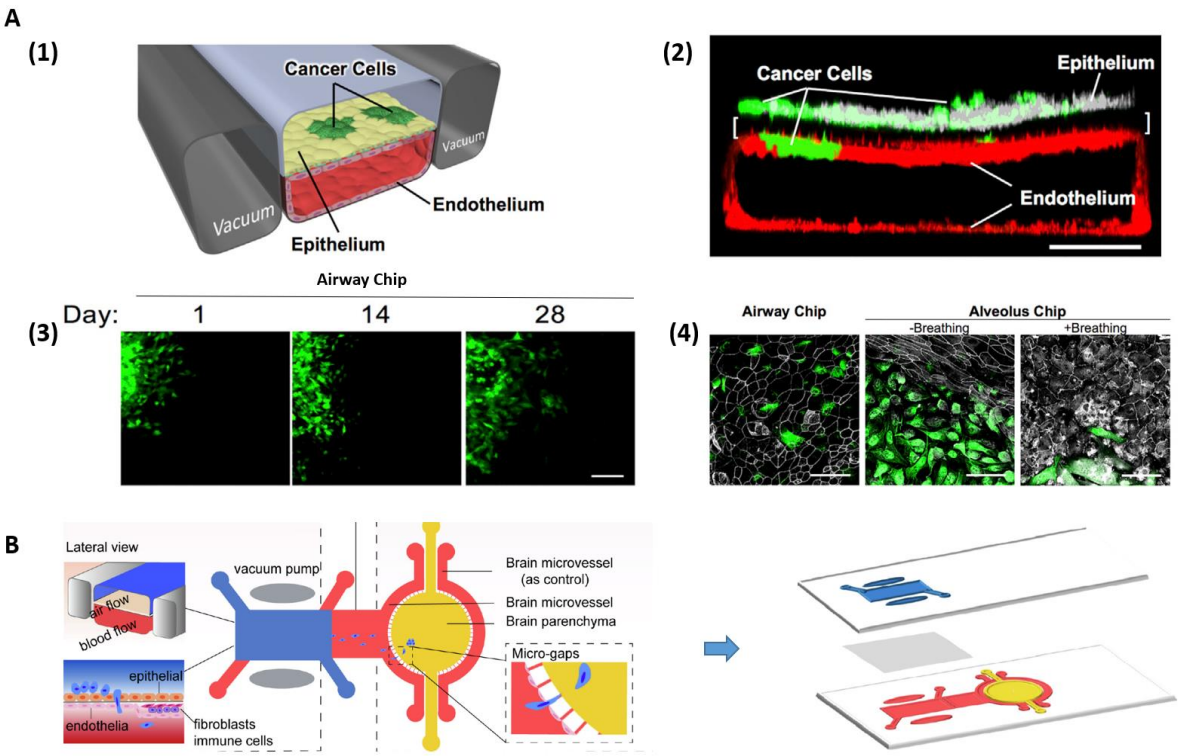

Figure 1: Examples of microfluidic OOC applications for lung cancer studies.

(A) Non-small cell lung cancer-on-a-chip: (1): Design of the chip. (2): Confocal fluorescence imaging of a cross-section in the chip showing the distribution of the different cell populations: lung cancer cells (green, anti-GFP); primary lung alveolar epithelial cells (white, antibodies against the tight junction protein ZO-1); primary lung microvascular endothelial cells (red, anti-VE-cadherin) (scale bar: 200 µm). (3): immunofluorescence imaging of NSCLC cells (green, anti-GFP) that showed slow growing rate over time when cultured in the airway mimicking chip (scale bar: 100 µm). (4): NSCLC cells (green, anti-GFP) showed different growing rates in the airway chip and the alveolus chip with and without breathing-like mechanical constraints (scale bar: 50 µm). Reproduced from [70], with permission from Cell Press. (B) Design of the microfluidic model developed by Liu *et al.*[75] for assessing brain metastasis of lung cancer. The chip consisted of two PDMS layers and a microporous membrane. Reproduced from[75], with permission from Elsevier.

3.2 Prostate cancer

Despite its epidemiological importance, there are few examples of prostate cancer-on-chip models compared to other cancer types. In 2019, Jiang *et al.* described a simple prostate-on-chip model that reproduced the stroma-epithelial interface[76]. This device consisted of two superposed PDMS channels separated by a polyester microporous membrane. Primary human prostate basal epithelial cells (PrECs) were used to form an epithelium on the lower membrane

surface while benign human prostate stromal cells (BHPrS1s) were cultured on the upper membrane surface to form the stroma compartment. This configuration allowed paracrine communication between the cancer cells and the stroma cells while preventing direct contact between the two cell types in order to study biochemical signaling pathways in the epithelial-stroma interface. Biochemical communication between epithelial, stromal and cancer cells is a key factor in prostate cancer pathophysiology. However, biomechanical cues in the TME also need to be considered[77]. For example, Ao *et al.* used a microfluidic chip to explore the impact of mechanical stress related to prostate cancer cell expansion on normal associated fibroblasts (NAFs)[78]. Their system was inspired by the alveolar-capillary membrane organ-on-OOC described by Huh *et al.*[41] with two superposed channels separated by a PDMS membrane which can be stretched through vacuum application on two lateral channels. Prostate NAFs were cultured on the top surface of the PDMS membrane and subjected to a consistent strain. Ao *et al*. showed that this mechanical stimulus could induce noticeable changes in NAFs behavior giving them a cancer associated fibroblasts or CAF-like phenotype[78]. Compared to non-stretched NAFs, stretched NAFs produced more aligned fibronectin network and showed higher ability to direct cancer cell migration. These results suggested that mechanical stress in the tumor microenvironment associated to cancer mass expansion can play a role in activating NAFs to CAFs which may promote cancer cell migration. Again, this study[78] could not have been carried out without using a microfluidic cancer model. However, this model has focused on a very specific aspect of prostate cancer pathophysiology. It can be improved by including other TME elements such as stroma cells and functional vasculature.

3.3 Breast cancer

Many pathophysiological and therapeutic aspects of breast cancer have been investigated using microfluidic systems in five main application fields: (1) Early stages of breast cancer initiation and development[79]; (2) Anticancer drug response evaluation[80] or drug delivery systems (DDS) testing[81] ; (3) Cancer cell-fibroblast interactions[50]; (4) Cell invasion[82] and (5) the development of metastatic models[58].

Breast cancer development is a long-term and highly complex process. Typically, the development of the cancerous lesion is preceded by an overgrowth of epithelial cells lining

the mammary ducts to form a non-invasive lesion called ductal carcinoma *in situ* (DCIS)[79]. Depending on several intrinsic and extrinsic factors, this non-invasive DCIS can develop into an invasive form (invasive ductal carcinoma (IDC))[83,84]. It is therefore, crucial to understand this transition from a localized to an invasive form. In this respect, Choi *et al.* described a DCIS microfluidic model[79], consisting of two superposed culture chambers (Fig. 2A). The upper chamber simulated the ductal lumen while the lower one mimicked stromal vascular capillary. The two chambers were separated by a thin vitrified collagen membrane that simulated basement membrane. On the upper membrane surface, epithelial mammary cells were first cultured to form an epithelium. Then, DCSI spheroids obtained from a 96-well hanging drop plate were seeded in this epithelial layer to mimic neoplastic masses. On the lower membrane surface, a fibroblast containing collagen gel was deposited to reproduce the stroma tissue in the mammary duct. This microfluidic model was then used to assess efficacy and toxicity of an anticancer drug (Paclitaxel) which was injected in the lower chamber to mimic intravenous administration. Although this model is a good representation of DCIS, it was only maintained in culture for one week. This is a very short time-window considering that DCIS can progress over years *in vivo* before potentially becoming a malignant tumor[85]. Devadas *et al.* described another microfluidic model of mammary gland to study complex interactions between epithelial and endothelial cells to better understand the evolution and propagation of cancerous lesions[84]. This model was composed of four microfluidic culture units in a 2X2 disposition (Fig. 2B). Each unit was formed of two parallel microfluidic channels connected by three migration ports. A partially polymerized Matrigel and collagen hydrogel was injected into the microfluidic channels then gently aspirated to form cylindrical lumens for the simulation of vascular and ductal lumens. The endothelial mimicking lumen was lined with endothelial cells. In the epithelial mimicking channel, three kinds of breast epithelial cells were tested, a non-cancerous breast epithelial line (MCF-10A), a non-invasive cancerous line (MCF-7) and a highly metastatic line (MDA-MB-231) (one cell type at a time in the culture unit). Using this device, it was possible to establish the migratory profile of the three breast cell lines and to show that the migration ability of MDA-MB-231 cells is potentiated when co-cultured with endothelial cells. While in this model no stroma compartment was taken into account, a complementary study was conducted by Nagaraju *et al.*[86] They focused on cancer cell intravasation into the blood stream and invasion of the surrounding stroma through the use a three layer concentric microfluidic model[86]. This model comprised an inner tumor region, an

intermediate stroma region, and a peripheral vascular region where endothelial cells embedded in a fibrin gel were allowed to spontaneously form an *in vivo* like capillary network (Fig. 2C). In both tumor and stroma regions, collagen was used to mimic the ECM while fibrin hydrogel was chosen to support capillary growth and organization in the vascular area. As for the previous example, it has been shown that the number of MDA-MB-231 cells migrating into the stroma was significantly increased in systems with endothelial cells capillary networks in comparison with control systems (without endothelial cells). Moreover, in the capillary network containing system, MDA-MB-231 cells were able to reach the fibrin matrix region and to penetrate inside the vascular network (intravasation phenomenon) while in control systems, they were incapable of reaching the peripheral fibrin matrix region. Using high resolution imaging techniques, it was possible to monitor real-time interactions between endothelial and cancerous cells, while the latter attached to the endothelial wall and crossed to the vascular lumen. Interestingly, it was demonstrated that cancer cell-endothelial cell interactions also take place in the other direction. Indeed, the authors showed that the vascular network grown in co-culture with MDA-MB-231 cells was highly permeable and smaller in diameter compared to that obtained in the control endothelial cell monoculture system. On the contrary, endothelial cell co-culture with MCF-7 (non-invasive breast cancer cell line) had no impact on the vascular network morphology or function. Here also, similar observations were made in *in vivo* animal models[86,87]. While Nagaraju *et al.* focused on the early stages of metastasis (intravasation)[86], other works have investigated more advanced stages of this highly complex process. In one example, Mei *et al.* designed a microfluidic device to elucidate the osteocytes mechanical regulation of breast cancer metastasis to the bone[88]. To accomplish this, they used a two-parallel channels microfluidic device. One channel had an endothelial cell coated lumen to mimic the vascular compartment. In the other channel, MLO-Y4 osteocyte cells were seeded to create bone compartment. The two compartments were connected by lateral channels (Fig. 2D). MDA-MB-231 breast cancer cells were introduced in the vascular-like channel. Their extravasation and migration through the side channels were then monitored in different conditions. In the control group, the observation was carried out under static conditions while in the experimental group, a physiologically relevant oscillatory fluid flow (1Pa, 1Hz) was applied to MLO-Y4 cells in the bone channel to mimic shear stress conditions applied to the bone during physical activity. The results showed that mechanically stimulated osteocytes have significantly reduced extravasation potential of the breast cancer

cells (reduction in the extravasation distance and the percentage of invaded side channels). It can be seen here that the use of a microfluidic system helped to understand how physical activity could influence bone metastasis of breast cancer through the ability to reproduce physiologically relevant dynamic conditions. In breast cancer research, microfluidic devices are also investigated as drug testing platforms. They can be used either for new drug development or for therapy optimization in personalized medicine. For new drug development, they are strongly thought to be more predictive than traditional 2D cultures. For therapy optimization, they offer a considerable time-effectiveness as compared to the use of patient derived xenografts (PDX) implanted in immunodeficient animals. To demonstrate this, Lanz *et al.* used a 96-microfluidic unit system to perform anticancer drug assays on cell lines and PDX-derived cancer cells. Cells cultured in 3D conditions under continuous flow showed high viability. Moreover, these 3D cultured cells showed higher drug resistance compared to these cultured in 2D conditions[89]. A more complex system was described by Predhan *et al*. who designed a vascularized perfused platform for assessing anticancer drug efficacy on non-invasive and invasive breast micro-tumor models[90]. In this platform, human breast tumor-associated endothelial cells (hBTECs) were first used to create a lumenized vascular network in microfluidic channels. This vascular structure ensured the perfusion of two superposed tumor chambers connected by a vertical channel (primary and secondary tumor chambers). For tumor formation, non-invasive MCF-7 or invasive MDA-MB-231 cells were suspended with fibroblasts in a poly(ethylene glycol)-fibrinogen hydrogel precursor. The cell containing suspension was then injected into the primary tumor chamber and photo-crosslinked using visible light[91] to form a “tumor-like structure”. The adaptation of the platform design made it possible to simulate two different perfusion levels (high perfusion and low perfusion level) to better capture the *in vivo* heterogeneity of different tumor regions. These platforms were then used for assessing anticancer efficacy and endothelial toxicity of two commonly used drugs (doxorubicin and paclitaxel)[90]. This demonstrates the possibility of using microfluidic devices for investigating both therapeutic and side effects of anticancer drugs. Moreover, this model shows numerous other advantages including the presence of a functional vascular network. The tumor structures could also be maintained in culture over a relatively long period of 28 days. Nevertheless, it remains far from reproducing all the complexity of an *in vivo* breast tumor, notably because of the absence of key TME actors such as adipocytes and immune cells[92,93].

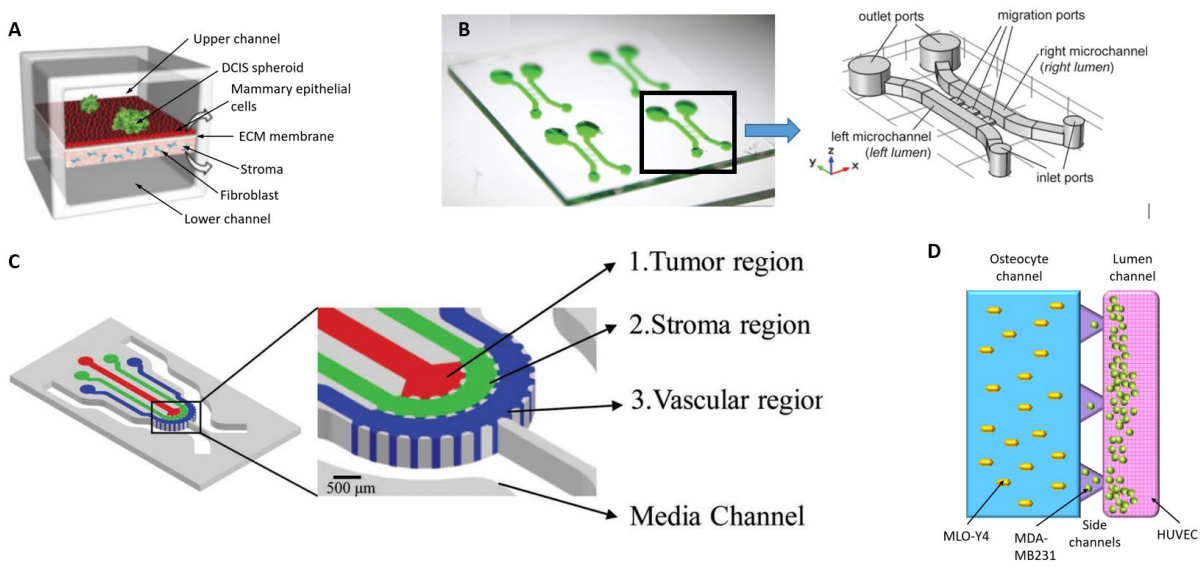

Figure 2: Application of COC technology for breast cancer modelling.

(A) Early stage breast cancer model. Ductal adenocarcinoma *in situ* (DCIS) spheroids were grown in the epithelized upper surface of a membrane. A fibroblast containing extracellular matrix (ECM) hydrogel was applied in the bottom surface of the membrane to mimic the stromal compartment. Reproduced from[94], with permission from Royal MDPI. (B) Device to study the interplay between cancer cells and endothelial cells in breast cancer. It was composed of 4 microfluidic units in a 2X2 configuration. Each unit comported 2 channels connected by 3 migration ports. Reproduced from[84], with permission from AIP Publishing. (C) Three-compartment microfluidic chip to study breast cancer cell dissemination in the stroma and intravasation into the vascular stream. Reproduced from[95], with permission from Elsevier. (D) Mei *et al.*[88] used a microfluidic device to simulate bone metastasis of breast cancer and the regulating role of osteocytes. The device they developed comprised 6 units. Each unit was composed of one vascular compartment and one osteocyte compartment. The two compartments were connected by side channels.

3.4 Colorectal cancer

For colorectal cancer (CRC) research, the potential of microfluidic approaches has been mainly exploited in the isolation, enrichment and analysis of circulating tumor cells for diagnosis and prognosis purposes [96 97 98]. Conversely, only few tumor[99] and metastasis[56 100 101] models have been reported so far. Nevertheless, microfluidic technology has already contributed to some of the recent advances in understanding CRC pathophysiology. In 2015, Vacchelli *et al.* conducted a complex study to investigate molecular mechanisms involved in cancer cell-

immune cell interactions and their implications in the development of chemotherapy-induced antitumor immunity[102]. In one step of this work, a microfluidic device was used to confirm that formyl peptide receptor 1 (FPR1) plays an important role in allowing and stabilizing the interaction between dying cancer cells and human immune cells, a crucial step in the antitumor immunity development. Besides being used to highlight such molecular and biochemical signaling pathways in CRC, microfluidic instruments were used, here again, to investigate the biophysical and mechanical aspects related to this disease. Recently, Armistead *et al.*[103] used microfluidic platforms for studying the impact of different shear stress levels in three different CRC cell lines, SW480, HT29 and SW620. A leukemia cell line HL60 was also used as a circulatory cell model for comparison with the CRC cell lines[103]. It was found that, under shear flow conditions, the metastatic cell line SW620 showed more deformation ability compared to the primary tumor-derived cell line SW480. This increased deformability has been shown to be related to an up-regulated expression of genes involved in cytoskeleton regulation[103]. The two examples cited above clearly illustrate the implication of microfluidics in CRC research but do not constitute tumor-on-chip models *per se*. In the framework of CRC modeling, one of the best works to be mentioned was published in 2016 by Sobrino *et al.* In this study, colorectal, breast, and melanoma cancer cells were used to create vascularized tumor models[99]. The tumor-on-a-chip design described in this work (Fig. 3A) consisted of three cell-culture chambers, delimited by two parallel microfluidic channels, with two media inlets and outlets. All these inlets and outlets were connected to a reservoir containing culture medium flowing across the microfluidic channels through the establishment of hydrostatic pressure gradient. To create a vascularized tumor model within this device, cancer cells (one type at a time), endothelial cells (Human endothelial colony forming cell-derived ECs (ECFC-ECs)) and stromal cells (Normal human lung fibroblasts (NHLFs)) were suspended in a fibrinogen solution. Thrombin was then added before the mixture was quickly loaded into the culture chambers and allowed for gelation. Within five to seven days, endothelial cells organized into a functional vascular network that merged with the outer channels and supported physiological flow. Among the cancer cells involved in this study, three CRC cell lines were used (SW480, SW620 and HCT116). Cancer cells were able to proliferate and form small spherical aggregates often in close proximity with the newly formed micro-capillaries. Interestingly, this platform maintained the specific phenotypic characteristics of the different cancer cell types: the SW480 non-invasive cell line formed tight structures while the SW620

metastatic cell line conserved its invasive growing profile. This tumor-on-a-chip device was also used to perform anticancer drug tests on the developed tumor models. For example, a routinely used drug combination in CRC treatment (5-Fluorouracyl, leucovorin, oxaliplatin) was assessed. The results confirmed its cytocidal (rather than cytostatic) action on CRC cells and highlighted its low toxicity on the capillary network. In this model, one limitation could be the lack of mechanical constraints simulating digestive peristalsis. A complementary CRC-on-chip model was reported in 2021 by Strelez et al[104]. It consisted of an epithelial-endothelial interface that could be submitted to peristaltic-like deformation. This device was used to study the early stages dissemination of CRC cancer cells namely intravasation. To investigate further CRC metastasis phases, one of the first microfluidic models was described by Skardal *et al.* in 2016[101]. It consisted of two connected microfluidic culture chambers (Fig. 3B). In one chamber, human intestine epithelial cells and HCT116 CRC cells (ratio 10:1) were used to simulate a primary tumor site. In the other chamber, a liver-mimicking structure was created by using human hepatoma cells (HepG2). In both cases, cells were suspended in a hydrogel and introduced into the microfluidic device. The hydrogel was photocrosslinked using UV-light, and fluid flow was generated across the microfluidic chip using micro-peristaltic pumps. After proliferation in the primary tumor site, HCT116 cells disseminated into the circulating media flow and reached the liver site where they formed secondary tumor aggregates. For comparison, the same procedure was conducted with another CRC cell line (SW480) which has less invasive potential than HCT116 cells. In this case, cells could grow in the primary site but never left it to spread elsewhere. This system was also used to study the impact of microenvironment mechanical properties on the metastatic behavior of CRC cells and to perform anticancer drug assays. In 2019, the same research team described an improved version of this metastasis-on-chip platform. It comprised a multi-organ-on-a-chip for the study of metastatic affinity of CRC cells[56]. This device contained four metastasis units equidistant from a primary tumor site (Fig. 3C). To form the latter, HCT116 CRC cells were incorporated into hyaluronic acid-gelatin gel to create a tumor-like structure. The same hydrogel was used to create liver, lung, and endothelial-based structures in three different metastatic units. A cell-free structure was created in the fourth metastatic unit to serve as a control. In this platform, CRC cells were shown to have a higher metastatic preference for liver and lung constructs. One drawback of these promising systems is the lack of an *in vivo*-like endothelial barrier to study the intravasation and extravasation phenomena that are crucial steps in the

metastatic process. Overall, the analysis of the previous examples shows the difficulty of combining different metastasis steps in a single model. Creating a complete metastasis device with a primary and a secondary sites each comprising a functional endothelial barrier remains challenging.

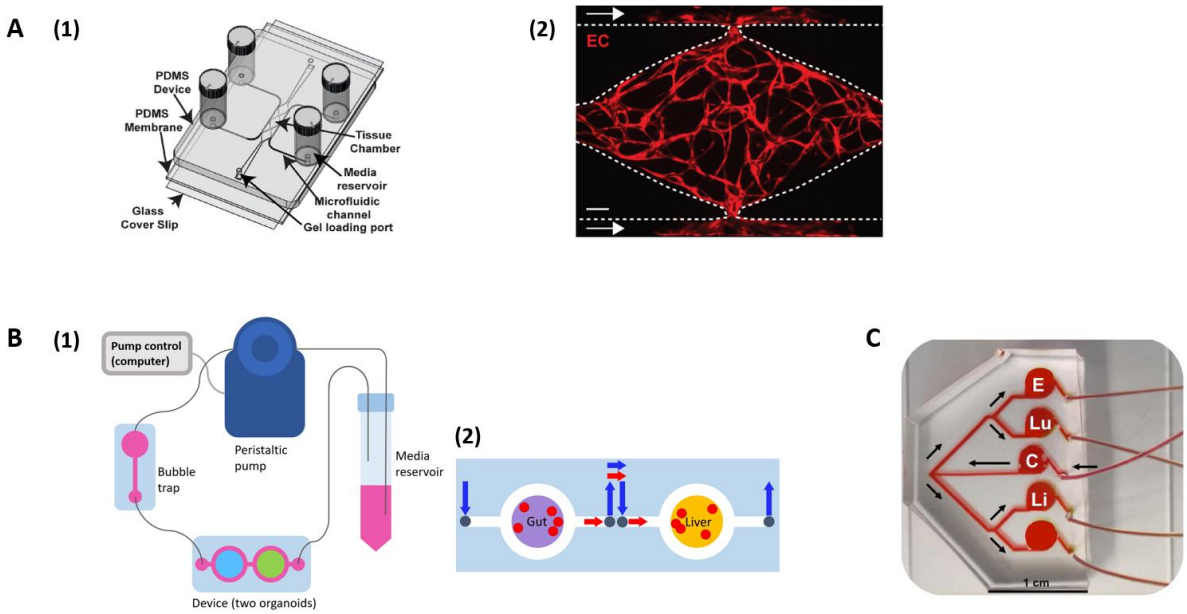

Figure 3: Cancer-on-chip (COC) technology applications for colorectal cancer.

(A) (1): Schematic of the microdevice used to grow vascularized micro-tumors. This device was composed of two perfusion channels and three culture chambers. (2): Confocal microscopy imaging of the vascular network formed by endothelial cells (Lentivirally-transduced ECFC-EC, red) after 7 days of culture. This network could connect to the two microfluidic channels. (Scale bar: 200µm). Reproduced from[99], with permission from Nature Publishing Group. (B) Overview of the experimental setup used as metastatic model of colorectal cancer (1). Photography (top) and schematic (bottom) of this metastasis model (2). It included two chambers (G for gut and LI for liver). Blue arrows show the sense of culture medium flow; Red arrow show the sense of potential migration of cancer cells from the primary tumor site to the metastatic site[101]. (C) Multi-organ metastasis device including a primary tumor site (C for colon) which is equidistant from 4 metastatic sites: E for endothelial; Lu for lung; Li for liver and a control site (cell-free chamber). Reproduced from[94], with permission from MDPI.

3.5 Pancreatic cancer

Pancreas is a vital organ that performs a double role, an exocrine function through the secretion of digestive enzymes, and an endocrine function through the secretion of hormones

such as insulin and glucagon for blood sugar regulation[105]. These two functions are related to different histological structures. Exocrine function is held by conical-shaped cells disposed around central lumens forming cell clusters named acini, separated by vascularized connective tissue[105]. The exocrine part constitutes the largest part of the pancreatic parenchyma[105]. It is also the birthplace of pancreatic ductal adenocarcinoma (PDAC), the most frequent tumor of the pancreas[106]. This cancer has focused most of the researchers' attention on microfluidic modelling of pancreatic tumors.

One of the first works in this context was published by Drifka *et al.* in 2013, who used a microfluidic device to investigate the complex interplay between cancer cells and stroma elements[107]. The model they described consisted of a microfluidic chamber with one outlet and three different inlets (one central and two side inlets) (Fig. 4A). PANC-1 pancreatic adenocarcinoma cells were suspended in an ECM-mimicking hydrogel (collagen-hyaluronic acid) and the suspension was introduced through the central inlet. Stromal cells (pancreatic stellate cells, PSC) were suspended in the same hydrogel and simultaneously introduced through the two side inlets. After hydrogel polymerization, a three-layered culture was obtained with a central tumor mass sandwiched between two stroma layers. This model was then used to assess the therapeutic response to the anticancer drug Paclitaxel. After exposure to Paclitaxel, cancer cells in the model showed reduced viability. Moreover, the specific structure of the stroma was disrupted. While this study focused on the interplay between cancer cells and stroma cells, endothelial cells have been included in other pancreatic cancer-on-a-chip models. For example, a recent work published in 2019 highlighted an important phenomenon called "endothelial ablation"[108] using a microfluidic device. This model consisted of a collagen matrix in which two parallel cylindrical channels were formed. One channel was lined with ECs to simulate a vascular capillary. The other channel mimicked a pancreatic duct and was seeded with primary mouse pancreatic cancer cells (PD7591) that formed an epithelial monolayer (Fig. 4B). A fetal bovine serum gradient in the vessel-like channel was then used to stimulate cancer cell migration through the collagen matrix. Under this stimulation, cancer cells were able to reach the vascular-like structure and penetrate its lumen after crossing the endothelial barrier. Moreover, it was shown that cancer cells do not just cross the vessel but partly occupy its lumen. In the vessel parts colonized by cancer cells, a high apoptotic tendency of endothelial cells, as well as the destruction of the collagen IV basal

membrane were observed. The authors used the term *"endothelial ablation"* to refer to this phenomenon. These findings were reproduced using other mouse and human pancreatic cancer cell lines and were also observable in *in vivo* PDAC models. Here, the use of a microfluidic device allowed to elucidate an important pathophysiological process. Endothelial ablation could indeed help explain why PDAC is highly invasive and at the same time poorly vascularized, which reduces its accessibility to systematically administrated chemotherapy[108]. Apart from endothelial ablation, other mechanisms involved in pancreatic cancer chemoresistance have been revealed through the use of microfluidic systems. In 2019, Kramer *et al*[109]. investigated the effects of intratumoral pressure on S2-028 pancreatic cancer cell responses to gemcitabine (a frequently used cytotoxic molecule in advanced stage pancreatic cancer[110]). They found these cells to be significantly more resistant to gemcitabine when exposed to intratumoral-like interstitial flow in comparison with cells cultured in 2D. This enhanced drug resistance was attributed to an increased expression and activity of multi-drug resistance proteins[109]. Along with other cancer types, pancreatic cancer is characterized by genetic heterogeneity and instability associated with a complex tumor microenvironment[111] making it even more difficult to realize *in vitro* relevant experimental platforms. Bradney *et al.* tried to capture part of this heterogeneity by incorporating murine PDAC cancer cells with different genomic subtypes into a microfluidic device[111]. In the same context, Haque *et al*[112]*.* designed a microfluidic platform to grow patient derived pancreas cancer organoids in the presence of key TME elements including stroma cells (pancreas stellate cells) and macrophages. This experimental platform was further used to highlight the stroma modulation of anticancer drug responses[112]. These works are interesting attempts to better capture the tumor heterogeneity and its impact on drug responses. They are also additional examples to illustrate the interest of microfluidics in pancreas cancer modelling.

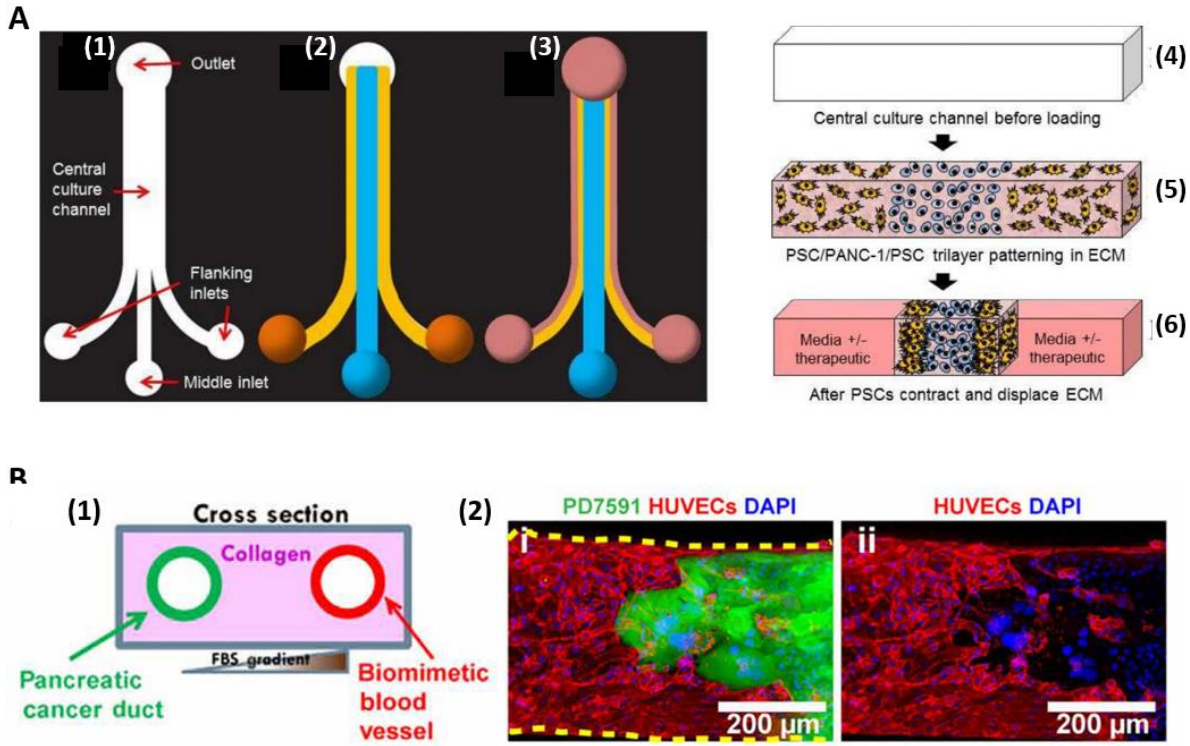

Figure 4: Application of cancer-on-chip technology for pancreatic cancer modelling.

(A) Microfluidic device used to study interactions between cancer cells (PANC-1) and stromal cells (PSC). An overall schematic of the device is shown in (1). The simultaneous introduction of cell-loaded hydrogels into the device is shown in (2): PANC-1 cell-loaded hydrogel (blue) was introduced through the middle inlet and PSC-loaded hydrogel (yellow) was introduced through the flanking inlets. As shown in (3), injection of culture medium (pink) through the flanking channels resulted in contraction of the three-layer cell construct. Cross section views of the process are shown in (4), (5) and (6). Reproduced from[107], with permission from Royal Society of Chemistry. (B) The device used to reveal endothelial ablation in pancreatic cancer. (1): A cross-section view of the device. (2): A confocal imaging of a section of the endothelial-lined channel showing its invasion by PD7591 cancer cells (i and ii). Endothelial cells (HUVECs) were marked with Anti-CD31 (red). PD7591 cells were labeled with FITC-conjugated anti-GFP (green fluorescent protein) antibody. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Reproduced from[108], with permission from American Association for the Advancement of Science.

3.6 Ovarian cancer

Ovary produces female gametes (ova) and female sex hormones (estrogens and progesterone). It consists of a cortical region, a central region (medulla) and a hilus[113]. The term ovarian cancer refers to several histologically and genetically distinct pathologies. High

grade serous ovarian cancer, also known as high grade serous carcinoma (HGSC)[114], is the most common and severe form. It accounts for 70% to 80% of the mortality associated with all ovarian cancer subtypes[115]. This probably explains why HGSC focuses most efforts on terms of microfluidic modelling of ovarian cancer.

Nowadays, it is hypothesized that premalignant lesions originating from the fallopian tubes may be involved in the genesis of HGSC[116 114]. To clarify this interplay, Fleszar *et al.* designed a microfluidic model of an ovarian cortical inclusion cyst (OCIC) to study its interactions with fallopian tube epithelial cells[116]. Before describing this OCIC model, we should remember that ovarian cortical inclusion cysts are cystic structures that form within the ovary when cells from the ovary surface epithelium (OSE) penetrate into the ovarian cortex after OSE rupture during ovulation[114].

Before establishing their OCIC model, Fleszar *et al.*[116] studied human ovarian sections and observed that a dense collagen band surrounds inclusion cysts with collagen fibers parallel to their boundaries. Then, they set up an *in vitro* OCIC experimental model consisting of a microfluidic cylindrical channel molded in a collagen-based hydrogel. This channel included a bottom large inlet, a top small inlet and two lateral inlets. Mouse fallopian tube epithelial cells (FTE) were then seeded in this OCIC model and formed a confluent monolayer over the channel surface. After this initial epithelialization phase, FTE cells spread into the collagen-based hydrogel showing an invasive behavior. Interestingly, the number of invading cells and their migration distance were influenced by the collagen type and concentration in the ECM-like hydrogel. The importance of ECM properties on cell behavior was thus demonstrated using a simple microfluidic device. While this study looks at early stages of HGSC development, other research teams have used microfluidic technology to develop metastatic models.

Indeed HGSC is a rapidly proliferating malignancy and it invades the peritoneal cavity often shortly after the apparition of the primary lesion[117]. This explains its late diagnosis and poor prognosis[114,117]. Unlike most solid tumors, which usually spread by hematogenous or lymphatic routes[118], ovarian cancer invades the abdominal cavity primarily through the peritoneal fluid. Cell aggregates (spheroids) derived from the primary lesion can circulate and further attach to secondary sites[117]. In an attempt to simulate this process, Li *et al.* used a microfluidic platform to reproduce the key structural and dynamic features of the peritoneum[119]. This “peritoneum-on-a-chip” device consisted of three parallel microfluidic

channels. Each channel was coated with fibronectin and seeded with primary human peritoneal mesothelial cells (HPMC) to form an epithelial monolayer on the bottom of its surface. SKOV-3 spheroids obtained in non-adherent 96-well plates were then introduced into the device. A syringe pump was used to apply physiologically relevant fluid flow across the microfluidic channels and cell spheroid behavior was observed over time. This simple model was far from capturing the *in vivo* complexity of the abdominal cavity but it already provided a good overview of microfluidics' potential to study ovarian cancer dissemination. Authors suggested improvements of their platform by incorporating other cell types to make it a more relevant experimental model for fundamental studies or for the development of new therapeutic tools. Other research teams have already reported the use of microfluidic platforms in the development of innovative therapeutic strategies. In one example, Wimalachandra *et al*. used a microfluidic experimental model to assess the efficacy and toxicity of anti-cancer nanoparticles[120]. For discussing this example, we will first have to describe these nanoparticles. They were designed to amplify the anti-cancer immune response by specifically targeting tumor cells and increasing recruitment of immune cells into the tumor region. For this goal, silica core-shell nanoparticles (NPs) were conjugated with folic acid and loaded with CCL21 (a chemokine known to stimulate the migration of dendritic cells and cytotoxic T lymphocytes). To validate these NPs, the authors developed a microfluidic platform consisting of a central channel connected to two side channels. The central channel was filled with a fibrin hydrogel containing human ovarian carcinoma cells (OVCAR-3) to mimic a tumor compartment. The two side channels were coated with fibronectin and seeded with HUVECs to generate an endothelial barrier. CCL21 loaded or CCL21 free NPs were added in one of the side channels. In this same channel, immune cells were then introduced and their migration across the vascular compartment toward the tumor site was observed. In comparison with CCL21 free NPs, CCL21 loaded NPs significantly increased immune cell migration into the tumor-endothelial interface and the tumor compartment. Other experiments showed that these CCL21 loaded NPs had no hemolytic activity and no disturbing effect on the coagulation processes which allowed to consider an intravenous administration. One important advantage of this system was the presence of an endothelial-like barrier to study the trans-endothelial passage of nanoparticles. However, only cancer cells were used in the tumor area and other important actors in the TME such as fibroblasts were not represented in this model.

3.7. Brain cancers

Among primary brain malignances, glioblastoma (GBM) is the most frequent and aggressive[121]. COC technology has been widely investigated in GBM modeling both for fundamental pathophysiological studies and for drug evaluation[122]. In 2022, Alves et al reviewed glioblastoma microfluidic models that have been used to evaluate GBM related therapeutic strategies over the past 10 years. This provided a good overview of the diversity and the evolution of the developed GBM-on-chip platforms. While lithography was the predominant manufacturing method and PDMS the most frequently used material, the designs of GBM-on-chip devices strongly varied from one study to the other depending on the model's goals. Among GBM specificities, the presence of the Blood Brain Barrier (BBB) is of major importance as its precise role remains to be clarified[123]. Du to their ability to simulate biological interfaces, microfluidic devices are particularly suitable to model the BBB for better understanding its implication in GBM pathophysiology and therapeutic outcomes. In this context, one interesting approach was described in 2022 by Straehla *et al*.[124]. They described a GBM-on-chip model consisting of tumor spheroids cultured with vascular cells, astrocytes and pericytes that self-organized to form a functional BBB-like structure. This model was used to assess the transport of therapeutic nanoparticles through the BBB. In vivo studies were performed to validate the predictivity of this GBM-BBB microfluidic model[124].

3.8. Hepatic cancers

While few primary liver tumor-on-chip models have been reported[125,126], microfluidic technology has been widely exploited in the study of metastatic phenomena involving the liver[55,127–129]. Moreover, being a crucial metabolic center, the impact of hepatic metabolism on the efficacy and toxicity of chemotherapy has also been studied using multi-site or multi-organ microfluidic models[130]. In this context, the ability of microfluidic systems to combine multiple sites while linking them through physiological-like circulatory systems is of crucial importance as it allows to take a step further in the global modeling of the human organism *in vitro*[131].

4. Challenges and future perspectives in microfluidic preclinical models

Although there has been a significant progress in the use of microfluidic technology for cancer modelling, further efforts should be undertaken not only to create more comprehensive models, but also to promote and accelerate their implementation at the industrial level. Though the analyses of the current state of the art, three main research areas can be indicated: (i) identifying and solving problems related to the fabrication and daily handling of microfluidic devices; (ii) addressing biological issues related to cancer-on-chip models; and (iii) accelerating the transfer from research to applications.

4.1 Fabrication and handling issues

Most OOC models are manufactured through soft photolithography process, which requires specific equipment, clean chambers, and qualified personnel. An interesting research perspective would be to develop alternative methods that are less restrictive and easier to implement while maintaining the precision and flexibility of photolithography. One recent attempt to address this problem was reported by Ferreira *et al*[132]. They described a rapid OOC manufacturing method based on xurography[132]. Xurography can be defined as the process in which the designed shape of each microfluidic channel is cut in a pre-cured thin PDMS layer. A cutting plotter was introduced to remove material from the PDMS layer to realize the desired shape. The layers were then bonded by plasma treatment for final assembly of the device. In contrast to soft lithography, this method does not require the prior fabrication of a mold. Nevertheless, PDMS layers with a minimum thickness of 200 µm should be used for a good manufacturing resolution[132]. In the same context, the use of 3D printing was also explored and may help to improve OOC manufacturing processes[133]. Another research prospect would be the identification of new materials for the fabrication of microfluidic platforms. Currently, PDMS is considered to be the "golden standard" material for OOC devices due to its interesting properties such as easy handling, gas permeability and optical transparency[134]. However, this material also has a high adsorption capacity for small hydrophobic molecules[31]. Therefore, it can bind therapeutic drugs, reduce their concentration, and affect their biological effect. In this context, Campbell *et al.* have recently reviewed materials that could be potential alternatives to PDMS for OOC production[31]. These

include elastomers, hydrogels, thermoplastic polymers, and inorganic materials[31]. Beyond manufacturing steps, the daily handling of microfluidic devices can also be challenging[18] because of the need of specific and cumbersome materials, such as peristaltic pumps or syringe pump units. Air bubbles remain another big issue to overcome while carrying out experiments on microfluidic devices[13].

4.2 Biological challenges

To consider COC application for the development of new anticancer molecules, a trade-off should be made between (i) their biological complexity and (ii) their ability to test multiple therapeutic molecules and multiple drug concentrations simultaneously. Indeed, the observation of current models shows that the above two parameters often evolve in opposite directions. In High throughput microfluidic devices, the biological part (tumor-like part) often consists of simple cancer cell structures without vascular network and without TME components[62,135,136]. On the contrary, complex cancer-on-chip devices with vascularization and TME elements are often difficult to adapt for large-scale drug screening applications[70,79]. High throughput thus remains a major challenge in current COC systems. An interesting attempt to combine sufficient biological complexity and high-throughput screening capability in a single device was reported by Phan *et al.*[137] They developed a microfluidic device comprising multiple tumor structures perfused by a functional vascular network. Cancer cells (HCT116) and stromal cells (human normal lung fibroblasts (NHLF)) were used to create the tumor structures. This platform was then used to assess efficacy and cancer cytotoxicity of multiple anticancer drugs including cytotoxic and anti-angiogenic molecules. While this system showed cell diversity (three cell categories) and large-scale drug screening applications, it was only used for short term assessment of drug effects (72 hours). Thus, an interesting research perspective would be to develop *in vitro* microphysiological systems that remain viable and functional over long term (several weeks to few months)[13] to study the kinetics of drug impacts. The use of machine learning is also being investigated. Artificial intelligence (AI) technology can indeed be combined with the OOC devices to improve and optimize their high throughput screening ability[138,139]. When developing OOC models, it is also crucial to identify the most appropriate cell source for the considered application. Overall, three main cell sources can be identified including primary cells, stem cells, and immortalized

cell lines[140]. Primary cells are already differentiated and functional besides maintaining patient-specific cues. However, they are often available in limited number and difficult to be cultured *ex vivo* while keeping their specific phenotype[141]. Human induced pluripotent stem cells (HiPSC) can be generated from easily accessible dermal fibroblasts and can be differentiated into different cell-types[141]. They are thus interesting alternatives to primary cells when establishing patient-specific OOC based tumor models[140]. Both primary cells and HiPSC present inter-donor variability which can affect result reproducibility. Using immortalized cell lines present some advantageous including cost effectiveness and availability. They also allow better reproducibility compared to primary cells[142]. Finally, while culturing multiple cell types in a single device is important to reflect *in vivo* cell diversity, a supplementary challenge is to set the culture medium composition for ensuring optimal growth and function for all these cell types.

4.3 Transfer acceleration from research to applications

Although several microfluidic based models have been reported[13] and used for testing drugs and therapeutic responses[143,144], most of them remain at the proof-of-concept level and do not find tangible applications in pharmaceutical research and development industry. We believe that one of the best ways to accelerate the development of OOC technology is to intensify collaboration between research laboratories and pharmaceutical companies. To optimize R&D investments, pharmaceutical companies need to have clinically relevant information as early as possible while developing a new drug molecule. This information must concern both the efficacy and the safety of the drug candidate for guiding the development decisions. In this context, OOCs have been shown to accurately reproduce clinically observed vascular[145], hepatic[146] and renal[147] side effects of new molecules developed by pharmaceutical companies. A systematic use of OOCs will thus allow earlier detection of these major side effects. For that, reproducibility issues related to these OOC devices also need to be addressed. Many variability sources can affect OOCs including cell sources and manufacturing process[140]. Manual manufacturing processes such as PDMS replica molding are very interesting for the development phases as they are versatile and cost-effective. However, hand-made microfluidic devices show important user dependency and laboratory dependency[140]. Before considering large-scale use of OOCs, the automation of manufacturing

processes is therefore necessary. Beyond technical issues, the scientific community, in collaboration with industry and international organizations, should undertake the effort to standardize the OOC technology, i.e., identify models, manufacturing processes, and handling conditions that would be systematically used at the international level. Promising attempts in this sense have already been reported[140]. Finally, we also believe that stricter regulation of animal use in pharmaceutical industry would also be a good boost to the development of the OOC technology. Beyond industry, OOC technology finds interesting applications in the daily medical practice especially for personalized medicine purposes in cancer treatment. OOC made up with patient specific cancer cells can be useful to evaluate therapeutic protocols for optimizing their efficacy and reducing their side effects before administration to the patient[148].

5. Conclusion

Innovative preclinical models based on 3D cell culture and microfluidic technology appear to be a relevant alternative to 2D cellular models whose reliability has been questioned by the high failure rate of new anticancer molecules. The aim of this work was to highlight the most important contributions in terms of cancer-on-chip (COC) models to provide the reader a global overview on the progress made recently, the current limitations of this technology and future research perspectives arising from them. Indeed, many research efforts are being undertaken for developing these models and it is sometimes difficult to find one's way considering the large number of works published each year and the lack of a clear and universal nomenclature. Therefore, more collaborative work is required in order to overcome the current technological challenges and accelerate larger-scale implementation of these new *in vitro* preclinical models.

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